

ABSTRACT

ISAZA, NHORA. Flower Promotion in *Pinus maximinoi* and *Pinus tecunumanii* in a Tropical Environment, and Artificial Screening of High-Elevation *P. tecunumanii* for Resistance to *Fusarium circinatum*. (Under the direction of Drs. Gary R. Hodge and William S. Dvorak.)

Pinus tecunumanii and *Pinus maximinoi* are two important commercial species for Smurfit Kappa Cartón de Colombia (SKCC). However, seed production in tropical regions can be very problematic for pine species. For SKCC, these problems create a severe limitation to cone and seed production of pine species used for commercial plantations. An effective and inexpensive method to increase seed crops would be very valuable. The literature suggests that application of exogenous gibberellin (GA_{4/7}) can be effective in promoting flowering in pines.

Two experiments involving stem injections of hormones to enhance female flowering were conducted in an 11-year-old *P. maximinoi* clonal seed orchard and in a 5-year-old low-elevation (LE) *P. tecunumanii* clonal seed orchard located in Colombia. Experiment 1 was conducted in August 2007 using 15 clones of both species and applications of Provide® 10 sg (active ingredient (AI) GA_{4/7}); Experiment 2 was conducted in September 2007 using 12 clones of both species and applications of Procone® (AI GA_{4/7}). Both products were manufactured by Valent Bioscience Corporation, Libertyville, IL, U.S.A. In both experiments one ramet per clone was randomly assigned to each of four gibberellin treatments: 0, 50, 100, or 300 mg/tree of AI. In Experiment 1, the solutions for all treatments were probably at saturation when injected into the stems, with approximately 9 mg of AI in solution which is lower than the target amount of the hormone. In addition, two branch girdling treatments were applied to investigate the effect of girdling in various locations within the crown on pollen production.

In Experiment 1, trees treated with GA_{4/7} produced significantly more female strobili than the controls for both species. Trees of *P. maximinoi* treated with 300, 100, and 50 mg of GA_{4/7} averaged 1193, 968, 1128 total female strobili per tree respectively vs. 870 on average for the controls. For *P. tecunumanii*, trees treated with the same doses of GA_{4/7}, averaged 353, 301, and 297 total female strobili per tree respectively vs. 211 for the controls. In Experiment 2, trees treated with GA_{4/7} produced significantly more female strobili than the

controls for *P. maximinoi*, but not for *P. tecunumanii*. *Pinus maximinoi* trees treated with 300, 100, and 50 mg of GA_{4/7} averaged 859, 878, 838 total female strobili per tree, respectively, vs. 623 on average for controls, an increase of 38 %. There was some evidence that branch girdling increased pollen production in the middle of the tree crown, but the practical importance of this increase is probably small.

Fusarium circinatum is a serious disease threatening many economically important pine tree species throughout the world. Fourteen open-pollinated families of high-elevation (HE) *P. tecunumanii* and three bulk seedlots (*P. patula* and *P. tecunumanii*) were screened for resistance to pitch canker using artificial inoculation on seedlings that were 16 and 18 weeks old. Consistent with previous results reported in the literature, *P. tecunumanii* (LE) shows essentially no stem dieback, *P. tecunumanii* (HE) shows intermediate resistance, and *P. patula* is very susceptible. Heritability estimates for the four variables used to assess response to pitch canker (stem dieback at 3 and 5 months after inoculation) were quite high, ranging from 0.48 to 0.58. There was little family x experiment interaction, with type B genetic correlation (r_{Bg}) values ranging from 0.77 to 0.91 for the four response variables. There was substantial genetic variation among the *P. tecunumanii* (HE) families for resistance to pitch canker infection; the range in general combining ability (GCA) predictions for percent stem dieback 5 months after inoculation was 12 % to 63 %.

Flower Promotion in *Pinus maximinoi* and *Pinus tecunumanii* in a Tropical Environment and
Artificial Screening of High- Elevation *P. tecunumanii*
for Resistance to *Fusarium circinatum*

by
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DEDICATION

This thesis is dedicated to:

My father Carlos Arturo,

my husband Hector Jaime,

my daughter Ana Maria,

and the rest of my family and friends who

have encouraged me to fulfill this great

dream.

BIOGRAPHY

Nhora Isaza was born in Medellín, Colombia, where she spent her childhood and her youth. She graduated from high school in 1978 in the Liceo Nacional Femenino Javiera Londoño. After high school, she attended the Universidad Nacional de Colombia and graduated in 1987 with a Bachelor degree in Forestry Engineering.

After her graduation, she worked in a private mining company as a soil manager, from 1987 to 1988. In 1989 she was hired to work with a private paper company, Smurfit Kappa Cartón de Colombia, as Seed Orchard Manager. She continues in this position until the present. In August 2006 she enrolled in the Department of Forestry and Environmental Resources at North Carolina State University to pursue her great dream: to get a Master degree in Forestry Science.

She is married to Hector Jaime Ramirez and is the mother of one child, Ana Maria.

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TABLE OF CONTENTS

LIST OF TABLES	ix
LIST OF FIGURES	xi
Chapter 1. Literature Review of Previous Work in Pine Reproductive Biology and Flower Promotion	1
I. Introduction	1
II. Reproductive Biology of Pines	2
A. Floral Initiation and Endogenous Controls	3
Cytokinins	4
Auxins	5
Abscisic Acid	5
Ethylene	5
Gibberellins (GAs)	6
B. Phenology	7
C. Meiosis and Pollen development	8
D. Pollen Dispersal	10
E. Pollen Germination.....	11
F. Fertilization	11
G. Seed Development	12
III. Geographic and Climatic Factors Affecting Cone Production	15
A. Environmental Controls of Flowering in <i>Pinus tecunumanii</i> , <i>Pinus maximinoi</i> , and Related Species	17
<i>Pinus tecunumanii</i>	17
<i>Pinus maximinoi</i>	18
<i>Pinus caribaea</i> var. <i>hondurensis</i>	19
<i>Pinus taeda</i>	20
<i>Pinus echinata</i>	21
<i>Pinus palustris</i>	21
<i>Pinus elliottii</i>	22
<i>Pinus ponderosa</i>	23
<i>Pinus jeffreyi</i>	23
IV. Artificial Induction of Flowering in Pines	24
A. Grafting	24
B. Girdling	25
C. Nutrition	25
D. Light and Temperature	26
E. Water	27
F. Growth Regulator Treatments	27
Methods of Gibberellin Application	30
Timing of Gibberellin Application	31

Gibberellin with Additional Treatments	32
Analysis of hormonal mechanisms of cone flowering	33
REFERENCES	34

Chapter 2. Flower Stimulation Treatments in *Pinus maximinoi* and *Pinus tecunumanii*

Seed Orchards in Colombia	42
I. Introduction	42
II. Materials and Methods	47
A. Experiments 1 and 2: Timing of Applications and Gibberellin Products	47
B. Plant Material	47
C. Hormone Treatments	48
Experiment 1	48
Experiment 2	49
D. Branch Girdling Treatments	49
E. Data Collection	50
F. Data Analysis	50
III. Results	52
A. Tree Size by Production Class	52
B. Weather	53
C. Experiment 1, Gibberellin Treatments	55
D. Experiment 1, August 2007, Provide® 10 sg treatments: Female Production	56
E. Experiment 2, September 2007, Procone® treatments: Female Production	61
F. Catkin Production, Experiments 1 & 2	66
Girdling Effects on Catkin Production	66
Hormone Rate Effect on Catkin Production	66
IV. Discussion	72
V. Conclusions and Recommendations	75
REFERENCES	76

Chapter 3. Responses of Seedlings of Open-pollinated Families of High-Elevation *Pinus tecunumanii* to Inoculation with *Fusarium circinatum*

.....	80
I. Introduction	80
II. Background	81
A. The Pathogen	81
B. Symptoms	83
C. Hosts and Geographic Distribution	84
D. Potential Management Strategies	87
III. Materials and Methods	89
A. Plant Material	89
B. Experimental Methods	90
C. Data Analysis	92
IV. Results	93

V. Discussion	98
VI. Conclusions	100
REFERENCES	101

LIST OF TABLES

Chapter 1

Table 1.1	Use and results of GAs in conifers	29
-----------	--	----

Chapter 2

Table 2.1	Climatic, geographic, and establishment description of the SKCC <i>P. tecunumanii</i> and <i>P. maximinoi</i> seed orchard sites in Colombia	44
-----------	--	----

Table 2.2	Seed production of <i>P. tecunumanii</i> and <i>P. maximinoi</i> in open-pollinated SKCC clonal seed orchards in Colombia.....	46
-----------	--	----

Table 2.3	Mean tree size prior to flower promotion treatments by species and production class in Experiment 1	53
-----------	---	----

Table 2.4	Mean tree size prior to flower promotion treatments by species and production class in Experiment 2.....	53
-----------	--	----

Table 2.5	Test of hypotheses for mixed model analysis of variance for female strobili production of <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – GA _{4/7} stem injections Experiment 1.....	58
-----------	---	----

Table 2.6	Test of hypotheses for mixed model analysis of variance for female strobili production of <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – GA _{4/7} stem injections Experiment 2.....	63
-----------	---	----

Table 2.7	Test of hypotheses for mixed model analysis of variance for catkin cluster production of <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 1	68
-----------	---	----

Table 2.8	Test of hypothesis for mixed model analysis of variance for catkin cluster production of <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 2.....	69
-----------	--	----

Chapter 3

Table 3.1	Most frequent hosts affected naturally by <i>Fusarium circinatum</i>	85
-----------	--	----

Table 3.2	Plant material included in the pitch canker resistance assessment – Experiments 1 and 2.....	90
Table 3.3	Chronological events for pitch canker screening of families of <i>P. tecunumanii</i> (HE) and control lots of <i>P. patula</i> , <i>P. tecunumanii</i> (HE and LE)	91
Table 3.4	Source of isolates used to screen families of <i>P. tecunumanii</i> (HE) and control lots of <i>P. patula</i> , <i>P. tecunumanii</i> (HE and LE).....	92
Table 3.5	Least square means for height and pitch canker resistance traits for <i>P. tecunumanii</i> (HE) open-pollinated families in the two experiments	94
Table 3.6	Test of hypotheses for mixed model analysis of variance for pitch canker resistance traits in OP families of <i>P. tecunumanii</i>	96
Table 3.7	Genetic parameter estimates for <i>Fusarium circinatum</i> tolerance traits for OP families of <i>P. tecunumanii</i> (HE) assessed at 3 and 5 months after inoculation	97
Table 3.8	Linear contrasts of species control lots versus <i>P. tecunumanii</i> (HE) open-pollinated families.....	97
Table 3.9	Rankings of <i>P. tecunumanii</i> (HE) open-pollinated families and three species bulk controls lots for pitch canker resistance traits	98

LIST OF FIGURES

Chapter 1

Figure 1.1	Biochemical structure of gibberellins (GAs).....	6
Figure 1.2	Stages of pollen and male strobili development in pines	9
Figure 1.3	Pollination and fertilization steps	10
Figure 1.4	Diagram of the proposed reproductive cycle of <i>P. tecunumanii</i> and <i>P. maximinoi</i> in Colombia	15

Chapter 2

Figure 2.1	Map of Colombia showing where SKCC has operational plantations and the locations of the <i>P. tecunumanii</i> and <i>P. maximinoi</i> seed orchards.....	45
Figure 2.2	Mean monthly historic precipitation in Cabuyerita farm, location of the <i>Pinus maximinoi</i> seed orchard from 1980 to 2003 compared with the data of 2007.....	54
Figure 2.3	Mean monthly historic precipitation in Aguaclara farm, location of the <i>Pinus tecunumanii</i> seed orchard from 1980 to 2003 compared with the data of 2007.....	54
Figure 2.4	Least square means for female strobili production for three levels of GA _{4/7} treatments (50, 100, 300 mg/tree) for <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 1	59
Figure 2.5	Least square means for female strobili production for three levels of GA _{4/7} treatments (50, 100, 300 mg/tree) and production class for <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 1	60
Figure 2.6	Least square means for female strobili production for three levels of GA _{4/7} treatments (50, 100, 300 mg/tree) for <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 2	64
Figure 2.7	Least square means for female strobili production for three levels of	

	GA _{4/7} treatments (50, 100, 300 mg/tree) and production class for <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 2	65
Figure 2.8	Least square means for catkin cluster production for three levels of GA _{4/7} treatments (50, 100, 300 mg/tree) for <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 1	70
Figure 2.9	Least square means for catkin cluster production for three levels of GA _{4/7} treatments (50, 100, 300 mg/tree) for <i>Pinus maximinoi</i> and <i>Pinus tecunumanii</i> – Experiment 2	71

Chapter 1 - Literature Review of Previous Work in Pine Reproductive Biology and Flower Promotion

I. Introduction

Success of a tree breeding program depends on early and consistent flowering in the seed orchards, neither of which is characteristic of conifers in general (Pharis et al. 1987). The constraints on flowering, especially in tropical species, are not very well understood (Owens 1995). Flowering promotion in *Pinaceae* is not a simple procedure; it requires the knowledge of the reproductive cycles from floral initiation through seed set, and also an understanding of different environmental factors that control flower initiation and development (Pharis 1977). Mechanisms that control flower initiation and development are still unclear; they are related to the carbohydrate budget of the tree, tree architecture, and are also influenced by external environmental stimuli and endogenous controls (Dick 1995). Borchert (1983) suggests that the shift of apical meristem from vegetative to the reproductive stage depends on internal and correlative factors that inhibit vegetative growth near the potential flower bud and favor accumulation of carbohydrates in the inductible meristem.

Flower induction research began in 1950 and peaked in 1980, with the bulk of research focused on temperate conifer species (Philipson 1985; Owens and Blake 1985; Longman et al. 1986; Wheeler and Bramlett 1991; Greenwood et al. 1993; Cherry et al. 2007; Rust 2007). The most reliable method of flower promotion in the *Pinaceae* has been found to be the application of plant growth regulators (PGRs) such as gibberellin (GA), cytokinins, auxins, abscisic acid, and ethylene in pines (Kong and von Aderkas 2004). In most experiments the best results have been achieved with applications of a GA_{4/7} mixture along with some additional treatment: girdling, root pruning, fertilization, or water stress (Pharis 1977; Pharis et al. 1987; Dick 1995). Results may vary from year to year and from clone to clone, and from experiment to experiment because each species will have particular requirements regarding type and dosage of hormone, timing of

treatment, and the best time to apply additional cultural treatments (Pharis 1977). It has been suggested that exogenously applied GA_{4/7} is first used for vegetative growth and any remaining is used for cone bud differentiation (Pharis et al. 1987; Dick 1995). Girdling, an old method of inducing trees to flower, has also been effective on several species of fruit trees and conifers (Longman 1985; White and Wright 1987). The physiological reasons for this are unclear, though it has been associated with an increase in carbohydrate concentration in the crown (Ebell 1971). Girdling plus GA_{4/7} has generally increased seed set over GA_{4/7} alone, but sometimes there has been a decrease in seed set, and sometimes permanent damage to valuable seed orchard trees (Philipson 1987; Pharis et al. 1987; Wheeler and Bramlett 1991).

The objective of this chapter is to review the current state of knowledge on experimental induction of flowering in pines with the goal to guide flower induction research on the sub-tropical species *P. tecunumanii* and *P. maximinoi*.

II. Reproductive Biology of Pines

Pines are monoecious, typically with male strobili located on tertiary or higher-order branches lower in the crown, and female strobili on vigorous main shoots in the upper part of the crown (Keeley and Zedler 1998). The primordia in *Pinaceae* are initially undifferentiated and may become either vegetative shoots or reproductive structures. Differentiation is determined by subsequent environmental conditions, but when the required conditions are not experienced the primordia abort (Pharis (1976) as cited by Slee (1977)).

The reproductive cycle of flowering plants starts with reproductive bud initiation and ends with maturation of the seeds (Bonner, n.d.; Owens and Blake 1985). The cycle length is a fixed period where many stages occur in a long chain of events. The occurrence of any weak link in this chain can result in poor seed production.

Identification of weak links is the key to determining the best methods for stimulating seed production (Owens and Blake 1985).

Three primary types of reproductive cycles have been identified in most temperate-zone forest trees (Owens and Blake 1985). In the tropics the annual cycle of strobili development in pines is not always very well synchronized compared with temperate pine species (Sirikul and Luukkanen 1987). The 2-year reproductive cycle is the most common type in pines. Reproductive buds form late in the growing season of the first year; pollination occurs in the next spring, followed briefly by fertilization (usually a few weeks). The embryo grows quickly, and seeds are mature by summer or early autumn of the second year. This is the cycle of most gymnosperms and angiosperms of North America (Owens and Blake 1985). The second type of reproductive cycle is the 3-year reproductive cycle. Reproductive buds form in late summer or early fall, followed by pollination the following spring. Pollen tube and ovule development stop in mid or late summer and resumes the following spring when fertilization occurs; seeds mature in the fall (Bonner n.d.). The third type of reproductive cycle occurs in a few conifers in the *Cupressaceae* family, and is somewhat similar to the second type. The difference is that fertilization occurs within a few weeks after pollination, so embryo and seed development are initiated, and enter into a dormant phase in late summer or early fall. Development resumes in the spring of the third year (Owens and Blake 1985).

A. Floral Initiation and Endogenous Controls

Floral initiation involves the transition of an indefinite vegetative apical meristem (apices) or axillary apices into determinant reproductive structures, angiosperm flowers or conifer strobili (megasporangiate and microsporangiate, often referred to as female and male strobili, respectively) (Owens and Blake 1985; Ledig 1998). In female strobili, two inverted ovules (megasporangia) form on the upper surface of each ovulate scale. Male strobili are abundant and short lived with a high production

of pollen (Bonner n.d.). Each pollen grain has two air sacs to promote wind movement (Dorman 1976).

Floral initiation is the first step in any reproductive cycle. In conifers, reproductive buds may be borne on terminal apices or axillary apices and are enclosed by bud scales (Owens 1980). Potential reproductive buds become defined over a large portion of the growing season, depending on their location on the branch and in the crown, and on species, site, crown form, age, and climate. In general, female strobili buds develop on vigorous shoots in upper regions of the crown (Varnell 1976). They overlap with male strobili buds on tertiary or higher-order branches lower in the crown (Owens and Blake 1985; Bonner n.d.). In the *Pinaceae*, female strobili are produced first, followed by male strobili several years later (Fraser, 1958).

Plant growth regulators (PGRs) may be involved in controlling processes affecting growth, bud differentiation, stem elongation, dormancy, flowering, sex, enzyme induction, and leaf and fruit senescence. Therefore, they play an important role in creating or maintaining the balance of multiple factors that control the initiation of the flowering process (Kong and von Aderkas 2004). There are five main classes of PGRs: cytokinins, auxins, abscisic acid, ethylene, and gibberellins.

Cytokinins

Cytokinins are compounds with a structure similar to adenine. Their major functions are to stimulate cell division, enlargement, and differentiation. They are involved in overcoming apical dominance and promoting lateral growth. Exogenous applications increase bud initiation and control bud release. Endogenous cytokinin levels rise in the xylem prior to bud burst (Kong and von Aderkas 2004). Cytokinin biosynthesis is thought to occur in the root tips, therefore, cytokinins stimulate flowering through treatments that increase root-tip density. There is evidence that applying the synthetic cytokinin, benzylamino purine (BAP), at specific floral developmental stages induces

gender conversion of male flower buds into female ones without affecting germination (Wakushima 2004).

Auxins

Another PGR is auxin that induces cell elongation in stems. Sites of auxin synthesis occur in young growing tissue: flowers, stem tips, root tips, and young leaves. Its role in flower induction is not very well understood. Effects on flowering have been shown to be related to ethylene synthesis and other physiological processes. Auxin indirectly influences flowering by improving nutritional status of the plant and mobilizing carbohydrates (Meilan 1997).

Abscisic Acid

In contrast, abscisic acid (ABA) is a growth inhibitor that is synthesized in older tissues. Initially it was discovered to play a role in leaf abscission in the autumn. More recently it has been associated with many physiological processes: transpiration, stress response, germination of seeds, induction of dormancy, root geotropism, and embryogenesis. ABA mediates the adaptation of the plant to stress, but its exact role in the flowering process is still unknown. Physical treatments such as girdling and pruning used to enhancing flowering in conifers can influence endogenous ABA concentrations in the stressed trees (Kong and von Aderkas 2004). It has also been shown that flower induction through gibberellin application results in increased endogenous ABA concentrations (Pilate et al. 1990).

Ethylene

Ethylene is a gaseous hormone that moves freely within the plant by diffusion, and never reaches high concentrations. Sites and modes of synthesis are still poorly understood. It plays an important role in fruit maturation and is also produced during stress responses, such as water stress, tissue wounding, high temperature, and fungal invasion. Major functions are: breaking dormancy, leaf abscission in response to

water stress, epinasty in flooded plants, and inhibiting cell elongation and bud growth. Flower promotion by ethylene in angiosperms is well documented; however no information is available on its effects in *Pinaceae* (Kong and von Aderkas 2004).

Gibberellins (GAs)

Gibberellins are one of the major groups of plant hormones and all gibberellins have a similar basic molecular structure derived from four isoprenoid units (Figure 1.1). Having 19 to 20 C atoms. By 2003 there were 136 GAs identified from plants, fungi, and bacteria differing in their biological effects, and with some types are more active than others. The GAs are named GA₁, GA₂, ...GA_n in order of discovery (Kong and von Aderkas 2004).

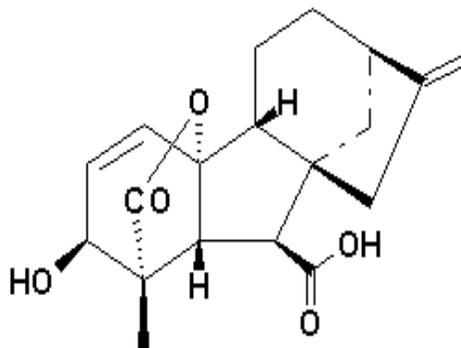


Figure 1.1. Biochemical structure of gibberellins (GAs) (Plant-Hormones.info n.d.).

Gibberellins were first discovered in 1926 when a Japanese scientist, E. Kurosawa, was studying the hyper elongation of stems of rice crops caused by a fungus of the genus *giberella*. He or she concluded four years later that the fungus secreted a chemical that stimulates shoot elongation, inhibits chlorophyll formation, and suppresses root growth (Plant-Hormones.info n.d.).

Gibberellin production occurs mainly in the roots, young leaves, and stems. As endogenous hormones, GAs stimulate growth in the stems (elongation and diameter growth) and leaves through cell division and cell elongation, but have little effect on

root growth. Exogenous applications increase internode elongation, affect apical form and apical dominance (enhancing it or decreasing it, depending on the species), and increasing diameter growth and shoot dry weight. In general, GAs cause sexual differentiation once they build up to a threshold concentration after plants have ended a vegetative growth (Pharis 1977).

Gibberellins are polar or non-polar, depending on the number of hydroxyl groups. Gibberellins with two or more hydroxyl groups are polar, while the non-polar forms have none (e.g. GA₉) or only one hydroxyl group (e.g. GA₄, GA₅, and GA₇). Non-polar gibberellins are the most effective in flower induction in *Pinaceae*. Gibberellins may be eliminated by the plant by oxidative metabolism, and it appears that conifers are very proficient at catabolizing gibberellins (Pharis 1977).

Plant hormones, especially the gibberellins, have been successfully used to promote flowering in trees of the *Pinaceae* family. Among the many GAs, the mixture of less-polar GA₄ and GA₇, and sometimes GA₉, have been found to be most successful in enhancing flowering of pines. Most commonly available commercial products with gibberellins contain GA₃, which promotes flowering in many species in the *Cupressaceae* and *Taxodiaceae* families, but not in *Pinaceae* (Owens and Blake 1985; Pharis et al. 1987; Sirikul and Luukkanen 1987).

B. Phenology

In hard pines (diploxylon) in northern temperate regions, female strobili occur on axillary apices, from May to October, and male strobili occur on axillary apices from August to October, (Ferguson 1904). In soft pines (haploxylon), female strobili occur on axillary apices from June to October, and male strobili from September to May, with a dormant period from October to February (Owens and Blake 1985). There is evidence that primordial differentiation in haploxylon pines occurs later and has a shorter developmental period than in diploxylon pines (Keely and Zedler 1998).

In tropical species, the time period between initiation of reproductive buds and anthesis is relatively short, and flowering may occur once, twice, or several times a year (Kramer and Kozlowski 1979). Continuous flowering is more noticeable in moist tropical environments forests where seasonal changes are much less than in dry tropical forests where flowering cycles are related to rainfall patterns (Willam 1985).

C. Meiosis and Pollen Development

Factors such as species, latitude, elevation, site, and climate define the time and duration of pollination. In *Pinaceae*, male strobili produce many microsporophylls, each with two microsporangia on their abaxial surface. Inside of each microsporangium, numerous sporogenous cells form, these cells undergo meiosis to form a tetrad of microspores, and each microspore develops into a pollen grain (Figure 1.2) (Owens 1982).

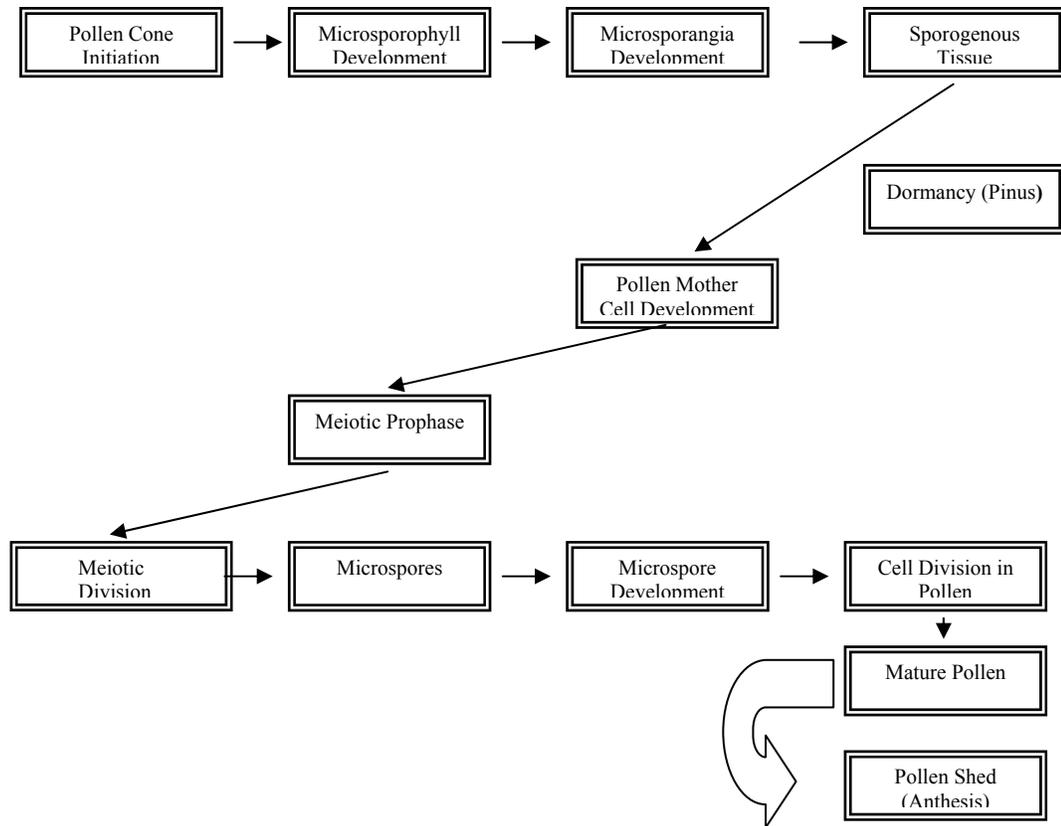


Figure 1.2. Stages of pollen and male strobili development in pines (Owens 1982).

Environmental conditions such as low temperatures, or physiological conditions, such as drought stress may cause meiotic irregularities such as pollen abortion or abnormal pollen development with low vigor or viability. The frequency of meiotic aberrations is generally low (Owens and Blake 1985).

The union of male and female gametes is the final stage in flowering that depends on two important processes: pollination and fertilization (Bonner n.d.). When male strobili have reached maturation, they release pollen grains for wind dissemination. When female strobili are completely developed, the scales separate and allow for pollination. Pollen grains enter the opening between the cone scales and rest in a position suitable for pollen tube growth. In pines, the ovule settles on the upper surface of the cone scale, with the opening of the micropyle facing the cone axis in a

downward position (Dorman 1976). Fertilization occurs when subsequent pollen tube growth facilitates the union of the sperm cell in the pollen with the egg cell in the ovule (Figure 1.3) (Bonner n.d.).

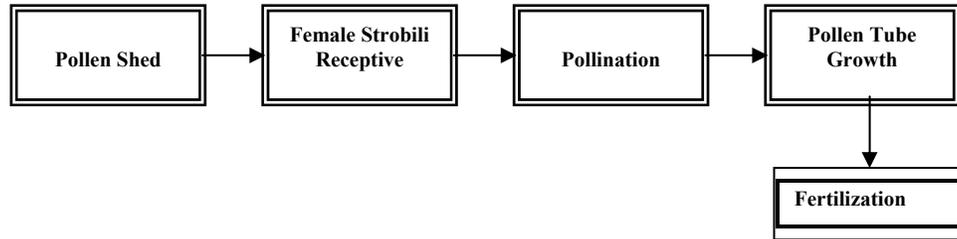


Figure 1.3. Pollination and fertilization steps.

During meiosis, the ovule becomes receptive to pollination. The cone scales open to nearly right angles to the cone axis to expose the ovules. The nucellus secretes a sticky droplet between the arms of the micropyle to which pollen grains adhere. The droplet is high in glucose, sucrose, and fructose (McWilliams 1958). The droplet is withdrawn and reabsorbed, moving the trapped pollen into the nucellar or pollen chamber in the tip of the nucellus. The middle cell layer in the micropyle enlarges, closing the neck. If pollen is abundant, between two to four pollen grains may be held in each pollen chamber, completing the pollination process (Ledig 1998).

D. Pollen dispersal

Conifers are mainly wind pollinated (anemophily). Pollen dispersal must occur at the time female strobili are receptive. Weather conditions strongly influence pollination; dry, warm weather will usually stimulate pollen dispersal by wind. However, rain or high humidity impedes anemophilous pollination; heavy rains during anthesis may sometimes cause seed crop failures (Bonner n.d.). Another important condition influencing anemophilous pollen dispersal is stand structure. Under near-calm conditions, pollen of many pines may disperse only a few dozen meters (Sedgley and Griffin 1989). While in turbulent conditions, it is possible to find pollen dispersed 1

km and more (Griffin 1980). For many different taxa at many different locations, the timing of the release of the wind-dispersed pollen can vary greatly, beginning in January at low latitudes and low elevations and prolonging into August at high latitudes and elevations (Young and Young 1992)

E. Pollen Germination

When the pollen grain germinates, it begins to penetrate the nucellar tissue. At the time of anthesis, the microspore has had two mitotic divisions. Meanwhile, the nucellus develops so that the pollen grain is further from the megaspore than it was at the time of pollination. The megaspore starts gametogenesis inside the nucellus. The archegonia are initiated on the surface of the megagametophyte. They are formed of neck cells and a large central cell which is divided to produce a small ventral canal cell and a large egg. A given ovule may produce only one archegonium, however the total number of archegonia is high, which is an important characteristic of the mating system of pines, and contributes to the high level of variability in pine species (Ferguson 1904; McWilliams and Mergen 1958; Owens 1980).

F. Fertilization

While the archegonia are expanding, the pollen tube grows quickly toward the megagametophyte, and the body cell divides forming two sperm. The pollen tube tip forces the neck cells of the archegonium to discharge its contents into a receptive vacuole in the egg. Several pollen grains may have been trapped in the pollen chamber, and each of the several archegonia formed on the megagametophyte may be fertilized and multiple embryos may develop. This phenomenon is called polyembryony (Ledig 1998). The zygote formed by the union of sperm and egg completes its development and the ovule becomes a seed. The seed has a hard outer layer, a wing, and contains the storage tissue (Dorman 1976). Development of the archegonia is complete less than one week before fertilization. In gymnosperms, the

elapsed time between pollination and fertilization ranges from three weeks for *Picea engelmannii* to 15 months for *Pinus radiata* (Bonner n.d.).

G. Seed Development

After pollination occurs, seeds develop quickly, mature and disperse around six months later, generally in the second autumn after pollination. A few species have a long seed maturation period such as *P. maximartinezii* and *P. pinea* with a likely four-year reproductive cycle from bud differentiation and *P. leiophylla* with a three-year cycle (Donahue and López 1995). Others exhibit a delay in seed dispersal ranging from a few months to many years, which is the case of strongly serotinous species (Keeley and Zedler 1998).

In most of the temperate pines, the conelet is more than a year old at fertilization. The food storage tissue in the female gametophyte is present at fertilization, and therefore embryo development dominates from that point. The embryo grows and differentiates into a small plant with a radicle, hypocotyl, plumule, and cotyledons (Chowdhury 1962).

After fertilization, carbon fixed during photosynthesis is translocated to the seeds in the form of sucrose. In the seeds, the sucrose is transformed into many components, but most goes into food reserves of carbohydrate, lipid, or protein (Bewley and Black 1994). Accumulation of food reserves is slow at the beginning, but is much more rapid when maturity and shedding approach. Soluble carbohydrates are transformed to insoluble fractions in starchy seeds, increasing the protein-nitrogen ratio at the expense of soluble forms. Therefore, during this period of development seeds are strong sinks for current photosynthate, reducing in some way the vegetative growth of the plant (Owens and Blake 1985). Simultaneously, when growing seeds are accumulating food reserves changes in hormonal concentrations occur affecting auxins, gibberellin (GA), cytokinins, and abscisic acid (ABA). PGRs play an

important role in the growth and development of seeds and fruits, but their roles are not totally understood. Gibberellins, cytokinins, and auxins have been found in high concentrations in immature seeds during the most rapid phase of development, and both gibberellins and cytokinins decline later, apparently becoming bound to other compounds. Auxins seem to be required for normal fruit development. In contrast, ABA concentration is low in immature seeds and highest at maturity (Bewley and Black 1994).

When seed dispersal from the cones occurs the embryo is generally both physiologically and morphologically mature. There are some exceptions in some pines that grow at extreme northern latitudes, such as *P. cembra*, *P. parviflora*, and *P. sibirica*, which require particular conditions for germination. Cones that demand more than one year to mature remain small during the first year in the interval between pollination and fertilization, but after that they grow quickly (Bonner n.d.).

Most literature reviews of pine reproductive cycles pertain to north-temperate species. The review performed by Owens and Blake (1985) did not include tropical and subtropical species because the reproductive cycles and factors which affect seed production may differ significantly from those of temperate forest species. Longman (1985) emphasized how knowledge of factors controlling the floral initiation and development in subtropical and tropical species is scarce.

Even though a thorough literature searches was performed, little information was found on the reproductive cycles of *P. tecunumanii* and *P. maximinoi* growing as an exotic near the equator. However, in its natural range in Central America and southern Mexico, *P. tecunumanii* produces male and female strobili from December through March, and cones mature 22 to 24 months after pollination in high elevation populations. Cones can be collected at both high elevation (above 1500 m) and low elevation (below 1500 m) populations from January through March (Dvorak et al.

2000a). The average reproductive cycle found in Colombia after three years of observation was 19 months, with flowers appearing throughout the year without a marked peak of production (Isaza et al. 2002). In South Africa, Zimbabwe, southern Brazil, and eastern Australia, cones mature from late June to August (Dvorak et al. 2000a). In the natural range of *P. maximinoi*, from western Mexico to northern Nicaragua, cones ripen each year at the end of March and disperse the seeds by the end of the second to third week in April at the beginning of the rainy season (Dvorak et al. 2000b). The length of the reproductive cycle of *P. maximinoi* is only 12 to 14 months (Camcore 2002a, Isaza et al. 2002), which makes this species unique in the western hemisphere (Camcore 2002b). Phenological observations made on natural stands of *P. maximinoi* at San Juan Sacatepéquez (Guatemala) showed that pollen dispersal occurred from January through March, and most of the female strobili were receptive between February and early April. Presumably most ovules were fertilized in March. At the end of March of the second year, cones were mature and began to disperse the seeds (Camcore 2002a). Based on the SKCC's experience a suggested reproductive cycle of *P. tecunumanii* and *P. maximinoi* is proposed in Figure 1.4.

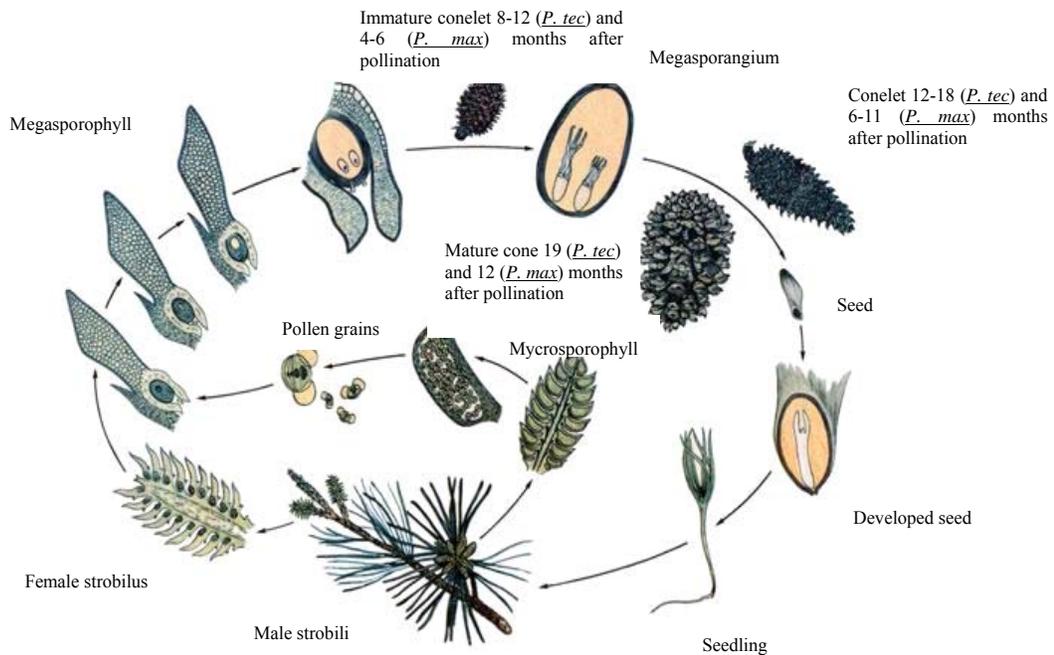


Figure 1.4. Diagram of the proposed reproductive cycle of *Pinus tecunumanii* and *Pinus maximinoi* in Colombia (amended from Tighe 2004 –adapted with permission from Carolina Biological Supply Company)

III. Geographic and Climatic Factors Affecting Cone Production

There are several factors affecting size of cone crops that are not well understood, especially in species with variable annual production. Abiotic factors such as temperature, precipitation, and radiation, and external biotic factors such as predation by insects or pathogens, and even internal competition for photosynthate and nutrients, can affect the size of cone crops at the different stages of strobilus initiation, pollination, and seed maturation (Owens and Blake 1985). Two independent studies with *P. ponderosa* and *P. resinosa* hypothesized that high temperatures during strobilus initiation were significantly correlated with cone crop size (Maguirre 1956; Lester 1967). Other studies have reported that water stress around the time of strobilus initiation has been correlated with high cone crops in *P. monticola* (Rehfeldt et al. 1971) and *P. taeda* (Dewers and

Moehring 1970). Environmental factors early in the reproductive cycle may be more critical than later in the cycle (Keely and Zedler 1998). Low levels of pollination may occur in years when the pollen crop is small, as between mast years, or because of pollen washout in a rainy season. Lack of pollination might lead to conelet abscission and the production of only wings. Genetic factors can also reduce seed yield due to homozygosity of lethal alleles following self-fertilization. Most pines are moderately self-fertile and produce fewer filled seed per cone when selfed. In *P. elliottii*, selfing reduced mean sound seeds per cone from 34 to nine (Lanner 1998). Sirikul et al. (1991) investigated cone setting in four provenance trials of *P. caribaea* var. *hondurensis* in Thailand. They found that for coastal-lowland provenances, cone setting decreased with increasing latitude and altitude. For inland-highland provenances, cone setting increased at higher altitude, while at lower altitudes more conelets were produced, but fewer reached maturity. This may be due to the high temperature and uniform equatorial day-length year round, which has been shown to inhibit the development of conelets into mature cones (Slee 1977). In a field evaluation of conservation stands of *P. caribaea* var. *hondurensis*, *P. oocarpa* and *P. tecunumanii* coordinated by the Food and Agriculture Organization (FAO) and Danida Forest Seed Center (DFSC) in Australia, Brazil, Cote d'Ivoire, India, Kenya, Tanzania, Thailand, and Zambia in the late 1970s and early 1980s, it was found that flowering and cone production were correlated with geographic and climatic parameters of the source of origin. These parameters included elevation, latitude, annual precipitation, difference in precipitation between driest and wettest month, mean annual temperature, and difference between hottest and coldest month. However, with the available data it was not possible to conclude how these specific climatic parameters influenced flowering and cone setting of the three species (DFSC and FAO 2001)

A. Environmental Controls of Flowering in *Pinus tecunumanii*, *Pinus maximinoi*, and Related Species

In the following sections, the available information related to the reproductive biology and factors influencing flower production in *P. tecunumanii* (subsection *Oocarpae*) and *P. maximinoi* (subsection *Ponderosae*, Group *Pseudostrobus*) is reviewed. Information on other species related to *P. tecunumanii* and *P. maximinoi* will also be reviewed: *P. taeda*, *P. echinata*, *P. palustris*, *P. elliotti*, *P. caribaea* var. *hondurensis* in the subsection *Australes*, closely related to the *Oocarpae* subsection (Dvorak et al. 2000), and *P. ponderosa* and *P. jeffreyi* in the subsection *Ponderosae*, related to *P. maximinoi* (Price et al. 1998).

The genus *Pinus* has been separated into three main subgenera: the subgenus *Strobus*, known as white pines or soft pines, characterized by scales without a sealing band, a terminal umbo, and adnate seed wings; the subgenus *Ducampopinus*, known as pinyon, bristlecone and lacebark pines, which features scales without a sealing band, a dorsal umbo, and articulate seed wings; and the subgenus *Pinus* known as typical pines, or yellow or hard pines, which have scales with a sealing band, a dorsal umbo, and articulate seed wings. Both subgenera *Strobus* and *Ducampopinus* are called haploxylon pines because they have one fibrovascular bundle per leaf, while the subgenus *Pinus* is called diploxylon pines because it has two fibrovascular bundles per leaf (Frankis 2002).

Pinus tecunumanii

Pinus tecunumanii Eguluz & J.P. Perry occurs from Mexico (central Chiapas) to central Nicaragua (Dvorak et al. 2000a). It belongs to the *Oocarpae* subsection and has evolved recently from Central American *P. oocarpa* (Dvorak et al. 2000). Based on elevation, two groups are recognized: trees from high-elevation (HE) populations occurring from 1500 to 2900 meter above sea level (masl) and low-elevation (LE) populations that occur from 450 to 1500 masl. *Pinus tecunumanii* has important

commercial advantages such as rapid growth in the nursery, rapid capture of site, higher productivity than *P. patula*, *P. oocarpa*, *P. elliotti*, and sometimes *P. caribaea* var. *hondurensis*, and acceptable wood properties for pulp, paper, and lumber. Disadvantages and limitations include susceptibility to stem breakage, root instability, and trees from HE populations have low seed production (Dvorak et al. 2000a).

Both female strobili receptivity and pollen dispersal occur between December and March throughout Central America and southern Mexico, depending on altitude. In HE populations, cones occur singly or in pairs and mature from January through March, 22 to 24 months after pollination. In LE populations, cones occur in clusters of two to four, and mature from January through March. In eastern Australia, southern Brazil, Malawi, South Africa, and Zimbabwe, cones mature from late June to August. In Colombia, cones mature throughout the entire year (Figure 1.4). Flowering begins in most seed orchards at 3 to 4 years of age. Seed production is best on the east coasts of Australia, Brazil, and South Africa between the latitudes of 18° and 28° S and in the highlands of Zimbabwe (i.e. 54 filled seeds per cone were obtained at 8 years) (Dvorak et al. 2000a). Seed production falls off drastically at low latitudes near the equator.

Pinus maximinoi

Pinus maximinoi H.E. Moore occurs from Sinaloa, Mexico to northern Nicaragua, over a wide range of microclimates and is the most common pine in Central America after *P. oocarpa*. It has important advantages such as rapid growth, excellent wood quality, and suitability for pulp and paper products. Disadvantages include high graft incompatibility, foxtails near the equator, top stem breakage, and low seed production in exotic environments (Dvorak et al. 2000b).

Information on the reproductive biology of *P. maximinoi* is limited compared to other pines. From Guerrero, Mexico to northern Nicaragua, cone collections occur from

the last week of March through the second to third week of April, just before the rainy season. The time span for collection is no more than 10 days. Cones occur at the end of long branches in clusters of three or four. Seeds are dispersed quickly in warm weather after the cones ripen. The reproductive cycle in its native range is about 12 to 14 months, with 15 filled seeds per cone at 8 years (Dvorak et al. 2000b; Camcore 2002a).

Pinus caribaea var. *hondurensis*

The distribution of *Pinus caribaea* Morlet var. *hondurensis* W.H.Barret & Golfari ranges from 18° 15' N at Ejido Caobas, Quintana Roo, Mexico to 12° 13' N in Nicaragua. It is most prominent below 500 m elevation, but is found from sea level to 1000 m elevation (Dvorak et al. 2000c). Phylogenetic assessments by Camcore have concluded that this species is intermediate between the *Oocarpae* and *Australes* subsections (Dvorak et al. 2000). It has important commercial advantages such as fast growth, availability of improved seeds, easy hybridization with Mesoamerican and southern US pines, and acceptable wood for a variety of uses. Disadvantages include high insect susceptibility, low wind firmness, and low seed production close to the equator (Dvorak et al. 2000c).

Production of male and female strobili usually occurs from November to January and occasionally as late as early February. Cones mature approximately 18 to 20 months after pollination between mid-May to mid-July. In natural stands it is common to get a high proportion of empty seeds. This could be caused by cone-boring insects or poor synchronization of female receptivity and pollen dispersal. Male and female strobili are produced as early as 3 years of age after grafting in optimum seed orchard locations and in areas that are extremely stressed. In the latter situation, heavy flowering may not occur again until age 8 or 9 years. Normally moderate flowering begins at age 5 years and the first cone collections are made around age 7. Cone collections are 19 to 20 months after pollination in September. In Brazil, flowering

occurs in June, July, and August and cone collections are in December and January, approximately 18 months later. Seed production is practically nonexistent at low elevations near the equator, but improves with increasing latitude and elevation of the seed orchard site (Dvorak, et al. 2000c).

Pinus taeda

Pinus taeda L. is widely distributed in the southeastern United States from southern New Jersey to central Florida and west to eastern Texas (Fowells 1965). Habitat ranges from mesic lowlands and swamp borders to dry lands. It is the most important commercial timber species in the southeastern United States; and has been introduced to other countries with variable success (Baker and Langdon 1990). Its most important uses are for lumber and pulpwood (Fowells 1965). *Pinus taeda* belongs to the *Australes*; based on a study of RAPD molecular markers, the *Australes* subsection appears to be descended from the Mesoamerican *Oocarpae* subsection, with *P. caribaea* var. *hondurensis* as the genetic link between them (Dvorak et al. 2000).

Flowering begins in the summer. The male strobili form in late July and the female strobili form in August, but they are not identifiable until late September or October. Buds remain dormant until the following spring when pollination takes place (Baker and Langdon 1990). The time of pollen release is variable and depends on springtime temperatures. The accumulation of 353 °C day-heat units above 13 °C after February 1 is required (Boyer 1978). Cones ripen in September and October of the second season. Seed dispersal begins in October and peaks in November and early December, about 26 months after the strobili were initiated. Latitude also influences flowering, which begins earlier at lower latitudes, occurring between February 15 and April 10 (Fowells 1965).

As *P. taeda* ages, the number of growth flushes decreases and flowering increases, although flowering has been promoted on young grafts with scions 3 years old.

Flowering is not only under genetic control but is also influenced by moisture (May-July rainfall) and nutrient stress (Baker and Langdon 1990).

Pinus echinata

Pinus echinata Mill. has the widest geographic range of any pine in the southeastern United States. It grows in 22 states from southeastern New York to northern Florida, throughout the Gulf States and inland to western Pennsylvania, Ohio, Illinois, Missouri, Oklahoma, and eastern Texas (Little 1971). Typically it is found at 3 to 910 m elevation, commonly in mixed stands with *P. taeda* (Fowells 1965). It is an important commercial species and ranks second only to *P. taeda* in standing timber volume. The wood is used for lumber, plywood, structural material, and pulpwood (Lawson 1990).

Male and female strobili emerge from late March to late April. Female strobili arise after male strobili form in the winter bud (Fowells 1965). After fertilization occurs in early spring or summer of the second growing season, cones develop rapidly and mature by late summer or early fall. The cone yields 25 to 38 seeds, and both number of seed per cone and number of seeds per tree can be increased by releasing seed trees from competition. A good cone crop occurs every 3 to 10 years in the North and every 3 to 6 years in the South (Lawson 1990).

Pinus palustris

Pinus palustris Mill. is found along the coastal plain from eastern Texas to southeast Virginia, extending into northern and central Florida. It grows at elevations from sea level to 600 m. Typical habitats are dry sandy uplands, sandhills, and flatwoods. It is a valued species for lumber and pulpwood and was once important for naval stores (Boyer 1990).

Male strobili may begin forming in their buds in July, while female strobili are initiated during relatively a short period in August. Apparently, climate conditions during the year of initiation affect the number of flowers produced; female production is enhanced under wet spring and early summer followed by a dry late summer (Shoulders 1967). Male strobili production is enhanced by abundant precipitation during the growing season (Boyer 1990). It has been known in *P. palustris* that a high percentage of female abortions results from excess ethylene production by foliage and shoots. Applications of anti-ethylene compounds soon after anthesis decreased female abortion, doubling seed yields (Hare 1987). Pollen buds emerge in November and remain dormant for a month. In turn, female buds emerge between January and February; their development depends on ambient temperature (Boyer 1990). Pollination occurs in the late winter or early spring, while fertilization occurs the following spring (Fowells 1965). The cones mature between mid-September and mid-October of the second year (Boyer 1990).

Pinus elliotii

Two varieties are classified, *Pinus elliotii* var. *elliotii* the most common and *Pinus elliotii* var. *densa*, found only in the southern half of Florida and in the Keys. *Pinus elliotii* Englem. has the smallest geographic range of the four important southern pines. It occurs on coastal plains from South Carolina to Central Florida, and west to Louisiana and is common on pine flatwoods throughout its range (Lohrey and Kossuth 1990). *Pinus elliotii* is an important timber species in the southeastern United States. Its strong, heavy wood is appropriate for construction. Due to its high resin content, the wood is also used for poles, railroad ties, and turpentine (McCune 1988; Lohrey and Kossuth 1990).

Male strobili begin to develop in June. These grow for several weeks, but then enter a dormant state until midwinter, and pollen is shed from January through February. Female strobili develop fully in late August. Cones mature in September,

approximately 20 months after being pollinated. Seedfall occurs in October but may be hastened by dry weather or delayed by wet weather. Good cone crops occur on average every 3 years for *P. elliotii* var. *elliotii*, while the var. *densa* produce good cone yields every 4 years (Lohrey and Kossuth 1990).

Pinus ponderosa

Two varieties have been differentiated, *Pinus ponderosa* var. *ponderosa* and *P. ponderosa* var. *scopulorum* (Peloquin 1984). *Pinus ponderosa* Dougl. ex Laws. is one of the most widely distributed pines in western North American. It has a natural range from southern Canada into northwestern Mexico, and from the Plain States of Nebraska and Oklahoma to the Pacific Coast (Oliver and Ryker 1990; Richardson and Rundel 1998). Ponderosa pines can be found in both high and low elevations and can also grow in a variety of soils (Fowells 1965). They are among the most valuable timber species with a great variety of uses such as poles, saw timber, railroad ties, mine timbers, fuel, livestock grazing, and enhancement of recreational sites (Schubert 1974).

Flowering in *P. ponderosa* is strongly correlated with the passing of freezing temperatures. At elevations from 910 to 1830 masl, flowering and pollen shed begin in spring and cones reach full size in the summer of the next year and seed is shed from September to November. This species does not have a regular periodicity in seed production (Fowells 1965).

Pinus jeffreyi

Pinus jeffreyi Grev. & Balf. occupies sites throughout much of California, southwestern Oregon, western Nevada, and northern Baja California (Critchfield and Little Jr. 1966). Its geographic distribution is strongly correlated with edaphic conditions in the northwest range and reflects climatic and altitudinal factors in the northeast, central, and southern portions. Commercially, there is no distinction

between the wood of *P. jeffreyi* and *P. ponderosa*. It is mainly used for lumber, and to a lesser extent, for piles, poles, posts, mine timbers, veneer, and railroad crossties. Currently, a considerable amount goes into the particleboard and paper industries (Jenkinson 1990).

Male strobili emerge from the bud earlier than female strobili. After pollination, conelets expand slowly and fertilization occurs 13 months after pollination. Cones reach maturity in summer of the second year and cones shed most of their seeds in September or October. Large seed yields occur every 2 to 8 years on pines that are 18 to 55 m tall (Krugman and Jenkinson 1974).

IV. Artificial Induction of Flowering in Pines

The production of genetically improved seed is one of the most important goals of a seed orchard, but flowering in conifer seed orchards may be insufficient and unpredictable to meet the requirements of the natural or artificial regeneration (Wheeler and Bramlett 1991). This condition is more complex in the tropics where environmental cues for flowering such as moisture, temperature, and photoperiod are weak (Sirikul and Luukkanen 1987). The long juvenile phase of trees is a severe constraint for traditional breeding programs (Meilan 1997). The two main objectives of research on flowering are: 1) developing techniques that stimulate flowering of selected genotypes to accelerate progeny testing, and 2) enhancing the production of genetically-improved seeds in seed orchards (Bonnet-Masimbert and Zaerr 1987). Therefore, considerable attempts have been made to induce cone flowering, particularly in *Pinaceae* using grafting, girdling, fertilizer treatments, induced moisture stress, and growth regulators, particularly the gibberellins (GAs) which have been increasingly successful (Owens and Blake 1985).

A. Grafting

Grafting juvenile scions onto the tops of reproductive trees has resulted in precocious flowering (Owen and Blake, 1985). Topgrafting is the grafting of scions of selected

genotypes into the crowns of sexually mature interstocks (such as flowering seed orchard ramets). This has proved extremely successful in producing both female and male strobili one to two years following grafting, and has become a useful tool to accelerate breeding cycles to only two or three years. Topgrafting has been much more effective than earlier accelerated breeding treatments, typically applied to indoor potted grafts, such as GA application, water stress, manipulation of nutrient status and photoperiod, and girdling. (Wheeler et al. 1980; Greenwood 1981; Bramlett 1997; Bramlett and Burris 1995; Meilan 1997; Medina Pérez et al. 2007).

B. Girdling

Branch or stem girdling is a kind of wound treatment that has been used with variable results to induce flowering on many species of fruit trees and conifers (Hare et al. 1979; Owens and Blake 1985; White and Wright 1987; Kosinski 1987; Woods 1989; Wheeler and Bramlett 1991; Bramlett et al. 1995; Meilan 1997). Its effect has been associated with an increase in the content of carbohydrate concentration, but its physiological reasons are unclear (Dick 1995). Results depend on the time and method of application, and the use of additional treatments such as GA (Owens and Blake, 1985; Pharis et al. 1987). Wheeler and Bramlett (1991) evaluated two methods for flower promotion in the Lyons *P. taeda* seed orchard: overlapping, saw-cut girdles and stem-injected GA_{4/7} alone and in combination. All treatments significantly enhanced female flower production, although girdling was the most effective single treatment.

C. Nutrition

The application of nitrogen is one of the most common floral induction treatments, but results are very variable for several reasons: time of treatment applications relative to the time of floral bud initiation, the nutrient conditions of the soil, and the type of fertilizer (Owens and Blake 1985). Schmidtling (1983) found that fertilizer application strongly affected flowering in southern pines when applied during bud

differentiation. He concluded that depending on the species, female flowering was enhanced when applications were performed in mid- to late summer, but male flowering was promoted when applications were performed in early summer. However, other fertilizer treatments have shown flowering promotion without coinciding with reproductive bud differentiation (Greenwood 1977). Another cause of variable results with fertilizer treatments was related to the nutrient condition of the soil; many experiments were carried out without prior soil analysis, so the nutrients that were deficient were unknown and the types and amounts of nutrients added may not have been adequate (Owens and Blake 1985). The type of fertilizer must also be considered; the form of nitrogen (ammonium, NH_4^+ or nitrate, NO_3^-) required to achieve flowering varies with the species. Ammonium increased total protein; in contrast nitrate increased amino acid levels (Owens and Blake 1985). Hare et al. (1979) applied $\text{NH}_4^+ \text{NO}_3^-$ fertilizer to *P. palustris* and found that bud length increased and concluded that fertilization was the most effective treatment to promote both male and female cones.

D. Light and Temperature

Unfortunately it has been difficult to separate photoperiod and temperature effects on flowering. In general, floral initiation is favored by high light intensities and temperature (Owens and Blake 1985; Bonnet-Masimbert 1987). Even so, photoperiod is generally considered not to have a direct effect on cone initiation in conifers (Owens and Blake, 1985), yet some reports suggest its influence. For example, Greenwood (1981) promoted both male and female strobili on *P. taeda* grown in a greenhouse by using an out-of-phase dormancy treatment under a 20-hr photoperiod beginning in October until early spring when the supplemental light was terminated. Reducing the photoperiod with or without lowering temperature slowed shoot elongation of the treated grafts and induced the formation of resting buds where strobilus initiation occurs (Greenwood 1980).

E. Water

The effect of water stress on flower induction throughout cultural treatments such as controlled irrigation, drought treatments, and root pruning (Owens and Blake 1985) has been very well known for many years. Several researches reported that the combination of water stress and GA_{4/7} in some species induce flowering, sometimes favoring female production over male production (Pharis 1977; Greenwood 1981; Owens and Blake 1985; Philipson 1990). It is important to note that use of this technique requires container-grown trees because controlled drought treatments would be difficult or impossible to perform in the field. Water stress can be monitored before dawn using the pressure chamber technique with a Scholander-Hammel pressure bomb, and pre-dawn moisture stresses of 1.2 to 2.0 MPa appear minimal for cone induction. Unstressed trees average about 0.7 to 0.8 MPa of water stress (Cade and Jackson (1976) cited by Owens and Blake (1985)).

F. Growth Regulator Treatments

Much research has been performed on flower induction using a variety of plant growth regulators with different concentrations, times, and methods of application (Table 1.1). This is one of the most effective methods for cone induction. The most commonly tested PGRs in cone induction are gibberellins (GAs), cytokinins, auxins, abscisic acid, and ethylene. Gibberellin A_{4/7} has shown promising results in pines (Kong and von Aderkas 2004).

Gibberellin A₃ is effective in inducing both cones and pollen in many species of the *Cupressaceae* and *Taxodiaceae* (Pharis et al. 1965; Pharis et al. 1970; Bonnet-Masimbert 1971; Owens and Pharis 1971; Owens and Molder 1977; Ross 1983). GA₃ is an inexpensive foliar spray, and the sex may be manipulated through timing of application (Ross 1983) or by varying the photoperiod during treatment (Owens and Molder 1977). Treatment time is typically for three weeks and the resulting pollen

and seed are of high quality. Cone production has been induced on seedlings as young as three or four months (Owens and Molder 1977).

Cone induction by GA application was begun in the *Pinaceae* in the mid-1970s, using the less polar mixture GA_{4/7} (Ross 1975). The best results were obtained when GA_{4/7} was applied with some additional environmental or cultural treatment, and responses were synergistic rather than additive (Greenwood 1980; Greenwood 1981; Philipson 1985; Longman et al. 1986; Pharis et al. 1987; Philipson 1990; Wheeler and Bramlett 1991). Gibberellin A_{4/7} applications have some limitations when compared with GA₃. Gibberellin A_{4/7} is not as readily available and is more expensive than GA₃. Treatments are not as easily applied (foliar spray may not be effective), the timing of treatment is more critical, the length of treatment may be longer, and the sex may not be manipulated (Owens and Blake 1985).

Table 1.1. Use and results of GAs in conifers.

Species	Growth Regulator	Method	Effects	Reference
<i>Picea glauca</i> <i>Picea mariana</i> <i>Picea rubens</i> <i>Picea mariana</i>	GA _{4/7}	Foliar spray and stem injection	Both promoted seed and male strobili, injection being more effective	Greenwood et al. 1993
<i>Picea mariana</i>	GA _{4/7}	Stem injection + root pruning	Increased seed and male strobili	Smith and Greenwood 1995
<i>Picea mariana</i>	GA _{4/7}	Stem injection	Increased female strobili	Brockhoff and Ho 1997
<i>Picea sitchensis</i>	GA _{4/7}	Stem injection + girdling	Increased female strobili	Longman et al. 1986
<i>Pseudotsuga menziesii</i>	GA _{4/7}	Stem injection + girdling	Enhanced pollen and female strobili	Cherry et al. 2007
<i>Pinus banksiana</i>	GA _{4/7}	Foliar spray and stem injection	Both promoted seed and male strobili, injection being more effective	Greenwood et al. 1993
<i>Pinus caribaea</i> var. <i>hondurensis</i>	GA _{4/7}	Bud base injection	Enhanced see cones in poor flowering clones	Harrison and Slee 1991
<i>Pinus elliotii</i> var. <i>elliottii</i>	GA _{4/7}	Foliar spray + topical bud	Promoted male strobili, foliar spray being more effective	Hare 1984
<i>Pinus kesiya</i> <i>Pinus merkusii</i>	GA _{4/7} +GA ₃	Foliar spray	Slightly promoted male strobili and inhibited female strobili production	Sirikul and Luukkanen 1987
<i>Pinus palustris</i>	GA _{4/7}	Foliar spray + topical bud	Promoted male strobili, being foliar spray more effective	Hare 1984
<i>Pinus ponderosa</i> <i>Pinus radiata</i>	GA _{4/7} GA _{4/7}	Stem injection Stem injection + topical bud	Increased female strobili Stem injection increased female strobili more than topical bud	Rust 2007 Siregar and Sweet 1996
<i>Pinus sitchensis</i>	GA _{4/7}	Stem injection + girdling	Increased seed and male strobili	Philipson 1985
<i>Pinus strobus</i>	GA _{4/7}	Foliar spray	Promoted pollen and female strobili	Ho and Schnekenburger 1992
<i>Pinus strobus</i>	GA _{4/7}	Foliar spray	Promoted pollen and female strobili on some genotypes	Pijut 2002
<i>Pinus taeda</i>	GA ₃ GA ₅ GA _{4/7}	Topical bud + girdling	GA _{4/7} promoted female strobili, GA ₃ was less successful, and GA ₅ was ineffective	Ross and Greenwood 1979
<i>Pinus taeda</i>	GA ₃ GA ₄ GA _{4/7} GA ₇	Foliar spray, stem injection, and topical bud	Increased female strobili by GA ₄ GA _{4/7} GA ₇ and GA ₃ was ineffective	Greenwood 1982
<i>Pinus taeda</i>	GA _{4/7}	Foliar spray + topical bud	Promoted male strobili, foliar spray being more effective	Hare 1984
<i>Pinus taeda</i>	GA _{4/7}	Stem injection + girdling	Increased female strobili	Wheeler and Bramlett 1991

Some studies have shown that GA_{4/7} is effective in increasing flowering in both poor-flowering and good-flowering clones. For example, Sweet (1979) with *P. radiata* and Harrison and Slee (1991) with *P. caribaea* var. *hondurensis* reported that GA_{4/7} increased flowering in poor relative to good flowering clones. However, the reverse pattern seems more common: increased production in good flowering clones and little effect in poor flowering clones was observed in *P. contorta* by Wheeler et al. (1980), in *Picea abies* by Dunberg (1980), and *Pseudotsuga menziesii* by Ross et al. (1985).

Methods of Gibberellin Application

The most commonly used method for GA application is a water-based foliar spray containing a dilute carrier and variable concentrations of GA. The GA concentration depends on species, frequency, and duration of applications. Foliar spraying is continued until solution begins to drip from the foliage. This can be costly, especially when applying non-polar GAs (Owens and Blake 1985). Even though this is the easiest method, its efficiency is limited because the cuticle layer on needles and shoots can inhibit the absorption of the GA solution (Bonnet-Masimbert 1987).

Stem injection of GA solution is most suitable for larger trees and field studies. The objective is to treat an entire tree and the amount of hormone that penetrates the tree can be controlled, although this does not provide a lot of information about its metabolism in the tree (Wample et al. (1975) cited by Bonnet-Masimbert (1987); Dunberg et al. 1983)). In a stem injection treatment, the solution is added into a hole drilled downwards into the stem of the tree using a hypodermic syringe. The GA is then translocated upward in the xylem from the point of injection (Owens and Blake 1985). In *P. sitchensis*, simple stem injections of GA_{4/7} into large, field-grown trees was very effective for producing pollen and female strobili, and was easy to apply (Philipson 1985). The concentration of GAs ranges from 50 to 100 mg/L in 0.5-5% ethanol (Bonnet-Masimbert 1987).

Topical application of GA solutions consists of direct application of small amounts of GA, often at fairly high concentrations, to the surface of young shoots (Ross 1975; Ross and Greenwood 1979; Greenwood 1981). The solution may contain a high concentration of ethanol (70 to 80%) that can be toxic to immature tissues. When application is made to young shoots, some kind of damage and partial girdling may occur, modifying the GA effect (Owens and Blake 1985). The hormone is applied to the bud or shoot close to the region where meristem differentiation takes place (Bonnet-Masimbert 1987).

Timing of Gibberellin Application

The time of reproductive bud determination is an important consideration in deciding the timing of GA application. Early attempts at flower induction were often unsuccessful due to improper timing; in later studies, timing was refined to coincide with known times of bud determination (Owens and Blake 1985). The ideal condition would be to know the biochemical changes leading to bud initiation because they begin before anatomical differences are visible (Dunberg 1979). However, in conifers this is unknown, so it is necessary to estimate the optimal time for GA application based on the onset of morphological differentiation (Owens and Blake 1985).

Floral stimulation treatments must precede reproductive bud determination. Phenological observations are essential to know in detail the stages of floral initiation and vegetative shoot growth in order to time induction treatments precisely (Owens 1995), and to understand the influence of the environment (Bonnet-Masimbert 1987). For example, Philipson (1983) showed that under cool wet conditions, GA_{4/7} was totally ineffective in inducing flowers in *Picea sitchensis*. In contrast, under warm, dry conditions, flowering was enhanced by GA.

Trees growing under tropical conditions do not follow the same reproductive pattern as when they are grown under temperate climates (Ibrahim 1977; Sirikul and Luukkanen 1987; Dick 1995). This is the case in Colombia, where the climate and photoperiod are uniform throughout the year. This lack of seasons results in the absence of a well-defined peak of flower or cone production during the year. In SKCC's seed orchards, most of the trees flower year round and it is possible to see flowers at different stages, green cones, and ripe cones, all at the same time on a single tree. Thus, it is difficult to say with confidence when would be the best time for GA applications. Experiments on the timing of application of GA to SKCC's seed orchards are needed.

Gibberellin with additional treatments

Cone induction has been successful using GAs alone in some forest trees, but an effective result has been from GA combined with one or more treatments which produces in most cases a synergistic effect (Owens and Blake 1985; Bonnet-Masimbert 1987; Smith and Greenwood 1995) and sometimes an additive effect (Longman et al. 1986). In the *Pinaceae*, GA_{4/7} treatments have been combined with girdling of the branch or stem, and also with water stress, yielding outstanding results (Table 1.1). The girdling generally led to an increase of the GA_{4/7} effect, although in some studies there was a decrease in seed set (Ross 1975; Ross and Greenwood 1979; Ross et al 1980). Considering that girdling may damage seed orchard trees, this combined treatment should be performed with caution or on trees that will be removed. Philipson (1985) mentions that girdling and GA_{4/7} application can both damage trees, but many trees can recover from the initial effects. Wheeler and Bramlett (1991) noted that while the combination of girdling and GA_{4/7} produced more flowers per tree in young *P. taeda*, the increased production would probably not be worth the increased cost. Other treatments such as root pruning, fertilization, high temperature, and changing photoperiod or light intensity, can also enhance

reproductive bud development (Owens and Blake 1985; Greenwood 1981; Philipson 1990; Smith and Greenwood 1995).

Analysis of hormonal mechanisms of cone flowering

Manipulation of sexual expression can be enhanced through different hormone combinations, crown pruning, treatment timing, and photoperiod control, but operational development requires a better understanding of the endogenous regulatory mechanisms involved. Currently this work is performed using mass spectrometry and techniques such as bioassay experiments, enzyme-linked immunosorbent assays, and radioimmunoassay. Today, gibberellins have been analyzed using gas chromatography single ion monitoring (GC-SIM), and interesting correlations between poor flowering clones and good flowering clones of *Picea abies* and GA metabolism have been found. Good clones lack GA₁, GA₃ is abundant in poor clones, and the ratio GA₉ to GA₁ was 10 fold greater in good clones than that in poor ones (Oden et al (1994) cited by Kong and von Aderkas (2004)).

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Chapter 2 - Flower Stimulation Treatments in *Pinus maximinoi* and *Pinus tecunumanii* Seed Orchards in Colombia

I. Introduction

Smurfit Kappa Cartón de Colombia (SKCC) initiated its tree improvement program in 1973 with introduced species (tropical and subtropical conifers), selecting the best phenotypes from Colombian commercial plantations based on high volume (height and diameter), bole straightness, high wood density, good crown form, and good health (Ladrach and Lambeth 1991). Currently, SKCC has 80 hectares of open-pollinated seed orchards composed of 9 species. *Pinus tecunumanii* and *Pinus maximinoi* are the two most important species due to their fast growth, good stem and crown form, high productivity, and wood density. However, they have a serious disadvantage: low seed production (Dvorak et al. 2000; 2000a).

A crucial part of any tree improvement program is the establishment and management of seed orchards of the most valuable species to produce genetically improved seeds to meet reforestation needs. However, seed production under tropical conditions can be very problematic for pine species. The reproductive biology and seed production of tropical pines is not very well known (Longman 1985). In tropical *Pinus* species, there is often poor synchronization between pollen dispersal and female strobili receptivity, and the pollen cloud, which is critical for good seed set, is often inadequate (Owens 1995; Ibrahim 1977; Sirikul and Luukkanen 1987). This is especially applicable to the seed orchards established with pine species outside of their natural range (Sirikul and Luukkanen 1987). For SKCC, these problems create a severe limitation to cone and seed production of pine species used for commercial plantations. An effective and inexpensive method to increase seed production would be very valuable.

SKCC's seed orchards are located in Southwest Colombia. The low-elevation (LE) *P. tecunumanii* seed orchard is located at 3° 41' N and 76° 32' W, at an elevation of 1585

masl. The average annual temperature and precipitation are 22 °C and 1166 mm respectively; soils are classified as Ultic Haplustalfs (Vertisols), derived from Andesites (Table 2.1 and Figure 2.1) (Planning Department SKCC, personal communication, 2007). Natural vegetation is dominated by several families including *Caesalpinaceae*, *Bignoniaceae*, *Fabaceae*, *Mimosaceae*, *Moraceae*, and *Myrtaceae* (Mahecha and Echeverry 1983). The latitude of the *P. maximinoi* seed orchard is 2° 31' N and 76° 34' W, at an elevation of 1810 masl. The average annual temperature and precipitation are 18 °C and 2125 mm respectively. Soils are classified as Acrudoxic Hydric Hapludans (Andisols), derived from volcanic ash (Planning Department SKCC, personal communication, 2007). The natural vegetation is dominated by several species: *Quercus humboldtii*, *Weinmania sp.*, *Miconia sp.*, and by a great number of heliconias, palms, and species of the families *Araceae* and *Melastomaceae* (Wille et al. 2000).

The forestry research department in SKCC, in response to the growing demand for seed with high genetic quality for its reforestation programs, began a strategy in 1988 that involved the increase in the area of seed orchards and the application of intensive management. Efforts to promote flower and fruit production focused on cultural treatments such as fertilization, weed control, manual supplementary pollination, and pest and disease control. After implementing these practices for over 12 years, a slight increment in total seed production was realized in both *P. tecunumanii* and *P. maximinoi*. Currently the *P. tecunumanii* orchard produces 1.3 kg/ha/year of seed, and averages 8 filled and 17 total seeds per cone, and the *P. maximinoi* orchard produces 1.0 kg/ha/year, and 11 filled and 18 total seeds per cone (Table 2.2) (Isaza et al. 2002). Potential seed production could be nearly doubled if pollen production was adequate (Sirikul and Luukkanen 1987). But near the equator, lack of pollen synchronization may limit filled seed production. For example, *P. patula* seed orchards have the best production on high elevation sites in South Africa (>1450 masl) and Zimbabwe (1900 masl), and produce 35 to 80 filled seed per cone (Barnes and Mullin 1974, Wormald 1975). In comparison, *P. patula* seed orchards in Colombia also have the best production at high elevations

(between 2,500 and 3,000 masl), but produce only 16 filled seed per cone (Lambeth and Vallejo 1988).

Table 2.1. Climatic, geographic, and establishment description of the SKCC *P. tecunumanii* and *P. maximinoi* seed orchard sites in Colombia.

	Seed Orchard Sites	
	Aguaclara	Cabuyerita
Species	<i>P. tecunumanii</i>	<i>P. maximinoi</i>
Latitude	3° 41' N	2° 31' W
Longitude	76° 32' W	76° 34' W
Elevation (masl)	1585	1810
Area (ha)	3.0	3.5
Rainfall (mm)	1166	2125
Temperature (°C)	22	18
Year planted	2003	1997
Soils	Vertisols	Andisols
Spacing (m)	10 x 5	10 x 10
Number of clones	38	36

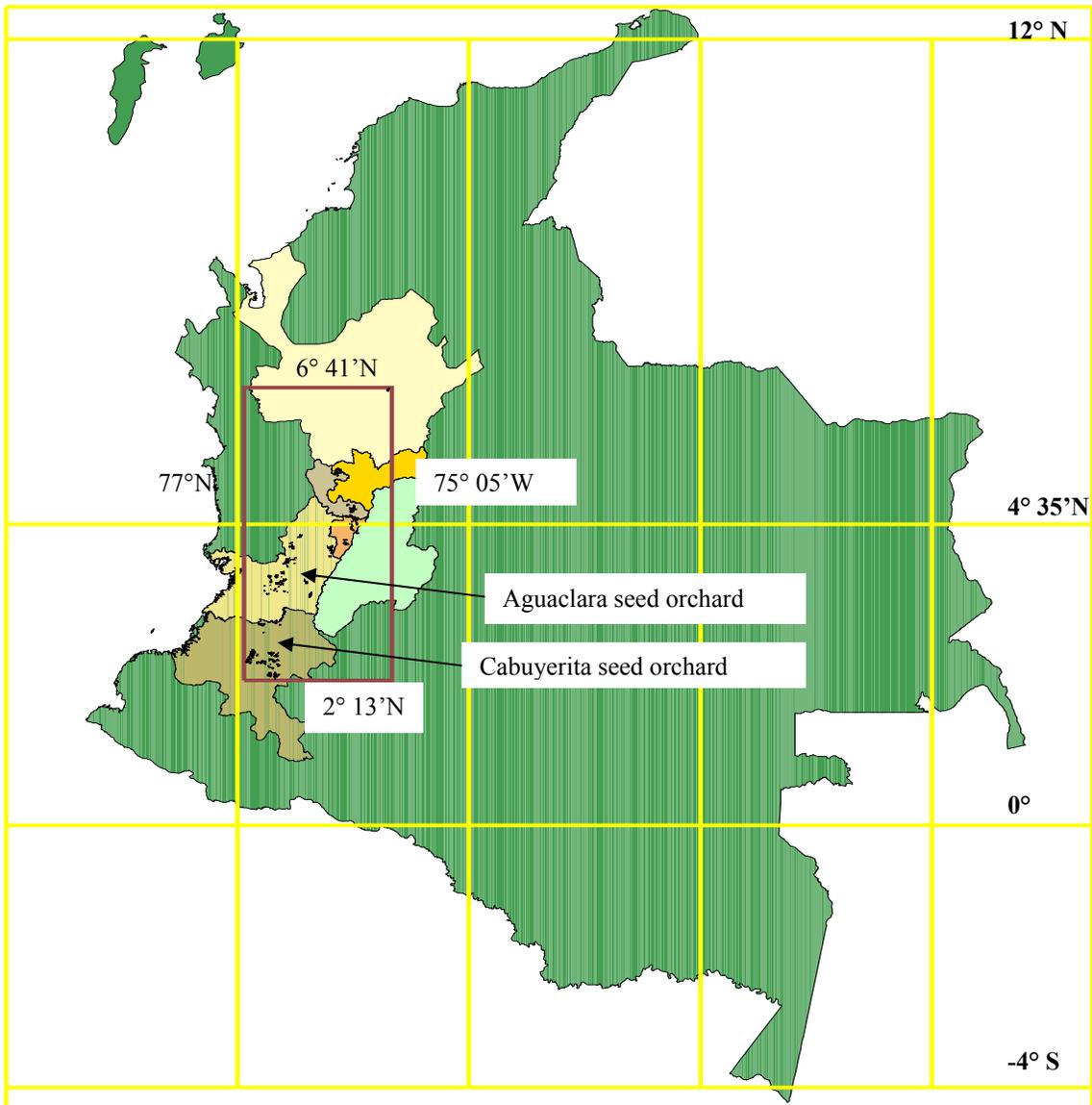


Figure 2.1. Map of Colombia showing the states (light areas) where SKCC has operational plantations (small dark areas) and the locations of the *P. tecunumanii* and *P. maximinoi* seed orchards. (Planning Department SKCC, personal communication, 2007)

Table 2.2. Seed production of *P. tecunumanni* and *P. maximinoi* in open-pollinated SKCC clonal seed orchards in Colombia.

Age (years)	Species	Orchard Location	Cone		Seed Production			
			Length (mm)	Width (mm)	Seed Potential	Sound Seed	Empty Seed	Total Seed
9	<i>P. maximinoi</i>	Cabuyerita	93	37	140	11	7	18
10	<i>P. tecunumanii</i>	La Suiza	64	37	123	8	9	17

Source: Isaza et al 2002

Both *P. tecunumanii* and *P. maximinoi* are yellow pines classified in the subsections *Oocarpae* and *Ponderosae*, respectively (Price et al. 1998). They are diploxyton with special features that play an important role in their reproductive strategies, such as greater dispersal abilities by reproducing at an early age, short intervals between large cone crops, and the production of abundant small seeds (Yeaton 1978). In Colombia there is lower and more variable seed and cone production in *P. tecunumanii* and *P. maximinoi* compared to their natural range (Dvorak and Lambeth 1992; Styles 1994; Zamora et al. 1983). This may be due to geographical factors such as latitude and photoperiod, and also the uniform climate with warm temperatures throughout the year, and lack of differentiation between dry and wet periods (Lambeth and Vallejo 1988). To increase seed production in Colombia in addition to the cultural practices already in use, it is necessary to evaluate additional alternatives such as growth regulators, either applied alone or in combinations with additional treatments.

Because they have been proven to enhance flowering in other related pines, the objective of this study was to examine the effectiveness of different applications of gibberellin (GA_{4/7}) stem injections and branch girdling treatments to enhance flowering on seed orchards of *P. tecunumanii* (LE) and *P. maximinoi* in Colombia.

II. Materials and Methods

A. Experiments 1 and 2: Timing of Applications and Gibberellin Products

In temperate pine species growing in the northern hemisphere, female and male strobili are initiated in the dry summer months (Shoulders 1967, Baker and Langdon 1990). Although the rainfall distribution in Colombia is relatively uniform throughout the year, there is normally a slight dry season in July and August, so the month of August 2007 was identified as a suitable time to test GA applications.

A commercial GA_{4/7} product called Procone®, manufactured by Valent Biosciences, is specially formulated for foliar spray and/or stem injection for the family *Pinaceae*. However, due to logistical problems, this product was not available for application on the SKCC orchards in August 2007. Another GA_{4/7} product called Provide® 10sg, also manufactured by Valent Biosciences was available in August 2007. Provide® 10sg is formulated for foliar spray application onto fruit trees. The active ingredient in both products is the same.

As a result, two experiments were conducted. Experiment 1 was conducted in August 2007 using Provide 10sg®, and Experiment 2 was conducted in September 2007 using Procone®.

B. Plant Material

In May of 2003 a clonal seed orchard of *P. tecunumanii* was planted using grafts of 38 clones (using 11 year-old scions) at La Cumbre (Aguaclara tract), Department of Valle del Cauca, Colombia located at 3° 41' N and 76° 32' W, at an elevation of 1585 masl. The spacing was 10 x 5 m following a systematic design. The average annual temperature and precipitation are 22 °C and 1166 mm respectively (Table 2.1) (Planning Department SKCC, personal communication, 2007). The average height of the trees was 6.4 m and the average breast high diameter was 11 cm at time of treatment in August 2007.

In December of 1997 a clonal seed orchard of *P. maximinoi* was established with grafts of 36 clones (using 10 year-old scions) at Popayán (Cabuyerita tract), Department of Cauca, Colombia located at 2° 31' N and 76° 34' W, at an elevation of 1810 masl (Table 2.1). The spacing was 10 x 10 m following a systematic design. The average annual temperature and precipitation are 18 °C and 2125 mm respectively (Planning Department SKCC, personal communication, 2007). The average height of the trees was 17.4 m and the average diameter breast height was 31.2 cm at time of treatment.

For Experiment 1, 15 clones of both species were selected for treatment, and for Experiment 2, 12 clones of both species were selected for treatment. The clones were stratified among good, average, and poor female strobili producers based on past production. One ramet per clone was randomly assigned to each of four gibberellin treatments.

C. Hormone Treatments

Experiment 1

Experiment 1 used three separate treatments of Provide® 10 sg (active ingredient GA_{4/7}: 10% w/w and inert ingredients: 90% w/w, manufactured by Valent Bioscience Corporation, Libertyville, IL, U.S.A). For the three hormone treatments, a volume of 750 mL was prepared by dissolving 18.75, 37.5, and 112.5 grams of Provide® 10 sg in 750 mL of distilled water. The goal was to produce solutions with concentrations of 2.5, 5.0 and 15.0 mg/mL of active ingredient. The three hormone solutions plus a control of distilled water were injected into the stems of the trees following the techniques described by Philipson (1985). Applications were performed between 6 am and 9 am on August 6-7, 2007 for *P. maximinoi*, and on August 9-10, 2007 for *P. tecunumanii*. For each treated tree, four 1 cm diameter holes were bored on opposite sides of the main stem above the graft union downward at a 60° angle to a depth of 7 cm. In each hole, 5 mL of solution was injected with a hypodermic syringe for a total

of 20 mL per tree. Controls received 20 ml of distilled water. After applying the hormone solution or the distilled water, the holes were plugged with a wood core. The target was for the trees in the treatments to receive 0, 50, 100, or 300 mg/tree of active ingredient.

Experiment 2

Experiment 2 used treatments of Procone® (active ingredient GA_{4/7}: 4% w/w and inert ingredients: 96% w/w, manufactured by Valent Bioscience Corporation, Libertyville, IL, U.S.A). The GA_{4/7} was stem injected between 6 am and 9 am on September 7, 2007 in *P. maximinoi* and on September 8, 2007 in *P. tecunumanii*. Two 1 cm diameter holes were drilled on opposite sides of the main stem above the graft union downward at 60° to a depth of 6 cm. The target was for the trees in the treatments to receive 0, 50, 100, or 300 mg/tree of active ingredient, so using a hypodermic syringe each treatment tree was injected with 1.19 mL of solution (50 mg dose), 2.38 mL (100 mg dose), or 7.14 mL (300 mg dose). The controls received 7.14 mL of distilled water. Half of the solution or distilled water was injected into each of the two holes, and the holes were plugged with a wood core.

D. Branch Girdling Treatments

In both experiments, a total of six clones (2 from each of the good, average, and poor cone producing classes) were selected to receive girdling treatments. For each selected clone, all of the ramets were girdled, i.e., one ramet in each of the four hormone treatments (0, 50, 100, and 300 mg/tree). In total, there were 24 trees that were branch girdled. Branch girdling consisted of two semicircular cuts on two first-order branches, one located in the lower part of the crown just above of the graft union and the GA injections, and the other one in the middle of the crown. Double overlapping band girdles, each about 6 mm wide, and spanning half the circumference of the branch, were applied near the base of the branch using a sharp knife. Bark was removed to the cambial zone in the two bands, placed about 5 cm

apart. Two branches located on the opposite side of the tree from the girdled branches were selected and labeled as controls.

Girdling treatments for Experiment 1 were performed approximately one week prior to the hormone application, on July 30 and August 1-2, 2007 for *P. tecunumanii* and *P. maximinoi*, respectively. For Experiment 2, girdling treatments were performed two to five days after the hormone application, on September 10 and September 12, 2007, for *P. tecunumanii* and *P. maximinoi*, respectively.

E. Data Collection

Prior to the hormone applications, all developing female strobili were marked with permanent tree paint so they would not be counted among strobili produced subsequent to hormone treatment. Six months after hormone application, complete inventories of all female strobili in the entire crown of every tree were completed between the end of February and March, 2008. Each female strobilus was classified as a conelet or as a developing female strobilus bud by experienced orchard technicians. Developing strobili are less than 2 months old, and conelets are from 2 to 5 months old. Dead and damaged strobili, aborted female strobili (counted every two months) were also included in the total inventory.

For the trees which received branch girdling treatments, pollen catkins on the girdled and control branches were counted three times, every two months from September 2007 through March 2008.

F. Data Analysis

Data were analyzed using analysis of variance with the GLM procedure, orthogonal contrasts, and least square means in SAS 9.1 (SAS Institute 1989). The response variables were total conelets (**Cones**), total developing female strobili buds (**Strob**), total dead, damaged, and aborted female strobili (**Dead**), total live female strobili (**FemLive** = the sum of **Cones** and **Strob**), and total female strobili produced

(**FemAll** = the sum of conelets, developing female strobili buds, dead, damaged, and aborted female strobili). The linear model for female cone and strobili response variables included:

ProduClass = cone production class

Clone(ProduClass) = clone nested within production class

Rate = GA_{4/7} treatment

ProduClass x Rate = production class x rate interaction

Volume = index of tree volume

NumBranch = total number of primary branches

All main effects were considered fixed except **Clone(ProduClass)** which was considered random. The terms **Volume** and **NumBranch** were included as covariates to account for the effects of tree size on strobili production. Volume index was calculated as $V = 0.0003 (\text{dbh})^2 \text{ Height}$. Preliminary analyses indicated that **ProduClass*Rate** interaction was not significant for any of the response variables for *P. maximinoi* in both experiments, so that term was deleted from the model for that species.

A reduced linear model was used to examine response to the different hormone treatments for each of the three cone production classes separately. The reduced linear model included the terms:

Clone = clone

Rate = GA_{4/7} treatment

Volume = index of tree volume

NumBranch = total number of primary branches

For the branch girdling treatments, the response variables were: total pollen catkin clusters produced on lower and middle branches (**MaleLow** and **MaleMid**, respectively). The linear model for the pollen catkin response variables included:

Clone = clone

Rate = GA_{4/7} treatment

Girdle = class variable, With and Without girdling

Volume = index of tree volume

Production Class was not included as it was based on female strobili production. Preliminary analyses showed that Rate*Girdle and Clone*Girdle interactions were never significant, and so those terms were eliminated from the final model.

For all linear models, least square means were calculated for **Produclass** and the treatment effects **Rate** and **Girdle**, where appropriate. Orthogonal contrasts were used to compare the treatments versus the controls. Differences with a p-value greater than 0.10 were considered Not Significant (NS).

III.Results

A. Tree Size by Production Class

Mean tree size by ProduClass for both species in Experiment 1 and Experiment 2 are presented in Tables 2.3 and 2.4, respectively. As might be expected, the *P. maximinoi* trees (age 11 years from graft) are much bigger than the *P. tecunumanii* trees (age 5 years from graft). Surprisingly, although the *P. maximinoi* trees are more than twice as tall and have more than twice the diameter of *P. tecunumanii*, they had only about 15 more branches. The subjective grouping of clones into production classes was reasonably accurate, with the Good production class producing the most female strobili (FemAll), and the Poor production class producing the fewest in every case (Tables 2.3 and 2.4). There was little variation in tree size among production classes, with the exception of *P. tecunumanii* in Experiment 1, where the Poor production class was quite a bit smaller than the Good or Average Production class (e.g., 7.3 m height vs 10.4 and 9.6 m, respectively (Table 2.3).

B. Weather

For the *P. maximinoi* orchard at Cabuyerita Farm, the precipitation pattern in 2007 was fairly typical, with a dry period from June to September (Figure 2.2). In particular, August and September 2007 (22 and 37 mm, respectively) were somewhat drier than the 23-year average from 1980 to 2003 (51 and 116 mm, respectively). For the *P. tecunumanii* orchard at Aguaclara Farm, precipitation in 2007 was much higher than average from June to August (Figure 2.3). In the month of August, there was 135 mm in 2007 compared to the 23-year average of 35 mm. The month of September was more typical, and just slightly below the 23-year average, 57 mm versus 71 mm.

Table 2.3. Mean tree size prior to flower promotion treatments by species and production class in Experiment 1.

Species	Produclass	Height (m)	DBH (cm)	Volume (m3)	Number Branches	FemAll
<i>P. maximinoi</i>	Good	17.3	30.3	0.492	60	1349
	Average	18.2	34.1	0.665	71	1348
	Poor	18.0	31.8	0.578	65	422
	Mean	17.8	32.1	0.578	65	1040
<i>P. tecunumanii</i>	Good	10.4	17.2	0.099	59	560
	Average	9.6	16.9	0.089	57	286
	Poor	7.3	10.1	0.027	39	26
	Mean	9.1	14.7	0.072	52	291

Table 2.4. Mean tree size prior to flower promotion treatments by species and production class in Experiment 2.

Species	Produclass	Height (m)	DBH (cm)	Volume (m3)	Number Branches	FemAll
<i>P. maximinoi</i>	Good	19	32.8	0.694	67	1306
	Average	17	31.2	0.526	62	549
	Poor	20	34.9	0.782	67	544
	Mean	19	32.9	0.668	65	800
<i>P. tecunumanii</i>	Good	8	13.5	0.058	52	296
	Average	8	13.1	0.053	53	147
	Poor	10	16.6	0.086	52	109
	Mean	9	14.4	0.066	52	184

Cabuyerita Farm (*P. maximinoi* seed orchard)

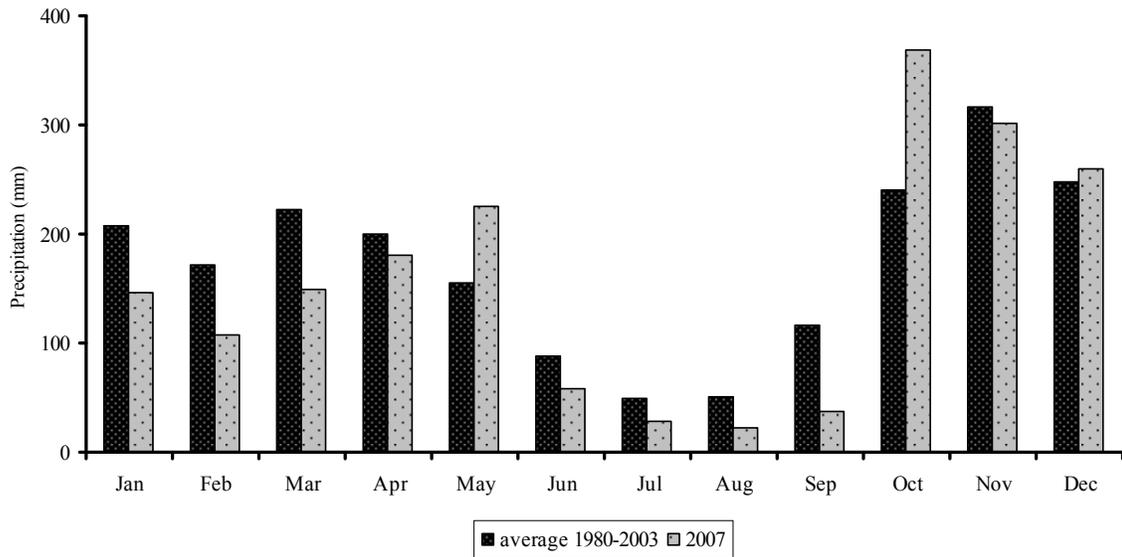


Figure 2.2. Mean monthly historic precipitation in Cabuyerita farm, location of the *P. maximinoi* seed orchard, from 1980 to 2003 compared with the data of 2007.

Aguaclara Farm (*P. tecunumanii* seed orchard)

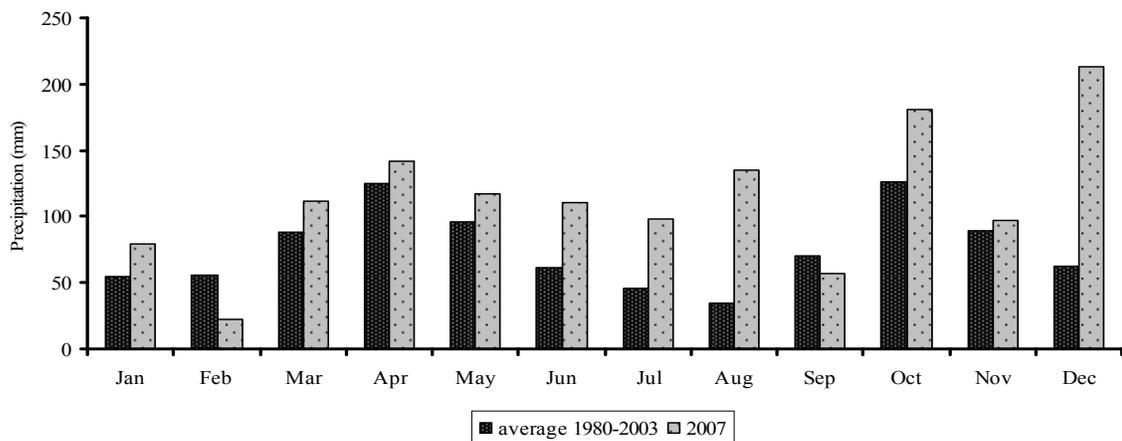


Figure 2.3. Mean monthly historic precipitation in Aguaclara farm, location of the *P. tecunumanii* seed orchard, from 1980 to 2003 compared with the data of 2007.

C. Experiment 1, Gibberellin Treatments

When the GA_{4/7} solutions were prepared for Experiment 1 using the product Provide® 10sg, it was apparent that not all of the product went into solution. In all three treatments, there was some white solid or precipitate that remained in suspension. Due to uncertainty over the amount of active ingredient that was in the treatment solutions, a quantitative analysis was performed by Dr. Lisa Dean, Research Food Technologist with the Market Quality and Handling Unit (USDA, ARS, SAA) at North Carolina State University. All solutions were analyzed using High Performance Liquid Chromatography (HPLC) interfaced to a mass spectrophotometer (MS) equipped with an electrospray ionization probe (ESI). The probe was operated in the negative mode. An authentic standard of GA₄ containing a trace amount of GA₇ was obtained from Sigma (St. Louis, MO). A standard curve was prepared and the samples were analyzed in triplicate using the single ion monitoring mode (SIM) at the negative ion of GA₄. No GA₇ was detected by this method in the standard or the samples. A water solution of Provide® 10 sg was prepared following the label directions, with an expected concentration of 25 mcg/mL of GA_{4/7}. The solution was clear, and analysis determined that the solution contained 25.13 ± 0.07 mcg/mL GA₄. Three other solutions were prepared using the Provide® 10 sg, with target concentrations of 2.5, 5.0, and 15.0 mg/mL of GA_{4/7}. All of these solutions were cloudy with a white precipitate, and analyses showed they contained 0.4616, 0.4961, and 0.3429 mg/mL GA₄, respectively, an average of 0.4335 mg/mL. The low values and the presence of the precipitate indicated that the limit of solubility for the compound had been reached even at the lowest target concentration of 2.5 mg/mL. A total of 20 mL of one of the hormone solutions was injected in the treated trees. The total amount of active ingredient in the solution + precipitate for the three treatment levels was 50, 100, and 300 mg/tree. However, the above analyses suggest that the maximum amount of active ingredient GA_{4/7} that was in solution when the trees were injected was around 8.7 mg / tree. It is unclear what might happen over time to the active ingredient in the precipitate injected into the tree stems.

D. Experiment 1, August 2007, Provide® 10 sg treatments: Female Production

There were low to moderate phytotoxic effects of GA_{4/7} seen in the form of yellowing or burnt needles a few days after treatment in both species, but more severe on *P. tecunumanii*. The symptoms disappeared after approximately two months.

A summary of the analysis of variance results for female strobili production in Experiment 1 is presented in Table 2.5. The covariate Volume had a statistically significant effect on FemAll, FemLive, and Strob (developing female strobili) for *P. maximinoi*, and for all response variables for *P. tecunumanii*. This was true even though ProduClass was in the model, and Good producers tended to be the largest trees and Poor producers the smallest trees. The covariate Number of Branches explained a significant amount of the variation for two of the response variables for *P. maximinoi* (FemLive and Cones), and for one of the response variables for *P. tecunumanii*. Clone(ProduClass) was significant for all response variables for both species.

In the analysis of variance, F-tests for Rate were mostly not significant; the only significant response was for the variable FemAll in *P. maximinoi*, significant at $p = 0.0794$. However, in both species there was a response when considering all three treatments versus the control. Trees of *P. maximinoi* treated with 300, 100, and 50 mg of GA_{4/7} averaged 1193, 968, 1128 total female strobili per tree respectively vs. 870 on average for the controls. For *P. tecunumanii* trees treated with the same doses of GA_{4/7}, the average total female strobili per tree found were 353, 301, and 297 respectively vs. 211 for the controls. Orthogonal contrasts of the three hormone treatments vs. the control showed a statistically significant increase in four of the five response variables for both *P. maximinoi* and *P. tecunumani* (Figure 2.4). For *P. maximinoi*, there was an increase of 26% in FemAll (total female strobili), and 21% in FemLive (live female strobili). For *P. tecunumanii*, the effect was proportionally greater than in *P. maximinoi*, an increase of 50% in FemAll and 48% in FemLive. Possibly the smaller size of the *P. tecunumanii* trees may have led to a

larger response to the hormone. For both species, there was a significant increase in Strob (developing female strobili), indicating a persistent effect of the hormone treatment on strobilus initiation even 4 months after the application.

Examining the results by ProduClass showed similar patterns in the two species. For *P. tecunumanii*, the orthogonal contrast of treatment versus control showed significant increase in female strobili production for the Good production class, with a 72% increase in FemAll, a 57% increase in FemLive, and a 61% increase in Cones (Figure 2.5). For the Average production class, the percentage increases were similar in magnitude, but these were not statistically significant (with p-values approximately 0.25 for FemAll, FemLive, Cones, and Strob). For the Poor production class, there were no significant contrasts of treatment versus controls (all p-values around 0.50), and no discernable response for any variable. The results for *P. maximinoi* showed a similar trend.

Table 2.5. Test of hypotheses for mixed model analysis of variance for female strobili production of *P. maximinoi* and *P. tecunumanii* –GA_{4/7} stem injections Experiment 1. Bold numbers indicate statistical significance at p = 0.10.

Species	Response Variable	Volume		Numbranch		Produclass		Clone(produclass)		Produclass*rate		Rate	
		F	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value
<i>P. maximinoi</i>	Femall	3.60	0.0651	1.08	0.3041	6.78	0.0105	7.06	<0.0001			2.43	0.0794
	Femlive	3.90	0.0553	3.44	0.0709	4.99	0.0263	8.67	<0.0001			2.21	0.1024
	Cones	0.53	0.4692	5.20	0.0280	5.01	0.0259	6.85	<0.0001			1.13	0.3476
	Strob	9.44	0.0038	0.57	0.4761	1.11	0.3615	5.26	<0.0001			1.87	0.1502
	Dead	1.23	0.2749	1.22	0.2766	2.04	0.1724	14.65	<0.0001			1.70	0.1815
<i>P. tecunumanii</i>	Femall	21.22	<0.0001	1.93	0.1733	1.44	0.2720	10.75	<0.0001	2.39	0.0494	2.08	0.1212
	Femlive	16.33	0.0003	1.96	0.1708	1.13	0.3523	10.33	<0.0001	1.90	0.1096	1.81	0.1633
	Cones	13.30	0.0009	3.33	0.0768	1.12	0.3549	10.05	<0.0001	1.48	0.2162	1.60	0.2085
	Strob	16.88	0.0002	0.20	0.6598	0.66	0.5317	10.65	<0.0001	2.62	0.0337	1.51	0.2298
	Dead	7.00	0.0122	0.07	0.7990	0.95	0.4122	5.79	<0.0001	1.47	0.2162	0.94	0.4338

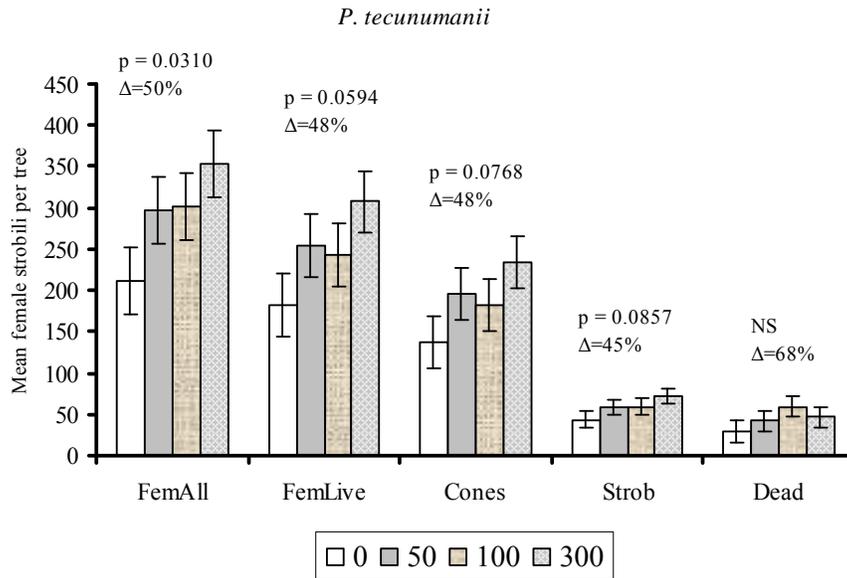
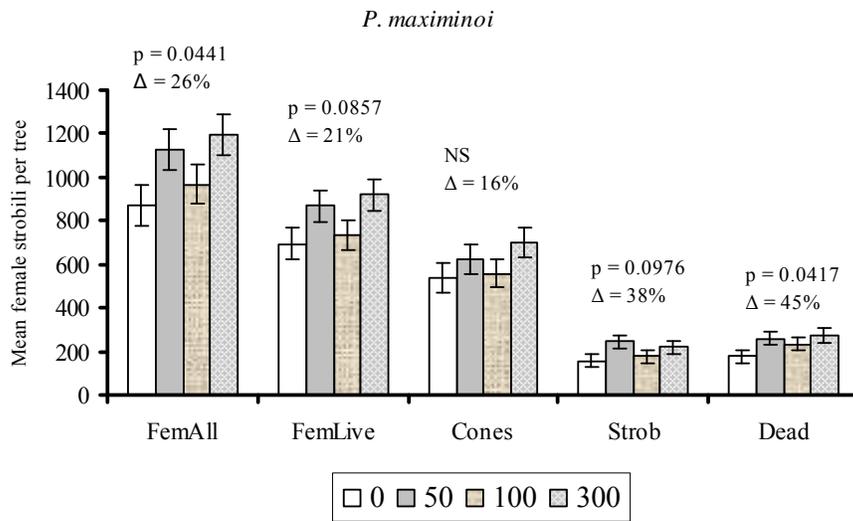


Figure 2.4. Least square means for female strobili production for three levels of GA_{4/7} treatments (50, 100, 300 mg/tree) for *P. maximinoi* and *P. tecunumanii* – Experiment 1.

- Variables are:
 - Femall = the sum of conelets, undeveloped female strobilus buds, dead, damaged, and aborted female strobili
 - Femlive = the sum of Cones and Strob
 - Cones = total conelets
 - Strob = total developing female strobili buds
 - Dead = total dead, damaged, and aborted female strobili
- Significance values are contrast of the three hormone treatments versus control
- Vertical line represents standard error of the mean.

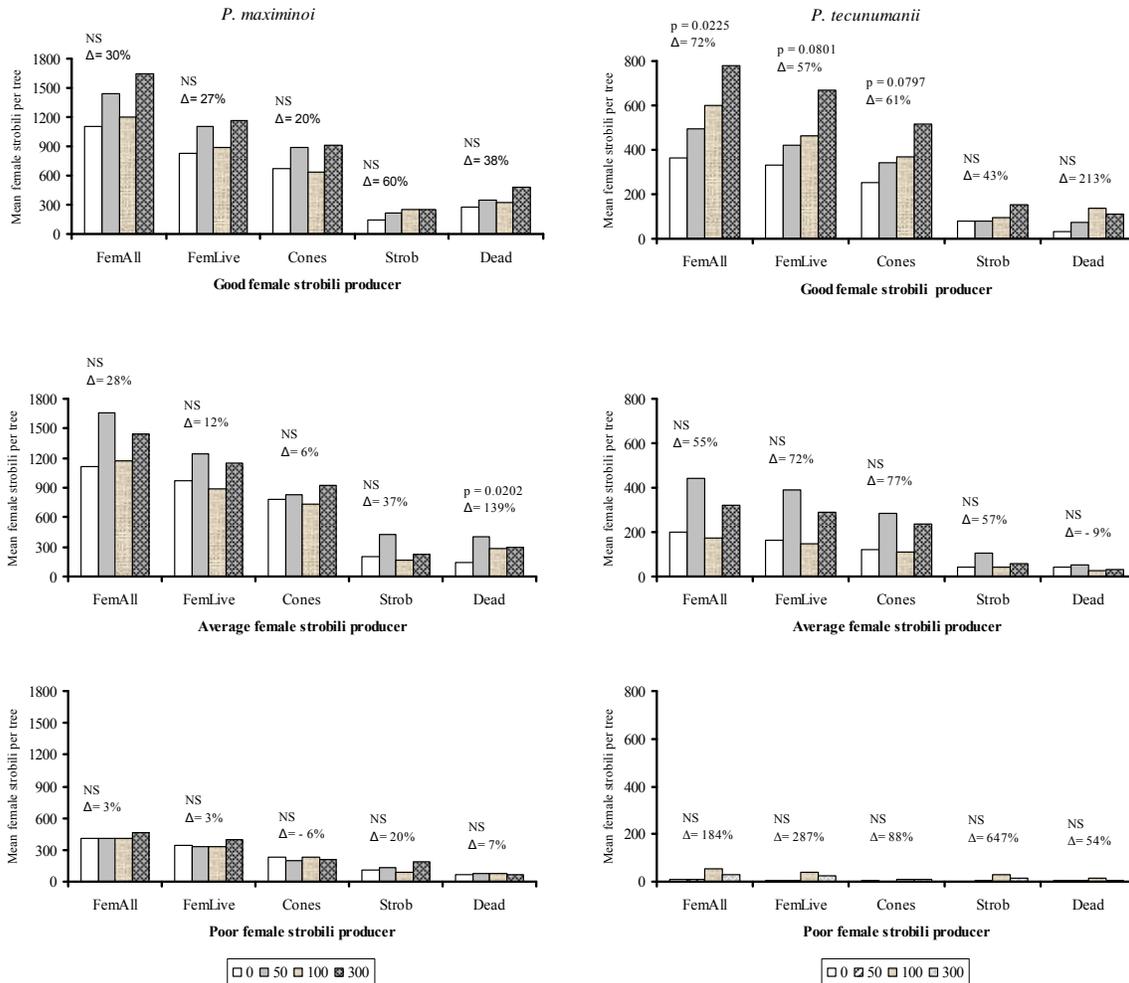


Figure 2.5. Least square means for female strobili production for three levels of GA_{4/7} treatments (50, 100, 300 mg/tree) and production class for *P. maximinoi* and *P. tecunumanii* – Experiment 1.

- Variables are:
 - Femall = the sum of conelets, undeveloped female strobilus buds, dead, damaged, and aborted female strobili
 - Femlive = the sum of Cones and Strob
 - Cones = total conelets
 - Strob = total developing female strobili buds
 - Dead = total dead, damaged, and aborted female strobili
- Significance values are contrast of the three hormone treatments versus control.

E. Experiment 2, September 2007, Procone® treatments: Female Production

As in Experiment 1, there were low to moderate phytotoxic effects from the GA_{4/7} observed in the form of yellowing or needle burn and slight loss of needles the first month after treatment. Symptoms were more noticeable in *P. tecunumanii* than *P. maximinoi*. The symptoms remained on some clones up to four months after treatment application, but thereafter the trees appeared healthy and the terminal buds were growing well.

Analysis of variance results are summarized in Table 2.6. Tree size covariates Volume and NumBranch were significant for many response variables in both species. Production class was significant for most response variables, while Clone(ProduClass) was significant for all variables in both species.

Once again, Rate was significant for only one variable, Strob (developing female strobili) for *P. maximinoi*. Comparisons of all treatments versus the control showed an important effect for *P. maximinoi*. Trees treated with 300, 100, and 50 mg of GA_{4/7} averaged 859, 878, 838 total female strobili per tree respectively vs. 623 on average for controls, an increase of 38% (Figure 2.6). Orthogonal contrasts of treatments versus control for *P. maximinoi* showed this to be a statistically significant increase. There was also a significant increase in Strob, from 72 developing strobili per tree to 152 per tree for the hormone treatments (Figure 2.6), indicating an effect from the hormone treatments persistent up to 4 months later. For *P. maximinoi*, there was also an indication of increased FemLive and Cones (35% and 23%, respectively), but these differences were not statistically significant.

Unlike for *P. maximinoi*, there was no evidence for any effect of the hormone treatments on female strobili production in *P. tecunumanii*. Orthogonal contrasts of treatments versus control for *P. tecunumanii* showed no significant differences for

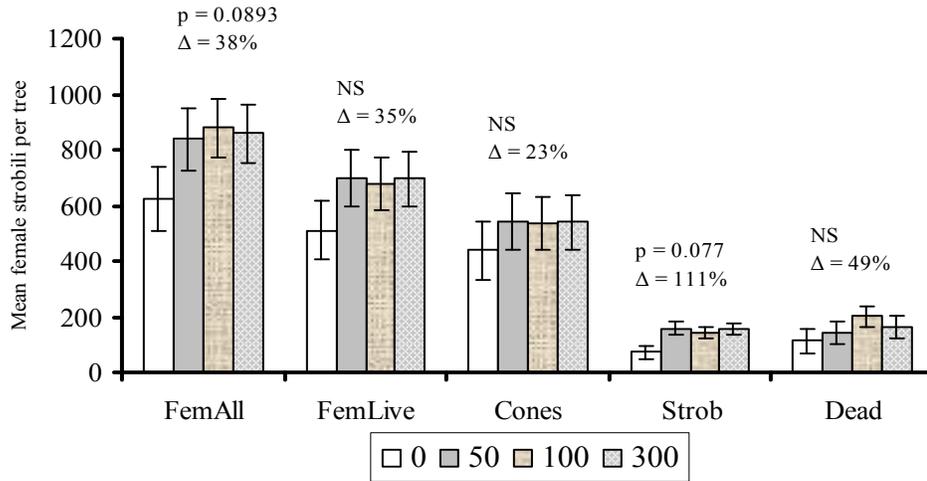
any variable, and in fact, the LS means for many of the response variables was lower for the hormone treatment than for the controls (Figure 2.6).

Examination of the results by production class was not informative. Most of the increase in female strobili production due to hormone treatments appeared to be concentrated in the Good and Poor production classes (Figure 2.7), although the orthogonal contrasts of treatment vs control were generally not significant. For *P. tecunumanii*, significant increases due to the hormone treatments was observed for the Average production class, but not for the Good and Poor production classes. As in Experiment 1, there was little evidence that GA_{4/7} treatments would stimulate a poor producing clone to produce substantially more female strobili.

Table 2.6. Test of hypotheses for mixed model analysis of variance for female strobili production of *P. maximinoi* and *P. tecunumanii* – GA_{4/7} stem injections Experiment 2. Bold numbers indicate statistical significance at p = 0.10.

Species	Response Variable	Volume		Numbranch		Produclass		Clone(produclass)		Produclass*rate		Rate	
		F	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value
<i>P. maximinoi</i>	Femall	4.31	0.0464	4.80	0.0361	6.78	0.0166	3.05	0.0100			1.10	0.3649
	Femlive	4.13	0.0509	4.14	0.0505	3.16	0.0923	4.66	0.0006			0.71	0.5537
	Cones	4.17	0.0497	3.23	0.0819	1.83	0.2165	4.60	0.0007			0.22	0.8792
	Strob	0.00	0.9967	1.18	0.2851	3.61	0.0712	4.94	0.0004			2.74	0.0599
	Dead	0.31	0.5840	0.73	0.3995	1.47	0.2800	5.20	0.0003			0.90	0.4536
<i>P. tecunumanii</i>	Femall	2.28	0.1438	5.84	0.0233	3.38	0.0791	3.67	0.0048	1.22	0.3270	0.16	0.9248
	Femlive	2.25	0.1458	7.31	0.0122	3.25	0.0851	3.43	0.0071	1.08	0.4026	0.18	0.9075
	Cones	3.67	0.0671	7.67	0.0104	3.71	0.0656	4.10	0.0025	1.87	0.1256	0.41	0.7483
	Strob	0.17	0.6794	3.05	0.0930	1.31	0.3154	2.22	0.0559	0.65	0.6935	0.52	0.6730
	Dead	1.08	0.3079	0.01	0.9240	1.83	0.2145	6.80	<0.0001	2.17	0.0799	0.38	0.7708

P. maximinoi



P. tecunumanii

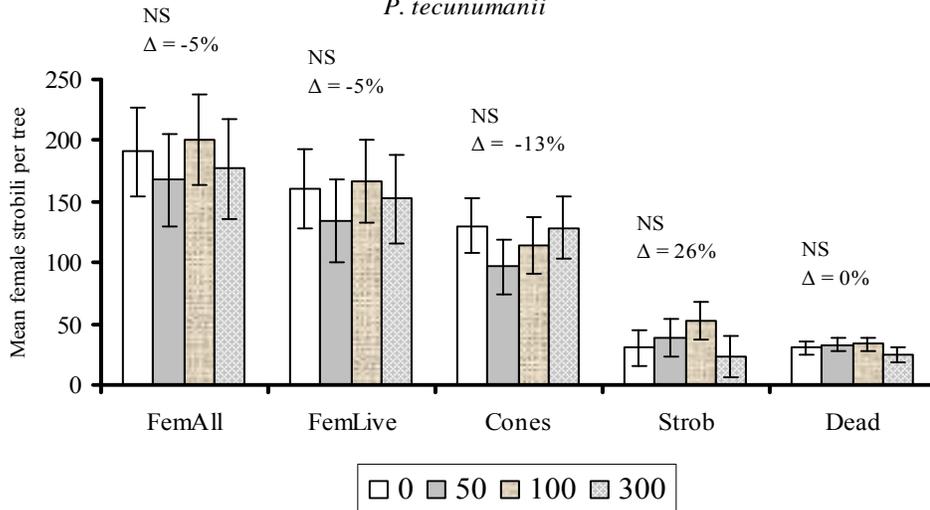


Figure 2.6. Least square means for female strobili production for three levels of GA_{4/7} treatments (50, 100, 300 mg/tree) for *P. maximinoi* and *P. tecunumanii* – Experiment 2

- Variables are:
 - Femall = the sum of conelets, undeveloped female strobilus buds, dead, damaged, and aborted female strobili
 - Femlive = the sum of Cones and Strob
 - Cones = total conelets
 - Strob = total developing female strobili buds
 - Dead = total dead, damaged, and aborted female strobili
- Significance values are contrast of the three hormone treatments versus control
- Vertical line represents standard error of the mean.

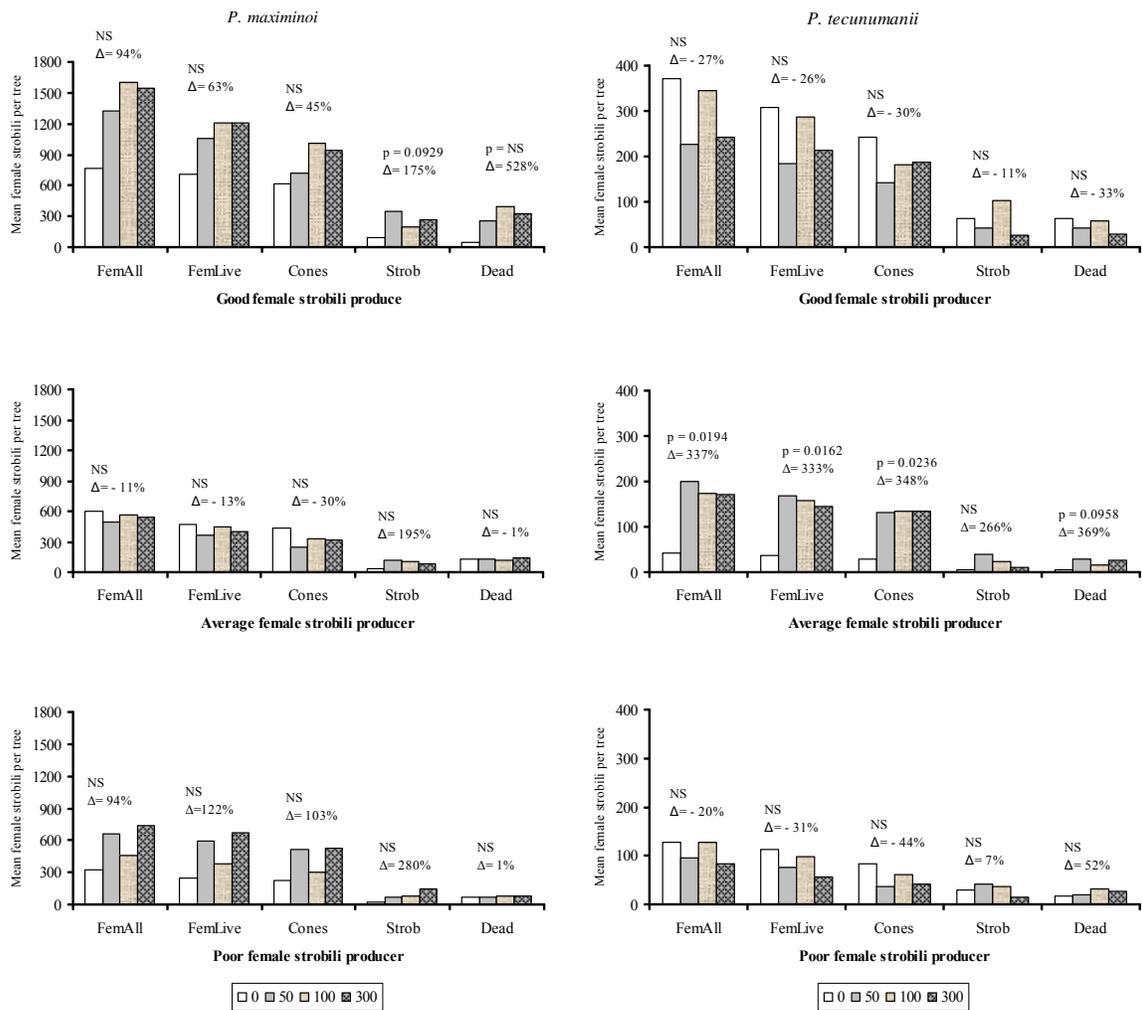


Figure 2.7. Least square means for female strobili production for three levels of GA_{4/7} treatments (50, 100, 300 mg/tree) and production class for *P. maximinoi* and *P. tecunumanii* – Experiment 2.

- Variables are:
 - Femall = the sum of conelets, undeveloped female strobilus buds, dead, damaged, and aborted female strobili
 - Femlive = the sum of Cones and Strob
 - Cones = total conelets
 - Strob = total developing female strobili buds
 - Dead = total dead, damaged, and aborted female strobili
- Significance values are contrast of the three hormone treatments versus control.

F. Catkin Production, Experiments 1 & 2

For catkin production, the response variables were MaleLow (number of catkin clusters per branch in the lower crown) and MaleMid (number of catkin clusters per branch in the middle crown). The analysis of variance results are summarized in Tables 7 and 8. The effect of Clone was statistically significant for both catkin production variables in both species in both experiments, and tree volume was a significant effect for *P. maximinoi* in both experiments (Tables 2.7 and 2.8). In general, the data suggest that there is only a small increase in production of male catkin clusters produced by application either of branch girdling or GA_{4/7}.

Girdling Effect on Catkin Production

Girdling produced a statistically significant response for only one of the eight catkin variables: in Experiment 1 for *P. maximinoi* for MaleLow (catkin production in the lower crown) (Table 2.7). In that case, the girdling treatment produced 20.9 catkin clusters per branch compared to 14.8 for the control.

Hormone Rate Effect on Catkin Production

The results from Experiment 1 were inconsistent and difficult to interpret. For MaleLow in *P. maximinoi*, despite the fact that the overall Rate effect was significant (Table 2.7), the individual contrasts of hormone level versus control did not show any significant differences. For MaleLow in *P. tecunumanii*, the 50 mg/tree Rate produced significantly less pollen catkin clusters than did the control (0.72 versus 1.54 catkin clusters/branch, Figure 2.8). However, for MaleMid for *P. tecunumanii*, the 50 mg/tree and the 300 mg/tree treatment produced significantly more catkin clusters than did the control (1.37 and 1.40 versus 0.59 catkin clusters per branch, Figure 2.8).

The results for Experiment 2 were somewhat more consistent. The effect of Rate on response variable MaleLow was significant for *P. maximinoi*, but not significant for

P. tecunumanii (Table 2.8); however, for both species, linear contrasts showed no significant differences between individual hormone rate treatments and the control (Figure 2.9). For response variable MaleMid, the overall effect of Rate was statistically significant for both species (Table 2.8). For *P. maximinoi*, all three hormone treatments (50, 100 and 300 mg/tree) produced significantly more catkin clusters than the control (9.0, 5.8, and 8.6 versus 2.6 catkin clusters per branch for the control, Figure 2.9). For *P. tecunumanii*, the 100 and 300 mg/tree treatments produced significantly more catkin clusters than the control (1.4 and 1.2 versus 0.5 catkin clusters per branch for the control, Figure 2.9).

Table 2.7. Test of hypotheses for mixed model analysis of variance for catkin cluster production of *P. maximinoi* and *P. tecunumanii* – Experiment 1. Bold numbers indicate statistical significance at $p = 0.10$.

Species	Response Variable	Volume		Clone		Rate		Girdle	
		F	p-value	F	p-value	F	p-value	F	p-value
<i>P. maximinoi</i>	Malelow	9.28	0.0028	7.63	<0.0001	2.14	0.0977	3.88	0.0509
	Malemid	4.64	0.0330	9.70	<0.0001	0.53	0.6632	0.06	0.8118
<i>P. tecunumanii</i>	Malelow	2.14	0.1464	3.25	0.0085	1.43	0.2372	2.34	0.1286
	Malemid	0.96	0.3286	5.60	0.0001	1.61	0.1902	0.18	0.6717

Table 2.8. Test of hypotheses for mixed model analysis of variance for catkin cluster production of *P. maximinoi* and *P. tecunumanii* – Experiment 2. Bold numbers indicate statistical significance at p = 0.10.

Species	Response Variable	Volume		Clone		Rate		Girdle	
		F	p-value	F	p-value	F	p-value	F	p-value
<i>P. maximinoi</i>	Malelow	24.68	<0.0001	3.98	0.0021	3.60	0.0153	0.03	0.8741
	Malemid	12.54	0.0006	11.16	<0.0001	8.94	<0.0001	1.27	0.2627
<i>P. tecunumanii</i>	Malelow	0.00	0.9563	4.98	0.0003	1.21	0.3107	2.28	0.1339
	Malemid	0.01	0.9102	6.11	<0.0001	3.24	0.0245	0.44	0.5068

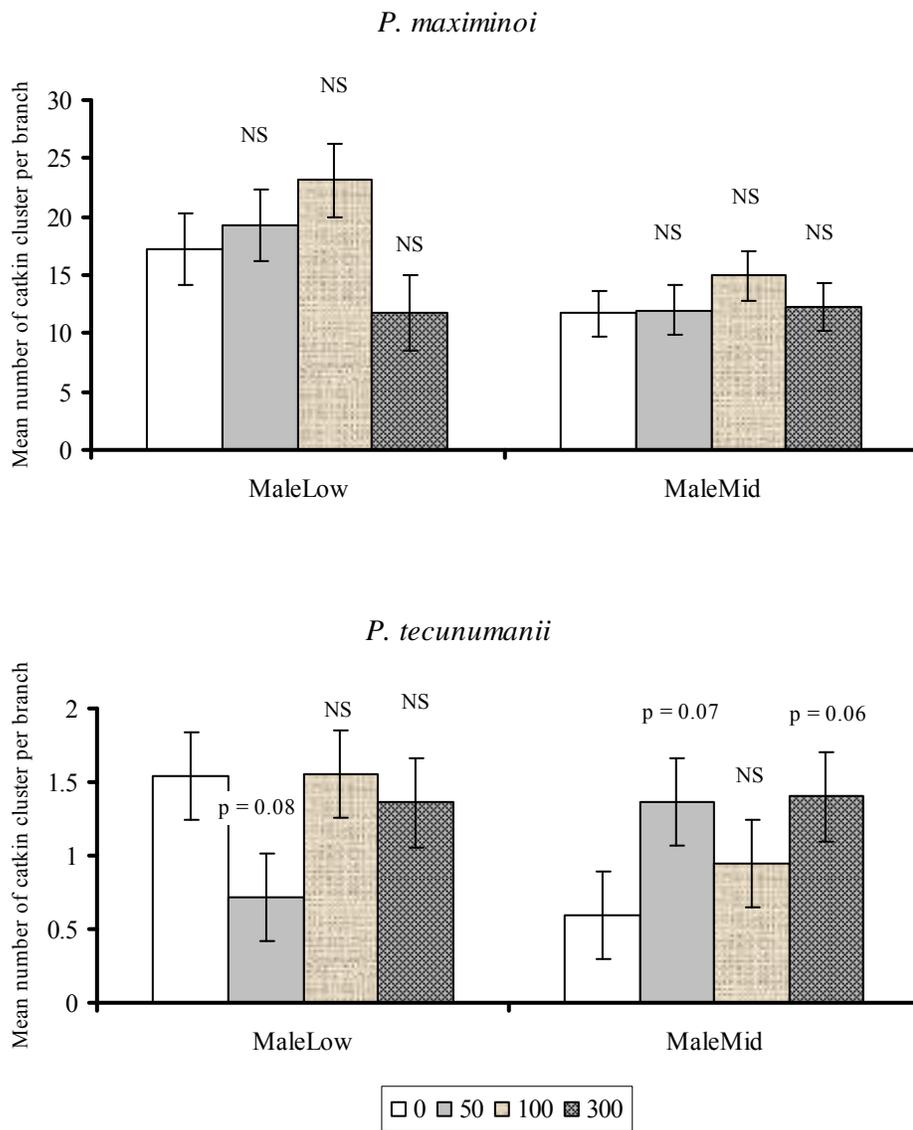


Figure 2.8. Least square means for catkin cluster production for three levels of GA_{4/7} treatments (50, 100, 300 mg/tree) for *P. maximinoi* and *P. tecunumanii* – Experiment 1.

- Variables are:
 - Malelow = total catkin produced on the lower branches
 - Malemid = total catkin produced on the middle branches
- Significance values are contrast of each separate hormone level versus control
- Vertical line represents standard error of the mean.

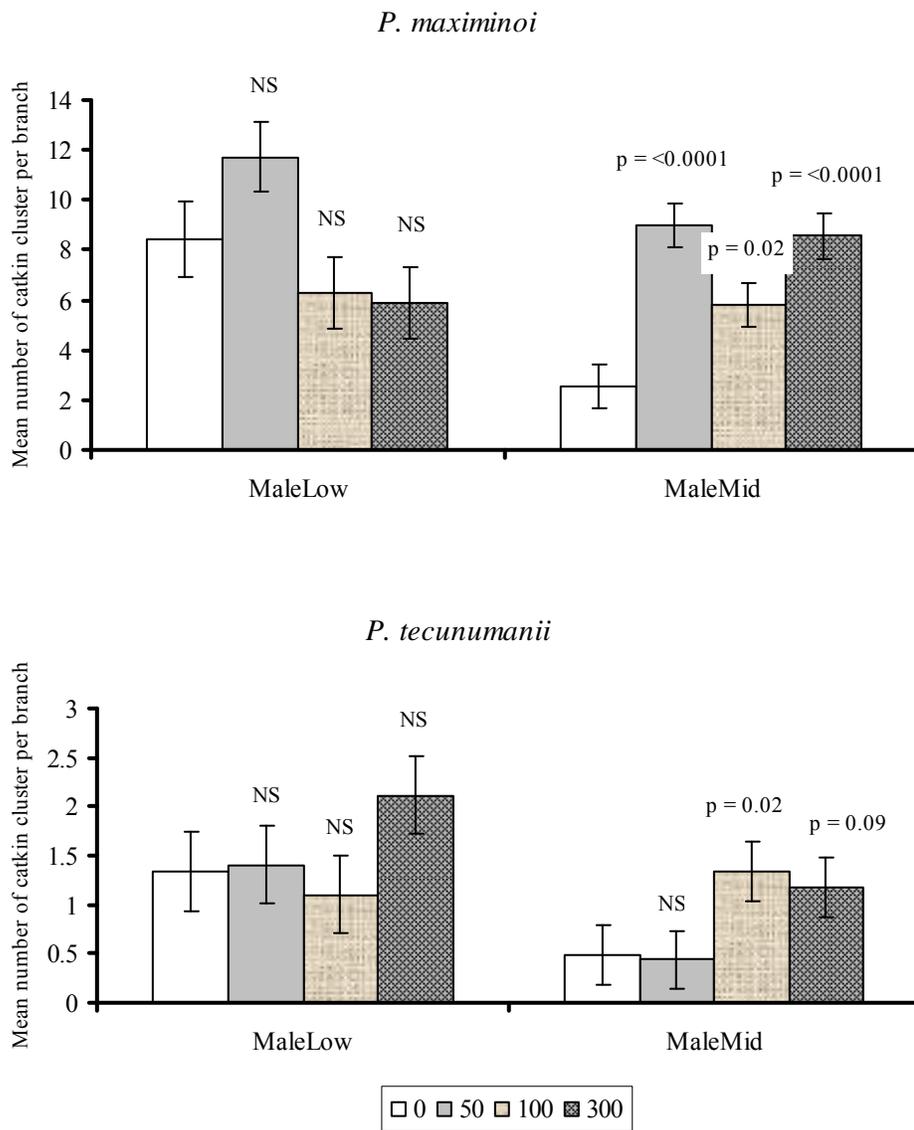


Figure 2.9. Least square means for catkin cluster production for three levels of GA_{4/7} treatments (50, 100, 300 mg/tree) for *P. maximinoi* and *P. tecunumanii* – Experiment 2.

- Variables are:
 - Malelow = total catkin produced on the lower branches
 - Malemid = total catkin produced on the middle branches
- Significance values are contrast of each separate hormone level versus control
- Vertical line represents standard error of the mean.

IV. Discussion

The variable FemAll (total female strobili = cones + developing female strobili + dead and aborted strobili) is probably the best variable to examine response to GA_{4/7} application. Female strobili that receive insufficient pollen will abort (McWilliam 1958; Owens 1995; Bramlett n.d.), but they could be useful in a breeding program or seed orchard through control pollination or supplemental mass pollination. In any case, FemAll and FemLive (live female strobili = cones + developing female strobili) showed very similar patterns for mean response and significance tests, suggesting that Cones and Strob were the main drivers of the response to GA.

This study indicates that simple stem injections of GA_{4/7} into large field-grown trees can significantly promote female strobili production up to six months after treatment in *P. maximinoi* and *P. tecunumanii*. In this study, both GA_{4/7} products (Provide[®] 10sg and Procone[®]) produced a response, suggesting good uptake of the hormone. This is in agreement with the consensus of recent research on the *Pinaceae* that stem injections of GA_{4/7} promotes female flowering in several temperate conifers (Philipson 1985; Wheeler and Bramlett 1991; Smith and Greenwood 1995; Siregar and Sweet 1996; Brockerhoff and Ho 1997; Eriksson et al. 1998; Pijut 2002; Cherry et al. 2007; Rust 2007), despite the fact that the metabolism of the hormone in the tree is not well understood (Dunberg et al. 1983). The technique is practical and economical in the use of GAs, and produces only moderate short-term phytotoxic effects.

In Experiment 1, trees treated with GA_{4/7} in the form of Provide[®] 10 sg produced 26% and 50% more female strobili than the control in *P. maximinoi* and *P. tecunumanii*, respectively. In Experiment 2, trees treated with GA_{4/7} in the form of Procone[®] produced 38% more female strobili for *P. maximinoi*, while there was no significant increase for *P. tecunumanii*. Other authors have reported inconsistent responses to flower induction treatments in field grown trees of pine species managed outside their natural range (Ibrahim 1977; Sirikul and Luukkanen 1987; Dick 1995). The effect of GA_{4/7} stem

injection treatments on female strobili production for temperate conifer species appears to be much larger than observed in this study with the tropical species *P. maximinoi* and *P. tecunumanii*. Increases in this study were on the order of 25 to 50%, compared to an increase of 62% in a 10-year-old *P. taeda* seed orchard (Wheeler and Bramlett 1991), over 200% in a 2-year-old seed orchard of *P. radiata* (Siregar and Sweet 1996), and over 500% in 14-year-old plantations of *P. mariana* and *P. banksiana*, respectively (Greenwood et al. 1993).

For Experiment 1, the nominal amount of GA_{4/7} delivered to the trees (50, 100, and 300 mg/tree) using Provide[®] 10 sg must be interpreted with caution. The results of the lab analyses suggest that the 100 and 300 mg/tree treatments probably had a similar amount of GA_{4/7} in solution. For these treatments, the 20 mL of the liquid injected into each tree probably contained around 8.7 mg/tree in solution, and the excess GA_{4/7} remained in solids in suspension. For Experiment 2, using Procone[®], the liquid injected into the stems contained the target amounts of GA_{4/7} in solution.

For the *P. maximinoi* orchard at Cabuyerita Farm, the precipitation pattern in 2007 was fairly typical, with a dry period from June to September. For this species, GA_{4/7} applications in both August and September produced an increase in female strobili production. For the *P. tecunumanii* orchard at Aguaclara Farm, precipitation in 2007 was much higher than average from June to August. This high rainfall may have limited the response of *P. tecunumanii* to the hormone treatments in Experiment 2. Philipson (1983) found that under cool and wet conditions, GA_{4/7} was completely ineffective in promoting flowering in *Picea sitchensis*, but under warm and dry conditions, flowering was enhanced by GA_{4/7}. Other reports also suggest that a combination of water stress and GA_{4/7} is effective in inducing flowering (Pharis 1977; Greenwood 1981; Owens and Blake 1985; Philipson 1990; Ho and Schnekenburger 1992).

The results of this study suggest that GA_{4/7} treatments increase female strobili production primarily in clones that are already producing flowers, but probably will not stimulate trees that have very few strobili to produce substantially more. Examining the data by ProduClass, the only significant differences from the control were found for the “Good” or “Average” production classes. These results contrast with Harrison and Slee’s (1991) findings in *P. caribaea* var. *hondurensis*, where GA_{4/7} treatments enhanced female strobili production in poor clones, and were ineffective on good flowering clones.

Girdling has been widely used to stimulate pollen production, and positive results have been reported by several authors on different species (Greenwood 1977; Ross et al. 1980; Philipson 1985; Owens and Blake 1985; Longman et al. 1986; Philipson 1987; White and Wright 1987; Wheeler and Bramlett 1991; Cherry et al. 2007). However, in this study, girdling was not particularly effective in promoting catkin cluster production. Some authors suggest that girdling may produce a carry-over stimulation in two subsequent years or even longer (Philipson 1985; Bonnet-Masimbert 1987; White and Wright 1987), while in the present study there is data only for the first six months after treatments were applied. The trees used in this study will be monitored over the next several years to assess the possibility of such a delayed response.

There was some evidence in this study for an effect of GA_{4/7} treatments on pollen production. Catkin clusters in the middle crown were increased for both species in Experiment 2, and for *P. tecunumanii* in Experiment 1. This is consistent with the results of Philipson (1985) who reported increased pollen production in *Picea sitchensis*, and with those of Sirikul and Luukkanen (1987), where spraying the branches of *P. merkusii* and *P. kesiya* with an aqueous mixture of GA_{4/7} + GA₃ promoted male strobili frequency (although they also reported lower female strobili production). However, Wheeler and Bramlett (1991) found that GA_{4/7} treatments produced no increase in pollen production in a *P. taeda* seed orchard.

Potted grafts of *Pinus taeda* and *P. strobus* growing indoors and treated with GA_{4/7} and water stress have been induced to flower at a very young age (Greenwood et al. 1979; Greenwood 1981; Ho and Schnekenburger 1992). Greenhouse studies on *P. maximinoi* and *P. tecunumanii* where environmental conditions such as photoperiod, water stress, temperature, and nutrient supply can be controlled might be useful to better understand optimum application conditions in the field in Colombia. In addition, greenhouses could be useful for accelerated breeding techniques, or to rapidly propagate elite genotypes for commercial use.

V. Conclusions and Recommendations

The results from the present study and previous research demonstrate that GA_{4/7} stem injection treatments increase female strobili production in tropical pine seed orchards in Colombia. There is some evidence that pollen catkin production is also increased slightly. The GA_{4/7} treatments do not appear to stimulate female strobili production in very poor flowering clones.

The stem injection treatments are easy and inexpensive, and should be implemented as part of standard seed orchard management practices in order to increase cone production and meet the seed requirements for reforestation purposes. Further research on optimum timing and dose is needed, and may lead to improvements in effectiveness.

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Chapter 3 - Responses of Seedlings of Open-pollinated families of High-Elevation *Pinus tecunumanii* to Inoculation with *Fusarium circinatum*

I. Introduction

Pitch canker, caused by the fungus *Fusarium circinatum* Nirenberg and O'Donnell syns. *Fusarium subglutinans* f. sp. *pini* and *F. moniliforme* var. *subglutinans*; teleomorph *Gibberella circinata* (Nirenberg and O'Donnell 1998), is a serious disease threatening many economically important pine tree species throughout the world (Dwinell 1999; Gordon et al. 2001, Wingfield et al. 2002a). The disease was first reported in the southeastern United States in 1946 (Hepting and Roth 1946), and it remains a chronic problem in plantations and seed orchards (Dwinell et al. 1985). It has since been reported on pine species in California (McCain et al. 1987), and around the world, including Haiti (Dwinell 1999), Japan (Kobayashi and Muramoto 1989), South Africa (Viljoen et al. 1994), Mexico (Guerra-Santos 1997), Spain (Dwinell 1999; Landeras et al. 2005), Chile (Wingfield et al. 2002), and most recently, Italy (Carlucci et al. 2007). The onset of the disease in new areas may have grave effects on native or exotic pine species (Viljoen et al. 1997).

Today forest plantations in Colombia total approximately 218,000 ha, with 76,000 ha of those planted with exotic pine species. This area includes 26,000 ha belonging to Smurfit Kappa Cartón de Colombia (SKCC) planted with *P. patula*, low and high-elevation *P. tecunumanii*, *P. maximinoi*, *P. oocarpa*, and *P. kesiya*. With the exception of *P. kesiya*, those species originate from Mexico and Central America (Price et al. 1998), the possible center of origin of the pathogen (Harrington and Wingfield 1998; Wikler and Gordon 2000; Britz et al. 2001; Gordon 2006).

Pinus radiata and *P. patula* are two of the most important commercial tree species in the world. The former is the second most extensively planted exotic conifer species after *P. taeda*, totaling about 4.5 million ha mainly in five countries: New Zealand, Chile,

Australia, Spain, and South Africa. Plantation areas by country are 1.43, 1.40, 0.75, 0.25, and 0.07 million hectares, respectively, with an additional 0.14 million ha in all other countries (Lavery and Mead 1998). *Pinus patula* totals about 1.0 million ha, principally in southern and eastern Africa, and to a smaller extent in the highlands of western South America (Dvorak et al. 2000). *Pinus radiata* has shown a very high level of susceptibility to *Fusarium circinatum* (Hodge and Dvorak 2000, Hodge and Dvorak 2007; Roux et al. 2007) which creates great concern in countries such as Australia, Chile, and New Zealand where plantations are focused primarily on this species. *Pinus patula*, which is the major species grown as an exotic in intensively managed plantations (Wingfield et al 2002a) in South Africa, has shown variation in susceptibility ranging from high susceptibility in South Africa (Roux et al. 2007) to intermediate levels of tolerance found among provenances from Mexico (Hodge and Dvorak 2000; Hodge and Dvorak 2007). There is great concern at SKCC that the pitch canker fungus might be introduced into the country. Currently, *P. patula*, *P. tecunumani*, and *P. maximinoi* plantations occupy 29%, 17%, and 4% of the company's land base, respectively. However, future planting efforts will probably decrease the percentage of *P. patula*, and increase the percentage of high and low elevation sources of *P. tecunumanii*, and *P. maximinoi*. High-elevation *P. tecunumanii* is more resistant to pitch canker disease than *P. patula*, but in general shows only moderate resistance (Hodge and Dvorak 2007), therefore screening selected material of these species should be a priority in the genetic strategies in SKCC. The objective of this study is to investigate the genetic variability of pitch canker resistance in high-elevation open-pollinated families of *P. tecunumanii* collected in the Catana clonal seed orchard, and to rank those selected families based on their tolerance to *Fusarium circinatum* for breeding purposes.

II. Background

A. The Pathogen

Pitch canker is caused by *Fusarium circinatum* Nirenberg and O'Donnell syns. *F. subglutinans* f. sp. *pini* and *F. moniliforme* var. *subglutinans*; teleomorph *Gibberella*

circinata (Nirenberg and O'Donnell 1998). It produces conidia on sporodochia (Worrall 2008). The major infection propagules of *Fusarium circinatum* are the asexual conidia (Harrington and Wingfield 1998). Sexual reproduction has been attained under laboratory conditions (Kuhlman et al. 1978), but has not been found in nature (Harrington and Wingfield 1998).

The population structure of *Fusarium circinatum* in the southeastern United States, where the disease is well established, is genetically diverse and represents a large number of vegetative compatibility groups (VCG). The California population has fewer groups (Correl et al. 1992), suggesting that it was recently established and is a clonally propagating population (Gordon 2006). The large number of VCGs found in the South African population by Viljoen et al. (1997a) implies a high level of genotypic diversity and indicates the presence of both mating types, which has been confirmed by the production of fertile perithecia in culture (Viljoen et al. 1997b). Thus, outcrossing may generate numerous VCGs in a short period of time. It also implies that South African populations are more similar to the southeastern United States populations than to the Californian. In contrast, the Californian populations, with a small number of VCGs, indicate that sexual reproduction does not occur (Viljoen et al. 1997a). However, the California and South African populations suggest that the fungus has been introduced from a variety of sources, so it is reasonable to expect that the disease will spread to new regions and become more severe in a few years (Viljoen et al. 1997). Another preliminary assessment of relationships between populations of *Fusarium circinatum* from different locations showed that the association of multiple VCGs with a common multilocus genotype indicates that VCG diversity may be due to mutation rather than recombination from sexual reproduction (Wikler and Gordon 2000).

In general, the ability of a pathogen to cause a disease depends on the availability of a susceptible host in a favorable environment. For pitch canker, determinant

environmental conditions rely on sufficient moisture when temperature is within a favorable range, and the presence of biotic and abiotic wounding agents (Gordon 2006).

The fungus spreads rapidly and may be air-borne, seed-borne, soil-borne, dispersed in rain splash, or by flying insects (Viljoen et al. 1997). The pathogen can infect wounds caused by silvicultural treatments, by wind and hail damage (Gordon 2006), by other pathogens such as fusiform rust, and by insects such as *Rhyacionia subtropica* (pine tip moth), *Pissodes nemorensis* (deodar weevil), and bark beetles (*Coleoptera: Scolytidae*) (Harrington and Wingfield 1998). Some authors call pitch canker a disease complex because the fungus infects a variety of vegetative and reproductive plant structures at different ages and causes a variety of symptoms. The involvement of several insects, the biotic and abiotic factors, and the interaction with other diseases are part of the complex (Dwinell et al. 1985).

B. Symptoms

Since the first description of pitch canker in 1946, the list of symptoms has lengthened. From 1946 to 1974, the infection was described mostly as a canker on the trunk or branches of planted pines in SE United States (Hepting and Roth 1946; Dwinell et al. 1985). In 1974, the disease manifested itself as shoot dieback and infections on reproductive structures, killing seed cones, first-year strobili, and mature cones and degradation of seeds of various pines species (Dwinell et al. 1985). In the 1990s, the fungus was reported to cause pre- and post-emergence damping-off and root rot in bare root and containerized seedlings (Viljoen et al. 1994; Dwinell 1999). The early symptoms include yellowing of the needles, wilting, and dieback of the branch tips; needles may turn red and drop from the tree (Phillips 2001). The classic progression of symptoms usually begins with cankers on small branches near the top of the tree that girdle and kill branch tips. Cankers may then occur on large branches and stems, indicating an advanced stage of the disease and a likelihood of

continued dieback and death. Cankers are typified by copious resin exudation and pitch soaking of the wood, often to the pith. Stem cankers may lead to tree death (Gordon 2006). Infection can occur on branches, shoots, cones, exposed roots and stems. Seed cones on infected branches often abort before reaching full size and fail to open, but there are also frequent reports of asymptomatic cones on infected trees which increase the probability of disease transmission via apparently healthy seeds (Britz et al. 2001). Symptoms may appear at any time of the year (Phillips 2001).

C. Hosts and Geographic Distribution

Hosts are primarily in the genus *Pinus*; they differ in their susceptibility, but most species seem to be susceptible to some degree, and even *Pseudotsuga menziesii* (Douglas-fir) has been recorded as a host with relative tolerance (Storer et al. 1994). The most important pine species affected naturally by pitch canker are listed in Table 3.1.

Early in the spring of 1945, several obvious symptoms such as dead branches and leaders, copious pitch flow with accumulations on and below the canker, were observed on many *Pinus virginiana* in the USDA Forest Service Bent Creek Experimental Forest in North Carolina. Isolations from natural cankers yielded a *Fusarium* as the causal agent. It was the first report of a new disease infecting southern pines and was called pitch canker due to the abundant pitch flow (Hepting and Roth 1946). It was hypothesized that the disease might have originated in Haiti (Wingfield et al. 2002a). In 1953, during a disease survey, pitch canker was observed on *P. occidentalis* in Haiti (Hepting (1953) cited by Dwinell (1999)).

Table 3.1. Most frequent hosts affected naturally by *Fusarium circinatum*

Host	Reported Year	Country	Source
<i>P. virginiana</i>	1946	North Carolina, USA	Hepting and Roth 1946
<i>P. occidentalis</i>	1953	Haiti	Dwinell 1999
<i>P. elliotti</i> var. <i>elliottii</i>	1974	Florida, USA	Dwinell et al. 1985
<i>P. taeda</i>	1974	North Carolina – Mississippi	Dwinell et al. 1985
<i>P. radiata</i>	1986	California, USA	McCain et al. 1987
	Late-1980s	Mexico	Guerra-Santos 1997
	1997	Northern Spain	Dwinell 1999
	2003-2004	Nothern Spain	Landeras et al. 2005
<i>P. luchuensis</i>	Mid-1980s	Japan	Kobayashi and Muramoto 1989
<i>P. halepensis</i>	Late-1980s	Mexico	Blanchette (1989) cited by Dwinell (1999)
	2005	Italy	Carlucci et al. 2007
<i>P. douglassiana</i>	Late-1980s	Mexico	Blanchette (1989) cited by Dwinell (1999)
<i>P. leiophylla</i>	Late-1980s	Mexico	Guerra-Santos 1997
<i>P. durangensis</i>	Late-1980s	Mexico	Guerra-Santos 1997
<i>P. estevesi</i>	Late-1980s	Mexico	Dwinell 1999
<i>P. arizonica</i> var. <i>stormiae</i>	Late-1980s	Mexico	Dwinell 1999
<i>P. patula</i>	1994	South Africa	Viljoen et al 1994
<i>P. pinaster</i>	2003-2004	Nothern Spain	Landeras et al. 2005
<i>P. pinea</i>	2005	Italy	Carlucci et al. 2007

By 1974, a shoot dieback caused by *Fusarium circinatum* reached epidemic proportions on planted *Pinus elliottii* var. *elliottii* in Florida and on *Pinus taeda* seed orchards in North Carolina and Mississippi, and since that time, the disease has become a chronic problem disturbing pines in the SE United States (Dwinell et al. 1977). In 1986, *Fusarium circinatum* was identified in California by McCain et al. (1987) causing a serious epidemic on ornamental *P. radiata* trees (Gordon 2006), and currently it has been found infecting nine additional pine species; the disease transmission is strongly correlated with insect vectors (Viljoen et al. 1997).

In the mid-1980s, pitch canker pathogen caused trunk cankers and dieback on *P. luchuensis* in Japan and is considered an endemic disease in this region (Kobayashi and Muramoto 1989). A preliminary assessment of relationships between populations of *Fusarium circinatum* from different locations showed that the

California and Japanese populations shared lineages with the southeastern United States population (Wikler and Gordon 2000).

In the late 1980s, pitch canker was observed in Mexico on planted *P. halepensis* and in natural stands of *P. douglassiana* (Blanchette (1989) cited by Dwinell (1999)), on planted *P. estevesi* and in natural stands of *P. arizonica* var. *stormiae* (Dwinell 1999). The disease was also frequent on planted *P. radiata* and in natural stands of *P. leiophylla* and *P. durangensis* (Guerra-Santos 1997). Guerra-Santos and Tovar (1991) cited by Viljoen et al (1997) reported that the most affected pine species in Mexico are *P. maximinoi*, *P. pringlei* and *P. pseudostrobus*, and that insects are implicated in the disease transmission. Even though pitch canker was only recently identified in Mexico (Guerra-Santos 1997), a number of genetic diversity studies suggest that the disease may have evolved in this part of the world (Harrington and Wingfield 1998; Wikler and Gordon 2000; Britz et al. 2001; Gordon 2006). This is supported by the fact that Mexico is the New World center of diversity of the pine genus with 40% of the world's described species (Perry et al. 1998), and that pitch canker occurs there, but causes relatively little damage in the native forests (Gordon 2006). It was also reported that branch tip dieback seems to be more frequent than stem cankers, and trees infected by the pathogen can be asymptomatic (Wikler and Gordon 2000). *Fusarium circinatum* in Mexico has a higher level of genetic diversity than in any other region examined (Wikler and Gordon 2000; Britz et al. 2001), which is expected for a pathogen's ancestral home (Gordon 2006).

In 1990, containerized seedlings of *P. patula* in a nursery in South Africa were heavily infected by *Fusarium circinatum* causing seedling root rot rather than pre- or post-emergence damping-off (Viljoen et al. 1994). To date this fungus has infected nurseries throughout the country, causing substantial losses. Also, it has been found in field-grown trees up to 3 years old but it is still unknown if these outbreaks are related to nursery infections (Roux et al. 2007). There is clear evidence that sexual

reproduction is occurring (Viljoen et al. 1997b) which favors the increase of genetic diversity of *Fusarium circinatum* (Wingfield et al. 2002a). In general, pitch canker is a disease that produces cankers on the stem and branches of established trees throughout the world; however, on seedlings in South Africa, it represents a different phase of the disease (Wingfield et al. 2002a).

In Chile, the disease is causing damage in seedling nurseries, but canopy dieback in established plantations has not yet been reported. A situation similar to that in South Africa is occurring in Chile where the pathogen is affecting *P. radiata* in nurseries but the symptoms are not typical of pitch canker (Wingfield et al. 2002). In 1997 in northern Spain, pitch canker infection also caused mortality of *P. radiata* seedlings in bare-root nurseries (Dwinell 1999). In Asturias (northern Spain) during the winter of 2003-2004, Landeras et al. (2005) found infected seedlings of *P. radiata* and *P. pinaster* with *Fusarium circinatum* and later in the year, pitch canker symptoms were observed in a 20-year-old *P. radiata* plantation in Cantabria (northern Spain): the isolations identified as *Fusarium circinatum* as the causal agent.

Since 2005, pitch canker symptoms such as dieback, and resinous cankers on twigs and branches have been observed in southern Italy on numerous trees of *P. halepensis* and *P. pinea* planted in urban parks and gardens. Several isolations from symptomatic tissue were identified as *Fusarium circinatum* using morphological and cultural characteristics, and this was confirmed by molecular techniques. This is the first definite evidence of the presence of pitch canker in Italy (Carlucci et al. 2007).

D. Potential Management Strategies

So far, no effective fungicidal or biological control measures for pitch canker are available. In the southeastern United States, forest management practice has reduced the incidence of pitch canker disease by minimizing injuries to the bark during pruning, thinning, and seed collection activities (Dwinell et al. 1985). In the western United States, where insects play an important role acting as infection vectors, efforts

to reduce insect populations are unlikely to be successful on disease incidence because chemical, biological, or cultural control of the beetle species is currently impractical, although more research on the biology of insects may provide insights to control the spread of the disease (Storer et al. 1997). Current efforts to control the infection caused by *Fusarium circinatum* in California have focused on public education and quarantines to avoid spreading the disease, disinfection of pruning tools, and restrictions on the transportation of wood (logs, chips, waste wood, firewood) and the movement of trees (Worral 2008). In South Africa the control relies mostly on nursery hygiene and cultural management (Roux et al. 2007). Using *P. radiata* in controlled greenhouse conditions and in the field, Bonello et al. (2001) demonstrated that repeated exposure to the pathogen led to an increase in resistance over time. This was the first report of systemic induced resistance (SIR) in a conifer. From an evolutionary perspective, SIR may be a means for long-lived trees to sustain populations long enough to provide time for genetic adaptation to new pathogens (Gordon 2006).

The harsh reality is that the pathogen is moving around the world and spreading rapidly; therefore, the long-term solution must be based on development of disease-tolerant stock to be used in subsequent breeding trials (Storer et al 1997; Gordon et al. 1998; Roux et al. 2007). The use of alternate species based on the high levels of tolerance to pitch canker shown for some pine species such as *P. oocarpa*, *P. jaliscana*, and *P. tecunumanii* (low- elevation) is an option to be considered (Hodge and Dvorak 2000), and the significant and important provenance variation found by Hodge and Dvorak (2007; 2007a) among *P. patula* and *P. tecunumanii* (high-elevation) and *P. leiophylla* for tolerance to pitch canker suggests that it may be possible to select for genotypes tolerant to *Fusarium circinatum*. In addition to a strategy based on breeding disease-tolerant trees in a pure species, pine hybrids offer great potential (Hodge and Dvorak 2000; Gordon et al. 2001; Wingfield et al. 2002a; Hodge and Dvorak 2007). Early results have showed encouraging signs of tolerance

of some hybrids such as *P. patula* x *oocarpa* and *P. elliottii* x *caribaea* in South Africa (Roux et al. 2007). However, since the pathogen is undergoing sexual mating in South Africa, it is important to consider that the fungus could adapt to new planting stock with time, thus an important alternative for the long term could be the development of genetically modified trees (Wingfield et al. 2002a).

III. Materials and Methods

A. Plant Material

The Catana *P. tecunumanii* (HE) seed orchard on SKCC land in Colombia is a grafted orchard containing 24 clones. These clones were selections made in first-generation Camcore provenance/progeny tests. There were two experiments conducted using primarily open-pollinated seedlots collected in this orchard. In Experiment 1, there were a total of 13 seedlots (Table 3.2):

- ten open-pollinated (OP) families of *P. tecunumanii* (HE) collected in the Catana seed orchard in Colombia,
- two seedlots of *P. tecunumanii* (LE), one collected in the Suiza seed orchard in Colombia, and the other a mix of eight provenances collected by Camcore within the natural range of the species, and
- a seedlot of *P. patula* collected in the Peñas Negras seed orchard in Colombia.

Germination of the seedlots for Experiment 1 was quite variable and four of the ten *P. tecunumanii* (HE) seedlots were represented by fewer than 40 trees. Additional seed was sown for Experiment 2 from nine of the ten families in Experiment 1, plus an additional five *P. tecunumanii* (HE). In summary, Experiment 2 contained 16 seedlots (Table 3.2):

- 13 OP families of *P. tecunumanii* (HE) collected in the Catana seed orchard,
- one seedlot of *P. tecunumanii* (LE) also used in Experiment 1, a mix of eight provenances collected by Camcore within the natural range of the species, and

- one seedlot of *P. tecunumanii* (HE), a mix of five provenances collected by Camcore in its natural range, and
- a seedlot of *P. patula* collected in the Peñas Negras seed orchard in Colombia.

Table 3.2. Plant material included in the pitch canker resistance assessment - Experiments 1 and 2.

Family	Species	Provenance or Source	Original Provenance of Selected Clone	Number of Trees		
				Expt. 1	Expt. 2	
13-149	<i>P. tecunumanii</i> (HE)	La Catana (SO)	Carrizal	78	90	
13-152			Juquila	21	104	
13-153			Chanal		104	
13-155			Juquila	83	50	
13-156			Carrizal		184	
13-157			Chanal		180	
13-158			Chanal	119	13	
13-161			Napite		26	
13-163			Chiul	34	82	
13-165			Pachoc	89	95	
13-167			Napite	120		
13-169			Chiul	62	29	
13-170			Pachoc	28	40	
13-171				Rancho Nuevo	18	165
Control 1			<i>P. patula</i>	Peñas Negras (SO)		120
Control 2	<i>P. tecunumanii</i> (LE)	La Suiza (SO)		120		
Control 3	<i>P. tecunumanii</i> (LE)	Bulk mix ¹		120	21	
Control 4	<i>P. tecunumanii</i> (HE)	Bulk mix ²			44	

¹ Mix of eight provenances: San Esteban, Cerro La Joya, Yucul, San Rafael del Norte, Culmi, Los Planes, Campamento, Locomapa.

² Mix of five provenances: San Jeronimo, San Vicente, Las Trancas, Chanal, Rancho Nuevo.

B. Experimental Methods

Seedlings were inoculated following the greenhouse-based protocols developed by Oak et al. (1987) at the USDA Forest Service Resistance Screening Center (RSC) in Bent Creek, North Carolina. Seedlings were inoculated with the pitch canker fungus to assess the relative resistance to infection. The chronological activities are summarized in Table 3.3. Seeds were soaked in cold water for 24 h prior to sowing, and seedlings were grown in Ray Leach® containers (115 ml) for 18 and 16 weeks for the Experiment 1 and Experiment 2, respectively, in a 3-4-4 mix of peat moss-

vermiculite-perlite. Seedlings representing a family-replication unit were grouped in small trays. The inoculum was an equal mixture of three proven pathogenic isolates, one originating from Florida and two from Georgia (Table 3.4). A bulk mix of conidia of *Fusarium circinatum* was prepared following the protocol described by McRae et al. (1985). The seedlings were injured by cutting the stem just below the apical meristem and removing the apical portion. The seedlings were then inoculated by spraying an aqueous spore suspension onto the fresh wounds with a concentration of 25,000 spores/mL. Each tree was sprayed twice, once from a distance of around 10 cm and the second time from 25 cm away.

Table 3.3. Chronological events for pitch canker screening of families of *P. tecunumanii* (HE) and control lots of *P. patula*, *P. tecunumanii* (HE and LE).

Activity	Experiment 1		Experiment 2	
	Date	Age	Date	Age
Sowing	April 16, 2007		June 6, 2007	
Inoculation	August 21, 2007	18 weeks old	October 2, 2007	16 weeks old
First Assessment	November 19, 2007	13 weeks post-inoculation	December 20, 2007	11 weeks post-inoculation
Second Assessment	January 14, 2008	21 weeks post-inoculation	February 25, 2008	21 weeks post-inoculation

The experimental design was a randomized complete block design with 3 and 5 replications in two blocks in the first and second experiment, respectively. Families had variable numbers of seedlings in replications due to poor germination (Table 3.2). Each replication was inoculated with a bulk mix of conidia.

All seedlings in Experiments 1 and 2 were inoculated at approximately 4 months of age (18 and 16 weeks, respectively) because previous studies have shown good differentiation among families using seedlings at this age (Hodge and Dvorak 2007). Following inoculation, the seedlings were returned to the greenhouse for 21 weeks during which pathogen colonization was allowed to occur. Two measurements of length of stem dieback were made in both experiments at 3 months and 5 months (13

and 21 weeks in Experiment 1, and at 11 and 21 weeks in Experiment 2). The total length of the stem from hypocotyl to the cut and total length of stem dieback was measured. From these data the percentage of live stem remaining was calculated.

Table 3.4. Source of isolates used to screen families of *P. tecunumanii* (HE) and control lots of *P. patula*, *P. tecunumanii* (HE and LE).

Isolate	Location	Host Species
S298	Gilchrist Co., FL	<i>Pinus elliottii</i>
S396	Bainbridge, GA	<i>Pinus taeda</i>
S397	Bainbridge, GA	<i>Pinus taeda</i>

C. Data Analysis

The data from the two experiments were analyzed in a combined analysis using the GLM and MIXED procedure in SAS[®] (SAS Institute 1989). There were five response variables: Stemkill at 3 and 5 months (**Stemkill3**, **Stemkill5**), dieback at 3 and 5 months (**Dieback3**, **Dieback5**), and height of the seedling at time of inoculation (**Ht**). The linear model included **Test** = experiment, **Rep(Test)** = replication nested within experiment, **Fam** = family, **Test*Fam** = experiment x family interaction, and **Fam*Rep(Test)** = family x replication nested within experiment interaction. The variables **Fam**, (**Test*Fam**) and (**Fam*Rep(Test)**) were considered random. The variable **Ht** was also included as a covariate in analyses for Stemkill and Dieback.

Using a data set with only the *P. tecunumanii* (HE) families, analysis of variance was conducted using GLM, and variance components for the random effects were estimated with PROC MIXED. Using the variance component estimates, the following genetic parameters were calculated:

- $h^2 = (3 * \sigma_f^2) / (\sigma_f^2 + \sigma_{fxt}^2 + \sigma_{f*r(t)}^2 + \sigma_e^2)$, and
- $r_{Bg} = (\sigma_f^2) / (\sigma_f^2 + \sigma_{fxt}^2)$

where:

- $h^2 =$ heritability

- σ_f^2 = family variance,
- σ_{f*t}^2 = family * test interaction variance,
- $\sigma_{f*r(t)}^2$ = family * replication nested within test interaction variance, and
- σ_e^2 = error variance.

The parameter r_{Bg} is called the Type B genetic correlation (Burdon 1977), and in this case it measures the genetic correlation between the two experiments. As the σ_{fxt}^2 = family x experiment interaction variance approaches zero, r_{Bg} will approach unity.

Best linear unbiased prediction (BLUP) estimates of family general combining abilities (GCA) were also estimated with PROC MIXED.

For purposes of comparison of the *P. tecunumanii* (HE) families and the controls, least squares means were calculated using the same linear model as above, but without height as a covariate. This was primarily because the *P. tecunumanii* (LE) controls grew much faster than the families (mean height was 253 mm versus 159 mm for the LE control and the HE families, respectively), and an adjustment for height was not appropriate. Linear contrasts to compare the three controls (*P. tecunumanii* (LE), *P. tecunumanii* (HE), and *P. patula*) versus the mean of the open-pollinated *P. tecunumanii* (HE) families were calculated.

IV. Results

The two experiments were conducted with the same protocols with the exception of some variation in the dates of sowing, inoculation, and measurement. At the time of inoculation, the seedlings in Experiment 1 were slightly taller than those in Experiment 2, 167.4 mm versus 151.1 mm, respectively (Table 3.5). This may be partially due to the fact that the inoculation was done at 18 weeks in Experiment 1, and 16 weeks in Experiment 2. There was also substantially less dieback observed in Experiment 2 than Experiment 1. For example, average dieback at 5 months was 47.4 mm in Experiment 1

and 32.7 mm in Experiment 2. This was likely due to lower temperatures in the greenhouse subsequent to the inoculation, which was August 21 for Experiment 1 and October 2 for Experiment 2. Normally, the staff at the Resistance Screening Center prefer to do pitch canker inoculations in the warmer months of July and August, if possible (Carol Young, former manager of the RSC, personal communication).

The analysis of variance showed that all terms in the linear model were significant for all four pitch canker response variables, with one exception (for variable Stemkill3, the rep(test) term was not significant) (Table 3.6). Seedling height at inoculation was a significant covariate for all response variables, with taller seedlings showing less dieback and stemkill. This may reflect a difference in physiological maturity or degree of succulence related to the ability of the pathogen to colonize the stem. Inclusion of height as covariate was much more important for stemkill than for dieback. Comparison of models without height and with height showed an increase in R^2 from 0.26 to 0.27 for Dieback5, while for Stemkill5, the increase was from 0.28 to 0.35.

Table 3.5. Least square means for height and pitch canker resistance traits for *P. tecunumanii* (HE) open-pollinated families in the two experiments.

Experiment	Height (mm)	Dieback3 (mm)	Stemkill3 (%)	Dieback5 (mm)	Stemkill5 (%)
1	167.4	36.8	24.7	47.4	36.4
2	151.1	13.2	9.0	32.7	21.8

Heritability estimates for the four pitch canker response variables were quite high, ranging from 0.48 to 0.58 (Table 3.7). Reports from similar studies using young seedlings at the RSC had much lower heritability estimates: for a study with 35 families of *P. radiata*, estimated heritability across four experiments was $h^2 = 0.14$ (Camcore 2001), and in a study with 46 families of *P. elliottii*, estimated heritability was $h^2 = 0.25$ (McRae et al. 1985). In contrast, Matheson et al. (2006) measured lesion length following stem inoculation of 1.5 year old *P. radiata*, and reported a heritability of 0.49, similar to the values observed in this study. There was little family x experiment

interaction, with r_{Bg} values ranging from 0.77 to 0.91 for the four response variables (Table 3.7), which is consistent with other results. For example, estimated r_{Bg} for 24-week stemkill of *P. radiata* families was 1.00 (Camcore 2001), and was 0.92 to 1.00 for 16- to 20-week stemkill and dieback of *P. tecunumanii* provenances (Hodge and Dvorak 2007). Since there is very little family x experiment interaction, including a set of families in one experiment at the RSC will be sufficient to produce precise rankings of those families. In the current study, the variable Stemkill5 appears to be the most useful of the four response variables for ranking families, as it has one of the highest heritabilities and the highest r_{Bg} (i.e., the lowest family x experiment interaction).

Linear contrasts of the pure species control lots versus the open-pollinated *P. tecunumanii* (HE) families showed a very clear pattern (Table 3.8). Compared to the HE families, *P. patula* was more susceptible to pitch canker for all four response variables, *P. tecunumanii* (LE) was more resistant for three of the four variables (Dieback3, Dieback5 and Stemkill5), and the native provenance *P. tecunumanii* (HE) seedlot was not significantly different for any of the response variables. The species ranks in this study are exactly consistent with previous results reported in the literature: *P. tecunumanii* (LE) shows essentially no stem dieback when inoculated as a seedling, *P. tecunumanii* (HE) shows intermediate resistance, and *P. patula* is very susceptible (Viljoen et al. 1995; Hodge and Dvorak 2000, Hodge and Dvorak 2007, Roux et al. 2007).

There was substantial genetic variation among the *P. tecunumanii* (HE) families for resistance to pitch canker infection (Table 3.9). For example, the range in GCA predictions for Stemkill5 was 12% to 63%. The 14 families ranked essentially the same regardless of which of the four response variables is used. The most resistant *P. tecunumanii* (HE) families approach the resistance of low-elevation *P. tecunumanii*, while the least resistant families approach the susceptibility of *P. patula*.

Table 3.6. Test of hypotheses for mixed model analysis of variance for pitch canker resistance traits in OP families of *P. tecunumanii*. Bold numbers indicate statistically significant at p = 0.10.

Response Variable	Test		Rep(test)		Ht		Rep*Fam(test)		Fam		Test*Fam	
	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value
Dieback5	6.60	0.0285	1.57	0.0992	6.25	0.0125	5.08	0.0274	5.08	0.0274	2.76	0.0078
Stemkill5	18.90	0.0013	1.91	0.0333	170.51	<0.0001	1.31	0.0242	7.93	0.0139	1.87	0.0699
Dieback3	25.11	0.0007	1.57	0.1001	31.83	<0.0001	1.43	0.0040	3.54	0.0545	4.02	0.0003
Stemkill3	40.54	<0.0001	1.24	0.2566	284.38	<0.0001	1.58	0.0003	4.45	0.0379	2.62	0.0112

Table 3.7. Genetic parameter estimates* for *Fusarium circinatum* tolerance traits for OP families of *P. tecunumanii* (HE) assessed at 3 and 5 months after inoculation.

Trait	Mean	h^2	r_{Bg}	σ_f^2	σ_{f*t}^2	$\sigma_{f*r(t)}^2$	σ_e^2
Height	159.3 mm	0.27	0.82	197.5	44.5	452.8	1538.3
Dieback3	25.0 mm	0.58	0.77	122.4	35.8	16.2	456.8
Stemkill3	16.9 %	0.55	0.85	76.1	14.0	26.1	312.8
Dieback5	40.1 mm	0.48	0.81	413.5	95.0	25.1	2039.4
Stemkill5	29.1%	0.56	0.91	225.6	16.6	35.6	944.9

* Genetic parameters are:

h^2 = heritability

r_{Bg} = the type B correlation of the family effects

σ_f^2 = family variance

σ_{f*t}^2 = family * test interaction variance

$\sigma_{f*r(t)}^2$ = family * replication nested within test interaction variance

σ_e^2 = error variance

Table 3.8. Linear contrasts of species control lots versus *P. tecunumanii* (HE) open-pollinated families. Values are p-values of tests for differences between least square means for pitch canker resistance traits, bold values are significant at $p = 0.10$.

Contrast	Stemkill3	Dieback3	Stemkill5	Dieback5
<i>P. patula</i> vs. Families	0.0041	0.0003	0.0004	< 0.0001
<i>P. tecunumanii</i> (HE) vs. Families	0.7729	0.6886	0.5026	0.4202
<i>P. tecunumanii</i> (LE) vs. Families	0.1652	0.0156	0.0458	0.0052

Table 3.9. Rankings of *P. tecunumanii* (HE) open-pollinated families (coded 13-xxx) and three species bulk control lots for pitch canker resistance traits.¹

Family	Original Provenance of Selected Clone	Dieback3 (mm)	Stemkill3 (%)	Dieback5 (mm)	Stemkill5 (%)
<i>P. tecunumanii</i> LE	Bulk	-1.5	-2.2	1.8	-0.2
13-167	Napite	7.1	5.2	17.0	12.0
13-156	Carrizal	14.3	11.2	18.0	13.4
13-158	Chanal	11.6	6.7	24.4	15.4
13-161	Napite	18.0	11.9	31.8	19.6
13-153	Chanal	17.2	15.5	25.2	20.8
<i>P. tecunumanii</i> HE	Bulk	17.1	14.3	25.8	21.6
13-155	Juquila	17.9	13.7	30.8	23.3
13-165	Pachoc	15.8	11.9	32.3	23.7
13-171	Rancho Nuevo	17.5	14.1	33.8	25.3
13-149	El Carrizal	18.6	15.2	37.1	27.6
13-170	Pachoc	27.7	21.1	49.2	35.8
13-157	Chanal	26.2	20.9	50.7	38.1
13-163	Chiul	32.6	23.8	57.2	41.5
13-169	Chiul	28.3	24.4	51.8	41.5
<i>P. patula</i>	Bulk	43.9	20.6	93.2	43.5
13-152	Juquila	45.4	35.5	85.2	63.8
Mean <i>P. tecunumanii</i> (HE) families		21.3	16.5	38.9	28.7

¹Seedlots are ordered by Stemkill. Values for *P. tecunumanii* (HE) families are best linear unbiased predictions using 4-month seedling height as a covariate. Values for control species are least square means.

V. Discussion

Open-pollinated seedlots of *P. tecunumanii* (HE) collected in the Catana orchard have been used to establish progeny tests and pilot plantations on SKCC land. Field inspections of these plantings have revealed the presence of some trees that have semi-pendulous needles, in appearance intermediate between *P. tecunumanii* and *P. patula*. In the region of the Catana orchard, most of the older plantations are *P. patula*, and the two species hybridize fairly readily (e.g., Roux et al. 2007). In this study, although the native provenance seedlot of *P. tecunumanii* (HE) and the OP families from the Catana orchard were not statistically different for the pitch canker resistance variables, the native provenances control did have somewhat lower dieback than the average of the OP

families (Table 3.9). This would be consistent with the presence of some *P. tecunumanii* x *P. patula* hybrid progeny among the OP families.

Two of the 14 families included in this study derived from selections made in progeny tests in Colombia from open-pollinated families originally collected in the Juquila provenance in the state of Oaxaca, Mexico. In original seed collections by Camcore (NC State University) in 1985, this provenance was classified as *P. tecunumanii* and it was planted in progeny tests with other *P. tecunumanii* provenances. Subsequent morphological research indicated that it was quite distinct from *P. tecunumanii* (Dvorak and Raymond 1991), although it more closely resembles *P. tecunumanii* than it does *P. patula*, *P. herrerae*, or *P. pringlei* (Dvorak et al. 2001; Dvorak 2008). In contrast, molecular studies indicated that the Juquila source is distinct from *P. patula* and *P. tecunumanii*, and more closely related to *P. pringlei*, *P. jaliscana*, *P. oocarpa*, or *P. herrerae* (Dvorak et al. 2001). All four of the latter species are in the *Oocarpae* subsection, and *P. pringlei*, *P. jaliscana*, *P. oocarpa* are in the group *Oocarpa*, while *P. herrerae* is in the group *Teocote* (Price et al. 1998). In an artificial inoculation study at the RSC, the *Oocarpa* species all demonstrated substantial resistance to pitch canker, very similar to low elevation *P. tecunumanii* (Hodge and Dvorak 2000), while *P. herrerae* was quite susceptible ($4 \pm 2\%$ LiveStem). The higher susceptibility of *P. herrerae* may have been influenced by the extremely small size of the *P. herrerae* seedlings relative to other species (Hodge and Dvorak 2000). In the current study, the selections derived from the Juquila provenance ranked 6th and 14th among the 14 orchard families (Table 3.9); this is consistent with a closer relationship with *P. herrerae*, a species with poorer pitch canker resistance.

Six of the 14 families in this study derived from selections made in the provenances Chanal, Napite, and Rancho Nuevo. In a study using OP seedlots of 15 native *P. tecunumanii* (HE) provenances, Chanal and Napite were just slightly below average for pitch canker resistance, while Rancho Nuevo was second to worst (Hodge and Dvorak

2007). Of the six families in this study deriving from those provenances, the Rancho Nuevo family (13-171) ranked fifth. As would be expected, there is a relationship between provenance rankings and family rankings. Among the 15 *P. tecunumanii* (HE) provenances examined by Hodge and Dvorak (2007), the most resistant were Monte Cristo, Rio Chiquito, Montebello, and Chiquival Viejo. In the current study, none of the 14 orchard families derived from the most resistant provenances, so the presence of very resistant families within average provenances (approaching the resistance of low-elevation *P. tecunumanii*) is quite promising.

VI. Conclusions

Artificial screening of high-elevation *P. tecunumanii* families for pitch canker resistance appears very reliable. Heritabilities for infection response variables are high ($h^2 \approx 0.50$ or higher), and there is little family x experiment interaction, so family ranks based on a single greenhouse experiment are expected to be precise. There is large genetic variation among families, and selection of the most resistant families should result in important genetic gain. Any organization planting high-elevation *P. tecunumanii* commercially should consider screening their genetic material using this type of approach.

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