Abstract

TIGHE, MICHAEL EDWARD. Comparison of 5 Pollen Storage Protocols for Subtropical Pine Species. (Under the direction of Dr. William S. Dvorak).

Storage of pine pollen for breeding and conservation purposes is essential to conserve valuable genetic material over time. Customized storage protocols are often necessary to address differing strategies and program goals for specific pine species. Subtropical pine pollen has been and will be important for forestry programs around the world, and the evaluation of pollen storage protocols for these species permits the determination of successful strategies to maintain viability over time. Germination of pollen grains using *in vitro* agar gel methodology has been shown to successfully predict *in vivo* germination of pollen tubes. Five methods commonly used for temperate pine pollen storage were evaluated for their efficacy for maintaining temperate (*P. taeda, P. radiata*) and subtropical species (*Pinus tecunumanii, P. oocarpa, P. maximinoi*) pollen after 6 months.

For best results, cryopreservation at -196°C should be used for subtropical pine pollen storage if available. When reliable resources for cryopreservation are limited, direct freezing at -20°C is recommended. Like the *P. taeda* and *P. radiata* pollen evaluated in this study, subtropical pine pollen exhibited increased *in vitro* germination rates when exposed to the lower temperatures evaluated after 6 months in storage. Temperate species pollen storage protocols evaluated in this study were used successfully for subtropical and tropical pine species.

The 3 subtropical pine species' pollen assessed diverged from temperate pollen behavior when only prestored pollen is considered. Cryogenic and direct freezing storage methods yielded higher *in vitro* germination rates for subtropical species than temperate

species (*P. taeda* and *P. radiata*). Vacuum drying/freezing and refrigeration, however, yielded higher *in vitro* germination rates for temperate species. Additional research into the physiology of these differences and the correlations with evolutionary phylogeny information is recommended to determine the cause of these differences.

Key words: subtropical pines, pollen storage, pollen cryopreservation, *in vitro* germination

COMPARISON OF 5 POLLEN STORAGE PROTOCOLS FOR SUBTROPICAL PINE SPECIES

by

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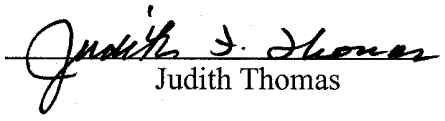

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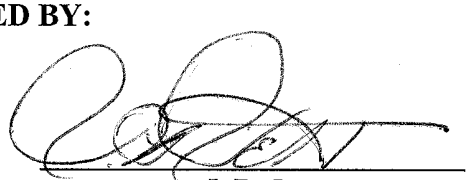

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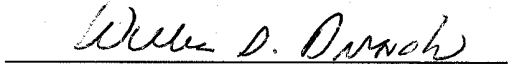

Chair of Advisory Committee
William S. Dvorak

Dedication

For Kirsten, my family, and loved ones,
who have patiently awaited the accomplishment of this dream as much as I have.

Biography

Michael Tighe was born on April 1, 1971 in Gaylord, Michigan, number 6 of 9 children. Soon thereafter, the family moved to the Upper Peninsula of Michigan where Michael spent his childhood. He attended Escanaba High School and graduated in 1989.

After high school, the author attended Michigan Technological University. He graduated in 1993 with a Bachelor's of Science in Forestry, and an Advanced Foreign Language Certificate (equivalent to a minor) in Spanish.

During college summers and for short periods after graduating, the author worked for various public and private entities in forestry-related positions. Among these were: the U.S. Forest Service in Custer, South Dakota and Sitka, Alaska, the City of LaPorte, Indiana, and the former MeadPaper Corporation in Escanaba, Michigan.

In 1994 Michael embarked on a journey to combine his interests in forestry and languages by joining the Peace Corps as an agroforestry volunteer in Paraguay. There he worked with small-scale farmers in agroforestry systems, soil conservation, citrus grafting, and beekeeping. In 1998 he accepted a short-term position with the U.S. Crisis Corps (a branch of the Peace Corps established in the 1990s to assist Peace Corps countries after natural disasters) in the region of Jinotega, Nicaragua soon after the devastation of Hurricane Mitch.

In 2000 Michael returned to the States and began graduate studies in applied forest genetics at North Carolina State University. In addition to his graduate degree program, in 2001 he began working full time for the Camcore Cooperative, where he is currently employed as a Research Forester.

Acknowledgements

I would like to thank all my committee members for their enthusiasm and persistence in seeing this project through. My special gratitude to Dr. Bill Dvorak, whose consistent motivation and ambition made this project possible.

I am especially grateful to the Camcore Cooperative for their funding and assistance in all aspects of this project. Representatives from Camcore-member companies such as Norha Isaza and Luis Osorio (Smurfit Cartón de Colombia) and Christine TeRiini and Andrew Rodwell (Trees&Technology) provided invaluable help on the collection and timely shipment of pollen.

I would like to extend a special thanks to members of the Camcore staff in both the Raleigh and Guatemala City offices. Dr. Gary Hodge and Jose Luis Romero (formerly of Camcore) provided daily encouragement and assistance that made this project happen from its very humble beginnings. Camcore staff members Willi Woodbridge, Juan Lopez, and Andy Whittier in Raleigh all helped in ways large and small to assist in the project. Mr. Elmer Gutiérrez and Mr. Mauro Gomez from the Guatemala City office provided essential knowledge, support, and encouragement during the pollen collection trip and all subsequent communication. Their encouragement and dedication led to the selection of this subject for my thesis.

Great appreciation to the staff of the NCSU Tree Improvement Cooperative for the use of many tools and pollen for this project. Dr. Bruce Zobel, Dr. Steve McKeand, and Dr. Bailian Li all provided support for this endeavor, while discussions and long pollen germination sessions with Mr. Mike Jett provided invaluable feedback and problem solving over the past 2 years.

Lastly, I would like to acknowledge the vital support, patience, and encouragement I received from Kirsten Cassingham. Her love and understanding during this challenge made the long hours worthwhile.

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I. Literature Review

The storage of pollen is essential for success in tree breeding programs around the world. Tree breeders use pollen to cross genotypes with desirable characteristics in order to maximize productivity, disease resistance, cold tolerance, drought resistance, or to improve wood quality. The rise in efficiency of intensively managed plantations using these products of tree breeding increases the supply of wood fiber to meet societal demands through the creation of compensatory plantations or "replacement forests," while easing reliance on natural stands for these products (Zobel, *et al.*, 1987). Enhanced productivity through tree breeding, coupled with efficient silvicultural practices, relaxes the wood fiber production burden for natural areas and is a necessary tool for achieving sustainability of this renewable resource.

Effective tree breeding is achieved through controlled pollination applied with an understanding of the reproductive biology of the tree species. To achieve controlled pollination, the male contribution to the cross is obtained by collecting the pollen of a desired individual. The selected pollen is then manually applied to a designated female strobilus to promote fertilization. Individual female reproductive organs are pollinated and subsequently shielded from foreign pollen entry to produce cones. Selected crosses of individuals with advantageous attributes can produce progeny with the desired characteristics, and later form the basis for a breeding population.

Logistical arrangements and pinpoint timing are necessary for successful crossing. Natural adaptations, climactic differences, and evolution have combined to create a complicated system for the timing of natural pollen production and flower receptivity. Research shows that peak pollen shed and greatest flower receptivity are usually simultaneous in pines (Boyer, 1981) (Bramlett, 1973). Variation of pollen production and receptivity even between individuals within the same orchard, however, can limit crossing or fertilization during open pollination. For successful crossing, pollen must be collected, extracted, stored, tested, and applied using the most efficient methods available. Better pollen quality results in better seed set, rather than the application of a higher quantity of pollen as previously practiced in many programs (Matthews and Bramlett, 1986). Pollen quality is defined as the combination of pollen viability (ability to germinate *in vitro*) plus pollen vigor (ability to germinate, grow, and set seed *in vivo*) (Moody and Jett, 1990) (NCSU-TIP, 2003). The combination of pollen quality and correct timing for pollination activities (understanding the reproductive biology) allows the tree breeder to make effective crosses and produce progeny of known parentage.

Reproductive buds are initiated during the growing season before the microsporangiate (male) and megasporangiate (female) strobili appear in pines. In pines, pollen is bisaccate and produced in the microsporangiate strobili (pollen sacs), at the base of the catophyll (Owens, 1993). Microsporangiate strobili are usually produced on slower-growing branches low in the crown of the tree, a natural evolution to avoid self-pollination. Female strobili are produced higher in the crown of the tree, and although conifers do not produce true female flowers (Nel, 2002), the term is a common descriptor among tree breeders to refer to the megasporangiate strobili.

The reproductive buds develop into megasporangiate ("female") or microsporangiate ("male") strobili over their first growing season. Ambient air temperatures, elevation above sea level, and precipitation are the most important factors in pollen development and dispersal. Strobili development increases in pace during the rainy season after dormancy, and pollen dispersal begins during the following dry season (dependent on temperature and moisture levels). These cycles are altered greatly in tropical environments, where species may produce male and female flowers year-round (Isaza, personal communication 2003) (Mirov, 1967), although peak production and receptivity times hastened by weather events (drought, seasonal rains) exist during the year that may provide better seed quality during certain periods.

The timing of peak pollen shed and peak flower receptivity are usually simultaneous in pines, although the date of occurrence for these peaks may vary significantly from year to year. Below-average temperatures slow pollen development, while above-average temperatures advance development for most species. Male flowers develop and ripen during summer or dry periods until the moisture level drops to a threshold value where the strobili begin to open. Pollen scales open and release pollen, which is dispersed by wind in pines (anemophilous species).

Pollen is dispersed from the male strobili and adheres to the pollen drop on the micropylar arms of the female strobilus. This indicates that pollination has occurred. When the pollen grain adheres to the pollen drop and is drawn into the ovule, the droplet is reabsorbed into the micropyle and the grain encounters favorable conditions for germination *in vivo*. The grain is germinated, and begins pollen tube development. The tube elongates until nearly reaching the

female gametophyte, stopping when it reaches a region of undifferentiated cells near the nucellus. Next, the pollen grain enters a period of slowed development, until approximately one year (for most species) has passed since the initial development of the strobilus. Then, the pollen tube begins rapid elongation 10-14 days fertilization. The tube reaches into the archegonia and discharges sperm. Fertilization has occurred, and the ovule begins embryogenesis. Seed is formed in the cones, completing the reproductive cycle.

In addition to timing of pollen shed and female strobilus receptivity, many additional obstacles complicate the effectiveness of controlled pollination. Large-scale tree improvement programs require extensive crosses each year, and tree breeders seldom have sufficient time to collect and process fresh pollen before the peak flower receptivity period passes (NCSU-TIP, 2000). Crosses may be performed using pollen from international germplasm exchanges or purchases, produced on separate continents with differing flowering and pollen flow periods. These controlled crossing systems require shipment of live pollen which may require storage until the mother trees are receptive for pollination activities. In industrial applications, species are often grown in plantations outside their native range and thus will not produce seed at certain elevations (Dvorak *et al*, 2000). Pollination must take place in other areas, requiring storage of the pollen for transport. Pollen of desirable polymixes can also be stored and used for crosses in subsequent years, with the amount and quality of available pollen known in advance (Bramlett and Matthews, 1991). This may result in storage of the pollen for more than one year in order to a) re-cross the pollen with the same flowers if the pollination was not successful, b) use the same good quality pollen for future crosses due to good viability, or c) for purposes of gene conservation. Additionally, many

laboratories are experimenting with the establishment of pollen banks for biotechnology purposes. Successful long-term storage of pollen requires little area, but preserves a full haploid compendium of genes for future gene transformation and can account for provenance variation by conserving pollen from throughout the species' range (Barnábas and Kovács, 1997) (Wang, *et al.*, 1993).

Research into controlled pollination practices and pollen storage has increased significantly with the advent of intensively managed plantations of species such as *Eucalyptus*, spp., *Pinus taeda*, and *Pinus radiata* in the 1930's (Bramlett and O'Gwynn, 1981). Private organizations endeavoring to increase yields in plantations performed proprietary research and also joined cooperative research groups aligned with research universities and federal agencies to study the potential of tree breeding. Through this effort, these species were studied extensively and their pollen management protocols were identified. The United States Forest Service Research Stations, private corporations, and land grant universities began studying pine pollen extensively in the 1950s. While some significant contributions were made prior to this, these first decades saw significant interest in tree improvement and the potential for high-yielding pine plantations both in the U.S. and worldwide. Early research into the genetic variation of species and tree improvement techniques was successful with pines because of the simplicity, stability, long life, and the prolific nature of gymnosperm pollen (Harrington, 1970). Detailed research began in the 1950'son *Pinus taeda* and *Pinus elliottii* in the U.S. Southeast region, as well as *Pinus radiata* and other species both in the U.S. and abroad to determine controlled pollination techniques and thereby increase plantation yields from areas planted with improved stock.

Before focusing on pollination techniques, the best collection and extraction techniques for processing pine pollen from the field to the laboratory were identified. Accepted standards for collection involve the gathering of microsporangiate strobili when pollen at the base of the most advanced individual strobili begins to shed (Beers, *et al*, 1981). Bramlett and Bridgwater (1989) examined the stages of strobilus development based on the female flower development system, and created the pollen development classification system (PDCS) which identifies preferred pollen collection stages more closely related to maturation (see Appendix 1). Rather than wait until the strobili begin to open and the corresponding loss of pollen, the PDCS allows the breeder to better identify pollen collection times to maximize viability. The moist pollen grains borne in the microsporangiate strobili are placed in kraft paper bags and subjected to elevated temperatures and air movement to facilitate opening of the pollen scales and the release of the pollen grains. Rustic methods with electric fans are effective for pollen extraction, as well as forced-air drying in a laboratory oven at 29-30°C (NCSU-TIP, 2000, also Appendix 2). Protocols for sophisticated pollen extraction and drying systems have also been identified using temperature-humidity controls (Jett *et al.*, 1993). These systems require some investment, but are very effective and currently used by industry and research programs in many countries.

From this early research it was determined that an accurate method to test pollen viability and ability to fertilize ovules was required. The evidence of seed set *in vivo* is the only sure method to assess pollen quality (Bajaj, 1987) (Moody and Jett, 1990). However, pollen viability must be determined before pollination to efficiently utilize the resource. Various *in*

vitro pollen germination tests have been developed to assess pollen viability and the effects on pollen vigor and resulting seed set. Germination tests sought to mimic conditions important for natural germination such as temperature, pH, oxygen, osmotic pressure, moisture, cations, anions, and carbohydrates (Stanley, 1967).

Initial testing procedures involved the selection and exclusion of individual chemicals or elements in the pollen growth medium thought to be vital for pollen term germination and growth. Many of these methods are detailed in Brewbaker and Kwack (1963), Shivanna and Rangaswarmy (1992), Kearns and Inouye (1993) and Goddard and Matthews (1981). Most methods involve dusting hydrated pollen over a medium containing boron and calcium and other elements, with 10-20% sucrose added as a carbohydrate source for development. The trace elements and sucrose content in the media has differed significantly, including the use of a honey solution for germination that offered better results in some studies (Zobel, B.J personal communication 2001). Methods such as the hanging drop technique, respiration rate analysis, leachate analysis, *in vitro* pollen tube growth, and nuclei staining (FCR test, blue dyes) have all been evaluated and proven effective for *in vitro* viability determination in many genera (Moody, 1988) (Heslop-Harrison and Heslop-Harrison, 1970) (Heslop-Harrison, *et al*., 1984) (Shivanna *et al*., 1991) (Rao, *et al*, 1992) (Shivanna and Rangaswarmy 1992). Additionally, *in vitro* pollen germination methods and media vary immensely by tradition and species tested. Correlation of *in vitro* and *in vivo* germination rates is essential to the success of any pollen testing program, and has been variable according to the species tested. Pollen viability, however, seems to be the single most important factor to indicate high seed yield per cone after pollination (Matthews and Bramlett, 1986).

A simple, inexpensive *in vitro* germination test used by many tree breeders employs the agar gel method. This method of germination evaluation was first identified by Goddard and Matthews (1981) and used in many subsequent experiments by other researchers (Moody and Jett, 1990) (Jett, *et al*., 1993) (Siregar and Sweet, 2000). This is the most commonly used method for checking pollen viability due to its relative simplicity and resulting quantitative values (Barnábas and Kovács, 1997). Pollen samples to be evaluated are spread thinly in a petri dish, rehydrated, then dusted evenly over a 0.5% wt./vol. distilled water medium. After 48 hours in incubation at 29°C-30°C, pollen tubes are evaluated for elongation and counted as "germinated" if length of tube exceeds the pollen grain diameter (Moody, 1988) (Bowes, 1990). After counting 200 grains, pollen resulting in 80% or better germination is considered excellent, while pollen down to 10% is considered usable by some (Bramlett, *et al.*, 1985). The agar gel method minimizes the rate of water uptake during germination. Rapid uptake can result in imbibational damage using other methods, which makes the agar gel method preferable (Hoekstra, 1992). In tests on pine pollen at NC State University, Moody and Jett (1990) found a high correlation between agar gel *in vitro* germination tests and *in vivo* seed set in *Pinus taeda* (coefficient of determination R^2=0.79). Webber and Bonnet-Masimbert (1993) obtained similar results using 3 viability assays on *Pseudotsuga menzesii*. Various *in vitro* germination tests were evaluated and agar gel germination was deemed an effective method of assessing *P. taeda* pollen viability and predicting seed set after pollination. Research has shown *in vitro* germination methods to be a good predictor of pollen viability and seed set in several species such as loblolly pine (Moody and Jett, 1990), and Monterrey pine (Siregar and Sweet, 2000) (Setiawati, 1994). This method of pollen testing is currently

employed at NC State University's Tree Improvement program due to its simplicity, quantitative nature, and correlation with seed set in *P. taeda*.

With reliable pollen viability testing procedures in place, effective methods for pine pollen storage were assessed. The benefits of tree improvement were well known, but previous controlled pollination activities employed only current years' pollen or anecdotal storage protocols before reliable pollen testing procedures were developed to measure their effectiveness. Experiments to assess the factors controlling pollen viability and vigor determined that moisture content (%MC) and storage temperature were the most important factors in determining pollen viability (Snyder and Clausen, 1974).

Importance of pollen storage

The differing objectives of tree breeders require different durations of storage for their pollen needs. Generally, the longer the storage period desired, the lower the storage temperature used (Siregar and Sweet, 2000). Species planted in the tropics and subtropics as exotics may show differences in peak pollen flow and peak flower receptivity, obliging the breeder to store the pollen until flowers reach target receptivity. This is typically a short-term storage situation of several months (up to 6 months). Other programs may have precocious pollen flows or flowering, or may simply develop a desirable polymix that they would like to replicate the following year. Programs that are unsuccessful in taking advantage of the appropriate biological pollination window and who must wait for the following period constitute medium-term pollen storage (6-18 months). Long-term storage (18 months or more) involves storage of live, dormant pollen for years, and is often involved with conservation efforts or pollen banking for safeguards.

1. Short to Mid-term storage needs

Storage temperature has a great effect on pollen viability in pines. Generally, the longer the desired length of storage, the lower the storage temperature. The main cause of pollen viability loss appears to be the continued metabolic activity of pollen in storage. This metabolic activity can be slowed with reduced storage temperatures and moisture levels in many ways (Wilson *et al*., 1979) (Layne and Hagedorn, 1963), allowing pollen to maintain its carbohydrate reserves and consequent viability for several years (Linskens and Pfahler, 1973). For short-term storage of pine pollen, refrigeration at 0-5°C has been used extensively, presumably due to its ubiquity of common household refrigerators that maintain this temperature (see Table 1). This strategy results in effective storage for up to approximately 1 year, but then tends to decrease rapidly thereafter. Siregar and Sweet (2000) report, "There is no advantage to storing pollen dried to 10%MC at -20°C (freezing—*author*) if only one year's storage is involved." Refrigeration is the best method for short gaps between pollen availability and flower receptivity or for crosses performed in areas distant from pollen collection areas.

Table 1. Short-term pine pollen storage temperatures in published studies.

Researcher	Year	Species	Refrigerated Storage Temperature
Duffield	1954	*Pinus radiata*	5°C
Mergen *et al.*	1955	*Pinus elliottii*	4°C
Duffield and Callaham	1959	*Pinus, spp.*	4°C
Callaham	1966	*Pinus, spp*	4°C
Nygaard	1969	*Pinus mugo*	4°C
Snyder and Clausen	1974	*Larix*, spp, *Thuja*, spp., *Pseudotsuga*, spp.	0-5°C
Sprague and Johnson	1977	*Pinus taeda*	2°C
Matthews and Kraus	1981	Southern pines	4°C
Matthews and Bramlett	1982	*Pinus taeda*	2°C
Matthews and Bramlett	1986	*Pinus taeda*	3°C
Moody and Jett	1990	*Pinus taeda*	3.3°C
Bramlett and Matthews	1991	*Pinus taeda*	3°C
Mercier	1995	*Pinus* spp., *Picea* spp., *Larix*, spp.	4°C
Vergara, *et al.*	1995	*Pinus radiata*	4°C
Siregar and Sweet	2000	*Pinus radiata*	2-4°C
Towill	2002	"tolerant species"	4°C

For medium to long-term storage of pine pollen, -18 to -21°C has been the standard temperature, presumably due to its ubiquity in household freezers. The most common storage method is achieved through freezing at -20°C (Duffield and Callaham, 1959) or vacuum drying/freezing (decrease %MC under vacuum, then store at -20°C). This practice is used operationally on many species worldwide (see Table 2). This is the best method if the pollination season is missed; as a simple reserve of pollen if environmental factors or user error resulted in poor pollination success the previous year.

Table 2. Medium-term pine pollen storage temperatures in published studies.

Researcher	Year	Species	Freezer Storage Temperature
Duffield and Callaham	1959	*Pinus,* spp.	-20°C (freeze dried)
Callaham and Steinhoff	1966	*Pinus,* spp.	-20°C (freeze dried)
Snyder and Clausen	1974	*Larix,* spp, *Thuja,* spp., *Pseudotsuga*, spp.	-20°C (freeze dried)
Sprague and Johnson	1977	*Pinus taeda*	-20°C
Matthews and Kraus	1981	Southern pines	-20°C (freeze dried)
Braggio, *et al.*	1990	*Pinus sylvestris*	-20°C
Bramlett and Matthews	1991	*Pinus taeda*	-18°C
Lanteri, *et al.*	1993	*Picea abies*, *Pinus,* spp.	-18°C
Connor and Towill	1993	*Pinus ponderosa*	-20°C
Bonnet-Masimbert and Webber	1995	Conifers—*Pseudotsuga menzesii*	-20°C
Towill and Walters	2000	"dessication-tolerant"	-20°C
Siregar and Sweet	2000	*Pinus radiata*	–20 to -25°C

2. Long-term storage needs

To achieve long-term storage with acceptable viability, pollen may be frozen or vacuum dried/frozen at -20°C in the same manner as medium-term storage. This has proven a reliable method for long-term storage in many species, and is readily available to most foresters worldwide (Hanna, 1994) (Duffield and Callaham, 1959) (NCSU-TIP, 2000) (Towill and Walters, 2000) (Jensen, 1964) (Sprague and Johnson, 1977).

Another effective manner used extensively for pollen storage in agriculture is cryogenic preservation. This method is best used for pollen purchased or collected at great expense, or pollen desired for long-term or permanent conservation activities. Many species' pollen have been successfully stored in cryopreservation, and a list of these species is provided in Hanna

and Towill (1995). A short list of conifer pollen stored cryogenically in published studies is shown in Table 3. Cryopreservation can be performed using ultrafreezers or cryogenic refrigerators. Ultrafreezers generally store the pollen at -80°C, while the cryogenic refrigerators use liquid nitrogen in either the immersion or vapor phase to achieve -196°C or -130°C, respectively. Both ultrafreezers and cryogenic refrigerators are effective storage structures, with one method requiring electricity to maintain the temperature (power outages could alter temperatures), and the other constant refilling to insure pollen stays immersed. For long-term cryogenic storage of pollen, liquid-phase immersion in liquid nitrogen at –180 to –196°C has been the standard. Although vapor phase storage at -130°C and the newer implementation of ultrafreezers at –80°C are also used (Towill, 2002), the majority of research has been with liquid immersion at -196°C.

Thawing and freezing methods are also essential, and slow-freeze and slow-thaw methods show better results, as suggested in Matthews and Kraus (1981). These findings on subtropical and temperate pine species are consistent with pine pollen cryopreservation results obtained at the National Tree Seed Laboratory (Connor and Towill, 1993) on *Pinus ponderosa*.

Table 3. Cryogenic storage of pine pollen in published studies.

Ichikawa and Shidei	1972	*Pinus,* spp., *Larix,* spp., *Cryptomeria,* spp.	-196°C
Jorgensen	1990	*Pinus sylvestris*	-196°C
Lanteri, *et al.*	1993	*Picea abies, Pinus,* spp.	-196°C
Mercier	1995	Conifers	-196°C
Bonnet-Masimbert and Webber	1995	Conifers—*Pseudotsuga menzesii*	-196°C

3. Influence of Moisture Content on Pollen Storage

Maintaining low moisture levels is the key to successful pollen storage (Jett and Frampton, 1990). Moody (1988) found that typical pine pollen should be less than 10-15% moisture content (%MC) to avoid significant pollen deterioration in storage (also cited in NCSU-TIP, 2000). Other recommendations range from 7-20 %MC (Table 4), depending on the species and desired length of storage (Snyder, 1957) (Jett and Frampton, 1990) (Bonnet-Masimbert and Webber, 1995) (Hoekstra, 1992) (Lanteri *et al*., 1993) (Siregar and Sweet, 2000). Hoekstra (1992) explains that respiration in pollen grains from numerous horticultural crops is undetectable at <20%MC. He also explains that at 10-15%MC there is no free water left for intracellular transport. If pollen is physiologically active in any method of storage, the viability declines quickly and all storage practices will be rendered useless. However, Connor and Towill (1993) state "if moisture is reduced so that at least the freezable water is removed, storage at low temperatures should be possible".

Moisture content control is essential for successful pollen storage. For most pine pollens, 6-8%MC is recommended for refrigerated storage, but frozen or cryogenically stored pollen appears to maintain viability longer when stored at 3-5%MC. Pollen moisture content below 16% also guarantees that pine sawfly larvae (*Neodiprion*, spp. or *Xyela*, spp.) if present, will be killed (Sprague and Snyder, 1981). Higher moisture content in storage also favors damaging fungal and bacterial activity, which can be suppressed for organic substrates if moisture content is at or below approximately 14% (Snyder, 1957) (Stanley and Linskens, 1974). The longevity of stored pollen is increased when the moisture content is decreased.

Through experiences at Camcore at North Carolina State University, tropical or subtropical pine pollen with moisture contents greater than or equal to 10% should be dried before storage to maintain viability. Many breeding institutions have reduced pollen moisture content with pine species through drying over silica gel (Sprague and Snyder, 1981) or freeze drying (Ching and Ching, 1964) (Layne and Hagedorn, 1963), but pollen must be closely monitored to avoid overdrying. Pollen moisture can also be reduced using simple methods such as line drying in kraft paper bags or low-heat drying in a forced-air oven. Jett and Frampton (1990) found that the pollen moisture regime closely mirrors that of wood samples, and the required drying times are comparable. Generally, drying pollen from 14%MC to 5%MC takes approximately 6 hours in a forced-air oven at 29°C (NCSU-TIP Coop., 2000). Pollen should be monitored during drying, and moisture content should be measured before removing samples from oven.

Table 4. Moisture contents of stored pine pollen in published experiments.

Researcher	Year	Species	Moisture Content in Storage
Duffield	1954	*Pinus radiata*	10,25,50,75%MC
Mergen *et al.*	1955	*Pinus elliottii*	15%MC
Ching and Ching	1964	*Pseudotsuga menzesii*	2-5%MC
Nygaard	1969	*Pinus mugo*	8.9 %MC
Snyder and Clausen	1974	*Larix*, spp, *Thuja*, spp., *Pseudotsuga*, spp.	less than 10-14%MC
Sprague and Johnson	1977	*Pinus taeda*	6-9%MC
Matthews and Kraus	1981	Southern pines	about 9%MC
Goddard and Matthews	1981	*Pinus taeda*	9-10%MC
Matthews and Bramlett	1982	*Pinus taeda*	2-3%MC
Bramlett, *et al.*	1985	*Pinus taeda*	8-10%MC
Moody	1988	*Pinus taeda*	2%MC
Moody and Jett	1990	*Pinus taeda*	6-8%MC
Bramlett and Matthews	1991	*Pinus taeda*	<10%MC
Connor and Towill	1993	*Pinus ponderosa*	4-9%MC
Jett, *et al.*	1993	*Pinus taeda*	<10%MC
Bonnet-Masimbert	1995	Conifers—*Pseudotsuga menzesii*	7-8%MC
Vergara, *et al.*	1995	*Pinus radiata*	6-8%MC
Siregar and Sweet	2000	*Pinus radiata*	7-10%MC
Towill	2002	"tolerant species"	5-10%MC

II. Introduction

With increased global competition in the forest products industry, many regional and international industry leaders are making significant investments in forest plantation projects in the southern hemisphere due to tremendous growth rates and low costs of production. The profitability and advancement of these projects in the tropical, subtropical, and temperate zones depends largely on the success of breeding programs and silvicultural techniques.

Many of the most successful of these programs and plantation projects have been established in Latin America. The successes in Chile, Brazil, Argentina, Colombia, and Venezuela, for instance, have established these countries as serious competitors and often leaders in the forest products industry worldwide.

These countries offer diverse environments and economic potential due to their locations and topography. Consequently, the organizations in these countries cultivate species ranging from short-rotation hardwoods for pulp and paper to longer-grown pines for utility poles and lumber. Many companies are also diversifying their species portfolio in these plantations to hedge their risks and improve yields. This often occurs through the establishment and evaluation of pilot plantations of exotic species, or through hybrid creation for increased yields or pathogen resistance. Successful hybrid creation requires a detailed understanding of the phenology and reproductive characteristics of each species, especially pollen management and flower development.

While the pollen management protocols for hybrid breeding in eucalypts and other hardwoods are well documented, little has been written regarding tropical and subtropical pine species pollen management. Many studies have been published on temperate species with advanced breeding programs in place (e.g. *Pinus radiata*, *P. taeda)*. Temperate pine species naturally have long breeding cycles to produce improved seed and assess the success of the cross, although some advanced breeding programs have shortened these cycles through intensive breeding. Tropical and subtropical pines have exhibited reproductive cycles as short as 12-14 months in warmer climates, enabling rapid assessment of genetic gains and shortened

breeding cycles if combined with intensive breeding methods (Dvorak, *et al.*, 2000). Many of these temperate and subtropical species are centered in industrial applications where species are grown in plantation on similar sites but often outside their native range, and thus will not produce seed at certain elevations. Storing pine pollen is especially important for cultivating exotics, due to the irregular flowering times of different species and provenances. They may coincide with or be totally opposite the flowering times in natural stands and may also result in asynchronous flowering in monoecious species (Young, 1990).

To address pollen storage concerns with pines planted as exotics, Camcore at NC State University began research in 2001 into the efficacy of pollen storage protocols for a variety of pine species. Camcore's mandate is for conservation and tree domestication worldwide, and *ex situ* conservation is a cornerstone of the program. Endangered and threatened species' seed and pollen is collected and conserved by the establishment of tree conservation banks protected on the lands of members around the world. After *ex situ* plantation establishment and protection, tree species are evaluated for their production value and their seed or pollen may be used for additional plantation diversification, hybridization, or selective breeding. Problems with differing flowering times compared to natural stands, asynchronous flowering, and the general challenges of forest management with exotic species are exacerbated when attempting interspecific hybridization with species from different continents. These are of special importance to achieve hybrids with intermediate characteristics or to assess the effects of hybrid vigor. The need exists to assess pine pollen storage protocols developed for temperate species and evaluate their effectiveness for subtropical pine pollen as used by Camcore members around the world.

Three of the subtropical pine species that have been extensively studied by Camcore and also express economic potential are *P. tecunumanii* Eguiluz & J.P. Perry, *P. oocarpa* Schiede ex Schlectendal var. *oocarpa*, and *P. maximinoi* H.E. Moore. These species' native ranges extend through Mesoamerica on a variety of sites, often overlapping geographically and across elevations. *Pinus tecunumanii* occurs from the mountainous section of Chiapas, Mexico through central Nicaragua in two distinct elevation patterns (Dvorak *et al.*, 2000). Populations of high elevation *P. tecunumanii* exist from 1500-2900m altitude, and low elevation populations are found commonly at 450-1500m. Due to human encroachment and exploitation however, individual provenances of this species are considered vulnerable to critically endangered in 90% of its native range using The World Conservation Union guidelines on conservation categories in Farjon and Page (1998). *Pinus oocarpa* var. *oocarpa* is the most common pine in Mesoamerica, occurring from Mexico to Nicaragua. Although it has been consistently exploited for decades, many populations exist and will continue to exist simply due to the extent of its range. *Pinus oocarpa* var. *oocarpa* populations throughout Central America, however, are considered vulnerable. *Pinus maximinoi* also occurs naturally from Mexico to Nicaragua, and is considered low risk in most of central Mexico but vulnerable to critically endangered from southern Mexico through Nicaragua. The desirable characteristics of these species (pest resistance, drought tolerance, etc.), combined with their adaptability and favorable wood properties, have increased their popularity as species for plantation diversification and breeding in Latin America.

The goal of this research is to test 5 methods of pine pollen storage commonly used for maintaining *Pinus taeda* pollen and evaluate their efficacy after 6 months on both temperate

and subtropical pine pollen. The objective is to assess these species using *P. taeda* testing procedures employed by NCSU-TIP. A secondary goal is to determine whether or not subtropical pollen showed the same patterns as the temperate species. Each species was evaluated under 5 storage methods: open-air storage, refrigeration, freezing, vacuum drying/freezing, and cryogenic storage. After storage, pollen was subjected to agar-based germination tests to determine viability.

Pollen was collected from distinct sources with differing collection methods and standardized to assess their comparative germination ability. The 6-month storage period does not attempt to explain the treatment effects on permanently stored pollen, but rather offers an indication of best storage methods to use for the tree breeder's specific needs. With this comparative information, the tree breeder can compare the similarities and differences between subtropical and temperate pine pollen behavior and evaluate these to meet the specific needs of their breeding program.

III. Preliminary studies to assess pine pollen storage methods

To learn more about subtropical pine pollen conservation and storage ability, pollen was collected and stored under several protocols. Pollen was germinated *in vitro* using agar-gel methodology after 6 months storage to assess its viability for medium- to long-term storage. Subtropical pine pollen was compared to temperate pine pollen behavior in storage to determine whether *P. taeda* pollen protocols are adequate for these species.

Pilot study 1.—Basic storage protocols

In 2001, a pilot project was initiated to assess pollen storage methods for subtropical pine species. A literature search of potential methods suggested vacuum drying and freezing as viable methods for long-term pollen storage. However, little information was discovered which assessed the behavior of tropical and subtropical pine species under this system. In order to draw comparisons based on *P. taeda in vitro* germination results at NC State, the protocols listed in the NCSU-TIP Tree Improvement Handbook (2003) were used to assess *in vitro* germination of the subtropical pine species. A preliminary study was initiated at NC State University to assess the viability of the two storage methods (vacuum drying/freezing and direct freezing) and the corresponding germination loss over time.

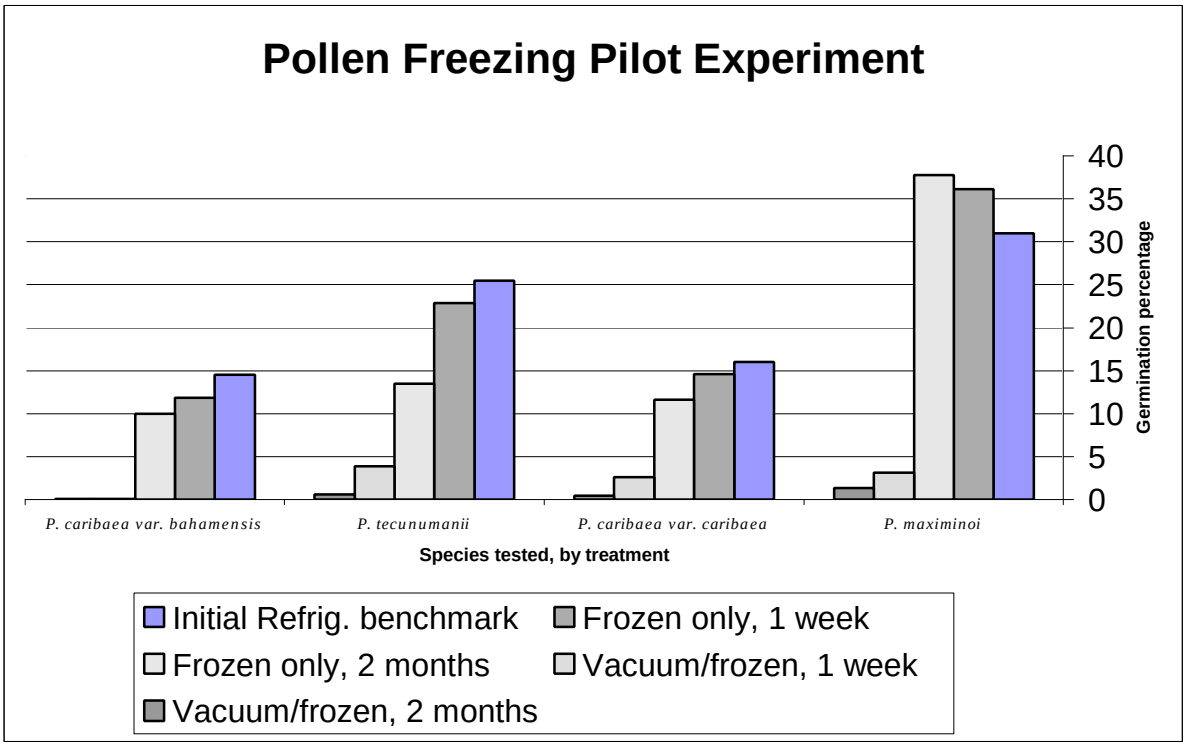


Figure 1. Results of pilot pollen freezing experiment with subtropical pine species.

For this experiment, 64 vials were filled with approximately 1cc of pine pollen harvested in 2001 with moisture content of 3-6%. One quarter of these vials were filled with the pollen from each of the following species: *Pinus maximinoi*, *P. caribaea* var. *caribaea*, *P. caribaea* var. *bahamensis*, and *P. tecunumanii*. Of these 16 vials per species, 8 were frozen directly and 8 were vacuum dried and frozen. The two treatments were then divided in half, with 4 vials of each treatment assessed at 1 week, and the other 4 vials assessed at 2 months, e.g. 4 vials of *P. tecunumanii* were vacuum dried/frozen and assessed after 1 week while 4 others of the same species and treatment were assessed at 2 months.

The data suggest that the vacuum drying/frozen treatment was not successful for the pollen from these species under the protocols designed for *P. taeda* (Figure 1). However, direct freezing seems an effective storage mechanism for all species tested, showing a maximum germination loss of 12% after 2 months and actually improving the germination of *P. maximinoi*. The results of this experiment and the dearth of public information available on this subject demonstrated the need for more research.

Pilot Study 2—Cryogenic Storage protocols

For the cryogenic preservation component of this experiment, storage protocols were less documented and were therefore examined before the initiation of the experiment. Because the cryogenic component was included to account for long-term storage options for pine pollen, the more extreme technique of liquid nitrogen immersion (-196°C) was selected. The options for immersion were assessed, and through consultations with various scientists and laboratory equipment suppliers it was determined that a cryogenic refrigerator would allow for this

method of storage. However, the majority of studies using cryogenic storage of pine pollen used the vapor phase of liquid nitrogen (-130°C) and therefore the protocols for liquid immersion phase had to be identified. Manufacturer resources and consultations with various researchers confirmed that for cryogenic storage in liquid nitrogen (LN) immersion, samples must be stored in polypropylene cryogenic vials (e.g. Nunc ^{TM} vials) that allow for expansion and contraction (Towill, Personal communication 2003) (Nalge Nunc International, 2001). Samples are then labeled and immersed directly in LN-filled cryogenic refrigerator. Samples were not supplementally dried before cryostorage, as rapid cooling upon immersion to –196°C prevents ice crystal formation (Sedgely and Harbard, 1993). Liquid nitrogen levels in cryogenic refrigerators must be maintained on the average of 2 times per week with standard laboratory-grade cryogenic refrigerators.

The goals of the initial cryogenic protocol evaluation were to establish the standards to be used in the larger experiment that resulted in the highest average *in vitro* germination percent for the pollen studied. First, the preferred volume of pollen in the cryogenic vials had to be determined; 0.5 ml or 1.0 ml. Second, the question of whether or not the remainder of the vial volume should be filled with non-absorbent cotton or not, as in refrigerated and freezer studies had to be determined. Third, literature from cryogenic equipment makers strongly encouraged the placement of cryogenic vials in specially designed sleeves to avoid rupture by crimping the sleeves and using heat to form the sleeves to the vials. The application of heat to the pollen was questionable, and thus assessed. Next, information exists that slower changes in temperature preserve pollen viability and plant cell integrity, both from warm to cold and vice-versa (Ichikawa and Shidei, 1972) (Kartha *et al*., 1988). Other studies suggest that an

abrupt change in temperature is adequate at least with angiosperm pollen (Bowes, 1990), so the slow freeze/slow thaw method was tested against the Bowes Method for pine pollen. Lastly, many angiosperm, gymnosperm, and agricultural pollen cryopreservation schemes suggest that a cryoprotectant such as glycerol or propylene glycol should be added to the pollen for protection from ice formation and ice-crystal damage during cryogenic storage (Jörgensen, 1990) (Lanteri, *et al.*, 1993. Pollen was assessed without any cryoprotectants simply to determine survival, and simple survival was evidence that the cryoprotectant was unnecessary as suggested in Bajaj (1987).

Samples of *P. taeda* pollen from South Carolina were provided by NCSU-TIP and prepared for a 24-hour liquid nitrogen immersion to test the suggested protocols before actual experiment methods were defined. In all, 30 vials were prepared while varying the content (0.5 ml, 1 ml), cotton or no cotton, heat-treated and non-heated cryo sleeves, then freeze/thaw method (slow method vs. Bowes Method.) Samples were removed from treatment and evaluated using loblolly pine *in vitro* pollen germination techniques.

The results of this pilot cryogenic storage experiment formed the protocols for the larger pollen storage experiment. There were no significant differences between 0.5 ml and 1 ml vial results, 50.3% germ to 51.9%. The slow freeze (prefreeze samples in –20°C 2 hours before cryo immersion) / slow thaw method (place samples in –20C freezer for several hours after cryogenic storage) gave nominally better results, although not significantly better—53.1% germination to 50.5%. Tests of heat shrink treatment vs. non-heated showed no real difference, 50.8% germ to 51.8%. Cotton seemed to have no effect, and subsequent

consultations with other pollen managers suggested that it was inert as expected. Based on these results, it was determined that samples for cryogenic storage should be prepared of 0.5 ml, no cotton is necessary, minimal heat shrink treatment can be applied, and samples should be placed in freezer a minimum of 4 hours at –20°C before and after cryogenic storage at –196°C. After successfully storing and establishing the cryopreservation protocols in the pilot experiment, samples were prepared for the larger experiment.

Based on the protocols identified in the pilot studies, the objectives of this experiment were to:

- Assess various storage methods for tropical and subtropical pine pollen to develop protocols for pollen storage in short, medium, and long term.
- Determine if certain species respond more favorably to specific storage protocols and why.
- Compare tropical and subtropical pine pollen behavior to temperate pollen to determine whether or not temperate protocols can be used.

IV. Materials and Methods

In 2003, a factorial storage experiment was begun to assess 5 storage treatments (Table 5) that represent the varying storage needs of tree breeders.

Table 5. Pollen treatments relative to duration of storage required.

Term	Method of Storage
Immediate storage	Open Air
Short-term storage	Refrigeration
Medium storage	Direct Freezing
Improved Medium storage	Vacuum Drying/Freezing
Long-term storage	Cryopreservation

Pollen of 5 pine species was received from 4 international and domestic sources. Subtropical pine pollen of 3 species each (*Pinus tecunumanii, Pinus oocarpa,* and *Pinus maximinoi*) was received from both Colombia and Guatemala. Pine pollen from temperate regions was received for controls; *Pinus radiata* pollen from Chile and *Pinus taeda* from the NCSU Tree Improvement Cooperative. The pollen arrived in varying conditions, with moisture contents ranging from 5-14%. All pollen was tested for moisture content and *in vitro* germination percent upon arrival, then dried at 29°C in a forced-air oven to standardize the samples at 5-7% MC for storage if necessary. This moisture content range was chosen to meet recommended *P. radiata* and *P. taeda* storage protocols of 6.5-8% (Siregar and Sweet, 2000) (Vergara, *et al*., 1995) (Moody and Jett, 1990).

An *in vitro* germination test was performed on each pollen species from each unique source upon arrival to serve as baseline data for this experiment. The *in vitro* test was performed on a 0.5% wt./vol. agar solution using *P. taeda* protocols developed by the NCSU Tree Improvement Cooperative based on local and published information (Goddard and Matthews,

1981) and correlated to *in vivo* germination by Moody and Jett (1990). Although *in vitro* methods have been unreliable in the evaluation of some species' pollen such as *Acacia*, spp. (Sedgely and Harbard, 1993), the method produced good correlations for seed set in *P. taeda* tests and was therefore selected as the standard test for comparison in this study. Loblolly pine (*P. taeda)* pollen from a seed orchard in Virginia, USA, was included as a standard to allow the comparison of this data to previous tests and in vivo germination rates using well-documented protocols.

To establish the baseline *in vitro* germination data, pine pollen was removed from storage and each bottle was mixed to insure homogeneity of the sample. Pollen was then dusted on the surface of a 7 cm sterile petri dish in a single layer and placed in a plastic hydration chamber. Pollen was hydrated for 2 hours in the closed, water-saturated environment of the hydration chamber to insure reliable germination (Moody and Jett, 1990) (Goddard and Matthews, 1981). While pollen was hydrating, fresh agar solution was prepared using Difco Bacto® granulated agar media, and cooled agar solution was poured into 64 petri dishes to cool. When cool (and after 2 hours in rehydration), pollen petri dishes were removed from hydration chamber and each pollen species/source was dusted carefully over 2 petri dishes and placed in an incubator at 29°C. Two agar germination plates were prepared per sample to avoid biases due to environmental variation within the incubator and avoid errors due to population effects when pollen is dusted non-uniformly. Germination petri dishes were randomized and placed in the incubator for 48 hours, then assessed for germination.

A. Sample preparation and treatments

To prepare the samples, pollen was taken from the 175 ml storage flasks and 0.5 ml was inserted into each Wheaton® vial or Nunc ™ cryogenic vial according to its desired treatment (Table 6). Wheaton-brand 5 ml screw cap vials were used as a standard for 4 of the treatments due to their ubiquity in tree improvement laboratories, although they are not approved for liquid nitrogen immersion and thus Nunc™-brand vials were used for cryogenic treatment (Hanna, 1994). No vial effect was expected between the two vials used due to minimal humidity uptake in Hanna (1994) (moisture content rose from 5%-6% in glass vials after 3 years freezing) and the extreme temperature in cryopreservation (water molecule crystallization prevents uptake). For controlled pollination purposes, smaller vials such as these are used to avoid repeated freezing and thawing pollen grains. This allows the tree breeder to only take the necessary amount of pollen out of storage and not affect the remainder. Repeated freeze/thaw cycles can be detrimental to pine pollen viability (Matthews and Kraus, 1981) (Bramlett and Matthews, 1991).

Pollen from each source was inserted into 36 Wheaton vials, then separated into 9 groups of 4 vials. After filling with 0.5 ml pollen, Wheaton-brand vials were loaded with non-absorbent cotton, topped with a rubber stopper, and closed tightly under a screw top lid. Only vacuum dry/frozen samples received additional treatment—caps were removed and vials were submitted to vacuum drying for 1 hour before being replaced and resealed. For cryogenic samples, 4 Nunc™-brand 1.8 ml roundbottom cryogenic vials were filled with 0.5 ml pollen from each species, then sealed and labeled by species/source. Groups composed of 4 vials were subjected to 5 different storage protocols.

Table 6. Description of treatment methods.

Treatment 1) Immediate storage: open air
Four 0.5 ml samples of 8 species/source combinations were placed in 5 ml Wheaton vials, stoppered, and placed in Bell jar with Drierite™ crystals in bottom on lab bench at ambient temperature (~22°C) for assessment after 6 months.
Treatment 2) Short-term storage: refrigeration
Four 0.5 ml samples of 8 species/source combinations were placed in 5 ml Wheaton vials, stoppered, and placed in refrigeration at 4?C. Six sets of 0.5 ml samples were stored for each species/source combination, and 1 set was evaluated with *in vitro* germination monthly for viability, culminating in the 6-month measure simultaneously with the other treatments.
Treatment 3) Medium storage: direct freezing
Four 0.5 ml samples of 8 species/source combinations were placed in 5 ml Wheaton vials, stoppered, and placed in a -20?C standard laboratory freezer. Each species/source was evaluated with *in vitro* germination after 6 months at end point.
Treatment 4) Improved medium storage: vacuum drying/freezing
Four 0.5 ml samples of 8 species/source combinations were placed in 5 ml Wheaton vials, stoppered, and placed on DuraDry® Vacuum Drier for 1 hour according to NCSU/TIP guidelines, then vacuum-sealed and stored at -20?C in laboratory freezer. Each species/source was evaluated with *in vitro* germination after 6 months at end point.

Table 6. Description of treatment methods (continued).

Treatment 5) Long-term storage: cryopreservation
Four 0.5 ml samples of 8 species/source combinations were inserted in Nunc™ vials and placed in 10-13mm Nalgene CryoSleeve™ tubing to avoid breakage when submitted to liquid nitrogen immersion. Tubing was crimped "sausage-style", and links of 6 Nunc™ vials in tubing were cut and placed in aluminum canes for immersion. Samples were refrigerated for12 hours, then stored at -20?C in a laboratory freezer for 4 hours. Samples were then immersed in Bio-Cane 20™ brand cryogenic refrigerator filled with liquid nitrogen at –196?C. Each species/source was evaluated with *in vitro* germination after 6 months at end point.

Vials from all treatments were entered into storage simultaneously, and assessed after 6 months. Refrigerated samples were comprised of 6 sets of samples, and 1 set was individually removed from treatment monthly to assess *in vitro* germination. The remainder was assessed for *in vitro* germination at 6 months.

B. In vitro germination procedure

Two petri dishes dusted with pollen were prepared on 0.5% wt./vol. agar medium for each sample. After the 48-hour incubation period at 29?C, petri dishes were removed from incubator and assessed for germination. Petri dishes were placed directly on stage of an Olympus CK2 phase-contrast light microscope and evaluated for pollen tube growth at 100X magnification. Pollen grains were considered "germinated" when the length of the pollen tube was equal to or greater than the width of the bisaccate pollen grain. One hundred pollen

grains were assessed randomly in a horizontal scroll across the plate, and the number of germinated grains was recorded. Next, one hundred grains were assessed in a vertical scroll across the plate and the number (%) of germinated grains out of 100 was recorded. These vertical and horizontal scroll numbers were averaged per dish to avoid errors. Pollen grains assembled in groups of more than 3 were not counted, due to a population or mutual stimulation effects documented in several studies (Moody, 1988) (Goddard and Matthews, 1981) (Brewbaker and Kwack, 1963) (Ahlgren and Ahlgren, 1978). The calcium leakage from nearby grains often results in erroneously high *in vitro* germination rates if these larger groups are included (Sari-Gorla and Frova, 1997).

C. Data collection and analysis

Data were recorded from the four samples for each species/source combination and averaged by species/source for trends using Excel©. However, all statistical analyses were performed on a dataset of 160 observations, representing "raw" data from each of the 4 samples per treatment of each species/source combination. These data were subsequently grouped or pooled to analyze the results in different manners.

Three species each (*P. tecunumanii, P. oocarpa,* and *P. maximinoi)* were collected from two different sources (Colombia and Guatemala) and were used for comparison as replications in this analysis to determine differences in pollen sources by species. Considering the 3 species/source combinations, 2 degrees of freedom (df) are garnered from the Colombian-source species, and 2 df from the Guatemalan-source species, yielding 4 df total. A general linear model was applied to these coupled results to test source significance at the a = .05

level. A Duncan's multiple range test was applied to group similar results and demonstrate differences.

Data were evaluated for treatment differences using SAS Statistical Software (SAS Institute, Inc., 1999-2001). For analysis, species are randomly selected within sources and used as an estimate of variability. Distinct pollen species were collected by distinct sources, and therefore do not compose true replications for the purposes of this experiment. Pollen species/source combinations were first evaluated individually to assess their individual characteristics. In order to assess storage method efficacy after 6 months, germination results were scaled according to baseline germination values for each individual species/source combination before analysis to provide a better estimate of treatment effects over time. Some post-storage germination values observed exceeded the original baseline germination and result in values greater than 100%. This phenomenon has also been observed in published studies with various pollen species (*Pinus*, spp. and *Picea*, spp.), but the causes of these effects are difficult to explain and not uncommon in the first months following cold storage (Ching and Ching, 1964) (Lanteri, *et al.*, 1995) (Hohtola, 1995) (Linskens, H.F. as cited in Ching and Ching, 1964). The scaling of the values to the baseline data permits greater insight into the pollen viability trends over the 6-month storage period by directly linking them to the initial germination before storage. All Excel© graphs and analyses were created using scaled data. Next, a general linear model was applied in SAS to assess differences between *in vitro* pollen germination results individually, with a Duncan's multiple range test applied to demonstrate statistically similar treatments.

Finally, all species were compared in a pooled comparison using the dataset in a general linear model where each species/source combination was evaluated against the entire group. This allows for more general comparisons to underscore significant trends within the experiment. A Tukey's Studentized Range test and Duncan's Multiple Range Test were performed on the dataset to observe data groupings.

V. Results

The agar gel *in vitro* germination method (NCSU-TIP, 2003) was used to evaluate pollen germination after 6 months in storage. Pollen showed statistically different results for germination after storage based on species and storage method. All species tested were exposed to similar storage protocols in 5 treatments: open storage, refrigerated storage, frozen storage, vacuum dry/frozen storage, and cryogenic storage.

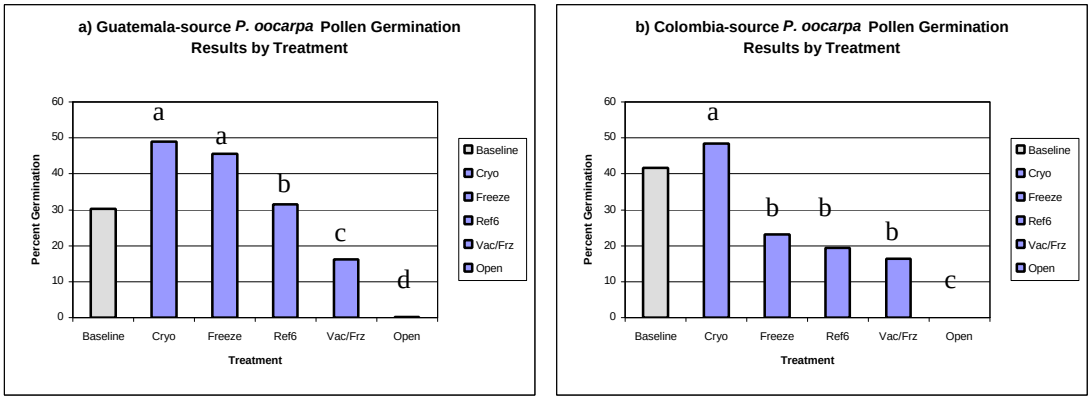
The residual plot of combined data seemed skewed initially, so square root and arc sine transformations were applied to the dataset to increase variances (sqrt), or randomize variances (arcsin) and consequently normalize the residual plot. However, in this study the transformations did not provide any significant improvement in residual plots and were therefore not used. Data were left untransformed for all analyses.

A. Pollen germination results after storage by species

Species response to each treatment was evaluated to determine which method proved best on a species level. While many subtropical pine species occur in the same geographical region in nature, their individual reproductive requirements and patterns may differ due to their distinct

evolutionary paths. Tree improvement programs may also work with only a single species of those evaluated, thus the *in vitro* germination rates for each species after 6 months storage were evaluated across treatments. Because only 1 species/source combination was used for *P. radiata* and *P. taeda*, these species were assessed separately using the "PROC GLM" function in SAS. All other species tested were collected from 2 sources and were compared as paired combinations to test source differences.

Pinus oocarpa

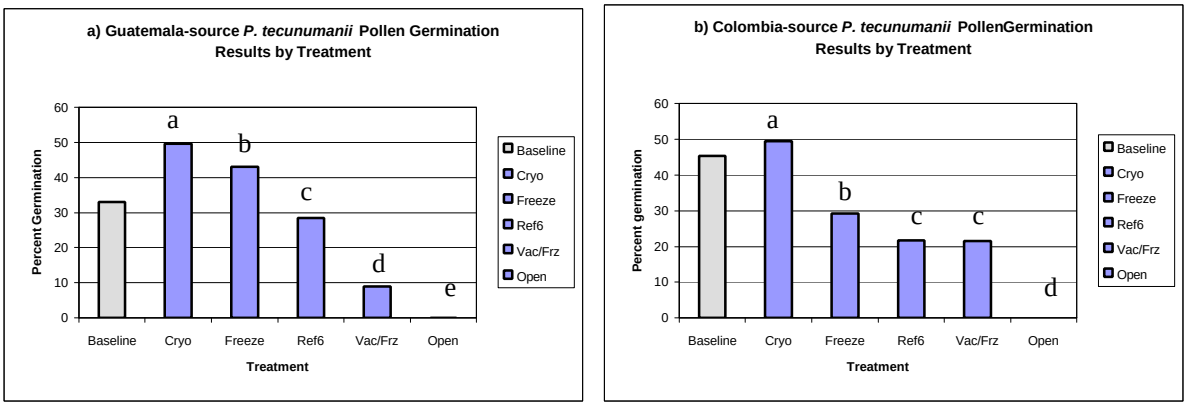


Figures 2a. and 2b. *Pinus oocarpa* pollen *in vitro* germination results versus baseline by source from a) Guatemala and b) Colombia. Columns with different letters are significantly different at a = .05 using Tukey's Studentized Range Test.

Both *P. oocarpa* pollen sources from Colombia and Guatemala exhibited the same rankings when assessed across treatments. The best *in vitro* germination value after 6 months was measured with the Cryogenic Method, then Freezing, Refrigeration, Vacuum Drying/Freezing, and the Open Method, respectively (Figures 2a. and 2b.). Combining the results from the two sources yields an average baseline germination rate of 36%. After storage, the species showed an average germination rate of 49% for Cryogenic storage, 35% for Freezing, 25% for Refrigeration, 16% for Vacuum Dry/Frozen, and 0% for the Open

Method. A Duncan's Multiple Range test combining the two *P. oocarpa* sources showed that "method" resulted in significantly different *in vitro* germination results for the *P. oocarpa* pollen sources.

Pinus tecunumanii

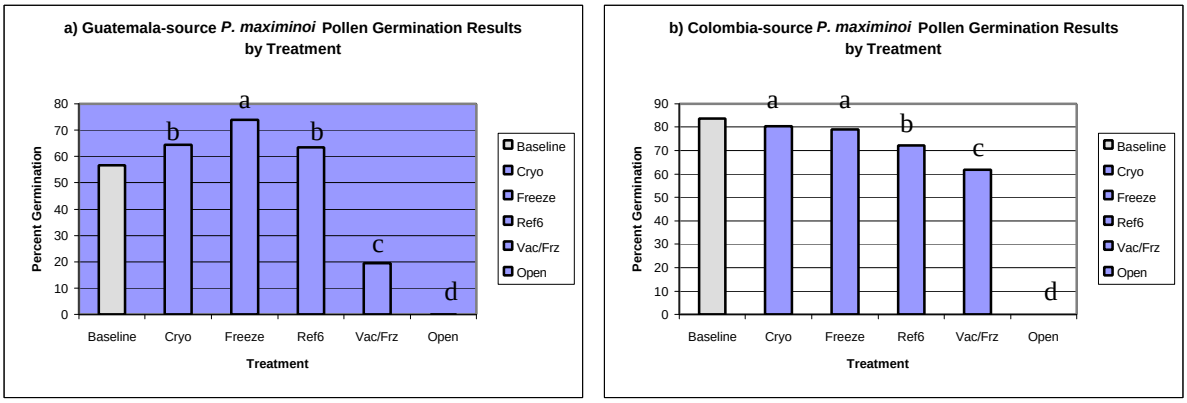


Figures 3a. and 3b. *Pinus tecunumanii* pollen *in vitro* germination results versus baseline by source from a) Guatemala and b) Colombia. Columns with different letters are significantly different at a = .05 using Tukey's Studentized Range Test.

Both *P. tecunumanii* pollen sources from Colombia and Guatemala exhibited similar germination results after 6 months in storage. Combining the results from the two sources yields an average baseline germination of 39%. After storage, the highest average germination value was measured in the Cryogenic Method (49.5%), then Freezing (36%), Refrigeration (25%), Vacuum Dry/Freezing (15.5%), and the Open Method (<1%) (Figures 3a and 3b). The only difference in the results between the two *P. tecunumanii* sources was that the Refrigeration and Vacuum Dry/Freezing Methods were equal in the *in vitro* germination results of the Colombian-source pollen (22%), while the refrigerated samples performed 19% better in the Guatemalan-source pollen than the Vacuum Dry/Frozen samples

(28% vs. 9%). A Duncan's Multiple Range test combining the two *P. tecunumanii* sources showed no statistical similarity between the *in vitro* germination tests by method.

Pinus maximinoi



Figures 4a. and 4b. *Pinus maximinoi* pollen *in vitro* germination results versus baseline by source from a) Guatemala and b) Colombia. Columns with different letters are significantly different at a = .05 using Tukey's Studentized Range Test.

The *P. maximinoi* pollen sources from Colombia and Guatemala exhibited very different results across the 5 storage treatments evaluated. For the Guatemalan-source stored pollen, the mean Freezing values (74%) showed better average *in vitro* germination than the Cryogenic Method (65%). In the fresher Colombian-source *P. maximinoi* pollen, this is reversed, with the Cryogenic Method yielding higher germination values (80%) than Freezing (79%). However, both of these methods were the top two treatments with respect to final germination rate, consistent with the other subtropical species tested. When averaged between sources, these methods were followed by the Refrigeration Method (68%), Vacuum Drying/Freezing (41%), and the Open Method (0%) (Figures 4a and 4b). A Duncan's Multiple Range test combining the two *P. maximinoi* sources showed that Freezing is the best method for 6-month storage of this species' pollen. The Cryogenic and Refrigerated

Methods, however, produced statistically similar results in *in vitro* germination tests. The other two methods reacted individually but lower than the aforementioned treatments.

A difference also exists in the general trends of the *in vitro* germination values between the sources of *P. maximinoi* pollen from Colombia and Guatemala. The stored Guatemalan pollen samples yielded germination values consistent with the other species tested; with the cryogenic, freezing, and refrigerated treatments actually resulting in values higher than the baseline germination. However, the fresher Colombian-source samples all yielded values less than the baseline germination, which although consistent with the theory of storage, differs from all other measures on the subtropical pine pollens in this experiment.

There was a very significant difference between the *in vitro* germination rates of the Vacuum Dry/Freezing samples between the two sources. The fresher Colombian-source samples of *P. maximinoi* pollen germinated much better (62%) than the stored Guatemalan samples (19%) under the vacuum dry/freezing protocols in this experiment.

Pinus radiata

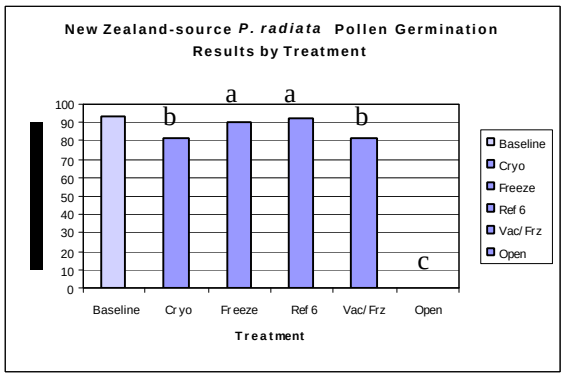


Figure 5. *Pinus radiata* pollen *in vitro* germination results versus baseline. Columns with different letters are significantly different at a = .05 using Tukey's Studentized Range Test.

The stored, temperate *P. radiata* samples evaluated in this experiment yielded germination results differing from the results of the subtropical pine pollen tested. *P. radiata* Refrigerated and Freezing samples showed almost no decline when compared to baseline germination values after 6 months (from 93% baseline germination to 93% and 91% for Refrigerated and Freezing, respectively), while Cryogenic Method samples actually shared the 3rd-place ranking with Vacuum Dry/Frozen (81%) (Figure 5). This is the only species for which the Cryogenic Method did not result in a germination value equal to or greater than the baseline germination rate. It is also the only species tested for which the Refrigeration Method showed the highest germination after 6 months. Thus, Refrigeration was the best method of storage after 6 months (93%), followed by Freezing (91%), then similar results for the Cryogenic Method and Vacuum Dry/Freezing (81%), followed by the Open Method (0%). As a species, *P. radiata* showed the least total decline in germination across the treatments after 6 months.

Pinus taeda

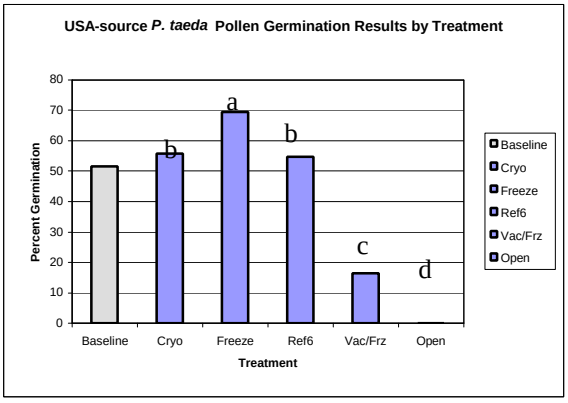


Figure 6. *Pinus taeda* pollen *in vitro* germination results versus baseline. Columns with different letters are significantly different at a = .05 using Tukey's Studentized Range Test.

The stored, temperate *P. taeda* samples evaluated in this experiment yielded germination results differing from the *P. oocarpa* and *P. tecunumanii* subtropical species tested. The best method of storage after 6 months (Figure 6) was Freezing (69%), followed by the Cryogenic Method (56%), Refrigeration (55%), Vacuum Drying/Freezing (17%), and the Open Method (0%). The *P. taeda* rankings mirror the rankings for stored *P. maximinoi* pollen from Guatemala, and are statistically similar in germination percentage.

Final Species Comparison results after 6 months:

The PROC GLM procedure in SAS results showed that the Method treatment was significant for all species/source combinations at the a = .05 level. Individual species/source analyses were performed on unscaled data, making species comparisons difficult because of differences in baseline germination. For instance, *P. maximinoi* from Colombia had a baseline mean in vitro germination rate =14% higher than any of the *P. tecunumanii*, *P. oocarpa*, or other *P. maximinoi* sources compared in this study. Germination rates after storage treatments were divided by their baseline germination values to determine a scaled value, then transformed into a percentage. Scaled values were assessed with a PROC GLM individually by species/source to assess method of storage significance (Table 7).

Table 7. ANOVA Tables to assess Method of Storage Significance from Individually assessed species/source combinations based on baseline-scaled data.

		Baseline Germ. %		Model Deg. of Freedom	Mean Square Error			Pr>F for Method
Camcore	*P. maximinoi*	56.67	78.02	4	13109.06	194.48	0.98	<.0001
SCC	*P. maximinoi*	83.67	70.1	4	6453.32	511.52	0.99	<.0001
Camcore	*P. oocarpa*	30.33	93.96	4	18275.99	140.43	0.97	<.0001
SCC	*P. oocarpa*	41.67	51.54	4	7051.98	50.9	0.93	<.0001
Trees&Tech	*P. radiata*	93.33	74.1	4	6974.23	742.69	0.99	<.0001
NCSU/TIP	*P. taeda*	51.67	76.14	4	13021.75	227.37	0.98	<.0001
Camcore	*P. tecunumanii*	33	78.9	4	16627.83	223.87	0.98	<.0001
SCC	*P. tecunumanii*	45.33	53.74	4	6135.52	55.44	0.93	<.0001

After determining that Method was significant for all species/source combinations tested, a PROC MEANS was executed to assess the differences between the species using the scaled data to determine if the initial differences observed in the raw data were evident after scaling (Figure 7).

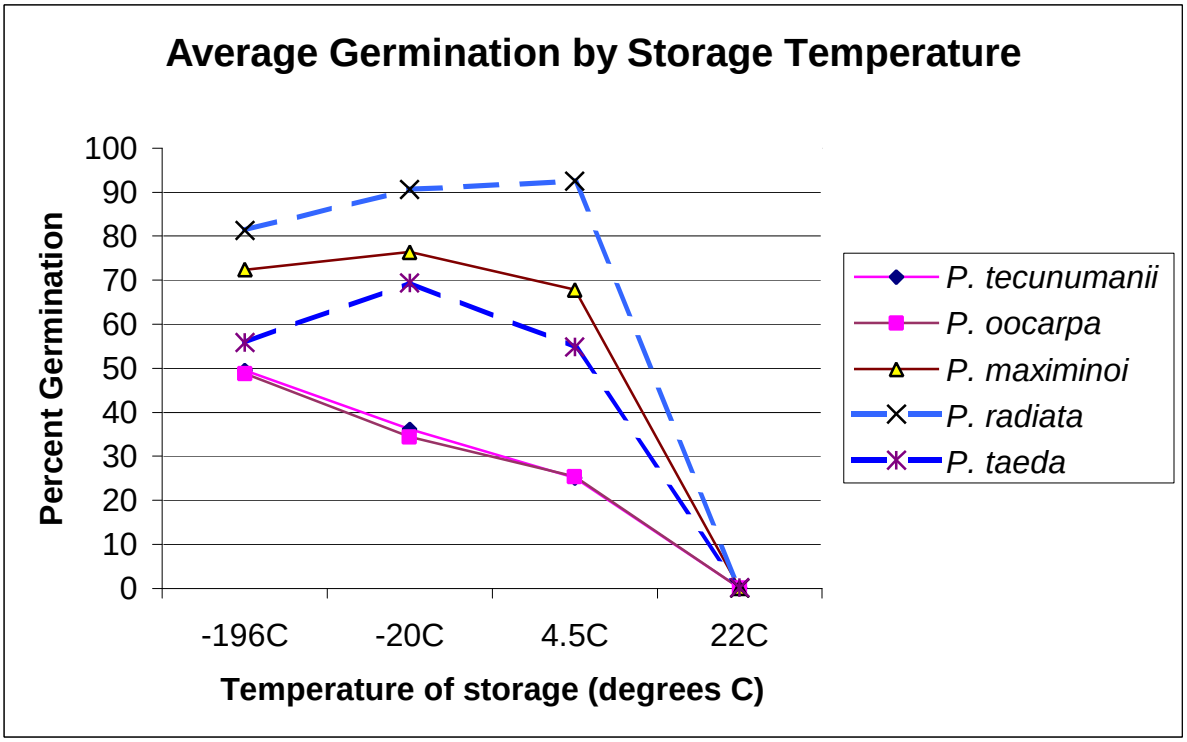


Figure 7. Average *in vitro* germination results by species by temperature (not including vacuum dry/freezing treatment at -20°C).

Pinus oocarpa and *P. tecunumanii* pollen from each source behaved similarly to each other for both the Guatemalan and Colombian-source pollen (Figure 7). Samples of *P. oocarpa* from Guatemala resulted in almost identical values to *P. tecunumanii* samples also from Guatemala across treatments. Likewise, *P. oocarpa* and *P. tecunumanii* samples from Colombia showed very similar germination results after storage. These species are grown and found naturally in similar environments in operational settings.

For the temperate pine pollen, *P. radiata* germination decreases severely after the first month in refrigerated storage. *P. taeda* pollen showed poor results under the Vacuum/Freezing Method, but much higher values under freeze only. This suggests that the drying aspect of the

Vacuum/Freezing treatment had more effect on the germination values rather than the freezing aspect.

B. Storage Method Comparison

In order to assess storage method efficacy after 6 months, germination results were scaled according to baseline germination values for each individual species/source combination. Thus, some post-storage germination values exceeded the original baseline germination and result in values greater than 100%. This phenomenon has also been observed in published studies with various pollen species (Lanteri, *et al.*, 1995) (Hohtola, 1995) and in addition to increased germinability, cold storage may also promote increased water-holding capacity and reduced deterioration by vacuum-drying (Ching and Ching, 1964). The causes of these effects, however, are difficult to explain and not uncommon in the first months following cold storage. The scaling of the values to the baseline data permits greater insight into the pollen viability trends over the 6-month storage period by directly linking them to the initial germination before storage.

The first treatment evaluated for pollen storage was referred to as the "Open Method". This method was included to ascertain the rate of decline if the pollen received no treatment beyond extraction and drying. The open method showed a dramatic change in *in vitro* germination capacity when assessed after 6 months, with all species germinating less than 1%. Stored pollen from Guatemala, USA, and New Zealand showed an average 0.24% germination under this storage treatment, while the fresher pollen from Colombia showed 0% germination for all species. Given the assumption of a high correlation between *in vitro* and *in vivo* pollen germination as previously discussed, this pollen would not be useful for

controlled pollination activities based on these low germination values and the method does not represent a viable storage option.

Refrigeration at 4°C is commonly used for short-term pollen storage in many operational tree improvement programs and is effective for short-term storage of pine pollen. After 6 months in refrigeration at 4°C, most species' germination rate decreased or maintained a rate similar to the baseline value before storage. The stored pollen samples of *P. tecunumanii* and *P. oocarpa* from Guatemala were stable, with germination consistent with baseline results after 6 months. However, the *P. maximinoi* from the same source (Guatemala) actually *increased* after 6 months in refrigerated storage from 57% baseline germination to 63% germination after 6 months in refrigerated storage. The fresher unstored samples from Colombia showed dramatic declines after 6 months in refrigeration (declined 19.3% on average), while *P. radiata* (no difference) and *P. taeda* (3% gain) showed little change.

The vacuum dry/freezing treatment is a common storage method for temperate pine pollen when used in tree improvement activities. This method is used operationally by the NCSU-TIP program with *P. taeda* pollen, and shows consistently acceptable results for their uses. However, when assessed for this study, **all** species evaluated showed a decline in viability after 6 months in storage (average decline=24.2% after 6 months), with almost no change in rank between species compared to baseline germination rates. Stored vs. fresh and subtropical vs. temperate species' pollen were compared, and no real differences were found in the rate of decline between sources.

Direct freezing at -20°C was evaluated after 6 months in storage as a low-cost method for medium- to long-term storage of pine pollen. Within this treatment, there was a significant change in rank and *in vitro* germination rates among the species. All stored pollen samples from Guatemala (*P. tecunumanii, P. oocarpa,* and *P. maximinoi*) increased after being frozen for 6 months, while all fresher samples from Colombia of the same species decreased in germination rate after freezing. The temperate control species also differed in their responses to direct freezing, as *P. radiata* was similar to its baseline germination (declined only 2%), while *P. taeda* actually *increased* almost 20% (showing a favorable response to freezing).

Cryogenic storage is regarded as a suitable method for long-term storage to minimize viability decline over time, and was expected to provide the best germination results after 6 months when compared to the other methods evaluated. In this experiment, all of the stored pollen samples from Guatemala (*P. tecunumanii, P. oocarpa,* and *P. maximinoi*) increased in germination rate, while the fresher samples of the same species from Colombia both increased and decreased. The Colombian-source *P. oocarpa* and *P. tecunumanii* increased in germination rate compared to baseline storage, while the Colombian-source *P. maximinoi* germination rate decreased in cryogenic storage. For the temperate controls, *P. taeda* pollen was relatively stable (actually increased 4%), while the *P. radiata* germination rate declined 12%.

Final Method Comparison results after 6 months:

The Cryogenic method yielded the highest average *in vitro* germination values after 6 months in storage (118%) (Figure 9). Direct freezing yielded the second highest *in vitro* germination

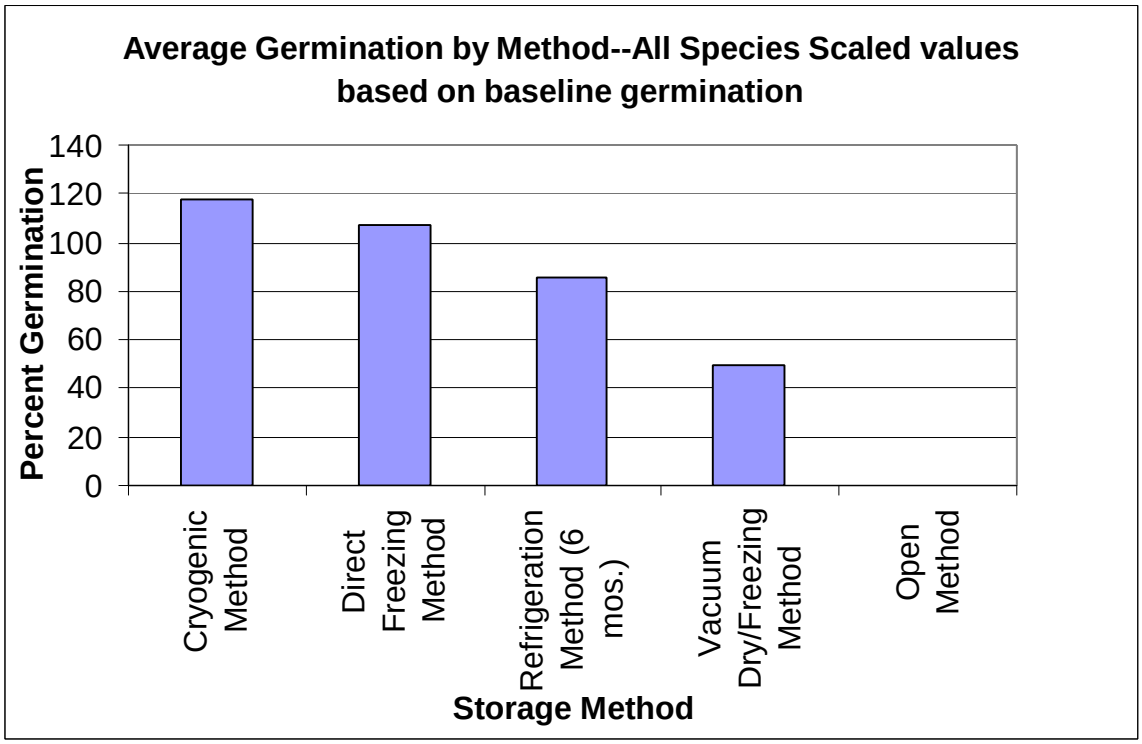


Figure 8. Average germination rates by method using values scaled to baseline germination.

rates after storage at 107%. Refrigeration (86%), Vacuum Drying/Freezing (49%), and the Open Method (0%) average values were consistently lower across all storage methods tested. The Cryogenic Method and Freezing Method are the two best storage methods across species, with the single exception of *P. radiata* (Refrigeration proved better than both the Cryogenic and Freezing methods with this species). The Freezing Method resulted in better germination than the Cryogenic Method in *P. taeda*, *P. radiata*, and *P. maximinoi*. Refrigerated storage has very similar values in some species, but is consistently lower than cryogenic and freezing methods. Generally, the Refrigerated sample mean shows no decline when considering all

samples at 6 months. Excluding the temperate species, mean fresh vs. mean prestored pollen germination values were significantly different (p=0.0008) for the subtropical species. When averaged and scaled to baseline germination, prestored pollen from *P. oocarpa*, *P. tecunumanii*, and *P. maximinoi* show average mean *in vitro* germination of 84%, compared to their fresh pollen analogs at 58%. The Open treatment showed poor results for pollen storage of both temperate and subtropical species. Refrigerated samples stayed the same or below baseline germination rates at 6 months. Cryogenic Method and Freezing samples generally increased in germination percentage above the baseline germination values.

Method of storage was significant when species were paired and assessed against each other. The method term had p-values of .0224, .0337, and .0307 for *P. maximinoi*, *P. oocarpa*, and *P. tecunumanii*, respectively at the a = .05 level (Table 8). This indicates that the method of storage significantly affected pollen germination over 6 months for these species, regardless of collector.

Table 8. ANOVA Tables to assess Method of Storage significance from paired species/source combinations to represent each species.

Species	Ave. Mean Germ% (scaled)	Model Degrees of Freedom		F-value	R^2	
P. maximinoi	74.06	9	17818.6	10.22	0.99	0.0224
P. oocarpa	72.75	9	22538.7	8.08	0.97	0.0337
P. tecunumanii	66.32	9	20376.9	8.54	0.97	0.0307

After 6 months in storage, the Cryogenic Method resulted in the highest average *in vitro* germination values for the subtropical pine species pollen, and also resulted in the highest average germination for all species.

VI. Discussion

A. Best Methods of storage across species

The results of the *in vitro* pollen germination tests suggest that cryogenic storage in liquid nitrogen immersion at -196°C provides the best *in vitro* germination rate for most species tested after 6 months. Exceptions were *P. maximinoi* from Guatemala (freezing at –20°C yielded 71% germination compared to 65% for cryo), *P. radiata* from New Zealand (refrigeration at 4.5°C yielded 93% germination compared to 81% for cryo), and *P. taeda* from the United States (freezing at –20°C yielded 69% germination compared to 56% for cryo). Results from storage experiments with *P. radiata* pollen in New Zealand also produced high *in vitro* pollen germination results after refrigerated storage (Siregar and Sweet, 2000). The cryogenic method employed for this experiment was total immersion in liquid nitrogen. Vapor phase storage (-135°C to -190°C) was not assessed although sometimes used as a method of storage in research laboratories, because only one cryogenic method was to be evaluated for this study to test cold temperature extremes. Vapor phase cryogenic storage also generates a temperature gradient within the storage unit, so that vials further away from the liquid nitrogen are stored at warmer temperatures than closer vials (Sigma Aldrich, 2005). Immersion (liquid phase) is preferred due to reduced variability and improved repeatability of results. The results from the cryogenic immersion and additional methods examined suggest that the lower storage temperatures (-196°C, -20°C) yielded pollen with higher average germination rates when compared to storage at higher temperatures (4.5°C, 22°C).

Higher pollen viability at lower storage temperatures is evident in this experiment. The two highest-rated pollen storage methods on average tested in this study were the two lowest temperatures, cryogenic storage at -196°C and direct freezing at -20°C. These results agree with experiments reported in Nel (2002) and Sedgely and Griffin (1989). The latter report identifies freeze drying and cryostorage as "the most successful methods for long term pollen storage of a range of plant species." Lower storage temperatures and moisture contents are generally accepted as proportional to the length in storage. Hoekstra (1992) suggests that "longevity is doubled at every 5-6° decrease in temperature", affirming that these colder storage temperatures will result in better average *in vitro* germination values over time. If reliable cryogenic storage facilities are unavailable, the results of this study suggest that direct freezing is an acceptable alternative for most pine species. Refrigerated samples stayed at or slightly below baseline germination rates after 6 months, and the open treatment showed poor results for pollen storage of both temperate and subtropical species.

Length of storage required and pollination equipment determines acceptable storage temperatures, moisture percentages, and storage vessels. Pollen stored for short-term needs can withstand warmer storage temperatures and higher moisture contents, up to 30%MC in refrigerated storage as reported in Siregar and Sweet (2000) for *P. radiata*. Pollen prepared for long-term storage should be tested and confirmed to have moisture percents less than or equal to 10% to insure dormancy and subsequent success in storage (Sprague and Johnson, 1977). Moisture content in this range is recommended for long-term storage of *P. radiata* and *P. taeda* pollen (6.5-9%) in several sources (Siregar and Sweet, 2000) (Vergara, *et al.*, 1995) (Moody and Jett, 1990) (Sprague and Johnson, 1977) and a range of 5-7%MC was successful

in this experiment. Storage under low-moisture, dormant conditions can be successful at very low temperatures for long-term storage. Smaller vial or vessel sizes are recommended for long-term storage so that small amounts can be withdrawn as needed without biologically activating the entire pollen lot and therefore encouraging reductions in the future viability of the pollen. Angiosperm pollen stored successfully in one study for one year at –196°C retained the ability to fertilize ovules and produce pod set, but thawing and refreezing of the pollen reduced viability to zero, emphasizing the importance of vessel selection for storage (Sedgely and Harbard, 1993).

For long-term storage, care must be taken that pollen is not over-dried, which will result in the mortality of pollen grains. Pollen moisture content less than 1% often results in poor germination under accepted *in vitro* germination protocols. This suggests that pollen either requires additional inputs or stimuli to reactivate from dormancy, or that pollen was physiologically damaged during drying and storage to result in poor quality pollen. *In vitro* protocols used for this experiment are shown to result in *in vivo* seed set with temperate species, and are presumably an adequate predictor of pollen vigor for successful fertilization. Therefore, *in vitro* tests which show low germination values for low-humidity pollen suggest that this pollen will consequently result in low seed set. Values at a minimum of 3% moisture seem to result in reliable germination in Camcore laboratory tests, but moisture contents less than this may be detrimental to pollen (Siregar and Sweet, 2000). In this experiment, pollen was standardized for storage at 5-7% MC.

Pollen that was stored under direct freezing at –20°C resulted in higher *in vitro* germination values than pollen stored under vacuum drying for 1 hour plus freezing at the same temperature. Pollen moisture content has been demonstrated to have a profound effect on pollen quality before and after storage. The same species and sources stored at the same temperature resulted in differential germination rates in this experiment. The only difference among these treatments was that the vacuum drying procedure reduced the moisture content from the average 5-7% used for all treatments to 1-3%MC after vacuum drying for 1 hour under approximately 0.15 mm Hg at 0°C vacuum (NCSU-TIP, 2000). Pollen was exposed to the same rehydration and *in vitro* germination procedures as used after storage in other methods, but produced lower germination results. Matthews and Kraus (1981) report that their vacuum drying in evacuated ampules reduced moisture content to 3-4% with good results, but laboratory tests on the equipment used for this experiment showed a further reduction in moisture content and poor germination. *In vitro* results show that pollen not submitted to vacuum drying but stored at the same temperature (-20°C) maintained higher average values after 6 months, 107% for direct freezing samples compared to 49% for vacuum-dried/frozen samples. These results coincide with vacuum drying/freezing experiments performed by Feret and Stairs (1970) and Wilcox (1965) on conifer and angiosperm pollen respectively, where vacuum dried pollen had significantly lower invitro germination results than undried pollen. The difference in germination values between the freezing and vacuum dry/freezing treatments suggest germination variance is a result of moisture differences, but it cannot be determined whether the differences are due to damage caused by the extremely low moisture content or processing in the vacuum dry treatment or if this pollen required additional rehydration time to reach conditions adequate for pollen tube

elongation. Air drying is recommended before vacuum drying; however, all pollen assayed in this experiment began at an already suitable moisture content for storage—5-7%.

Pollen samples from the same species in Colombia and Guatemala were collected and compared for this study. The Guatemalan species' pollen was collected in previous years and taken from refrigerated storage, but the Colombian pollen was collected specifically for this experiment and processed directly after collection. The Guatemalan samples are therefore referred to as "prestored" samples and the Colombian samples as "fresh". In this experiment, the prestored pollen samples from Guatemala seemed to have much higher germination rates after treatment under freezing than the fresh pollen samples from Colombia. The fresh pollen samples, however, had higher germination rates than the prestored samples after cryogenic storage. Thus, freezing is preferred for previously stored pollen samples, and cryogenic storage results in higher germination rates for fresh samples. Mean fresh versus mean prestored pollen germination values were significantly different ($p=0.0008$) for the subtropical species tested. When averaged by source and scaled to baseline germination, the mean *in vitro* germination of prestored pollen from *P. oocarpa*, *P. tecunumanii*, and *P. maximinoi* was 84%, compared to their fresh pollen analogs at 58%. This reflects the dynamic of higher germination after the first months following cold storage observed in other studies (Ching and Ching, 1964) (Lanteri, *et al.*, 1995) (Hohtola, 1995), but the causes of these effects are difficult to explain. Higher *in vitro* germination values however, do not succinctly confirm that these prestored pollen lots will provide higher *in vivo* germination and seed set. Studies linking *in vitro* and *in vivo* germination have also indicated that older, stored pollen may provide poor germination values in the lab, but later result in adequate seed set

(Kraus and Hunt, 1970) (Feret and Stairs, 1970). *In vivo* germination protocols provide a good indicator of viability, but *in vivo* germination is the true measure and must be assessed to confirm these results for subtropical pine pollen.

The open treatment (lab-bench storage at 22°C in a bell jar) assessed in this study provided the lowest germination values for all pollen tested. While this result reflects the predicted ranking of storage methods, it was expected that the overall germination rates would be somewhat higher. Hohtola (1995) agrees, saying, "At room temperature, the viability of pollen lowers and disappears within a few days". *Pinus taeda* pollen stored under 5 storage methods by Sprague and Johnson (1977) showed variable success in storage for all treatments except in a cotton-stoppered dessicator, in which all pollen clones were dead at the end of the experiment. In an experiment with *P. radiata* pollen, open-stored pollen had 30-40% lower *in vitro* germination values than pollen stored at 4°C and -20°C for 0.9 years (Siregar and Sweet, 2000). These results resemble the pollen tested for this experiment, where the open treatment showed minimal success with the previously stored Guatemalan, New Zealand, and USA pollen lots (~1%), but completely killed all fresher pollen samples from Colombia.

B. Species-dependent Responses to Pollen Storage Methods

Results of the *in vitro* pollen germination tests suggest that pollen responds differently to storage based on species. Pollen from the subtropical species *P. tecunumanii, P. oocarpa,* and *P. maximinoi* were collected in both Colombia and Guatemala. The average germination of these pollen lots was statistically different based on species and method of storage, so data from each species/source combination was compared to determine which pollen was

influencing the results. The Colombian-source *P. maximinoi*'s mean *in vitro* germination rate is much higher than any of the *P. tecunumanii*, *P. oocarpa*, or other *P. maximinoi* compared in this study for all treatments. Coincidentally, it also had the highest baseline germination rate of any of the subtropical species tested. Although the causes of this successful storage are not evident from these results, it should be noted that the pollen that arrived with highest viability maintained this advantage after storage. This emphasizes the need for good pollen collection, handling, and storage methods for success management of breeding programs.

Two subtropical species' pollen reacted similarly to each other across treatments after storage: *P. oocarpa* and *P. tecunumanii* (Figure 10). These pollen lots reacted similarly both inter-species and inter-source for both the Guatemalan and Colombian-source pollen. Samples of *P. oocarpa* from Guatemala resulted in almost identical values to *P. tecunumanii* samples also from Guatemala across treatments. Likewise, *P. oocarpa* and *P. tecunumanii* samples from Colombia showed very similar germination results after storage. These species are grown and found naturally in similar environments in operational settings.

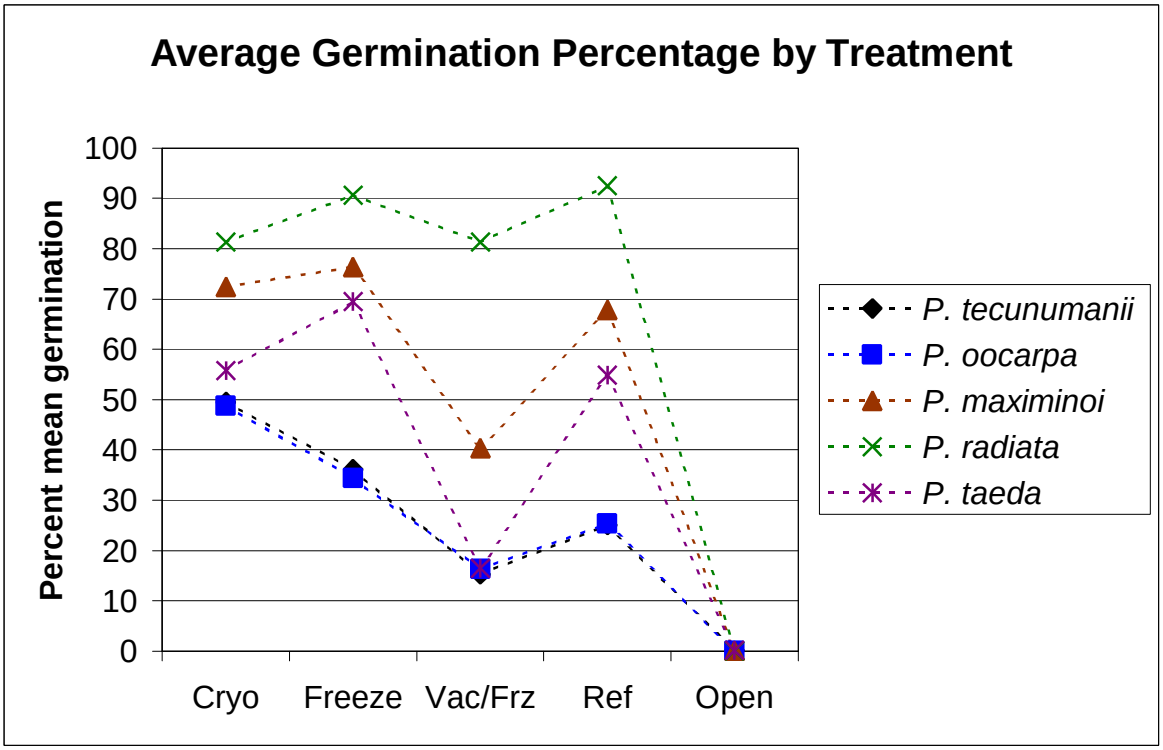


Figure 9. Average Germination Percent by Treatment by species. Treatments ranked by storage temperature, lowest temperature (L) to highest (R).

For the temperate pine pollens tested, the germination result of *P. radiata* samples from New Zealand most closely resembled Colombian *P. maximinoi* values. Pollen samples that arrived with higher-viability baseline germination (New Zealand *P. radiata*—93%, Colombian *P. maximinoi*—84%) followed the predicted curve of decreasing germination over the 6-month storage period, but sources with lower baseline germination tended to increase with cryopreservation and direct freezing treatments (such as *P. taeda*). *P. taeda* from U.S-sourced Virginia seed orchards behaved most similarly to the Colombian samples after treatment, posting the highest *in vitro* germination values after freezing. *P. taeda* pollen showed low results under the Vacuum/Freezing Method, but much higher values under freeze only. This suggests that the drying aspect of the Vacuum/Freezing treatment had more effect on the

germination values rather than the freezing aspect, and that some species are more sensitive to moisture deficiencies than others. Species are known to respond distinctly to moisture and storage temperatures, and the pollen of many species are tolerant of complete desiccation (Hoekstra, 1992). Temperate species resulted in high values for the 4 successful storage treatments (cryopreservation, direct freezing, vacuum dry/freezing, and refrigeration), and responded more favorably than most subtropical species averages to all but vacuum dry/freezing.

The storage protocols used for the vacuum dry/freezing treatment reflect suggested protocols used by tree improvement programs around the world, but were not successful in this experiment. The method has been used successfully for many species, including the storage of several species of *Acacia*, spp. pollen (Sedgely and Harbard, 1993). Results from experiments at NC State with *P. taeda* pollen showed that vacuum drying may not be needed, but resulted in higher viability after 3 years in storage and also produced the most consistent results (Sprague and Johnson, 1977). The treatment as previously detailed in the materials and methods section, however, resulted in lower *in vitro* germination values for all species tested in this experiment. Only *P. radiata* stored reasonably well under this regimen, losing only 12% germination from its baseline germination percentage after 6 months storage. All other species declined 24% on average from baseline germination; including a 37% decline in Guatemalan-source *P. maximinoi* and 35% decline in *P. taeda*. These results conflict directly with research performed by several scientists, but are more likely the result of overaggressive

vacuum drying and moisture reduction. Lower *in vitro* germination values for vacuum-dried

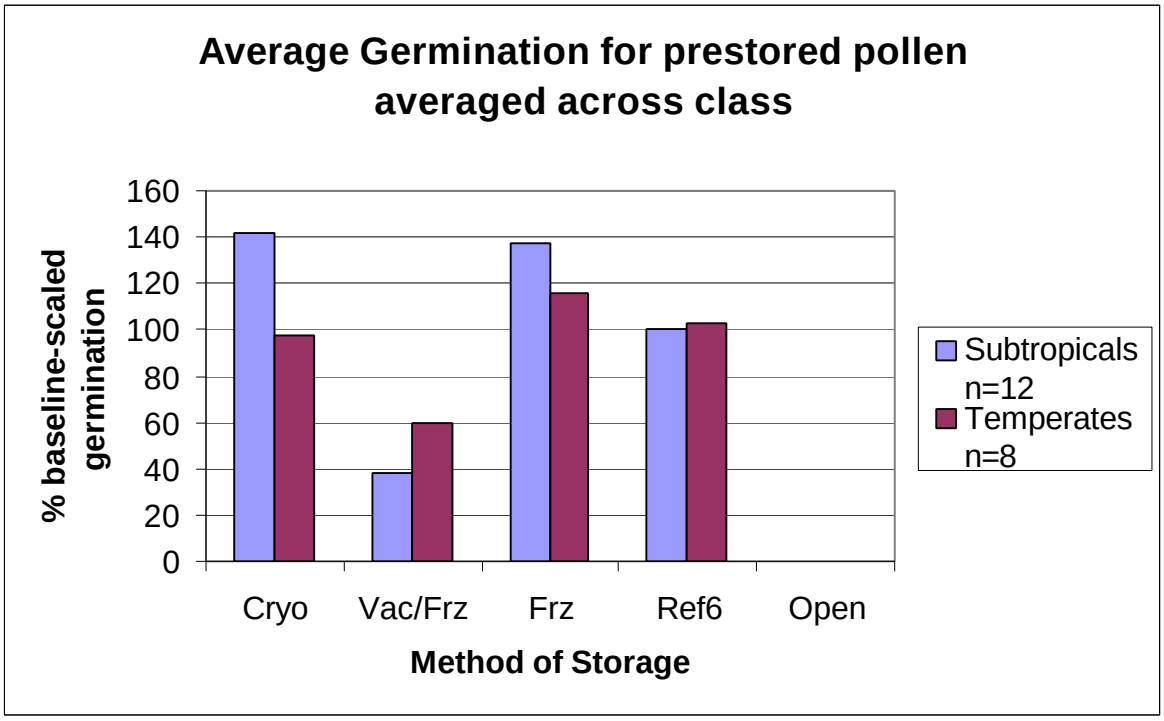


Figure 10. Average *in vitro* germination rates for prestored pollen lots averaged across class (subtropical or temperate) using baseline-scaled data.

pollen were also found by Feret and Stairs (1970) on white spruce pollen, but these *in vitro* results did not correlate to seed set and seed germination upon subsequent investigation. This suggests that these low *in vitro* germination results for vacuum dry/frozen *P. taeda* pollen should be tested to confirm that they are indeed correlated to seed set at these vacuum ratings.

In this experiment, prestored pollen from subtropical species was compared against prestored pollen from temperate species (Figure 11). Pollen from the two classes of pine species behaved similarly; somewhat better under some storage methods and worse under others. Thus, temperate pine pollen storage protocols can be successfully used for subtropical species.

C. Effects of Initial Pollen Quality on Viability

Initial quality after pollen harvest greatly affects behavior and results during storage. Recommended strobili collection times for pine species are 3.6 to 3.9 on the PDCS (Bramlett and Bridgwater, 1989) (Appendix 1) and represent the developmental stage when pollen grains are mature and contain lower moisture levels. Harvested at this stage (approx. 3.9), pollen in Guatemala was found to have ~22% MC in natural state (author, 2003—unpublished data). Catkins were placed in well-ventilated room in closed kraft bags one level deep for initial airdrying and minimization of fungal activity. Upon return to the laboratory, pollen was further airdried for 24 hours, then extracted from the catkins and dried in a forced-air oven until it maintained 5-7%MC. When harvested in this manner, pollen quality is maintained and pollen storage is more effective. First, pollen is collected when biologically mature to provide the highest per-grain germination potential. Next, pollen is dried immediately to remove water to avoid fungal growth and reduce insect activity. Removal of moisture also stimulates dormancy of the pollen, the desired state for pollen storage. The resulting clean, mature, dormant pollen stores better over time and provides better *in vitro* germination results.

Problems with initial pollen quality are exacerbated in storage, and lead to low viability or unusable pollen over time. Pollen harvested outside of the recommended stages of development leads to lower germination capacity for all uses. Catkins or extracted pollen left for extended periods before drying leads to the progression of carbohydrate-consumptive metabolic activity in the grains, and therefore depletes the pollen's resources for germination. Reduction of pollen moisture and temperature stimulates dormancy, which protects these

energy reserves for pollen tube elongation after reactivation. Reductions in moisture also reduce the activity of bacterial or fungal contaminants naturally present in the pollen. Given suitable growing conditions of moisture and temperature, spores of fungi such as *Cladesporium cladesporioides, Aspergillus, spp., Penicillium, spp., or Fusarium, spp.*,can germinate and the developing mycelium may have detrimental effects on the pollen (Hodges, personal communication 2005). At the least, the fungal hyphae can result in caking of the pollen making it difficult to use for controlled pollinations. Many of the fungal spores found in pollen during the current study are common cosmopolitan saprophytic fungi; e.g. *Cladesporium cladesporioides*, which is "amongst the most common fungi to be isolated from air and soil" (David, 1997) and therefore may colonize the pollen during any exposure. Storage methods that inhibit the growth and development of these fungi, therefore, are paramount for maintaining viability.

Collection and storage of pollen with the highest available pollen quality is essential for international germplasm exchange. Cross-continental shipments of pollen for breeding purposes are occurring with increasing frequency for both hardwood and softwood species due to international markets and research programs. These shipments require high initial quality, and are often shipped by third-party pollen vendors, enduring several days in transit to arrive at their destination. Germinative capacity is generally diminished during shipment due to changes in temperatures or delays in governmental clearance under sub-optimal conditions. Studies show that high viability pollen stored under low moisture conditions can tolerate elevated temperatures incurred in transport if stored properly (Nel, 2002). Well-prepared pollen samples with identified moisture contents and *in vitro* germination rates

(under established protocols) can greatly aid in successful shipment. Accepting that these pollen lots are commercial merchandise and a nominal loss of germination in transit notwithstanding, initial quality must be respected to guarantee success for breeding programs using this pollen.

Studies with diluted pollen have been performed to evaluate the minimum pollen viability necessary for successful pollination. A study performed at the Institute of Forest Genetics by Callaham (1966) evaluated the success of pollination with diluted pollen for hybridization of pines when pollen is limited or expensive. Results showed that viable pollen can be diluted with dead pine pollen without ill effects for seed set, seeds per cone, or proportion of sound seeds using 30% or greater viable pollen in the pollen mix. This assumes, however, that the proportional viable pollen in the diluted mixture maintains high vigor and viability (Matthews and Bramlett, 1986). Pollen grains subjected to suboptimal conditions will diminish in germinative capacity evenly, and therefore do not behave like diluted pollen. Although used successfully when pollen is limited or expensive, pollen dilution does not mimic low quality pollen and therefore does not suggest that pollen can be used successfully at low viability proportions. Pollinating more flowers is a more effective way to increase seed yields with low quality pollen rather than using higher quantities of pollen as practiced in many breeding programs (Matthews and Bramlett, 1986).

Assuming adequate initial pollen quality, pollen responds somewhat differently based on the duration of previous storage. Pollen that has been stored for 1 year will respond differently to *in vitro* germination protocols than pollen stored for 1 week, although stored under the same

conditions. In this experiment, pollen from Guatemala was prestored for approximately

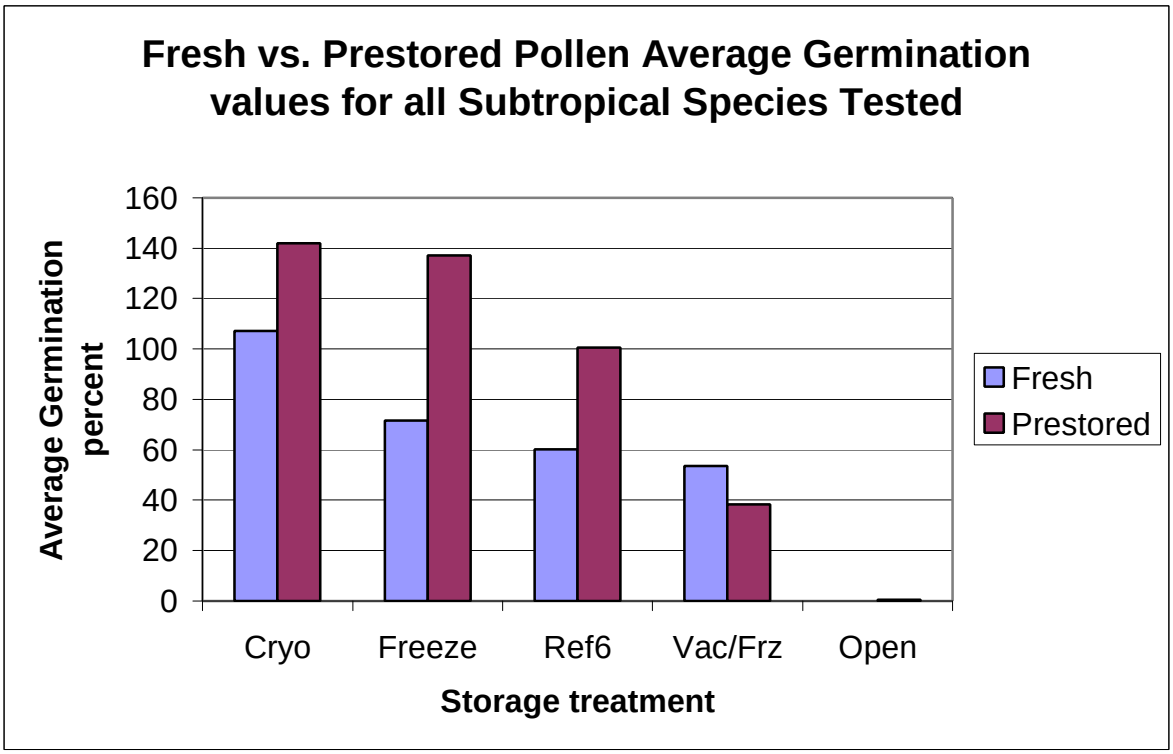


Figure 11. Fresh vs. Prestored Pollen Average Germination values for all subtropical species tested.

one year before analysis, while pollen from Colombia was harvested and received mere days before pollen storage began. On average, prestored pollen from Guatemala resulted in much higher *in vitro* germination values than the same subtropical species pollen from the fresher Colombian-source (Figure 12). When adjusted to baseline germination rates for calibration, prestored Guatemalan samples of *P. tecunumanii*, *P. oocarpa*, and *P. maximinoi* stored at or above baseline germination rates for 3 of the 5 treatments. The only treatment for which the fresher Colombian samples resulted in higher germination rates was for vacuum drying/freezing. The fresher samples responded better to the vacuum drying treatment than the stored samples. As noted in previous references, pollen intended for medium- to long-term storage should be stored at lower moisture levels and temperatures. The correlation

between the *in vitro* germination rates for both pollen origins and their *in vivo* germination rates should be assessed to better quantify the success of these protocols.

D. Causes of experimental variation

After storage under the 5 treatments, pollen was tested to assess viability and produced variable results. In several species (Guatemalan *P. tecunumanii*, *P. maximinoi*, *P. oocarpa*; Colombian *P. tecunumanii*, *P. oocarpa*, USA *P. taeda*) significant variability in germination rates was observed between baseline and germination values after storage. Although the average germination results decline from baseline to 6 months, many individual species showed increases or significant variability instead of the expected decreasing response curve. This suggests that there was variability in the *in vitro* germination protocols used. Increases in *in vitro* germination rate were also observed in Sprague and Johnson (1977), which the authors attribute to experimental error in the sampling techniques.

Variation in *in vitro* pollen germination tests on agar gel is expected and can be caused by multiple variables whose influence may be enhanced when using pollen from differing sources and collection ages. Pollen for this study was collected on different continents at distinct times, and arrived under differing moisture regimes. While all pollen received the same treatment before storage, the history of pollen collection might influence the long-term storage capacity of the individual pollen collection. All pollen normalized for treatment in this experiment was subsequently re-dried during the vacuum dryer treatment to a lower %MC and resulted in low germination values for most species, compared to high or highest rankings for direct freezing without vacuum drying. Also, cryogenic storage and direct

freezing resulted in the highest *in vitro* germination rankings after storage, but a single moisture content level was established for all treatments and moisture is therefore confounded with these rankings. Perhaps pollen at 5-7%MC is suitable for cryogenic and direct freezing storage, but pollen at higher moisture contents may result in better results for certain treatments. Sedgely and Harbard (1993) suggest that drying to this moisture level (5-7%) is not necessary prior to cryostorage for angiosperm pollen and therefore may not be an acceptable protocol for other treatments.

Additional potential sources of variability are the agar gel, differential temperatures within the incubator, and human error. For each germination test, fresh agar gel was created from granular agar and consequent culture media were the same for all tests. However, some difference was noted within the incubator among Petri dish lid condensation rates from the lower to the upper racks within the incubator. Replications were randomized within the incubator and 6-month germination values are therefore unbiased for this source of variation. Although the circulation of air within the incubator may be a problem for future tests, it was beyond the scope of this study and will require future analysis. All pollen was considered "germinated" when the length of the pollen tube was equal to or greater than the width of the pollen grain. Pollen grains in groups of 4 or more were ignored for germination assessments to avoid the population effect or "mutual stimulation effect" (Goddard and Matthews, 1981) which may promote germination of pollen grains in close proximity to each other. Research indicates that calcium leakage from nearby grains often results in erroneously high *in vitro* germination rates (Sari-Gorla and Frova, 1997). While these groups of 4 or more pollen grains were not counted for this study, distances between grains were not measured to objectively ensure the absence of calcium leakage and prohibit biased germination values.

V. Conclusion / Recommendations

For conservation of subtropical pine pollen for 6 months, the results of this study suggest that cryopreservation at -196°C should be used if available (Table 9). Reliable resources for cryopreservation are limited in some geographic regions where subtropical species are planted, and therefore direct freezing at -20°C is recommended where liquid nitrogen and reliable electricity are scarce or cost prohibitive. Traditionally, lower temperature and lower moisture methods are employed to maintain good viability for long-term storage. Like the *P. taeda* and *P. radiata* pollen evaluated in this study, subtropical pine pollen responded well to these lower temperatures after 6 months in storage, and these protocols can be used successfully.

The 3 subtropical pine species' pollen assessed in this study (*Pinus tecunumanii, P. oocarpa, P. maximinoi*) diverged from the temperate pollen behavior when only prestored pollen is considered.

Table 9. Treatment(s) providing highest *in vitro* germination rate after 6 months storage at 5-7 % MC (Ave. across source) in this study.

Species	**Treatment(s) Providing <u>Highest</u> *In Vitro* Germination Rate**
P. maximinoi	Direct Freezing Storage at -20°C / Cryogenic storage at -196°C
P. oocarpa	Cryogenic storage at -196°C
P. radiata	Direct Freezing Storage at -20°C / Refrigerated storage at 4.5°C
P. taeda	Direct Freezing Storage at -20°C
P. tecunumanii	Cryogenic storage at -196°C

Cryogenic and direct freezing storage methods yielded higher *in vitro* germination rates for subtropical species than temperate species (*P. taeda* and *P. radiata*). Vacuum drying/freezing and Refrigeration, however, yielded higher *in vitro* germination rates for temperate species. Additional research into the physiology of these differences and the correlations with evolutionary phylogeny information is recommended to determine the cause of these differences.

VI. Literature Cited

Ahlgren, C.E., and Ahlgren, I.F. 1978. Viability and fertility of vacuum dried pollen of 5-needled pine species. *For. Sci.* 24: 100-102.

Bajaj, Y.P.S.(ed.) 1987. Cryopreservation of Plant Cell, Tissue, and Organ Culture for the Conservation of Germplasm and Biodiversity. Biotechnology in Agriculture and Forestry 32: Cryopreservation of Plant Germplasm I. Springer-Verlag Berlin Heidelberg.

Bajaj, Y.P.S. 1987. Cryopreservation of Pollen and Pollen Embryos, and the Establishment of Pollen Banks. *International Review of Cytology*. Vol. 107, Pp. 397-420.

Barnábas, B. and Kovács, G. 1997. Ch. 14--Storage of pollen. In: Shivanna, K.R. and Sawhney, V.K., 1997. Pollen Biotechnology for Crop Production and Improvement. Cambridge University Press. Cambridge, UK. 448 pp.

Beers, W.L., Bivins, J., and J.E. Mocha. 1981. Pollen Collection. In: E.C. Franklin, E. Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 30-31.

Bonnet-Masimbert, M. and Webber, J.E. 1995. From flower induction to seed production in forest tree orchards. *Tree Physiology*, 15, 419-426.

Bowes, S.A. 1990. Long-term storage of *Narcissus* anthers and pollen in liquid nitrogen. *Euphytica* 48:275-278. Kluwer Academic Publishers, the Netherlands.

Boyer, W.D. 1981. Pollen Production and Dispersal as Affected by Seasonal Temperature and Rainfall Patterns. In: E.C. Franklin, Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 37-39.

Braggio, G.M, Cornara, L., and Dellacha, E. 1990. The cryopreservation effect on pollen viability. *Boll. Soc. Ital. Biol. Sper.* 66: 821-868.

Bramlett, D.L. 1973. Pollen Phenology and Dispersal Pattern for Shortleaf Pine in the Virginia Piedmont. USDA Forest Service Res. Paper SE-104. SE For. Exp. Sta., Asheville, NC.

Bramlett, D. L. and Bridgwater, F.E. 1989. Pollen development classification system for loblolly pine. Proc. 20th So. For. Tree Improv. Conf., June 26-30. Charleston, SC, p.116-121.

Bramlett, D.L., and Matthews, F.R. 1991. Storing Loblolly Pine Pollen. *Sou. Journal of App. For.* 15, Pp. 153-157.

Bramlett, D.L. and O'Gwynn, C.H. 1981. Controlled Pollination. In: E.C. Franklin, E. Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 44-51.

Bramlett, D.L., Bridgwater, F.E., Jett, J.B., and Matthews, F.R. 1985. Theoretical impact of pollen viability and distribution on the number of strobili to use for controlled pollinations in loblolly pine. Proc. 18th So. For. Tree Improv. Conf., May 21-23. Long Beach, Mississippi. Pp.194-203.

Brewbaker, J.L., and Kwack, B.H. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *Am. Jour. Of Botany*, 50:859-865.

Callaham, R.Z. 1966. Hybridizing pines with diluted pollen. Proceedings of the 8th Southern Forestry Tree Improvement Conference, June 16-17, Savannah, GA. Southern Forest Tree Improvement Committee Pub. #24.

Callaham, R.Z., and Steinhoff, R.J. 1966. Pine pollen frozen five years produces seed. USDA Forest Service Res. Paper NC (6), 94.

Ching, T.M., and Ching, K.K. 1964. Freeze-drying pine pollen. *Plant Physiology* 39:5, pp. 705-709.

Connor, K.F. and Towill, L.E. 1993. Pollen-handling protocol and hydration/dehydration characteristics of pollen for application to long-term storage. *Euphytica* 68:77-84. Kluwer Academic Publishers, Dordrecht, Netherlands.

David, J.C. 1997. *A contribution to the systematics of Cladesporium*. International Mycological Institute. CAB International Information Press, Eynsham, England. 157pp.

Duffield, J.W. 1954. Studies of Extraction, Storage, and Testing of Pine Pollen. Zeit. Forstgen. Forstpflanz. 3:39-45.

Duffield, J.W. and Callaham, R.Z. 1959. Deep-freezing pine pollen. *Silvae Genetica* 8:22-24.

Dvorak, W.S., Gutiérrez, E.A., Gapare, W.J., Hodge, G.R., Osorio, L.F., Bester, C., and Kikuti, P. 2000. *Pinus maximinoi*. In: Conservation & Testing of Tropical & Subtropical Forest Tree Species by the Camcore Cooperative, College of Natural Resources, NCSU, Raleigh, NC. USA. Pp: 106-127.

Dvorak, W.S., Gutiérrez, E.A., Osorio, L.F., Hodge, G.R., and Brawner, J.T. 2000. *Pinus oocarpa*. In: Conservation & Testing of Tropical & Subtropical Forest Tree Species by the CAMCORE Cooperative. College of Natural Resources, NCSU, Raleigh, NC. USA. pp. 129-147.

Dvorak, W.S., Hodge, G.R., Gutiérrez, E.A., Osorio, L.F., Malan, F.S., and Stanger, T.K. 2000. *Pinus tecunumanii*. In: Conservation & Testing of Tropical & Subtropical Forest Tree Species by the CAMCORE Cooperative. College of Natural Resources, NCSU, Raleigh, NC. USA. pp. 129-147.

Farjon, A. and Page, C.N. 1998. Conifers. Status survey and conservation action plan. IUCN/SCC Conifer Specialist Group. IUCN, Gland, Switzerland and Cambridge. 121 pp.

Feret, P.P. and Stairs, G.R. 1970. Vacuum-dried spruce pollen: pollen viability and seed germination. *Univ. of Wisconsin Forestry Research Notes.* No. 153. Madison, WI.

Goddard, R.E., and Matthews, F.R. 1981. Ch. 9--Pollen Testing. In: E.C. Franklin, (ed.) Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 40-43.

Hanna, W.W. 1994. Pollen storage in frostless and conventional frost-forming freezers. *Crop Science*. 34:1681-1682.

Hanna, W.W., and Towill, L.E. 1995. Long-term pollen storage. In: Janick, J. (ed.), Plant Breeding Reviews, Vol. 13. p. 179-207. Wiley and Sons, New York.

Harrington, J.F. 1970. In: O.H. Frankel and E. Bennett (eds.) Genetic Resources in Plants—Their Exploration and Conservation. Pp. 501-521. Blackwell, Oxford.

Heslop-Harrison, J., and Heslop-Harrison, Y. 1970. Evaluation of pollen viability by enzymatically induced fluorescence: Intracellular hydrolysis of fluorescein diacetate. *Stain. Technol*. 45:115-120.

Heslop-Harrison, J., Heslop-Harrison, Y., and Shivanna, K.R. 1984. The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. *Theo. App. Genetics* 67:367-375.

Hodges, C.S. Personal communication, 2005.

Hoekstra, F.A. 1992. Stress Effects on the Male Gametophyte. In: Cresti, M. and Tiezzi, A. (ed.) Sexual Plant Reproduction. Springer-Verlag, Berlin. pp. 193-201.

Hohtola, A. 1995. II. 11 Cryopreservation of Scots Pine (*Pinus sylvestris*, L.). In: Biotechnology in Agriculture and Forestry 32. Y.P.S. Bajaj, Editor. Springer Publishing, Pp. 205-214.

Ichikawa, S. and Shidei, T. 1972. Fundamental studies on deep-freezing storage of tree pollen. *Bulletin Kyoto Univ. For*. 44, p. 47.

Isaza, N. 2003. Smurfit Cartón de Colombia personnel. Personal communication.

Jensen, C.J. 1964. Pollen storage under vacuum. Royal Veterinary and Agricultural College Yearbook. Copenhagen, Denmark. Pp 133-146., as cited in: Towill and Walters, 2000 (see below).

Jett, J. B., and Frampton, L.J., Jr. 1990. Effect of Rehydration on In Vitro Germination of Loblolly Pine Pollen. *South. J. App. Forestry*. 14(1):48-51.

Jett, J.B., Bramlett, D.L., Webber, J.E., and Eriksson, U. 1993. Ch. 5—Pollen Collection, Storage, and Testing. In: Advances in Pollen Management. USDA Forest Service Agriculture Handbook 698. Washington, D.C.

Jörgensen, J. 1990. Conservation of valuable gene resources by cryopreservation in some forest tree species. *Jour. Of Plant Phys.* 136: 373-376.

Kartha, K.K., Fowke, L.C., Leung, N.L., Caswell, K.L., and Hakman, I. 1988. Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). *Jour. Plant Physiol.* 132:529-539.

Kearns, C.A., and Inouye, D.W. 1993. Techniques for Pollination Biologists. University Press of Colorado. Niwot Ridge, CO. 583pp.

Kraus, J.F. and Hunt, D.L. 1970. Fresh and stored pollen from slash and loblolly pines compared for seed yields. USDA Forest Research Note SE-143, Southeast Forest Experiment Station, 5 pp.

Lanteri, S., Belletti, P., and Lotito, S. 1993. Storage of Pollen of Norway Spruce and Different Pine Species. *Silvae Genetica*, 42,2-3. Pp. 104-108. J.D. Sauerlander's Verlag. Frankfurt Am. Main.

Layne, R.E.C., and Hagedorn, D.J. 1963. Effect of Vacuum –Drying, Freeze-Drying and Storage Environment on the Viability of Pea Pollen. *Crop Science*, 3:433-436.

Linskens, H.F., and Pfahler, P.L. 1973. Biochemical composition of maize (*Zea mays*, L.) pollen: Effects of allele x storage interactions at the waxy (wx), sugary (su_1) and shrunken (sh_2) loci, on the amino acid content. Theo. Appl. Genetics 43:49-53. *As cited in*: Barnábas, B. and Kovács, G. 1997. Ch. 14--Storage of pollen. In: Shivanna, K.R. and Sawhney, V.K., 1997. Pollen Biotechnology for Crop Production and Improvement. Cambridge University Press. Cambridge, UK. 448 pp.

Matthews, F.R., and Bramlett, D.L. 1986. Pollen Quantity and Viability Affect Seed Yields from Controlled Pollinations of Loblolly Pine. *Sou. Journal of App. For.* 10, Pp. 78-80.

Matthews, F.R., and Bramlett, D.L. 1982. Pollen storage methods influence filled seed yields in controlled pollinations of loblolly pine. Proc. Of the Second Biennial Southern Silvicultural Research Conference. Atlanta, GA. November 4-5, 1982.

Matthews, F.R., and Bramlett, D.L. 1986. Pollen quantity and viability affect seed yields from controlled pollinations of loblolly pine. Southern Journal of Applied Forestry **10**: 78-80

Matthews, F.R., and Kraus, J.F. 1981. Pollen Storage. In: E.C. Franklin, Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 37-39.

Mercier, S. 1995. The role of a pollen bank in the Tree Genetic Improvement Program in Québec (Canada). *Grana* **34**:367-370. Scandinavian University Press.

Mergen, F., Rossoll, H., and Pomeroy, K.B. 1955. How to control the pollination of slash and longleaf pine. USDA Forest Service SE For. Exp. Sta. Paper 58, Asheville, NC 14 pp.

Mirov, N.T. 1967. The Genus *PINUS*. The Ronald Press Company. 602 pp.

Moody, W.R. 1988. Evaluation of loblolly pine pollen quality. Thesis (M.S.)--North Carolina State University. 68 pp.

Moody, W.R., and Jett, J.B. 1990. Effects of pollen viability and vigor on seed production of loblolly pine. Southern Journal of Applied Forestry. 14:1, 33-38. South Carolina Forestry Commission, Tillman, SC.

Nalge Nunc International. 2001. Nalgene™ and Nunc™ Product Catalog. Nalge Nunc International Corporation, Rochester, New York.

NCSU-TIP Coop. 2000. December 5, 2000 Contact Meeting Notebook. North Carolina State University Industry Tree Improvement Cooperative. Raleigh, NC. 20 pp.

NCSU-TIP Coop. 2003. Tree Improvement Manual. North Carolina State University Industry Tree Improvement Cooperative. Raleigh, NC.

Nel, A. 2002. Factors Influencing Controlled Pollination of *Pinus patula*. Master's Thesis. University of Natal, Pietermaritzburg, South Africa. 100 pp.

Nygaard, P. 1969. Studies on the Germination of Pine Pollen (Pinus mugo) in vitro. I. Growth Conditions and Effects of pH and Temperature on Germination, Tube Growth and Respiration. *Physiologia Plantarum*, Vol. 22, Pp. 338-346.

Owens, J.N. 1993. Chapter 1— Pollination Biology. In: Advances in Pollen Management. USDA Forest Service Agriculture Handbook 698. Washington, D.C.

Rao, G.U., Jain, A., and Shivanna, K.R. 1992. Effects of high temperature stress on *Brassica* pollen: viability, germination and ability to set fruits and seeds. *Annals of Botany* 69:193-196.

Sari-Gorla, M. and Frova, C. 1997. Ch. 16--Pollen tube growth and pollen selection. In: Shivanna, K.R. and Sawhney, V.K., 1997. Pollen Biotechnology for Crop Production and Improvement. Cambridge University Press. Cambridge, UK. 448 pp.

SAS Institute, Inc. 1999-2001. SAS® User's Guide: Statistics. Version 8.02. SAS Campus Drive, Cary, NC. 27513.

Sedgely, M. and Griffin, A.R. 1989. Sexual Reproduction of Tree Crops. Academic Press, London.

Sedgely, M. and Harbard, J. 1993. Pollen Storage and Breeding System in Relation to Controlled Pollination of Four Species of Acacia (Leguminosae: Mimosoideae). *Australian Jour. Of Bot.* **41**, Pp. 601-609.

Setiawati, Y.G.B. 1994. Study of seed yield and physical quality in *Pinus radiata* D. Don seed orchards. Master of Forestry Science thesis, University of Canterbury, New Zealand. In: Siregar, I.Z., and Sweet, G.B. 2000. The Impact of Extraction and Storage Conditions on the Viability of Radiata Pine Pollen. *Silvae Genetica*, 49, 1. p. 10-14.

Shivanna, K.R., and Rangaswarmy, N.S. 1992. *Pollen Biology: A Laboratory Manual*. Springer-Verlag, Heidelberg, Germany.

Shivanna, K.R., Linskens, H.F., and Cresti, M. 1991. Pollen viability and vigor. *Theo. Appl. Genetics*, 81:38-42.

Sigma Aldrich, 2005. www.sigmaaldrich.com website. Fundamental Techniques in Cell Culture…A Laboratory Handbook. **7.0 Cryopreservation and storage of cell lines.**

Siregar, I.Z., and Sweet, G.B. 2000. The Impact of Extraction and Storage Conditions on the Viability of Radiata Pine Pollen. *Silvae Genetica*, 49, 1. p. 10-14.

Snyder, E.B. 1957. Pollen Handling. Proceedings of the 4th Southern Forestry Tree Improvement Conference. Jan 8-9, Athens, GA. Southern Institute of Forest Genetics, USDA Southern Forest Experiment Station. pp. 111-115.

Snyder, E.B. and K.E. Clausen, 1974. Pollen handling. In: C.S. Schopmeyer, ed., Seeds of woody plants in the United States. USDA Agric. Handbook 450.

Sprague, J.R., and Snyder, E.B. 1981. Extracting and Drying Pine Pollen. In: E.C. Franklin, Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 33-36.

Sprague, J.R. and Johnson, V.W. 1977. Extraction and storage of loblolly pine (*Pinus taeda*) pollen. Proceedings of the 14th Sou. For. Tree Imp. Conference. Pp. 20-27.

Stanley, R.G. 1967. Factors affecting germination of the pollen grain. Proceedings, IUFRO 14th Congr., Munich, West Germany. Vol. 3. Pp. 38-59.

Stanley, R.G. and Linskens, H.F. 1974. Pollen. Springer-Verlag, New York. 307pp.

Tighe, M.E. 2004. Unpublished data from research pursuant to a Master's of Science degree at NC State University, Raleigh, North Carolina, USA.

Towill, L.E. personal communication 2003.

Towill, L.E. 2002. Cryopreservation of Plant Germplasm: Introduction and Some Observations. In: Biotechnology in Agriculture and Forestry 50: Cryopreservation of Plant Germplasm II. Springer-Verlag Berlin Heidelberg.

Towill, L.E. and C. Walters. 2000. Cryopreservation of pollen.. In: F. Englemann and H. Takagi (eds.) Cryopreservation of tropical plant germplasm: Current research progress and applications. International Plant Genetic Resources Institute, Rome. Pp. 115-129

Vergara L., R., Ipinza C., R., and Pérez P., Erika. 1995. Manual de Cruzamientos Controlados para *Pinus radiata* D. Don. Cooperativa de Mejoramiento Genético UACH/CONAF/Empresas Forestales. Univ. Austral de Chile, Valdivia. 50 pp.

Wang, B.S.P., Charest, P.J., and Downie, B. 1993. *Ex situ* storage using pollen, Ch. 3 in: *Ex Situ* storage of seeds, pollen and *in vitro* cultures of perennial woody plant species. Food and Agriculture Organization of the United Nations Forestry Paper #113. Rome. 83 pp.

Webber, J.E., and Bonnet-Masimbert, M. 1993. The response of dehydrated Douglas-fir (*Pseudotsuga menziesii*) pollen to three *in vitro* viability assays, and their relationship to actual fertility. *Ann. Sci. For.* 50: 1-22.

Wilcox, J.R. 1965. Vacuum storage of yellow-poplar pollen. U.S. Forest Service Res. Paper NC-6. N. Central For. Expt. Sta., pp. 102-103.

Wilson, A.T., Vickers, M., and Mann, L.R.B. 1979. Metabolism in dry pollen: a novel technique for studying anhydrobiosis. *Naturwissenschaften* 66:53-54. In: Barnábas, B. and Kovács, G. 1997. Ch. 14--Storage of pollen. In: Shivanna, K.R. and Sawhney, V.K., 1997. Pollen Biotechnology for Crop Production and Improvement. Cambridge University Press. Cambridge, UK. 448 pp.

Young, H.J. 1990. Ch.11—Pollination and reproductive biology of an understory neotropical aroid. In: K.S. Bawa and M. Hadley (eds). 1990. Reproductive Ecology of Tropical Forest Plants. Unesco Man and the Biosphere Series. Parthenon Press. Paris, Carnforth, Lancs, UK. Park Ridge, NJ, USA. 421 pp.

Zobel, B.J. Personal communication 2001.

Zobel, B.J, Van Wyk, G., and Stahl, P. 1987. Growing Exotic Forests. John Wiley & Sons. New York, Chichester, Brisbane, Toronto, Singapore. 508 pp.

VII. Appendices

Appendix 1. PDCS--Pollen Development Classification System

Stage 1.0 Catkins can be distinguished in the fall as individual buds on the vegetative shoot but are enclosed by bud scales.

Stage 2.0 Individual catkins emerge from their protective bud scales and slowly elongate from November to February.

Stage 3.0 Individual microsporophylls develop as catkins increase in length over an extended period. Pollen catkins exude a clear fluid when pressed between the fingers.

Substage

3.3 Pollen catkins exude a yellow liquid when pressed between the fingers, this stage occurs approximately 7-10 days before pollen release.

3.6 Catkins increase further in length and exude a clear liquid when pressed between the fingers. This stage occurs approximately 3 to 5 days before pollen release.

3.9 Very little if any fluid can be pressed from the catkin. Microsporophylls are separating. Catkins bend easily and spaces are visible between sporophylls. Catkins feel light and "rubbery" when bent. This stage occurs approximately 1 to 2 days before pollen release.

Stage 4.0 Microsporophylls begin to release pollen. Pollen release begins at the proximal end of the catkin and progresses acropetally. Pollen released from cluster is less than 10 percent of the total.

Stage 5.0 Maximum pollen release for the catkin cluster. The majority of individual catkins within a cluster are releasing pollen. Pollen release can be divided into substages based on a visual estimate of the pollen released from the catkins cluster.

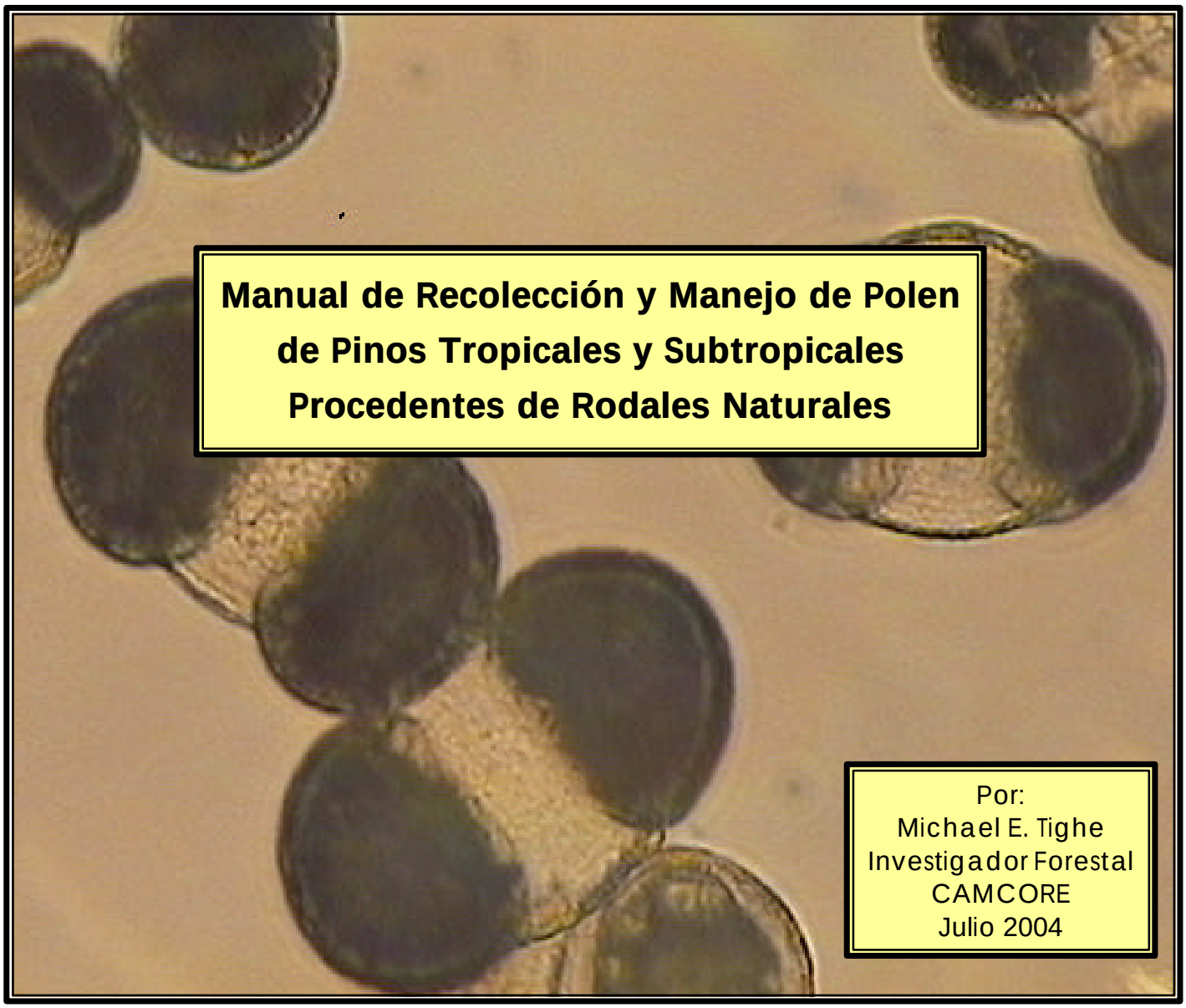
Substage

5.2 Twenty percent of pollen released from the catkin cluster.
5.4 Forty percent of pollen released from the catkin cluster.
5.6 Sixty percent of pollen released from the catkin cluster.
5.8 Eighty percent of pollen released from the catkin cluster.

Stage 6.0 Pollen release completed. All microsporophylls have opened and released pollen. Catkins are light to dark brown in color and are dry.

Bramlett, D.L., and Bridgwater, F.E. 1989. Pollen development classification system for loblolly pine. Proceedings 20th Southern Forest Tree Improvement Conference. In: NCSU-TIP Coop. 2003. Tree Improvement Manual. North Carolina State University Industry Tree Improvement Cooperative. Raleigh, NC.

Appendix 2. Manual de Recolección y Manejo de Polen de Pinos Tropicales y Subtropicales Procedentes de Rodales Naturales (Español/Spanish)



Manual de Recolección y Manejo de Polen de Pinos Tropicales y Subtropicales Procedentes de Rodales Naturales

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Julio 2004

NC STATE UNIVERSITY

Bruce and Barbara Zobel Endowment for International Forestry Studies

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Prefacio

La información presentada en las siguientes páginas es el resultado de una gira técnica a Guatemala financiado a través de una beca de la Donación Zobel (Zobel Endowment), experiencia profesional con CAMCORE, experimentos realizados conforme con estudios de postgraduado en la Universidad Estatal de Carolina del Norte en Raleigh, NC, USA, y revisión de material publicada.

Mientras existe mucha literatura que detalla los protocolos específicos para el análisis de polen y prácticas de polinización, muy poca literatura existe en Latinoamérica en español que de una perspectiva amplia de los protocolos básicos para el manejo del polen de pino. Este manual fue escrito específicamente para brindarle a los profesionales forestales hispanohablantes en Latinoamérica y otros lugares una guía de referencia, detallando métodos actuales para la recolección, secado, y almacenamiento de polen procedente de pinos tropicales y subtropicales en rodales naturales. Aunque existen muchos programas sofisticados de mejoramiento genético en esta región geográfica, éste manual intenta ofrecer recomendaciones y una orientación simplificada para los profesionales forestales interesados en programas de hibridación o mejoramiento genético forestal. Una referencia excelente para cruces controlados con *Pinus radiata* D. Don fue realizada en español por una unión cooperativa universidad/gobierno/industria forestal en Chile por Vergara, et al. (1995), cuya citación aparece en la bibliografía. Este manual trata específicamente con *Pinus radiata*, pero provee descripciones detalladas de actividades de mejoramiento genético forestal comunes para muchas especies de pino. Si desea información adicional, puede ser obtenida por medio de las referencias incluidas, o contactando al autor en la dirección dada al final del manual.

Contenido

Recolección y Manejo de Polen de Pinos Tropicales y Subtropicales Procedentes de Rodales Naturales

1. Introducción.

Con el aumento de la competencia global en la industria forestal, muchos líderes industriales regionales e internacionales están haciendo inversiones significativas en proyectos de plantaciones forestales en el hemisferio Sur debido a las altas tasas de crecimiento y bajos costos de producción. La rentabilidad y avance de estos proyectos en las zonas tropicales, subtropicales y templadas depende en gran parte del éxito de los programas de mejoramiento genético y las técnicas silviculturales. Muchos de los proyectos de plantaciones más exitosos han sido establecidos en Latinoamérica. Por ejemplo los éxitos en Chile, Brasil, Argentina, Colombia, y Venezuela, han definido estas compañías como fuertes competidores y frecuentemente líderes en la industria de productos forestales al nivel mundial.

Debido a sus ubicaciones y topografía, estos países ofrecen ambientes y potenciales económicos diferentes. Consecuentemente, las organizaciones en estos países cultivan especies desde latifoliadas de corta-rotación para pulpa y papel hasta pinos de mayor rotación para postes y madera. Muchas empresas también están diversificando sus portafolios de especies en estas plantaciones para balancear el riesgo y aumentar los rendimientos. Esto ocurre muchas veces por medio del establecimiento y evaluación de plantaciones pilotos de especies exóticas o la creación de híbridos para rendimientos mejorados y resistencia a plagas y enfermedades.

El desarrollo de híbridos dentro de un género se logra operacionalmente por medio de polinización controlada. El polen debe ser recolectado y en algunos casos almacenado para poder realizar los cruces deseados en la época de receptividad máxima. Mientras los protocolos están bien identificados para el manejo de polen relacionado con mejoramiento genético en eucaliptos y otras especies de latifoliadas, existe poca información relacionada con el manejo de polen de pinos tropicales y subtropicales. Las características deseadas de estas especies (resistencia a plagas, resistencia a sequías, etc.), combinadas con su adaptabilidad y propiedades de la madera favorables, han aumentado su popularidad como especies para la diversificación de plantaciones y mejoramiento en Latinoamérica.

La recolección y manejo de polen se desarrolla como una estrategia importante para el mejoramiento genético forestal y manejo intensivo de plantaciones forestales. A través de polinización controlada y técnicas de polinización masiva suplementaria, profesionales forestales pueden seleccionar individuos que expresen características deseables y para así controlar los árboles padres y realizar el cruce deseado. La contribución masculina al cruce se realiza por la recolección del polen del individuo deseado, para luego aplicarlo sobre un estróbilo femenino escogido para la fecundación y la producción de semilla. Cruces seleccionados de individuos deseables pueden producir progenie con las mismas características, y luego formar la base de selección para generaciones avanzadas.

La recolección de polen para usos agrícolas y alimenticios ha sido bien documentada a través de la historia, originados en antiguos documentos del Egipto, China, Grecia, Rusia, y Persia,

entre otros (Binding, 1980). Sin embargo, en la mayoría de los casos estas citas se refieren a la recolección de polen con respecto a miel de abeja, prevención de enfermedades, y sobre todo a la protección de la salud humana. Esto contrasta mucho con la ciencia de recolección y manejo de polen para especies forestales relativamente nueva. El interés en estos temas está aumentando diariamente, al ritmo de los avances en la tecnología y el desarrollo de nuevas prácticas.

Este manual se dirige específicamente a los profesionales forestales hispanohablantes en Latinoamérica y otros lugares para proveer una guía referencial detallando métodos actuales para la recolección, secado, y almacenamiento de polen procedente de pinos tropicales y subtropicales en rodales naturales. Aunque muchos programas sofisticados de mejoramiento genético existen en esta región geográfica, éste manual intenta ofrecer recomendaciones y una orientación simplificada para profesionales forestales interesados en mejoramiento genético forestal. Si desea, información adicional puede ser obtenida a través de las referencias incluidas o contactando al autor en la dirección dada al final de este manual.

2. Recolección del Polen.

La primera etapa en la recolección de polen es el entendimiento de la fisiología y fenología de la especie forestal. Adaptaciones naturales, diferencias climáticas, y evolución se han combinado para crear un sistema complejo de sincronización entre la producción de polen y la receptividad de las flores. La investigación científica muestra que generalmente la época de mayor dispersión del polen y también de mayor receptividad de las flores ocurre simultáneamente en pinos naturales (Boyer, 1981)(Bramlett, 1973). Sin embargo, la variación entre la producción de polen y receptividad, hasta entre individuos dentro del mismo huerto, puede limitar el cruzamiento o fecundación en la polinización abierta.

Normalmente las yemas reproductivas se inician un año antes de la aparición de los estróbilos microesporangios (masculinos) y megaesporangios (femeninos) en pinos. En todo el género, el polen se caracteriza por un cuerpo central de forma ovoide flanqueado por dos sacos aeríferos, y está producido en los sacos polínicos en la base de las hojas catafilares fértiles. Los nombres comunes para estas estructuras reproductivas son “catkins”, “flores masculinas” o “estróbilos masculinos”. Los estróbilos masculinos se producen normalmente en las ramas de lento crecimiento en la copa inferior del árbol, una evolución natural para evitar auto-polinización. Se produce los estróbilos femeninos en la copa superior del árbol y aunque las coníferas no producen flores verdaderas (Nel, 2002), se utiliza este término popularmente entre profesionales forestales para referir a los estróbilos megaesporangios. Para evitar errores y expresiones regionales, los términos “estróbilo masculino” y “estróbilo femenino“ se usarán para lograr las metas de este reporte.

Las yemas se desarrollan para formar estróbilos megaesporangios femeninos o microesporangios masculinos durante su primer año (Figura 1). La temperatura del aire ambiental, la elevación sobre nivel del mar, y la precipitación son los factores más importantes en el desarrollo y la dispersión del polen. Lluvias tempraneras que ocurren antes del comienzo de la época lluviosa (similares a las lluvias veranéales tardías en zonas templadas) pueden favorecer el desarrollo de estróbilos masculinos sobre el desarrollo de los estróbilos femeninos en muchas especies de pinos (Bengston, 1969). El ritmo del desarrollo de los

estróbilos masculinos (y femeninos) aumenta durante la siguiente época lluviosa, después del estado latente, y la dispersión del polen comienza durante la época seca siguiente (dependiendo de la temperatura y la humedad ambiental). Estos ciclos cambian mucho en ambientes tropicales, donde las especies pueden producir estróbilos masculinos y femeninos todo el año (Isaza, comunicación personal 2003), aunque existe una época de producción y receptividad máxima debido a las influencias climáticas (sequías, lluvias temporales).
Como se mencionó previamente, la sincronización de la máxima dispersión de polen y la receptividad del estróbilo femenino es generalmente simultánea, aunque la fecha de ocurrencia de estos picos pueda variar apreciablemente cada año. Las temperaturas inferiores al promedio retardan el desarrollo

Figura 1. Ciclo de vida del pino. **Reprinted with permission from Carolina Biological Supply Company. Translated from the original English into Spanish by Michael E. Tighe**

del polen considerablemente, mientras las temperaturas por encima del promedio avanzan el desarrollo anticipado para la mayoría de las especies. Los estróbilos masculinos se desarrollan y maduran durante el verano o en épocas secas hasta que el nivel de humedad disminuye a un valor predeterminado por la naturaleza y los estróbilos masculinos comienzan a abrir. Las escamas de polen abren y liberan el polen, el cual en pinos es dispersado por el viento (polinización anemófila). Se han utilizado varios métodos para calcular el tiempo de la dispersión máxima del polen, incluyendo el método de días-grados utilizado extensamente en agricultura. Un método bien investigado para determinar el desarrollo del polen y la época para su recolección fue desarrollada por Bramlett y Bridgwater (1989), el cual describe las etapas del desarrollo de los estróbilos masculinos que puede ser comparadas al sistema del desarrollo del estróbilo femenino. Las épocas del año preferibles para la recolección del polen se basan en las etapas del desarrollo de los estróbilos masculinos por medio del sistema de clasificación y desarrollo del polen (SCDP) en Tabla 1.

Tabla 1. SCDP-Sistema de Clasificación y Desarrollo de Polen (*Pinus taeda—autor*)*

Etapa 1.0 Se puede distinguir los estróbilos masculinos en el otoño como yemas individuales por el brote vegetativo, pero están encerradas por las escamas foliáceas.

Etapa 2.0 Los estróbilos individuales emergen de sus escamas foliáceas protectoras, y se alargan lentamente desde noviembre a febrero.

Etapa 3.0 Las microsporofilas individuales se desarrollan mientras los estróbilos aumentan en longitud sobre un período extendido. Estróbilos masculinos de polen emiten un fluido transparente cuando son apretados entre los dedos.

Figura 2. Estróbilos masculinos *Pinus maximinoi* en aproximadamente Etapa 3.3 en Guatemala (Foto cortesía de Elmer Gutiérrez).

Subetapa 3.3 Los estróbilos masculinos de polen exudan un fluido amarillo cuando son apretados entre los dedos. Esta etapa ocurre 7-10 días antes de la dispersión del polen.

Subetapa 3.6 Los estróbilos masculinos aumentan aún más en longitud y emiten un fluido transparente cuando son apretados entre los dedos. Esta etapa ocurre 3-5 días antes de la dispersión del polen.

Subetapa 3.9 Muy poco (o ningún) fluido puede ser forzado del amento. Las microsporofilas ya están dividiéndose. Los estróbilos masculinos pueden ser doblados fácilmente y es posible notar espacios entre las esporofilas. Los estróbilos masculinos son livianos y al doblarse dan una sensación de goma. Esta etapa ocurre aproximadamente 1-2 días antes de la dispersión del polen.

Etapa 4.0 Las microsporofilas comienzan a dispersar el polen. La dispersión del polen comienza en la base y se desarrolla hacia la cumbre del estróbilo. El polen dispersado del racimo es menos del 10 por ciento del total.

Etapa 5.0 Máxima dispersión de polen del racimo de estróbilos masculinos. La mayoría de los estróbilos individuales dentro del racimo están dispersando polen. La dispersión puede ser dividida en subetapas basado en una estimación visual del por ciento del polen dispersado del racimo de estróbilos masculinos.

Figura 3. La dispersión de polen *Pinus oocarpa* en Colombia en aproximadamente Etapa 5.6 (Foto cortesía de Norha Isaza, Smurfit Cartón de Colombia).

Subetapa 5.2 Veinte por ciento del polen está dispersado del racimo de estróbilos.

Subetapa 5.4 Cuarenta por ciento del polen está dispersado del racimo de estróbilos.

Subetapa 5.6 Sesenta por ciento del polen está dispersado del racimo de estróbilos.

Subetapa 5.8 Ochenta por ciento del polen está dispersado del racimo de estróbilos.

Etapa 6.0 La dispersión de polen esta completa. Todas las microsporofilas han abierto y dispersado su polen. Los estróbilos masculinos están de color castaño claro a castaño oscuro y están secos.

* Excerpto de: Bramlett, D.L., and Bridgwater, F.E. Pollen Development Classification System for Loblolly Pine. Proceedings from the 20th Southern Forest Tree Improvement Conference. 1989 as cited in NCSU-TIP, 2000. --Traducido por Michael Tighe, 2004.

Después de haber dispersado de los estróbilos masculinos por el viento, el grano de polen se adhiere a la gota micropilar en el estilo del estróbilo femenino. Esto indica que la polinización ha ocurrido. Cuando el grano de polen se adhiere al estróbilo femenino y encuentra condiciones favorables para la germinación, se germina y comienza el desarrollo de la escama polínica. Posteriormente, el grano de polen comienza un período de desarrollo lento, hasta que pasa aproximadamente un año (en la mayoría de las especies) desde que aparecen los estróbilos. Luego, el ritmo de desarrollo aumenta y la escama polínica comienza a alargarse rápidamente. Se logra la fecundación, y el óvulo comienza el proceso de embriogénesis. La semilla se forma en los conos, completando el ciclo reproductivo.

La duración del ciclo reproductivo en pinos varía de acuerdo a la especie y su ubicación, y puede durar desde 9 meses hasta 4 años. La especie asiática *Pinus merkusii*, por ejemplo, tiene un ciclo reproductivo corto de 9 meses en Indonesia (Dvorak, com. pers., 2004), mientras la misma especie tiene un ciclo de 12 meses en el noroeste de Tailandia (DFSC, 2000). En el caso contrario, en los rodales naturales de *Pinus maximartinezii* en México se ha comprobado que se demoran hasta 4 años para completar el ciclo (Donahue y Mar-López, 1995) (Dvorak *et al*. , 2000). Estas adaptaciones naturales pueden ser debidas a una variedad de factores, entre ellos: las diferencias climáticas en la época de mayor crecimiento debido a la ubicación geográfica (*P. merkusii* es el único pino que se encuentra al sur del ecuador), a la competencia establecida por la presencia de malezas, incendios, o la predación de semillas (animal). Estas diferencias en la duración del ciclo reproductivo refuerzan la necesidad de entender la fenología de la especie antes de empezar las actividades de mejoramiento genético forestal.

El ritmo de la recolección de polen es muy importante, ya que la colección de estróbilos masculinos inmaduros puede resultar en la reducción de viabilidad de los granos y bajos rendimientos de semilla (Snyder y Clausen, 1974). La etapa ideal para la recolección de polen queda entre Etapas 3.6-3.9, cuando el polen está maduro, pero todavía firmemente sujetado a los estróbilos masculinos (Bramlett, como citado en NCSU TIP, 2000). El polen se comienza a dispersarse desde las escamas seminíferas hacia la base de los estróbilos más avanzados, mientras la mayoría del polen se mantiene todavía en los estróbilos normales del huerto (Brees, *et al.*, 1981). Sin embargo, cuando se llega a esta etapa puede ser demasiado tarde para lograr máximos rendimientos de polen, y la recolección tempranera puede ser adelantada artificialmente utilizando las técnicas disponibles en Dorman (1976) o en Snyder (1957). Los tiempos adecuados para la recolección de polen en rodales naturales varían por especie, elevación, clima, y geografía. A medida que el rodal se encuentra más cercano al ecuador, las épocas de recolección son mucho más irregulares y hasta pueden presentarse todo el año (Mirov, 1967). Tabla 2 muestra ejemplos de las fechas distintas de recolección en rodales naturales de pino en varios países.

Tabla 2. Épocas de recolección de polen en rodales naturales.

Especie	*País*	*Meses Aproximadas de la Recolección de Polen***
Pinus tecunumanii	Guatemala	febrero-abril
Pinus taeda	Estados Unidos (Zona Sureña)	abril-junio
Pinus greggii	México	abril-mayo
Pinus oocarpa	Guatemala	noviembre-diciembre
Pinus maximinoi	Guatemala	febrero-abril
Pinus oocarpa	México (zona sureña)	noviembre-enero
Pinus patula	México	enero-abril

**Las fechas de la recolección de polen fueron obtenidas por medio de documentación y contacto directo con miembros de CAMCORE en los respectivos países.

Los pinos tropicales y subtropicales muestran variación significativa en relación con la época de dispersión de polen en rodales naturales, como se ve en la Tabla 2. Abajo, la Tabla 3 muestra los distintos meses de recolección de polen para una sola especie, *Pinus caribaea* var. *hondurensis* (PCH) plantada por fuera de su región natural de ocurrencia. Esta especie es autóctona de la zona de Centroamérica y Quintana Roo, México, y muestra patrones de desarrollo muy distintos cuando es sembrada como exótica en otras regiones. Se encontró que el desarrollo del polen de PCH tiene pobre sincronización con la presencia de flores cuando plantado es en los llanos cálidos de la zona oriental de Venezuela, pero mejor sincronizado (producía más semilla con polinización abierta) cuando es plantado en altas elevaciones o en zonas más alejadas del Ecuador (Dvorak, *et al*., 2000). Se encontraron problemas similares con la producción de semilla cuando PCH fue plantado en Monte Dourado, Pará, Brasil (Brune, 1990).

Tabla 3. Fechas de recolección de polen de *Pinus caribaea* var. *hondurensis* cuando es plantado como exótico en distintos países.

País	**Meses Aproximados de la Recolección de Polen****
Brasil	mayo-julio
Venezuela	noviembre-enero
Sudáfrica	febrero-abril
Argentina	mayo-junio
Belize	noviembre-diciembre

**Las fechas de la recolección de polen fueron obtenidas por medio de documentación y contacto directo con miembros de CAMCORE en los respectivos países.

La humedad del polen varía entre los estróbilos masculinos debido a su exposición y maduración. Bramlett (como citado en NCSU-TIP, 2000) sugiere que "Recolecciones de polen no deben incluir los racimos que están en las primeras etapas del desarrollo aunque unos de los racimos del mismo árbol se están dispersando." En un estudio no publicado en Guatemala por el autor, la humedad de los estróbilos masculinos fue analizada y relacionada a la tasa de germinación in vitro del polen de *Pinus tecunumanii*. En rodales naturales, la recolección de polen se lleva a cabo a través de la escalada de árboles seleccionados y la cosecha de los estróbilos masculinos de la copa inferior. En este caso, el escalador cosechó

todos los estróbilos cerca del fuste y los puso directamente en la bolsa de colección, mientras que los que estaban más cerca de los extremos de las ramas fueron cosechados con una podadora de mango largo (*pole pruner*) y procesados abajo manualmente. Debido a la germinación variable del polen cosechado en años anteriores, el polen fue dividido en bolsas separadas e identificado por su método de recolección para el análisis. El polen recolectado en la copa (cerca del fuste) fue inspeccionado y se encontró obviamente en una etapa más desarrollada de maduración (SCDP, Tabla 1.) que la mayoría del polen recolectado en las puntas para procesamiento abajo. Para evaluar la diferencia entre las dos etapas de polen, se realizaron dos estudios pequeños basados en el polen de estos árboles.

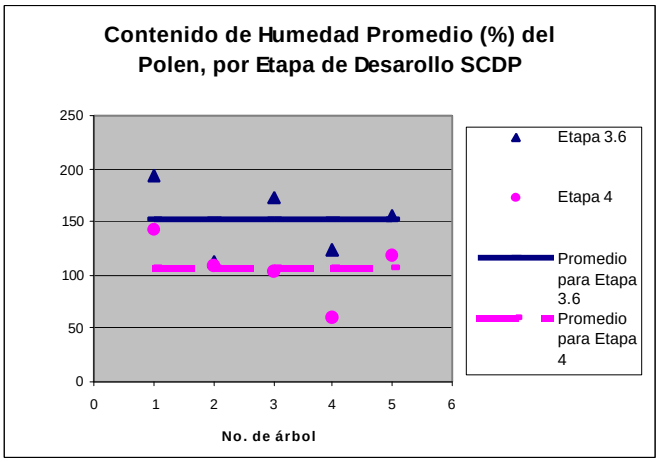

Figura 4. Contenido de humedad promedio encontrado en los estróbilos masculinos recolectados en Etapa 3.6 y 4 según el sistema SCDP en Bramlett y Bridgwater, (1989).

El primer estudio se dedicó a la evaluación de la diferencia entre el contenido de humedad de los estróbilos masculinos en las dos etapa s (Etapas 3.6 y 4 en Tabla 1). Por esta razón, los estróbilos fueron cosechados asignándoles el número de su etapa de desarrollo, y puestos en bolsas plásticas bien selladas. El polen cosechado en la copa (cerca del fuste) fue casi siempre de Etapa 4, más maduro que el polen de la punta de las ramas. La Figura 2 muestra el contenido de humedad promedio de las muestras en ambas etapas. Estos datos fueron recolectados para ayudar en el asesoramiento de la conexión entre el contenido de humedad (%CH) y la germinación, cuando se compararon con los datos de germinación del polen *P. tecunumanii* recolectado en las mismas etapas SCDP. Esta información servirá consecuentemente para enfocar las actividades de recolección. El polen de etapa 3.6 (recolectado de las p untas de las ramas con la podadora de mango largo) fue mucho más húmedo que el polen de Etapa 4 recolectado en la copa del árbol cerca del fuste.

3. Cosecha del Polen.

El segundo experimento se concentró en una comparación entre la humedad del polen y su germinación para usos operacionales en programas de mejoramiento genético forestal. Por lo cual, el polen fue tratado de igual forma, y se sometió al aire ambiental solamente lo suficiente para inducir la abertura de los estróbilos para liberar su polen (48 horas). Luego, el polen fue embotellado individualmente y evaluado por su contenido de humedad individual y promedio, tanto

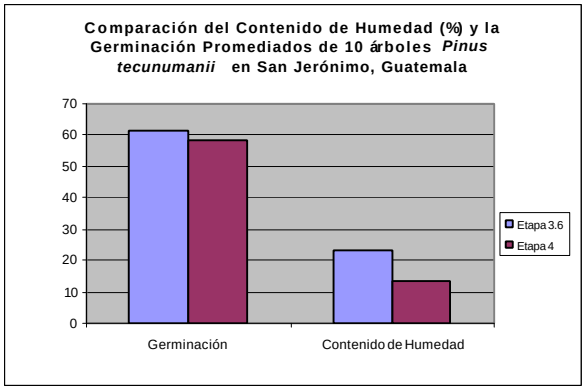

Figura 5. Comparación de la germinación y contenido de humedad para polen cosechado en Etapas 3.6 y 4 como identificado en Bramlett y Bridgwater (1989)

como su porcentaje de germinación in vitro. Figura 5 muestra los promedios de estos cálculos. El polen de la Etapa 3.6 (recolectado de las puntas de las ramas con una podadora) retuvo mayor humedad que el polen de Etapa 4 recolectado cerca del fuste, aún después de haber sido expuesto de forma iguala la humedad ambiental en el proceso del secado al aire libre. Sin embargo, las muestras húmedas de la Etapa 3.6 tenían esencialmente el mismo porcentaje de *germinación* que el polen más seco y maduro de la Etapa 4. Entonces, los resultados de este estudio sugieren que las veces que sea necesario recolectar polen a gran escala, parece ser aceptable cosechar todos los estróbilos posibles de ambas zonas del árbol, y mezclarlos para el secado. Esto resulta en una mezcla aceptablemente homogénea, y permite al escalador cosechar una cantidad de polen suficiente para un programa de mejoramiento escalando el árbol de una sola vez (mayor eficiencia).

El polen para mezclas en bulto debe ser recolectado de (como mínimo) 20 árboles para mantener la mayor proporción de la variación genética. Este número es procedente de estudios de investigación sobre la variación alélica en estudios de semilla (Dvorak, *et al*., 1999) y se considera suficiente para analizar todos los alelos que ocurren en frecuencias de 5% o más dentro de poblaciones grandes y pequeñas. Se debe recolectar el polen de árboles esparcidos naturalmente por el sitio para capturar las diferencias genéticas y representar las diferencias climáticas entre micrositios que puedan influir la calidad del polen.

Los estróbilos masculinos deben ser recolectados en racimos, y puestos en bolsas construidas de un material permeable que permita la salida y extracción natural de la humedad (como una bolsa de papel Kraft). Se deben poner los racimos en la bolsa con no más de 2.5 cm de estróbilos de espesor para mejorar el secado y disminuir la probabilidad de pudrición debido a la presencia de hongos (NCSU-TIP, 2000). Es imperativo que cada bolsa esté bien rotulada, con una identificación única, y que ésta información esté guardada en los archivos. De esta manera, los árboles que producen polen consistentemente bueno o de baja calidad pueden ser cosechados de manera preferencial en los años posteriores. Esto también provee información adicional para programas de mejoramiento genético sofisticados que se pueden beneficiar del conocimiento estricto de la genealogía, tal como el manejo de consanguinación o autofecundación (Griffin, 1990). Estas etiquetas únicas de identificación deben seguir el material hasta el fin de la producción de semillas y cruzamiento para asegurar la información de linaje y la historia genética adecuada. La atención a los detalles y la representación suficiente de la población son las claves en la investigación genética forestal.

4. Extracción y Secado del Polen.

En zonas remotas de difícil acceso, la extracción de polen de los estróbilos masculinos se puede lograr de varias maneras. Un método económico comienza con las bolsas Kraft (*Kraft paper bags*) cargadas de racimos de estróbilos de no más de 2.5 cm de espesor como se mencionó previamente en la Sección 2 (véase Figura 6.). Se deben cerrar bien las juntas o

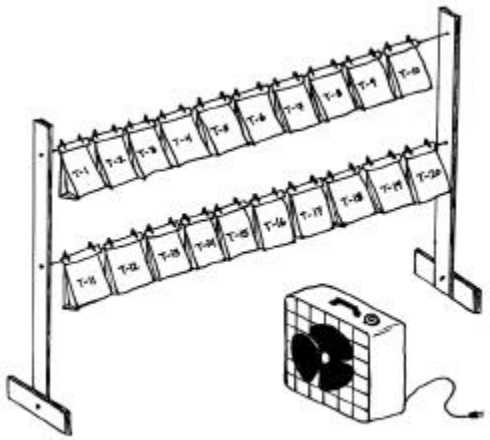

Figura 6. Un método rústico para la extracción de polen utilizando bolsas de papel Kraft, aire cálido y ventilación suficiente.

uniones de las bolsas Kraft con cinta adhesiva para evitar el escape de polen mientras los estróbilos liberan su polen. Posteriormente, se cuelgan las bolsas en un salón cálido (32-38°C) con la humedad relativa baja (menor del 40%) y con un ventilador eléctrico (*standard oscillating fan*) para propiciar el movimiento del aire (NCSU-TIP, 2000) (Beers, *et al.*, 1981) (Sprague y Snyder, 1981). Dependiendo de las condiciones ambientales, las escamas seminíferas se deberían abrir y comenzar a liberar el polen después de 2 días. En este momento, el contenido de las bolsas debe ser descargado en el colador de suelo—conocido técnicamente como "tamiz de suelo" (*soil sieve*)--cuidadosamente; cortando la esquina de la bolsa Kraft y vertiéndola sobre el colador. Luego se vierte el contenido sobre un colador No. 35 (.0197" malla) para separar el polen de los restos del estróbilo. Se pueden agitar los estróbilos suavemente con un pincel blando hecho de fibras naturales (*natural hair brushes*), para alcanzar rendimientos altos (Figura 7.). Después, el polen previamente pasado por el colador No. 35 debe ser pasado por el más fino No.60 (.0098" malla) y luego el colador No. 120 (.0049"malla) colador (Gutiérrez, 2004, com. personal). Estas etapas extraen aún más de los restos y escombros del estróbilo, pero también sacan la mayoría de las larvas de la peligrosa mosca de sierra ("sawfly", *Neodiprion*, spp.) y otros insectos. Estas etapas son importantes tanto para mantener la viabilidad como para evitar la distribución de plagas dañinas. El polen debe ser almacenado en un contenedor temporáneo y hermético después de la limpieza. Los proveedores y los costos aproximados para realizar estas actividades de extracción se encuentran en este manual en el Apéndice 1.

Figura 7. Un método simple para la limpieza de polen. Después de la extracción primaria, se pasa el polen por tejidos sucesivamente más finos para sacar restos y larva de insectos. (La foto muestra polen *Pinus tecunumanii* en Guatemala.)

Sistemas adicionales para la extracción de polen también son efectivos, aunque pueden requerir de infraestructura e inversión adicional. Varios mecanismos como bolsas de aislamiento (se pueden usar "sausage casings"), cámaras secadoras, etc. están descritos en Sprague y Snyder (1981) y NCSU-TIP (2000). Estos sistemas pueden ser más aptos para programas sofisticados y resultan en un producto más limpio y seco con menor inversión en la mano de obra requerida.

Cuando el polen ha sido separado de todos los desechos externos, el contenido de humedad debe ser calculado antes de su almacenamiento. Esta es una etapa muy importante, la cual determina si el polen requiere secado adicional o no. El mantenimiento de niveles de humedad bajos es clave para el almacenamiento exitoso del polen (Jett y Frampton, 1990). Existen métodos sofisticados y baratos de evaluación del contenido de humedad, pero un método simple requiere sólo una bandeja de aluminio para pesar (resistente al calor), una balanza estándar Mettler® (que lea hasta 4 lugares decimales) y un horno secador de laboratorio (*convection oven*).

Primero, el horno secador debe ser prendido a una temperatura de 100°C (usando un termómetro de laboratorio—*laboratory thermometer*). Segundo, la bandeja de pesar

(*aluminum weigh boats*) se pesa en la balanza Mettler y se tara su peso. Se debe apuntar el peso, luego cargar la bandeja de pesar con aproximadamente 0.5-1 gramos de polen, esparcido uniformemente, y anotar el peso total. Esto medirá el peso total "verde" (el polen natural más el peso de la bandeja de pesar). Después, la bandeja de pesar con el polen debe ser introducida en el horno secador por una hora (como mínimo). Eso extraerá toda la humedad de la muestra. La bandeja junto con el polen debe ser pesada inmediatamente después de sacarla del horno para asegurar que la muestra no absorba humedad ambiental. Este peso representa el peso total "seco". Luego, se utilizan estos números en la formula siguiente:

$$\text{\% Contenido de humedad} = \frac{(\text{peso de polen 'verde'}) - (\text{peso de polen seco}) \; X \; 100}{\text{peso de polen seco}}$$

Para almacenar, Moody (1988) comentó que en general para evitar el deterioro significativo del polen almacenado (también citado en NCSU-TIP, 2000), el polen de pino debe tener menos del 10-15% de contenido de humedad (%CH). Otras recomendaciones varían entre 7-20%CH, dependiendo de la especie y la duración de almacenamiento deseada (Snyder, 1957) (Jett y Frampton, 1990) (Bonnet-Masimbert y Webber, 1995) (Hoekstra, 1992) (Lanteri *et al.*, 1993) (Siregar y Sweet, 2000). Hoekstra (1992) explica que no se puede detectar la respiración fisiológica del polen con contenidos de humedad menor del 20%CH. También explica que en polen con un CH entre 10-15% no existe agua libre (disponible) para transporte intracelular. Si el polen es activo fisiológicamente bajo cualquier método de almacenamiento, la viabilidad disminuye rápidamente y las prácticas de almacenamiento se vuelven inútiles. El control del contenido de humedad es esencial para el almacenamiento exitoso del polen. En la gran mayoría de los pólenes de pinos, 6-8%CH es recomendado para almacenamiento bajo refrigeración, pero el polen almacenado entre 3-5%CH parece mantener su viabilidad mejor cuando es congelado o almacenado criogénicamente (vea Sección 5). Un contenido de humedad inferior al 16% también asegura que las larvas de *Neodiprion*, spp., en el caso de estar presentes, serán eliminadas (Sprague y Snyder, 1981).

Por medio de experiencias en CAMCORE, aprendimos que si el contenido de humedad en polen de pinos tropicales o subtropicales es mayor o igual al 10%CH, el polen debe ser secado antes del almacenamiento. Muchas instituciones dedicadas al mejoramiento genético han logrado bajar el contenido de humedad en el polen de especies de pino por medio de un secado sobre sílica gel (Sprague y Snyder, 1981) o liofilización con un "freeze dryer" (Ching y Ching, 1964) (Layne y Hagedorn, 1963), pero el polen debe ser monitoreado cuidadosamente para evitar su sobresecado. La humedad del polen puede ser reducida también de manera similar a la de la Sección 4—Extracción y Secado del Polen. El polen debe ser vertido en bolsas de papel Kraft con las juntas selladas y puestas en un horno secador con una temperatura muy baja (29°-30°C). Un horno secador de aire forzado (*forced-air oven*) funciona mejor (Figura 8.), forzando el aire cálido por encima de las bolsas de papel para absorber y extraer la humedad entre las fibras del papel Kraft. Jett y Frampton (1990) encontraron que el régimen de humedad en el polen es muy semejante al régimen en muestras de madera, y sus necesidades para el secado son similares. Generalmente, para secar polen de pino desde 14%CH a 5%CH de esta manera demora aproximadamente 6 horas

Figura 8. Un horno secador de aire forzado de marca Precision® aceptable para el secado de polen en 29-30° C.

(Tighe, datos no publicados). El polen se debe monitorear durante el secado, y el contenido de humedad se debe medir antes de remover las muestras del horno.

Cuando el polen alcanza el %CH deseado para almacenamiento, se descarga el contenido de las bolsas de papel en los contenedores de almacenamiento permanentes. Estos contenedores deben ser herméticos y sus orillas bien selladas con Parafilm™ u otro sellador similar para evitar la absorción de humedad durante el almacenamiento.

5. Almacenamiento del Polen.

La sincronización de la dispersión del polen y el período de receptividad floral, junto con las necesidades para hibridización entre especies, y el deseo de reintentar polinizaciones no exitosos son algunos de los factores que determinan la necesidad para el almacenamiento del polen. Estos factores también demuestran las distintas necesidades en los programas de mejoramiento genético forestal, desde el almacenamiento de corto plazo a mediano y largo plazo. El polen puede ser reservado por unos cuantos meses, o guardado por años dependiendo de las metas del programa de mejoramiento.

5.1. Selección de frascos para el Almacenamiento.

Cuando el polen ha sido secado hasta el contenido de humedad deseado, el polen debe ser colocado en un frasco hermético para su almacenamiento. La selección del frasco depende del método de polinización controlada a ser utilizado. Se puede lograr la polinización utilizando bolsas de aislamiento (*natural or microfiber bags*) y pinceles de pelo de camello, jeringas, (Moody y Jett, 1990) (Bramlett y O'Gwynn, 1981), u otros polinizadores distintos desarrollados por programas forestales de investigación. Se puede encontrar una buena descripción de varios métodos en Bramlett y O'Gwynn (1981). Un ejemplo de esto es el polinizador de ciclón ("cyclone pollinator", Figura 9) desarrollado por el USDA Laboratorio del Servicio Forestal juntos con Weyerhaeuser, y provee un método eficiente de polinización. Cada vez que el bulbo de goma es apretado, el aire generado expele aproximadamente 1cc de polen en la bolsa de aislamiento en una nube grande, con un movimiento giratorio que mejora la distribución del polen. El depósito del polen plástico del polinizador permite que el usuario lo cargue con aproximadamente 10cc de polen por vez. Entonces, si quiere polinizar con este instrumento, frascos de 10cc (*10cc screw top vials*) serán eficientes para el almacenamiento del polen, permitiendo el usuario cargar el depósito del polinizador y polinizar aproximadamente 10 bolsas de aislamiento con exposición mínima al aire y humedad ambiental.

Figura 9. Polinizador de ciclón armado en CAMCORE, basado en los diseños de la Cooperativa NCSU-TIP y MeadWestvaco.

Similarmente, la capacidad del polinizador debe ser determinada antes de la selección de los frascos para todos los instrumentos, para asegurar que solo la cantidad justa de polen será sacada del almacenamiento diariamente. El polen utilizado para polinización en masa (en cantidad) puede ser almacenado en volúmenes más grandes, tales como los frascos de polen de 175cc (*marca Nalgene*®) que se muestran en la Figura 10. Cada vez que el polen se expone a temperaturas más altas y a ambientes húmedos disminuirá la viabilidad del polen y anulará los efectos del almacenamiento del polen. El contenido de humedad y la temperatura son los dos factores que más influyen en la longevidad del polen en su almacenamiento (Wang, *et al*., 1993).

5.2. Duración del Almacenamiento Deseado.

Las distintas metas y necesidades de varios programas forestales requieren distintos períodos de almacenamiento del polen para lograr sus fines. Generalmente, a medida que se extiende el período de almacenamiento deseado, la temperatura utilizada para su almacenamiento disminuye (Siregar y Sweet, 2000). Las especies plantadas en las zonas tropicales, subtropicales, y templadas pueden exhibir diferencias en su máxima dispersión de polen con respecto a y su máxima receptividad floral, obligando al profesional forestal a almacenar el polen hasta que los estróbilos femeninos alcancen su período de receptividad máxima. Normalmente, esto requiere almacenamiento a corto plazo de sólo unos meses (hasta 6 meses). Otros programas pueden tener producción precoz de polen y de estróbilos femeninos, o simplemente desarrollan un polimix de pólenes que desean replicar el próximo año. También incluidos aquí son los programas que pierden la época de polinización y deben esperar hasta el próximo período. Estos constituyen almacenamiento a mediano plazo (6-18 meses). La última situación incluye el almacenamiento de polen vivo en estado de latencia por años, y se usa frecuentemente con esfuerzos de conservación o bancos genéticos de polen para protección permanente. Esto es almacenamiento de largo plazo (18 meses-perpetuidad). Los tres métodos son evaluados en detalle abajo, respaldados con datos preliminares de ensayos de germinación realizados por el autor.

Figura 10. Frascos de 175cc de polen tropical, subtropical, y templada en almacenamiento, CAMCORE.

5.2.1. Almacenamiento a Corto Plazo.

El polen en estado de latencia puede ser almacenado en el corto plazo por varios métodos (Layne y Hagedorn, 1963). Con el polen de pino, la manera más efectiva para realizar su almacenamiento a corto plazo es bajo refrigeración a 4°C. Esto resulta en un almacenamiento efectivo hasta aproximadamente un año, pero luego tiende a disminuir rápidamente. Siregar y Sweet (2000) reportan, "No existe una ventaja en almacenar polen secado al 10%CH a -20° C (congelamiento—*autor*) si sólo se requiere un año de almacenamiento." Este es el mejor método para intervalos cortos entre la disponibilidad de polen y la receptividad de estróbilos femeninos, o para cruces realizados en zonas alejadas de la zona de recolección de polen.

5.2.2. Almacenamiento a Mediano Plazo.

El almacenamiento a mediano plazo puede ser logrado exitosamente por medio de congelación a -20°C (Duffield y Callaham, 1959) o secado al vacío / congelación (disminuir el %CH bajo vacío, luego almacenar a -20°C) con un "vacuum dryer". Aunque los experimentos del autor muestran resultados pobres para las muestras expuestas al secado bajo vacío, ésta practica se usa operacionalmente con varias especies de manera exitosa (NCSU-TIP, 2000). Se evita el congelamiento y el deshielo del polen por medio del uso de frascos pequeños. Ciclos repetidos de congelamiento / deshielo pueden dañar la viabilidad del polen de pino (Matthews y Kraus, 1981). Este es el método preferible a aplicar si se pierde la época de polinización, como una reserva simple de polen si factores ambientales o errores humanos causaron pobres resultados en la polinización el año anterior.

5.2.3. Almacenamiento a Largo Plazo.

Para lograr almacenamiento a largo plazo con viabilidad aceptable, el polen puede ser congelado o secado al vacío / congelamiento a los -20°C de la misma manera que almacenamiento a mediano plazo. Esto ha sido probado como un método confiable para el almacenamiento a largo plazo en muchas especies, y está disponible para la mayoría de los profesionales forestales (Hanna, 1994) (Duffield y Callaham, 1959) (NCSU-TIP, 2000) (Towill y Walters, 2000) (Jensen, 1964).

Figura 11. Frascos criogénicos de marca Nunc® (Izq.), y una heladera criogénica (Der.) para almacenamiento en nitrógeno liquido.

Otra manera efectiva y muy utilizada en la agricultura es la preservación criogénica. El polen de muchas especies ha sido almacenado exitosamente por criopreservación y una lista de estas especies está disponible en Hanna y Towill (1995). Criopreservación puede ser realizada por medio de Ultrafreezers o refrigeradores criogénicos (*cryogenic refrigerators*). Los Ultrafreezers generalmente almacenan el polen a los -80°C, mientras los refrigeradores criogénicos usan inmersión bajo nitrógeno liquido para llegar a los -196°C. Ambos métodos son efectivos, uno de los métodos requiere electricidad para mantener la temperatura baja (cortes de electricidad podrían causar daños), y el otro requiere monitoreo constante para asegurar que el polen se mantiene en inmersión. El autor hizo un estudio utilizando un refrigerador criogénico (Figura 11), el cual dio buenos resultados después de 6 meses a los -196°C (Tighe, datos no publicados 2004). Se evaluaron también los métodos de congelación y deshielo, y resultaron mejor las prácticas de congelación lenta y deshielo lento como se sugiere en Matthews y Kraus (1981). Estos resultados con especies de pinos tropicales, subtropicales y templados son consistentes con resultados de criopreservación de polen de pino obtenido con *Pinus ponderosa* en el Laboratorio Nacional de Semillas Forestales (Nat. Tree Seed Lab.) en Colorado, EEUU (Connor y Towill, 1993).

Este método es adecuado para polen comprado o recolectado de gran valor, o polen deseado para actividades a largo plazo o de conservación permanente.

6. Recomendaciones.

El polen en estado de latencia de pinos tropicales y subtropicales procedentes de rodales naturales puede ser almacenado exitosamente por períodos extendidos bajo un manejo responsable del polen. Por lo general, el conocimiento del comportamiento típico del polen de especies de pino parece ser aceptable para estas especies también. Sin embargo, se puede hacer unas recomendaciones para ayudar al profesional forestal en el manejo del polen:

1. Aunque de naturaleza rústica, el polen del pino responde de manera desfavorable a cambios bruscos de temperatura o humedad. Los mejores resultados parecen lograrse con cambios lentos en el ambiente del polen.
2. Una vez que se saque el polen de su almacenamiento y éste vuelve a la temperatura y humedad del ambiente, no se recomienda su re-almacenamiento. El deshielo y el congelamiento repetitivos deben ser evitados porque disminuyen la viabilidad del polen (NCSU-TIP, 2000). Es importante sacar del almacenamiento solamente el polen que se vaya a usar durante el día, y mantenerlo fresco en una nevera bajo sombra durante el día laboral.
3. Los protocolos adecuados para evaluar la viabilidad de polen por medio del análisis de germinación de grano se encuentran en Moody (1990). Existen distintas opiniones con respecto al límite mínimo de la germinación del polen para considerarlo viable, pero el 30% parece ser del rango mínimo. Debido al ciclo reproductivo largo (~24 meses) de los pinos, el polen con un porcentaje de germinación por debajo de este valor debería ser evaluado cuidadosamente, y utilizado sólo si no hay otra fuente disponible para asegurar una polinización exitosa. Callaham (1966) afirma que el polen con germinación tan baja como al 10% puede ser utilizado para obtener buenos resultados de semillas viables, pero puede ser que el polen en estas condiciones no representará la misma composición genética del lote original (Towill, 1985).

Muchos matices del manejo de polen para pinos tropicales y subtropicales todavía están por ser determinados, pero la creciente importancia de estas especies en la silvicultura de plantaciones al nivel mundial estimula investigación adicional. Este manual pretende proveer un resumen de prácticas aceptables, experiencias personales, y citas literarias para ayudar al profesional forestal en el manejo y recolección del polen procedente de rodales naturales.

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8. Literatura Citada.

Beers, W.L., Bivins, J., y J.E. Mocha. 1981. Pollen Collection. In: E.C. Franklin, E. Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 30-31.

Binding, G.J. 1980. About Pollen: Health Food and Healing Agent. Thorsons Publishers Limited, Wellingborough, Northamptonshire, England. As cited on www.graminex.com.

Bonnet-Masimbert, M. y Webber, J.E. 1995. From flower induction to seed production in forest tree orchards. *Tree Physiology*, 15, 419-426.

Bramlett, D. L. y Bridgwater, F.E. 1989. Pollen development classification system for loblolly pine. Proc. 20th So. For. Tree Improv. Conf., June 26-30. Charleston, SC, p.116-121.

Bramlett, D.L. y O'Gwynn, C.H. 1981. Controlled Pollination. In: E.C. Franklin, E. Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 44-51.

Brune, A. 1990. Reproductive Biology and Tropical Plantation Forestry, Ch.24 in: Reproductive Ecology of Tropical Forest Plants. Edited by K.S. Bawa and M. Hadley. UNESCO Man and the Biosphere Series, Parthenon Publishers, Park Ridge, NJ. 421 pp.

Callaham, R.Z. 1966. Hybridizing pines with diluted pollen. Proceedings of the 8th Southern Forestry Tree Improvement Conference, June 16-17, Savannah, GA. Southern Forest Tree Improvement Committee Pub. #24.

Ching, T.M., y Ching, K.K. 1964. Freeze-drying pine pollen. *Plant Physiology* 39:5, pp. 705-709.

Connor, K.F. y Towill, L.E. 1993. Pollen-handling protocol and hydration/dehydration characteristics of pollen for application to long-term storage. *Euphytica* 68:77-84. Kluwer Academic Publishers, Dordrecht, Netherlands.

DFSC, 2000. Conservation of genetic resources of *Pinus merkusii* in Thailand. DFSC Series of Technical Notes. TN56 Danida Forest Seed Centre, Humlebaek, Denmark.

Donahue, J.K., y Mar-López, C. 1995. Observations of *Pinus maximartinezii* Rzed. *Madroño*. 42(1): 19-25. Published by the California Botanical Society.

Dorman, K.W. 1976. The genetics and breeding of southern pines. USDA For. Serv. Agric. Handbook #471., Washington, D.C. 407 pp.

Duffield, J.W. y Callaham, R.Z. 1959. Deep-freezing pine pollen. *Silvae Genetica* 8:22-24.

Dvorak, pers. comm. 2004. Personal communication with Dr. William S. Dvorak, CAMCORE.

Dvorak, W.S., Hamrick, J.L., y G.R. Hodge. 1999. Assessing the sampling efficiency of ex situ gene conservation efforts in natural pine populations in Central America. *Forest Genetics* 6(1): 21-28.

Dvorak, W.S., Stanger, T.K., y Romero, J.L. 2000. *Pinus maximartinezii*. In: Conservation & Testing of Tropical & Subtropical Forest Tree Species by the CAMCORE Cooperative. College of Natural Resources, NCSU, Raleigh, NC. USA. pp. 96-105.

Griffin, A.R. 1990. Effects of inbreeding on growth of forest trees and implications for management of seed supplies for plantation programmes, Ch.25 in Reproductive Ecology of Tropical Forest Plants. Edited by K.S. Bawa and M. Hadley. UNESCO Man and the Biosphere Series, Parthenon Publishers, Park Ridge, NJ. 421 pp.

Gutiérrez, E. 2004. Personal communication regarding practices at the CAMCORE - Guatemala office.

Hanna, W.W. 1994. Pollen storage in frostless and conventional frost-forming freezers. Crop Science. 34:1681-1682.

Hanna, W.W., y Towill, L.E. 1995. Long-term pollen storage. In: Janick, J. (ed.), Plant Breeding Reviews, Vol. 13. p. 179-207. Wiley and Sons, New York.

Hoekstra, F.A. 1992. Stress Effects on the Male Gametophyte. In: Cresti, M. and Tiezzi, A. (ed.) Sexual Plant Reproduction. Springer-Verlag, Berlin. pp. 193-201.

Jensen, C.J. 1964. Pollen storage under vacuum. Royal Veterinary and Agricultural College Yearbook. Copenhagen, Denmark. Pp 133-146., as cited in: Towill and Walters, 2000 (see below).

Jett, J. B., y Frampton, L.J., Jr. 1990. Effect of Rehydration on In Vitro Germination of Loblolly Pine Pollen. *South. J. App. Forestry*. 14(1): 48-51.

Mirov, N.T. 1967. The Genus *PINUS*. The Ronald Press Company. 602 pp.

Moody, W.R., y Jett, J.B. 1990. Effects of pollen viability and vigor on seed production of loblolly pine. Southern Journal of Applied Forestry. 14:1, 33-38. South Carolina Forestry Commission, Tillman, SC.

Moody, W.R. 1988. Evaluation of loblolly pine pollen quality. Thesis (M.S.)--North Carolina State University. 68 pp.

Lanteri, S., Belletti, P., y Lotito, S. 1993. Storage of Pollen of Norway Spruce and Different Pine Species. *Silvae Genetica*, 42,2-3. J.D. Sauerlander's Verlag. Frankfurt Am. Main.

Layne, R.E.C., y Hagedorn, D.J. 1963. Effect of Vacuum –Drying, Freeze-Drying and Storage Environment on the Viability of Pea Pollen. *Crop Science*, 3:433-436.

Matthews, F.R., y Kraus, J.F. 1981. Pollen Storage. In: E.C. Franklin, E. Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 37-39.

NCSU-TIP Coop. 2000. December 5, 2000 Contact Meeting Notebook. North Carolina State University Industry Tree Improvement Cooperative. Raleigh, NC. 20 pp.

Nel, A. 2002. Factors Influencing Controlled Pollination of Pinus Patula. Master's Thesis. University of Natal, Pietermaritzburg, South Africa. 100 pp.

Siregar, I.Z., y Sweet, G.B. 2000. The Impact of Extraction and Storage Conditions on the Viability of Radiata Pine Pollen. *Silvae Genetica*, 49, 1. p. 10-14.

Snyder, E.B. y K.E. Clausen, 1974. Pollen handling. In: C.S. Schopmeyer, ed., Seeds of woody plants in the United States. USDA Agric. Handbook 450.

Snyder, E.B. 1957. Pollen Handling. Proceedings of the 4th Southern Forestry Tree Improvement Conference. Jan 8-9, Athens, GA. Southern Institute of Forest Genetics, USDA Southern Forest Experiment Station. pp. 111-115.

Sprague, J.R., y Snyder, E.B. 1981. Extracting and Drying Pine Pollen. In: E.C. Franklin, E. Pollen Management Handbook, Agric. Handbook 587, USDA For. Serv., Washington, D.C., p. 33-36.

Tighe, M.E. 2004. Unpublished data from research pursuant to a Master's of Science degree at NC State University, Raleigh, North Carolina, USA.

Towill, L.E. 1985. Low temperatures and freeze/vacuum drying preservation of pollen. In: Kartha, K.K., ed. Cryopreservation of plant cells and organs. CRC Press, Inc. Boca Raton, FL. 276 pp.

Towill, L.E. y C. Walters. 2000. Cryopreservation of pollen. In: F. Englemann and H. Takagi (eds.) Cryopreservation of tropical plant germplasm: Current research progress and applications. International Plant Genetic Resources Institute, Rome. Pp. 115-129

Vergara L., R., Ipinza C., R., y Pérez P., Erika. 1995. Manual de Cruzamientos Controlados para *Pinus radiata* D. Don. Cooperativa de Mejoramiento Genético UACH/CONAF/Empresas Forestales. Univ. Austral de Chile, Valdivia. 50 pp.

Wang, B.S.P., Charest, P.J., y Downie, B. 1993. *Ex situ* storage using pollen, Ch. 3 in: *Ex Situ* storage of seeds, pollen and *in vitro* cultures of perennial woody plant species. Food and Agriculture Organization of the United Nations Forestry Paper #113. Rome. 83 pp.

Apéndice A. Proveedores y Equipo Básico Usado en el Mejoramiento Genético Forestal

Abajo se encuentra una lista de instrumentos y equipo básico para realizar actividades de mejoramiento forestal tales como las mencionadas en el texto. Esta lista pretende dar solamente una idea de las herramientas necesitadas, unos proveedores, y el costo actual publicado en julio del 2004 para los instrumentos para la venta en los EEUU. No incluye flete, y unas herramientas no se encuentran para venta internacional de estos proveedores. La lista sirve para darle una idea del producto al profesional forestal, con su respectivo sitio web y número de catálogo para encontrar imágenes de las herramientas mencionadas y entonces identificar el proveedor adecuado según los recursos y la ubicación del programa forestal.

Artículo	Proveedor	Información de contacto	Unidades	Número de Catálogo	Costo Aprox. (US$)
Pine Life Cycle Chart (from Non-flowering plants CD)	Carolina Biological Supply Company	2700 York Rd. Burlington, NC 27215-3398. URL: www.carolina.com	1 set (with other charts incl.)	39-8924	$109.95
Soil Sieve set	Fisher Scientific	www.fishersci.com	3 sizes (#35, #60, #120)	04-881Q 04-881U 04-881Y	$57.22 $61.42 $58.80
Cyclone pollinators	CAMCORE, USFS, or MeadWestvaco	CAMCORE, Tel: (919)-515-6424, Email: info@camcore.org	Each	-----	$30
Sausage casings (permeable fibrous casings)-3.75” x 15”	Devro Teepak	Teepak ATTN: Janie Hackett 915 N. Michigan Ave. Danville, IL 61832 1-800-637-8121 www.teepak.com	Pack of 1000	F2633600002 0000	$464.94
Kraft paper bags 12” x 7” x 17”	ReStockIt.com	www.restockit.com	1 case of 500	#80076	$39.57
Snap-cut Pole pruner	Forestry Suppliers, Inc.	www.forestry-suppliers.com	Each	81090	$75.00
Precision© brand forced-air oven—4.5 ft^3 capacity	Fisher Scientific	www.fishersci.com	Each	13-254-27	$1,702.07

Artículo	Proveedor	Información de contacto	Unidades	Número de Catálogo	Costo Aprox. (US$)
Isotemp® Laboratory Convection oven	Fisher Scientific	www.fishersci.com	Each	13-247-625G	$1,240.70
Standard oscillating fan	Local suppliers	Local suppliers	Each	----	$20.00
Small, natural-hair paintbrushes	Fisher Scientific	www.fishersci.com	Pack of 12	03-670	$23.20
Nalgene® 175ml plastic flasks (for pollen)	Fisher Scientific	www.fishersci.com	1 case—72 bottles	03-311-2B	$127.70
Aluminum weigh boats	Fisher Scientific	www.fishersci.com	Pack of 144	08-732	$18.90
Parafilm® wax sealing tape	Fisher Scientific	www.fishersci.com	Each	13-374-16	$20.25
10cc glass screw-top vials	Fisher Scientific	www.fishersci.com	Pack of 144	03-339-21E	$43.90
Cryogenic refrigerator	VWR International	www.vwr.com	Each	55709-982	$945.00
Nunc® brand Cryo vials	VWR International	www.vwr.com	1 box of 500	66021-985	$310.00
-80°C, 85L Ultrafreezer	VWR International	www.vwr.com	Each	14230-142	$7,366.66
BenchTop 6K Freeze Dryer, ES® vacuum dryer	Virtis	www.virtis.com	Each	#405072	$7,000.00
Conventional (-20°C) freezer, compact 5 ft^3	Local suppliers	Local Suppliers	Each	----	$200.00
Conventional refrigerator (4°C), compact 4.4ft^3	Local Suppliers	Local Suppliers	Each	----	$150.00
Laboratory thermometer, (-20-150°C)	VWR International	www.vwr.com	Each	61013-040	$8.40