Pollen Management Handbook
Pollen
Management
Handbook

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98 p.

Results of practical experience and a limited amount of applied 
research on production, collection, processing, storing, and 
utilization of pollens of forest trees were reported by an international 
collection of authorities. Emphasis is on pollens of the southern 
pines, but chapters on southern hardwoods, European black alder, 
and eucalypts are included.

KEYWORDS: pollen collection, pollen processing, pollen storing, 
pollen utilization, forest tree pollens, southern pine pollen.

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This group is to be commended for supporting one of the 
early efforts to expand knowledge about producing, 
processing, and applying southern pine pollen on a large 
scale. In 1976, the United States Department of Agriculture 
Forest Service assumed full financial support of the research, 
so the cooperative was disbanded. But at the final meeting the 
representatives were unanimous in their appeal for a 
comprehensive state of the art reference document on pollen 
management. When I approached the Southern Forest Tree 
Improvement Committee shortly thereafter, they agreed to 
form a Pollen Management Subcommittee to support the 
Forest Service’s effort in producing such a book, and to 
promote other activities in pollen research. The cooperation 
of these two groups has largely been responsible for the 
completion of the Pollen Management Handbook.

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of thanks.

E. Carlyle Franklin
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Introduction

As research and development programs in forest tree improvement have advanced, a wealth of practical experience has been gained on production, collection, processing, storage, and utilization of forest tree pollens. Unfortunately, only a small amount of applied research has been done to support this important aspect of tree improvement technology. As a result, many differing methods and processes were developed by numerous practitioners, all of which worked to some degree but among which there was little or no comparability. This book does not solve that problem—we still need a concerted research effort on forest tree pollens. This book attempts to record a synthesis among researchers and practitioners based on research and knowledgeable experience as to what are some of the best approaches, methods, equipment, and procedures for handling forest tree pollens. Authors represent a wide diversity of background and experience. Emphasis is primarily on the southern pines, but chapters are also included on southern hardwoods, European black alder, and eucalypts. In many instances, alternative methods are given to provide a wider latitude of application. The purpose of this book is to provide a set of standard procedures to which various alternatives may be compared. New and better methods for certain procedures were probably developed before this ink dried. So these are not the final words; they are just the ones recorded here.
Chapter 1
Pollination Production and Dispersal as Affected by Seasonal Temperature and Rainfall Patterns
by William D. Boyer

This chapter is a brief review of what is known about the effects of temperature and rainfall patterns on pollen production and dispersal. Most of what we know about the flowering of forest trees concerns conifers, primarily pines. Research on reproduction of pines has been devoted mainly to cones because they produce the seeds. Seasonal variations in temperature and moisture can differentially affect initiation and development of conelets and catkins. In southern pines, large conelet crops are not necessarily accompanied by large catkin crops. Peak pollen shed and greatest conelet receptivity are usually simultaneous. This suggests that conelet and catkin development may be similarly influenced by prevailing temperature and moisture conditions.

Moisture Influence on Flower Initiation
Both catkins and conelets of southern pines are initiated during the growing season before the year they appear. Catkins of slash pine (Pinus elliottii Engelm.) in Florida began forming in late June and continued into February (Gregory 1968). Complete conelet production was observed within a month. The ratio of catkins to conelets than do other treatments. Abundant late summer irrigation may suppress conelet initiation. Continuous irrigation, however, supplying at least 1 inch per week, increases production of catkins but not of conelets in slash pine (Bengtson 1969). Year-round irrigation gives a greater ratio of catkins to conelets than do other treatments.

Rainfall patterns greatly affect initiation of both catkins and conelets, although requirements differ. Abundant late summer rainfall depresses conelet initiation, probably because vigorous exposed shoots continue to grow. Catkins are usually borne low in the tree crown, where shoots cease growing earlier in the year than do in the upper crown, where conelets are most commonly located. So, best pollen production depends on adequate soil moisture from catkin and conelet initiation until pollen ripening.

Irrigation and fertilization supplementing rainfall and natural fertility were shown to have little effect on catkin production in two loblolly orchards (Gregory 1968). Complete control of moisture and nutrient levels can markedly affect abundance of catkin production (Greenwood 1977a).

Winter Dormancy and Bud Break
Catkin buds of some southern pines go through a varying period of winter rest or dormancy. Catkins of others continue development toward maturity without a pause after bud emergence in November, though cold weather does slow development. With favorable temperatures, catkins of sand pine (Pinus clausa (Chapm.) Vasey) may reach maturity and shed pollen in December or even late November, while nearby shortleaf pines (P. echinata Mill.) rarely shed their pollen before the end of March. Catkin buds of loblolly pine (P. palustris Mill.) are dormant about a month before development resumes. Loblolly and shortleaf pines are dormant about 2 months. Catkins of slash pine continue development without a break, as to those of both sand and spruce pines (P. glabra Walt.); all three species shed their pollen before or during winter. The reproductive phenology of these species suggests an inherent adaptation to tropic or subtropic conditions.

Dormancy requirements for longleaf, loblolly, and shortleaf pines are inferred from the average starting date for accumulation of heat sums that effectively promote catkin development (Boyer 1978). According to Greenwood (1977b), conelets of loblolly pine are dormant in winter and will not resume development without chilling (2,000 hours at 4°C). Vegetative buds and catkins resume development without a cold treatment when temperatures are favorable. Mirov (1956) reported that day length does not affect the flowering of pines.

Development of loblolly pine catkins in southwest Alabama (2 to 4 catkins on each of 5 to 10 selected trees) was observed 4 successive years, 1969-70 through 1972-73 (Boyer, unpublished data). Bud break on individual trees occurred as early as the 3d week in December in 1972 and as late as the 1st week of February in 1970. Some trees were consistently early or late while others varied. For the 4 years, date of bud break of the earliest tree averaged January 2 and of the latest tree February 7, a 5-week spread. For longleaf pine, at least, catkin buds are quiescent for several weeks after they first emerge in November. The causes for this dormancy are still unknown. Bagging of selected bud-bearing shoots for 4 weeks in December, to increase heat load, did not affect date of bud break.

Heat Sums and Date of Pollen Shed
Flowering date of most spring-blooming plants is associated with air temperatures. Below-average temperatures retard, and above average temperatures advance the rate of development of most species. Among conifers, however, catkin response to temperature differs among species and, to a lesser extent, among individuals in the same species. Pollen ripening of Monterey pine (Pinus radiata D. Don) in Australia, for example, was affected directly by air temperature, but also indirectly by soil temperature (Millett 1944). For longleaf pine, air temperature around catkins was most directly related to date of peak pollen shed (Boyer and Woods 1973). Temperature summations have long been used as an index of plant development rate in agriculture (Holmes and Robertson 1959, Pearson 1924, Seely 1917, Voigts 1949), and are also useful in following flower development in trees. Boyer (1973) discussed problems associated with using heat sums to predict pollen shed in conifers.
Use of Degree-Hour Heat Sums. The best measure of the effective heat sum for a particular day is that area on a thermograph chart above the appropriate base or threshold temperature for the plant process being observed and below the temperature trace. Given a thermograph trace, one can determine daily degree-hours by planimetering the area between the trace and base temperature (or by using a digitizer). More easily, one can sum for each hour the positive difference between actual and base temperatures. The planimeter method differs from hourly sums by less than 1 percent in daily degree-hour values.

Daily degree-hours can be estimated from daily maximum/minimum temperatures, which are available from nearby weather stations. Daily degree-hour heat-sum estimates obtained by Lindsey and Newman's (1956) method and Baskerville and Emin's (1969) method were compared with degree hours taken from thermograph traces for January, February, and March in each of 7 years from 1966 through 1972. Both methods provided good estimates of actual daily degree-hours. Though estimates can be badly off for any single day, degree-hour sums for several weeks are usually close to those derived from a thermograph trace (fig. 1).

Because of a small intercept and a slope coefficient near 1.0, the Baskerville-Emin method gives the closest direct estimate of daily degree hours.

Boyer (1973) used 15 years of data from southwest Alabama and 3 years of data from other locations in testing use of heat sums to determine time of pollen shed. Degree-hour heat-sum values were estimated (Lindsey-Newman) from daily temperature data for a nearby weather station. Ranges of base temperatures and starting dates were tested. Results showed which combination would minimize the year-to-year variation in the degree-hour heat sum required for peak pollen shed in each of the 10 years from 1957 through 1966. The best combination was a base temperature of 50°F (10°C) and a date of January 1 for beginning accumulation of degree-hour heat sums. Degree-hours required for peak pollen shed declined as the season advanced. The regression Y = 19009 – 89.26X (Y = degree-hour heat-sum above 50°F and X = days from January 1 to peak pollen shed) accounted for nearly all annual variation in degree-hour heat sum over the 10 years, with an average deviation of observed from expected date of peak pollen shed of 0.3 day. In eight other observations of pollen shed, including two in North Carolina and one in North Alabama, the same regression gave an average deviation of 1.6 days between observed and expected date of peak pollen shed. Variations are probably due to annual departures of actual from average starting date for heat-sum accumulation. This factor will be more important for those species with longer apparent winter dormancy.

The relationship between degree-hour heat sums and date of pollen shed has not been established for other forest tree species. Long-term pollen-shed data are needed first, from which the best starting date and threshold temperature for heat accumulation can be determined. Once a relationship has been established, however, an observer can monitor catkin development merely by using a thermograph or estimates derived from daily high-low temperatures to sum daily degree-hours of effective heat. Instruments are available that will accumulate and record daily heat sums above selected base temperatures (Buckley 1979). The Forestry Weather Interpretations System (FWIS) expects soon to provide current data on heat accumulation for any threshold temperature, starting date, and location.

Use of Degree-Day Heat Sums. Degree-days is the positive difference in degrees between the selected threshold temperature and the maximum temperature for the day. Use of degree-day heat sums to follow catkin development is easier though less accurate than use of degree-hours. Degree-days do not allow for impact of minimum temperature on effective heat for a given day. With the same high, say 75°F, the low might be 64°F on one day and 30°F on another. While the degree-day sum above 50°F is 25 in each case, degree-hours for 64°F would be 468 and for 30°F only 167. Use of degree-day heat sums assumes that effects of varying daily low temperatures will average out and will also be about the same from year to year.

Degree-day heat sums to peak pollen shed have been determined from 6 years of pollen shed data each for slash, loblolly, and shortleaf pines and 19 years for longleaf pine (Boyer 1978). Degree-day heat sums accounted for most yearly variations in date of pollen shed. Deviations of observed from expected date of peak pollen shed averaged 2.5 days for longleaf, 3.2 days for slash, 2.7 days for loblolly, and 4.3 days for shortleaf. Base temperature for longleaf was 50°F (10°C). The best base for the other three species was 55°F (13°C). Using the 50°F base instead of 55°F for all species was more convenient and affected annual variability very little. With a 50°F base for all four pine species, best starting dates for heat-sum accumulation and average degree-day sum to peak pollen shed are:

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<th>Species</th>
<th>Starting date for degree-day accumulation</th>
<th>Degree-days to peak pollen shed</th>
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<tr>
<td>Slash pine</td>
<td>December 5</td>
<td>1,023</td>
</tr>
<tr>
<td>Longleaf pine</td>
<td>December 31</td>
<td>1,208</td>
</tr>
<tr>
<td>Shortleaf pine</td>
<td>January 31</td>
<td>1,462</td>
</tr>
<tr>
<td>Loblolly pine</td>
<td>February 1</td>
<td>840</td>
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Data on pollen shed by both longleaf and slash pine were obtained for years 1970 through 1978. Data for loblolly and shortleaf pine were obtained for years 1970 through 1977. Actual number of calendar days from January 1 to peak pollen shed (fig. 2A) shows a much wider range of variability than the same data expressed as departure in days of observed date of peak pollen shed from the expected date, when appropriate degree-day heat sum was reached. Thus, by using those starting dates or better, some developed for the local region and species in question, prediction of peak pollen shed could be done within about plus or minus 10 days, as shown by the following results. Slash pine ranged 54 calendar days in annual date of peak pollen shed. Use of degree-day heat sums
reduced this spread to 11 days. A 40-day range for longleaf heat sums reduced this spread to 11 days. A 40-day range for longleaf was reduced to 17 days, and a range of 23 days for loblolly was reduced to 14 days. Shortleaf pine was affected very little, as use of degree-day heat sums reduced a 20-day range only to 18 days.

Figure 1.—Comparison of two methods of estimating degree-hours from daily maximum-minimum temperatures with values taken from a thermograph trace.
Although longleaf and loblolly often shed their pollen at about the same time, longleaf's range in date of peak pollen shed was nearly twice loblolly's. This difference is probably because catkin development in longleaf, as in slash, is affected by highly variable January temperatures. Late winter and springtime temperatures are less variable than those in December and January. Also, average daily heat sums are more consistent in spring than winter, so annual flowering dates vary less.

Individual Tree and Clonal Variations in Time of Pollen Shed

Variation in heat requirements for flowering among individual conifers seems to be genetically controlled. Some individuals are consistently early from year to year and others consistently late, and the order of flowering among individual trees is usually consistent (Sluder 1977, Bingham and Squillace 1957, Rooser 1942). Pollen-shed patterns characteristic of an individual tree also apply to all clonal material derived from that tree. So, in seed orchards some combinations among individuals or clones may not occur naturally because of differences in flowering date. For example, Eriksson and others (1973) calculated that 66 of 190 possible combinations among 20 clones of Picea abies would occur at frequencies of less than 0.1 percent.

Wasser (1967) reported on the phenology of pollen shed in a Virginia seed orchard with 12 clones of shortleaf pine and 30 clones of loblolly pine from both Piedmont and Coastal Plain sources. In 1967, peak pollen shed by loblolly pine was mostly reached during the 3d week of April, and shortleaf pine during the 1st week of May. Onset of peak pollen shed ranged 9 days among loblolly pines and 5 days among shortleaf. Wasser (unpublished data) repeated these observations in 1970 for loblolly pine. Peak pollen shed was reached during the last week of April, with a range among trees of only 4 days in onset of peak shedding. Most of the variation in date of peak pollen shed was clonal, although some variation was found among ramets of the same clone.

When 10 longleaf pines were closely observed in a study in southwest Alabama (Boyer and Woods 1973), peak pollen shed by individual trees occurred between March 19 and April 1. These trees had heat-sum requirements for peak pollen shed ranging from 11,262 to 16,126 degree-hours above 50° F, accumulated from January 1, 1968. Some of this variation results from use of a single starting date (January 1) for heat...
accumulation. Tree-to-tree variation in date of bud break suggests that each tree has its own starting date because of varying periods of dormancy. Trees with early bud break were among the early flowering trees, and trees with late bud break were usually late in flowering. Variations among trees in flowering date can be attributed to differences in (1) dormancy requirements, (2) response to temperature during development, and (3) microclimate about individual trees.

Pollen shed of the first and last two trees to shed pollen did not overlap. Observed catkin clusters shed all their pollen in about 6 days. On individual trees, different clusters reached peak pollen shed within 2 days, regardless of location on the tree. The general period of pollen shed was slightly longer than average, with 15 instead of 13 days needed for shedding of 80 percent of the pollen. Overlap of pollen shed among early and late flowering trees might have occurred if the pollen shedding period was condensed from 15 to 6 or 7 days. Later observations on the same 10 trees confirmed that trees that flowered early in 1968 were consistently early, and those that flowered late were consistently late. But flowering order of intermediate trees often changed from year to year.

In all years with an extended period of pollen shed, opportunities for crossing between early- and late-flowering individuals of longleaf pine will be limited at best. This same situation probably also applied to other southern pines. Loblolly, shortleaf, and slash pine all demonstrated similar year-to-year variations in duration of pollen shed (Boyer 1978).

**Annual Variations in Pollen Crop Size**

Little is known about yearly variations in size of pollen crops, although these variations are important to seed-crop size. Lack of effective pollination is one factor causing conelet abortion (White and others 1977, McLemore 1977, Sweet 1973). Boyer (1974) reported a strong correlation between pollen density during the height of shedding and cone production the next year.

Rainfall patterns associated with catkin initiation in southern pines differ from those favoring initiation of conelets, so large crops of male and female flowers need not always coincide. Ten years of observation on the Escambia Experimental Forest did not reveal any correlation between sizes of conelet and pollen crops in longleaf pine, or between conelets and subsequent number of mature cones. The number of mature cones was strongly correlated \( r^2 = 0.76 \) with size of pollen crops over 15 years, indicating that in longleaf pine, pollen crop-size may limit cone production.

For 22 years, annual longleaf pine pollen counts per square centimeter in longleaf pine stands ranged from 0.6 thousand to 24.5 thousand, and averaged 8.0 thousand. Seven of the twenty-two years had pollen crops exceeding 10 thousand grains per cm², for an average of one large crop every 3 years. No large crops have occurred since 1973. Before 1973 no more than 3 years intervened between successive good pollen crops. Climatic factors responsible for large or small pollen crops in pines deserve more study. Based on the correlation between pollen and cone crops in longleaf pines, until we know more about climatic effects, size of the pollen crop can serve as one index of the size of the seed crop the following year.

**Synchrony in Flowering**

Despite differences in developmental patterns and presumed differences in dormancy requirements between conifer catkins and conelets, their flowering is remarkably well synchronized within individual trees or clones. Bramlett (1973) found that pollen dispersal and receptivity of conelets of shortleaf pine were well synchronized even when the flowering season was early or late. Grano (1973), after observing flowering by loblolly pine for 7 years, reported that pollen shed and conelet receptivity coincided every year. Phenological data were obtained in 1973 from 49 slash pine clones in a seed orchard near Perry, Florida (Beers 1974). Twenty-one clones reached peak pollen shed during the receptive period of their own conelets, which 20 clones reached greatest pollen shed before and 7 clones after the time of peak receptivity. Synchrony of male-female flowering has also been reported for firs (Franklin and Ritchie 1970) and many other pine species (Wright 1953, Barnes and Mullin 1974).

**Weather Factors and Pollen Viability and Germinability**

Normal yearly variations in weather patterns apparently do not greatly affect germinability of viable pine pollen. McWilliams (1959) found no evidence that temperatures will limit pine pollen germination under natural conditions. Temperatures above 46° C (115° F), which can occur on bagged strobili, will kill germinating pollen.

Bramlett (1973) found that for 6 years pollen viability of shortleaf pine in Virginia was consistently high. In 8 years, viability of longleaf pine pollen collected in southwest Alabama varied little from year to year. In certain years, exceptionally early or late freezes can disrupt the normal meiotic cycle of pine pollen development and cause the production of nonviable pollen grains. In such cases, pollination and cone development may proceed normally but seedcoats will be extremely small, indicating little or no ovule development. Evidence of this phenomenon in southern pines is sparse, but extensive observations of European and Japanese larch and the hybrid have confirmed the process and the consequences (Eriksson 1970).

**Duration of Pollen Shed**

Duration of pollen shedding by pines is highly variable from year to year. Observations of slash pine have suggested a marked correlation between rising temperatures and increasing amounts of pollen output. High humidity and low temperatures, however, usually delay flowering and prolong the pollen shed period (Wang and others 1960).

Most pollen shed by some western conifers, mainly Douglas-fir (Pseudotsuga menziesii), occurred during the day, with greatest release between 10 a.m. and 4 p.m. (Ebell and Schmidt 1964). This pattern was related to daily fluctuations...
in temperature and humidity. The entire pollen dispersal period ranged from 7 to 43 days, though most pollen was shed in only 4 to 5 days, not necessarily consecutive.

Reporting on red pine (P. resinosa), Fowler (1965) observed that late flowering telescoped flowering period and masked differences among individual trees.

Longleaf pollen shed on the Escambia Experimental Forest was monitored from 1957 by traps kept in a longleaf forest. These records tell how much pollen is dispersed annually and also give information on rate and duration of pollen shed. Because pollen shed often begins slowly, builds to a peak, and then tapers off, sometimes for a long time, duration of pollen shed was arbitrarily set at the fewest consecutive days needed for dispersal of 80 percent of the total. Dispersal took as few as 5 days, as many as 21, and averaged 13 for 22 years.

Temperature patterns combined with tree-to-tree differences in heat requirements for pollen maturation appeared to be the most important variables affecting duration of pollen shed in the stands. Cool weather extended the pollen shed period, and warm weather shortened it.

In 1972, longleaf pine needed 21 days to shed 80 percent pollen, but only 5 days were needed in 1973. These figures are extremes. Figure 3 shows pollen shed patterns for each year. About the same amount of pollen was trapped in the forest each year: 10.8 thousand grains per cm² in 1972, 11.6 thousand in 1973. Temperatures during the pollen shed period were strongly related to dispersal pattern, and nighttime lows were more important than daytime highs. For the 21 days of pollen shed in 1972, daily high temperatures averaged 75.3 °F, and lows averaged 42.4 °F. For 12 of the 21 days, nightly lows were 40 °F or lower.

Daily high temperatures averaged 79.3 °F for the 6 days of pollen shed in 1973 or 4 °F higher than the 1972 average. Daily lows, however, averaged 64.0 °F, or 21.6 °F higher than the average in 1972. With low temperatures above the threshold for catkin development, no slowdown or interruption in development occurred. One disadvantage of degree-day heat sums is that they do not differentiate between high and low daily minimum temperatures. For the 21 days in 1972, degree-hours averaged 259 per day (Lindsey and Newman 1956). For the 6 days in 1973, degree-hours averaged 520 per day. Figure 3 shows degree-hour heat sums above 50 °F for the period of pollen shed. Days with rain (more than 0.05 inch) are also shown.

Several pollen shed peaks occurred in 1972 and were associated mostly with the variable rate of heat accumulation. Rainy weather may also have reduced pollen dispersal. Fielding (1957) found that high humidity affects pollen dispersal by retarding rupture of sporangia walls.

Figure 3.—Rate of longleaf pine pollen dispersal in 1972 and 1973 associated with daily degree-hours above 50 °F and rainfall.
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Chapter 2
Effectiveness of Wind Pollination in Seed Orchards
by David L. Bramlett

In southern pine seed orchards, emphasis is normally placed on the production and management of female conelets, cones, and seed; however, successful pollination of female conelets is fundamental to seed production. Production and dispersal of pollen has important biological and genetic ramifications for the quantity and genetic value of seed produced. Methods for the observation and measurement of pollen production along with data on dispersal patterns from natural stands and seed orchards are presented in this chapter.

Pollen Sampling

Effectiveness of pollination implies some measurement of the quality, quantity, and distribution of pollen within an orchard. The quantity or distribution of pollen can be assessed through sampling methods that use direct counts of catkins on individual trees or by collection traps that measure density of pollen per unit volume of air or per unit surface area.

Direct Measurement. Actual counts of male catkins or clusters of catkins are only feasible for very small trees or a few individual trees (Bramlett and Belanger 1976, Madgwick 1968). Annual rates of pollen on young grafts can be measured by recording the number of catkins produced on individual trees for several years. Pollen production normally lags behind female flower production in young orchards and may not reach its potential until age 15 to 20 years. It is unlikely that direct counts of pollen would be possible after an orchard age of 8 to 10 years.

Sarvas (1962) measured the catkin residue in natural stands of Scots pine (Pinus sylvestris L.) to evaluate pollen production. Catkins were trapped in the stand as they were shed. This sampling procedure would be difficult in seed orchards with the wide spacing of individual trees, but it could be used on individual trees to estimate annual or clonal variation in the orchard.

Volumetric Samples. Because pine pollen is transported by air movement, the most representative trapping systems collect pollen from a measured volume of air. Volumetric traps have been designed to sample spores, pollen, and other airborne particles. Husain (1963) used an automatic suction-type trap that deposited airborne particles on an adhesive-coated transparent film. The film was mounted on a rotating drum disk and faced the intake and exhaust nozzles. Grano (1966) devised an 8-day volumetric pollen sampler specifically for pine pollen. The sampler provided accurate round-the-clock estimates of atmospheric pollen as number of pollen grains per cubic meter of air.

Deposition Samples. Pollen can also be sampled by counting the pollen grains that are deposited on the surface of a sticky material. The simplest type of pollen trap is a glass microscope slide or other flat object that is coated with a thin layer of petroleum jelly. Pollen grains from the air adhere to the surface coating until counted. These traps can be positioned either horizontally or vertically to quantify either the pollen drift or pollen deposition (Boyer 1966) and may be placed at various positions in or between tree crowns.

Modifications of the simple glass slide were made by Grano (1958) and Hoekstra (1965). Hoekstra used a simple and inexpensive pollen trap consisting of a flat plastic strip with two holes ¼-inch (6.35 mm) in diameter. The holes exposed a sticky surface on a celluloid tape that was positioned on the lower side of the strip. Hoekstra's trap can be mounted at a 45-degree angle or exposed vertically or horizontally.

Sarvas (1962) and Koski (1970) used various devices to measure pollen distribution within the tree canopy including a petroleum-coated surface on a 30-mm globe that recorded pollen grains per millimeter deposited on the sphere. Shields were used to protect the pollen trap from rain. On the other hand, Kenady (1968) devised a water trap to collect pollen continuously during rainy weather.

The cost of complex sampling devices negates their use at many locations. Simple traps give less accurate absolute readings than the complex types and never represent a unit volume of air; however, their cheapness, size, and light weight mean that more traps can be installed in a stand or seed orchard.

Pollen Distribution

Natural Stands. Shedding and dispersal of pollen has recently been reviewed by Stanley and Kirby (1973). Time and rate of pollen shedding are controlled by external environmental conditions and internal genetic control. Species, families, and clones vary in actual date of pollen release from year to year but hold the same relative position in relation to other trees (see chapter 1). When pollen is released, it is carried by air movement away and eventually downward from the source. Colwell (1951) released radioactive pollen at a height of 12 feet (3.65 m) and found that the pollen density rapidly decreased as the distance from source increased.

Observations of pollen distribution patterns in natural stands confirmed Colwell's observations. Buell (1947) observed that most pollen fell within the forest stand. In a shortleaf (Pinus echinata Mill.) stand in North Carolina, Buell trapped only 17 percent as much pollen 528 feet (161 m) from the stand as within the stand and only 10 percent as much 1,320 feet (402 m) from the forest edge.

Boyer (1966), observing pollen dispersal from a single longleaf pine (P. palustris Mill.), found that pollen density rapidly dissipated from the tree and that a large amount of background pollen in the area obscured the dispersal from the tree. He captured considerably more pollen on vertical than horizontal traps. This pattern indicated that pollen drift was exceeding pollen deposition.

Wang and others (1960) evaluated pollen dispersal of slash pine (P. elliottii Engelm.) in isolated individual trees, isolated natural stands, and plantations. They reported that the pollen frequency at a distance of 400 to 500 feet (122 to 152 m) is 2 to 5 percent of the source frequency.
Other researchers have shown similarly rapid decrease in pollen density with increasing distance from the source (Silen 1962). Bramlett (1973) observed the pollen distribution in a natural shortleaf pine stand for 6 years. Traps were mounted at a 45° angle at heights of 10, 20, and 30 feet (3.0, 6.1, and 9.1 m). Four traps per location were placed facing north, east, south, and west. Considerable variation was observed in the annual date of pollen release and the quantity of pollen trapped (fig. 4). Location, elevation, and orientation had significant effects on observed pollen density. McElwee (1970) used P^{32}-labeled pollen to study pollen distribution within natural stands. Pollen spread was dependent on wind direction at the time of pollen release. Stand density modified pollen flight, with the majority of pollen deposited within 100 to 250 feet (30.5-76.2 m) in fully stocked stands and up to 500 feet (152.4 m) in open stands. All positions of the crown received about equal amounts of pollen released by adjacent trees.

Seed Orchards. Distribution of pollen within a seed orchard is important to the production of large seed crops that are to be pollinated by a representative mixture of orchard clones. Future seed orchards may use special pollen producers in strategic locations.

Most pollen produced within a seed orchard is deposited in the orchard (Wang and others 1960). Background pollen also may be an important source of pollen, but Florence (1956) found that a seed orchard received only small amounts of pollen from a source 2½ miles (4.3 km) away. Squillace (1967) found seed yields in an emasculated seed orchard to be comparable with those in natural stands in the same area. Lanner (1966) stressed the importance of long-distance transport in pollen dispersal and described weather conditions that could facilitate mass movement of pollen. From studies on pollen distribution, McElwee (1970) concluded that, within the orchard, the majority of pollen reaching the conelets of an individual tree originated from its closest neighbors. He therefore recommended that seed orchard ramets of a single clone should be separated by four or five nonrelated genotypes. Sorensen (1972) modeled pollen distribution in a seed orchard on a hypothetical example. He estimated that about 65 percent of outcrossed seeds would be sired by near neighbors, about 5 percent by other orchard trees, and about 30 percent by background pollen. Therefore, the most important source of pollen for a single tree is its nearest neighbors, according to Sorensen’s model.

Pollination Effectiveness

Pollen Grains Per Ovule. Although pollen has not been directly measured in many southern pine orchards, annual production of large seed crops is evidence of successful pollination as are relatively high yields of seed per unit of cones.

Research studies show that unpollinated ovules abort during the first year of development (McWilliam 1959). These ovules are mere remnants of the original ovules and most frequently appear in the mature cone as a wing without a seed. If many of these 1st-year aborted ovules are found in the mature cones, pollination failure could be suspected. Unfortunately, other factors besides the lack of pollen may cause 1st-year ovules to abort. DeBarr and Ebel (1974) found that immature seed bugs can destroy 1st-year ovules and may frequently damage many ovules per cone. Therefore, the presence of many 1st-year aborted ovules can result from lack of pollen or damaging insects. Careful observation, insect protection, and microscopic examination are needed to determine the exact cause of reduced seed yields from aborting ovules.

Pollen density assessed by traps can be related to seed yields, but the most accurate and meaningful measure of pollination is the counting of pollen grains within the pollen chamber of the ovules. Sarvas (1962) published data from Scots pine in Finland showing that ovules contained about two pollen grains per pollen chamber. Lill and Sweet (1977) reported an average of 2.5 pollen grains per ovule in Monterey pine (P. radiata D. Don.) in New Zealand. Bramlett
and Johnson examined 194 separate ovules from 11 clones in a mature slash pine seed orchard. The number of pollen grains in the slash pine ovules ranged from 0 to 4 and averaged 1.9. Of the 194 observed ovules only 3 percent had no pollen present; 27 percent had a single pollen grain present; 43 percent had two grains; 25 percent had three pollen grains; and 2 percent had four pollen grains (fig. 5).

**Seed Development After Pollination.** Production of pollen per se is not the final measure of pollination's effectiveness. For seed development, pollen in the pollen chamber must also be viable before ovule, embryo, and seed development can continue to maturity. Without viable pollen, the female gametophyte deteriorates with subsequent ovule abortion. Bramlett and Johnson (see footnote 2) determined the total pollen effectiveness for a slash pine orchard by microscopically examining pine ovules and observing the number of pollen grains present and the percentage of grains that germinated. They quantified the pollination effectiveness (PE) for the seed orchard:

\[
PE \text{ (percentage)} = \frac{SP - UPO - UGO}{SP} \times 100 \text{ percent}
\]

where:
- \(SP\) = Seed potential, or number of potential seeds in the sample conelets.
- \(UPO\) = Number of unpollinated ovules per conelet; calculated as the percentage of unpollinated ovules in the sample times the seed potential.
- \(UGO\) = Number of ovules per conelet with ungerminated pollen grain(s) present; calculated as the percentage of ungerminated ovules in the sample times the seed potential.

For the observed seed orchard, the pollination effectiveness was calculated:

\[
PE = \frac{165 - 0.09(165) - 0.01(165)}{165} \times 100 \text{ percent}
\]

\[
PE = \frac{165 - 15 - 2}{165} \times 100 \text{ percent}
\]

\[
PE = 90 \text{ percent}
\]

where:
- \(SP = 165\); percent unpollinated = 9 percent; and, percent ungerminated = 1 percent.

In this study, the two most important factors in pollination effectiveness, quantity and quality of the pollen supply, were favorable in the slash pine seed orchard. These observations indicated that wind distribution of pollen is well synchronized with receptivity of female flowers and that nearly all pollen within the pollen chamber readily germinates.

The same procedure can be used in young orchards to evaluate pollination effectiveness during early years of development. If projected pollination effectiveness were at less than an acceptable level, for example 40 percent, supplemental pollination could be used to boost seed production until pollen production and effectiveness increased.

**Apparent Pollination Effectiveness.** Apparent pollination effectiveness (APE) can be calculated by the ratio of developed seed to the seed potential. This is valid only when the cones have been protected from insects during the seed development period, so that losses are entirely or primarily due to lack of pollen or pollen viability (Debarr and others

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Figure 5.—Number of pollen grains per ovule in the Arrowhead Slash Pine Seed Orchard, February 20, 1975.
APE is the most realistic evaluation of pollen performance available in the orchard, evidence that polUnation was successful. Conversely, when yields of seed per unit of cones from seed orchards are low, it shows actual effectiveness of pollination. The ratio would underestimate polliinatation effectiveness if ovules aborted before seedcoat formation from other causes, such as insects, developmental problems, etc. With absolute insect protection, APE is the most accurate measure of successful pollination.

Conclusions
Production of seed in southern pine seed orchards is directly related to effectiveness of wind pollination. High yields of seed per unit of cones from seed orchards are evidence that polliination was successful. Conversely, when low seed yields are observed, poor pollen effectiveness can be suspected as one of the causes. If low seed yields are observed, the pollen supply should be quantified to determine if the amount of pollen is limiting the development of the seed crop. Pollen quantities can be measured by direct counts of pollen catkins, volumetric pollen traps, deposition pollen traps, or observations in pine ovules. The most accurate and informative measure of pollination effectiveness is actual observation of pine ovules after pollination. The number of pollen grains per ovule and their viability are the basic measures of successful pollination. As research on the subject continues, the indirect measures of pollination effectiveness can be correlated with actual ovule counts. By providing evidence of the pollen density available in the orchard, pollen counts can be related to actual seed yields. Caution must be taken that other causes of low seed yields are not confused with suspected low pollen supplies. For example, insects, fungi, or other variables can, instead, be the causes of seed failure.

The most serious pollen problem in seed orchards occurs during the early years of establishment when pollen catkin production lags behind female conelet production. In these young orchards, conelets are pollinated primarily from background pollen that reduces the genetic gain from the orchard. Two solutions may alleviate the early pollen shortage. First, known precocious and prolific pollen producers of good general combining ability can be interspersed in the orchard for early pollen production and later rogued as orchard pollen supply increases. These trees can be first-generation selections that have a history of early and abundant pollen production. Care should be used in family selections to avoid excessive inbreeding of first- and second-generation selections. Second, supplemental pollination can be used when female cone production is high enough to warrant collection, but orchard pollen is inadequate. (Methods of supplemental pollination are presented in chapter 11.)

Once pollen production reaches moderate to high levels, the distribution of pollen in the orchard is more important to genetic gain than the formation of seed per se. Little work has been done on the distribution of pollen from specific clones or neighborhoods within the seed orchard, but the current concept is that most trees are pollinated primarily from their nearest neighbors.

It is important that, if pollen supply problems are suspected because of low seed yields, seed orchard managers should use some method to measure the pollen that is available to conelets in the orchard.

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Wang, C., Thomas O. Perry, and Albert G. Johnson.
Chapter 3
Proportion of Pollen From Nonorchard Sources
by A. E. Squillace and Ernest M. Long

Pollen can travel long distances, so orchard planners try to reduce the amount of wild pollen entering seed orchards by providing an isolation zone. Pollen of southern pines tends to settle at a fairly predictable rate, most of it settling out at about one to two times the height of the tree. Therefore, it is customary to remove all trees (the species occurring in the orchard) from a 400-foot (122 m) zone around the orchard. Under some circumstances, substantial amounts of pollen may be carried beyond the normal setting range. This has caused the effectiveness of isolation zones to be questioned. If contamination is appreciable, expected genetic gains can be reduced. This chapter (1) briefly reviews pollen dispersal patterns and summarizes knowledge on extent of contamination, (2) presents results from a recent study of contamination in a slash pine (Pinus elliottii Engelm.) seed orchard, (3) shows the probable effects of contamination on genetic gain, and (4) suggests methods for estimating and reducing contamination.

Pollen Transport

Colwell (1951) stated that pollen distribution patterns are influenced by local turbulence, topography, ballistics of the particular pollen, temperature, and relative humidity. Of these, topography at a given location and ballistics of a particular pollen are fixed and would be the same throughout pollen release. The other factors are highly variable and would be expected to change within a given day and between days. Seldom would all variables exactly duplicate conditions found at a specific time. Colwell showed that pollen of Coulter pine (P. coulteri D. Don) and some other species settled rapidly after release. Practically all pollen was collected downwind within the first 160 feet (49 m) from the release point. Similar patterns of downwind pollen dispersal were reported by Wright (1953).

Wind velocity, temperature, relative humidity and time since inception of anthesis all had significant influences on dissemination patterns of loblolly pine (P. taeda L.) pollen. Of lesser importance were wind direction, distance from source, time of day, and height above ground of the collection station (McElwee 1960).

Release of pollen from a fixed point and subsequent sampling of settling may not provide an adequate index of the amounts of pollen actually available for pollination. Silen (1962) found that isolated Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) trees, which produce large pollen grains with about the same theoretical sedimentation rate as loblolly and slash pine pollens, had released pollen that settled at about the expected rates, with the highest pollen counts recorded at 50 feet (15 m) downwind from the tree. He reported a range of pollen counts of 5,100 to 7,3000 grains per square inch at 50 feet (790 to 1,130 per cm² at 15.2 m); however, at 2,000 feet (610 m) from the tree about 2,000 grains per square inch (310 per cm²) were counted. This pollen, far from the trees being studies, was believed to have originated from more distant trees.

Several European authors have reported that pine pollen can be transported great distances (Lanner 1966, Koski 1970). Recently, van Buijtenen (1973) found substantial amounts of pine pollen settling at College Station (Bryan), Texas. The nearest stands that could have produced the quantities of pollen observed were more than 20 miles (32 km) away. Existence of atmospheric thermal shells, which may lift pollen vertically and carry it long distances by wind, perhaps accounts for long-distance transport (Cone 1962, Lanner 1966).

Reports on distance of pollen dispersal are often contradictory. A major cause of disagreement seems to be differences in sampling techniques, especially differences in the height levels at which samples are measured. Other factors are probably involved. But there is sufficient evidence to conclude that, under certain conditions, pollen can be transported long distances.

Estimating Degrees of Contamination

Pollen dispersion studies have resulted in conflicting views as to the extent of contamination that might be expected in seed orchards (see chapters 1 and 2). Empirical estimates of the degree of contamination are rare. In one study, Squillace (1967) emasculated a young 5-acre (2 ha) slash pine seed orchard having a 400-foot (122 m) isolation zone. Cones collected the following year yielded about 40 viable seeds per cone. Therefore, nonorchard pollen was sufficient to pollinate female flowers in the orchard. Using monoterpene composition, Squillace (1977a, 1977b) later verified that contamination was large in this same orchard even when the pollen crop was good and not removed (see next section). Likewise, a heavy seed crop was obtained from a well-isolated 10-year-old Scots pine (P. sylvestris L.) seed orchard after complete emasculation (Hadders 1973).

In slash pine, pollen frequencies at 400 to 500 feet from the source were 2 to 5 percent of source frequency. Isolation strips of 500 feet should be considered minimal for slash pine seed orchards. With that isolation, percentage of extraneous pollen will be negligible when the orchard reaches full pollen production potential, according to Wang and others (1960).

In a hypothetical example, Sorensen (1972) estimated that about 30 percent of seeds in a Douglas-fir orchard would be sired by pollen from trees outside the orchard (background pollen). Jonsson and others (1976) periodically measured the patterns of pollen shed in quantity and time of shedding in a Scots pine orchard in relationship to pollen dispersal. An appreciable nonconformance between peaks of the two patterns would indicate high contamination, while a conformance in peak time would prevent conclusions. Results suggested that contamination during one year was considerable, while results for the two following years were inconclusive.

Koski (1970, 1975) determined that the quantity of background pollen depends greatly upon the relative
abundance of the tree species in the region of interest. For Scots pine in south Finland, he estimated that background pollen was about 60 percent of that which occurs within pure tended stands. The quantity of pollen in seed orchards may be greater than that in pure stands he used as a base; nevertheless, his results suggest that contamination can be extensive.

**Contamination in a Slash Pine Orchard**

An unusual situation in a demonstration slash pine seed orchard at Olustee, Florida, permitted us to make estimates of both the number of selfed offspring and the amount of pollen contamination. Results have limited application because the orchard was atypical, but the study shows the importance of contamination and demonstrates a technique for making estimates.

The 5-acre orchard was established in 1957. All slash pines within 400 feet (122 m) of the orchard were removed. The orchard contained nine clones, which were all related as either half-sibs or full sibs. We knew the genotypes of all clones with respect to four simply inherited monoterpenes occurring in the cortical oleoresin (table 1). Clone 5 was selected to estimate the number of selfed offspring produced in the orchard because, upon controlled selfing, 1/16 of this tree's progeny were of the type bmlp (see table 1). This type could not be produced from matings with other clones in the orchard; therefore, the frequency of type bmlp from offspring of clone 5 provided direct estimates of frequency of selfed offspring. Wind-pollinated seeds were collected from six ramets of this clone in 1973, 1974, and 1975, and monoterpenic composition was determined in seedlings grown from them.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>1</td>
<td>BB Mm II Pp</td>
</tr>
<tr>
<td>2</td>
<td>BB Mm II Pp</td>
</tr>
<tr>
<td>3</td>
<td>BB mm II Pp</td>
</tr>
<tr>
<td>4</td>
<td>BB Mm II Pp</td>
</tr>
<tr>
<td>5</td>
<td>Bb mm II Pp</td>
</tr>
<tr>
<td>6</td>
<td>BB Mm II Pp</td>
</tr>
<tr>
<td>7</td>
<td>BB mm II Pp</td>
</tr>
<tr>
<td>8</td>
<td>Bb mm II Pp</td>
</tr>
<tr>
<td>9</td>
<td>BB Mm II Pp</td>
</tr>
</tbody>
</table>

*B, M, L, and P represent high amounts of beta-pinene, myrene, limonene and beta-phellandrene in cortical oleoresin, while lowercase letters represent low amounts. High is dominant over low in all cases (Squillace 1977a, 1977b).

Of the 844 progeny 11 (1.3 percent) were of the bmlp phenotype. Therefore, one could estimated that 16 x 1.3 = 21 percent of the wind-pollinated progeny of clone 5 were selfs. However, examination of all data indicated that some of the phenotypes could not have been sired by pollen from the orchard and must have been sired by pollen from outside sources (table 2). Therefore, some of the bmlp types were likely not selfs, and the estimate of selfed offspring may be too high.

To obtain an estimate of the proportion of contamination, we first computed gene frequencies for wild trees in the general vicinity of the seed orchard using data from Gansel and Squillace (1976). From these results, we determined the phenotypic frequencies that would be expected if all pollen came from nonorchard trees (table 2, last column). From the genotypes of table 1, we also computed the frequencies of progeny phenotypes expected if all matings with clone 5 were with self pollen and also if all matings were with pollen from clones other than clone 5 in the orchard (table 2, columns 3 and 4).

Progeny having high limonene (Bmlp and Bmlp) were necessarily contaminants because none of the orchard trees contained high limonene. Types bMlp and bMlp were likewise contaminants, because they could not be formed from matings of clone 5 with other orchard trees. Other types likewise contained contaminants, but because they could also be formed from selfing or mating with other clones, we could not distinguish individual contaminants.

To estimate the proportion of the three possible pollen sources siring progeny, we proposed that the observed number of progeny of each phenotype was comprised of a proportion of each of the three corresponding expected values. The problem was to determine an average proportionate contribution of each of the three pollen types. A multiple regression analysis was used to obtain these proportions:

\[ Y = a + b_1 X_1 + b_2 X_2 + b_3 X_3 \]

where

- \( Y \) = observed number of progeny of each phenotype,
- \( a \) = regression constant to be estimated,
- \( b_1, b_2, \) and \( b_3 \) = regression coefficients to be estimated,
- \( X_1, X_2, \) and \( X_3 \) = expected numbers of progeny sired by self pollen, other orchard pollen, and nonorchard pollen.

The regression equation fitted to the data of table 2 was:

\[ Y = .004 + .062X_1 + .057X_2 + .835X_3. \]

The coefficient of determination was 0.996, indicating a good fit to the data. The coefficients (0.062, 0.057, and 0.835) reflect the proportionate share of seedlings resulting from self pollen, other orchard pollen, and nonorchard pollen. Because these values do not add up to one, we normalized them, yielding 0.065, 0.060, and 0.875 for the three pollen types. Therefore, we can estimate that 6.5 percent of the progeny were selfs, 6 percent were sired by orchard pollen, and 87.5 percent were sired by nonorchard pollen.

These estimates do not take into account that all of the nine clones in the orchard were related. Matings among
Table 2.—Observed phenotypic frequencies in wind-pollinated progeny of clone 5 in a slash pine seed orchard, and expected frequencies under three hypothetical pollination conditions

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Observed</th>
<th>Expected, when pollen is entirely from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Clone 5) (selfing)</td>
</tr>
<tr>
<td>BMLP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BMLp</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BM1P</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>BM1p</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>BmLP</td>
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<tr>
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<td>474</td>
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<tr>
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<td>159</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>bmLP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>bm1p</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>bm1P</td>
<td>35</td>
<td>159</td>
</tr>
<tr>
<td>bm1p</td>
<td>11</td>
<td>52</td>
</tr>
<tr>
<td>Total</td>
<td>844</td>
<td>844</td>
</tr>
</tbody>
</table>

related trees tend to yield fewer seeds per cone, with less germination, than do progeny of outcrosses. The expected average inbreeding coefficient among progeny of matings of clone 5 with all other clones was computed as 0.156. From this value and also from data on effects of inbreeding (Squillace and Kraus 1963), we estimated that these matings would yield only 60 percent viable seed in comparison with outcrossing. Therefore, we adjusted the estimated percent of progeny that would have been sired by orchard pollen if the trees had been unrelated: \(\frac{6.0}{10} = 10\) percent. We estimated that if the orchard has contained unrelated clones, the proportions of selfing, orchard crossing, and nonorchard crossing would have been 6.5, 10.0, and 83.5 percent.

In considering these results, we must note that our orchard was very small, contained few clones, and also had considerable variation in time of pollen shedding. Larger orchards with more clones would undoubtedly receive less contamination. But the high degree of contamination found here suggests that contamination might be appreciable in some southern pine orchards.

Effects of Contamination on Genetic Gain

When seeds from an orchard are sired by pollen from wild, unimproved trees, the genetic gain from such seed is one-half of that expected under no contamination. The net overall gain \(G_n\) is therefore:

\[ G_n = G - \frac{GC}{2} \]

where:

\(G\) = the gain expected under no contamination, and
\(C\) = the proportion of viable seed sired by pollen from unimproved trees (nonorchard pollen).

For example, if one expects 20-percent gain under no contamination, the expected gain under 40-percent contamination would be: \(20 - \frac{20 (0.04)}{2} = 16\) percent.

Expected net gains for other conditions are given in Table 3.

<table>
<thead>
<tr>
<th>Extent of contamination(^1)</th>
<th>Expected gain with no contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>14</td>
</tr>
<tr>
<td>80</td>
<td>12</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^1\)The percent of viable seed-orchard seed sired by pollen from unimproved nonorchard sources.
On the question of how much contamination is acceptable, opinions are likely to vary, but it is reasonable that contamination in excess of 20 percent should cause concern.

What To Do About Contamination

How can one determine whether or not a particular orchard is subjected to appreciable contamination? A determination such as the above would be desirable, but the costs normally are prohibitive. Measurement of background pollen densities as done by Koski (1970) might be feasible. One could also make some good guesses from conditions in the orchard. If, for example, the isolation zone is only 400 feet (112 m), and if the species in question is relatively abundant within the region of the orchard, contamination could be high. Other situations that would increase the likelihood of contamination would be high variation in phenology of flowering among clones in the orchard and a scarcity of pollen production.

If there is a contamination problem, here are some ways of alleviating it:

1. Increase the size of the isolation zone. (This may not be effective if there is an abundance of neighboring trees of the species used.)
2. Increase the size of the orchard, which will increase the quantity of orchard pollen relative to background pollen.
3. Avoid use of clones that are exceptionally early or late in flowering (either male or female). If their female flowers are late or early, they will probably be pollinated by nonorchard pollen and if their pollen is early or late, the clones probably will not be of value to the orchard.
4. Establish about equal numbers of ramets per clone, which will probably increase the number of compatible matings in the orchard.
5. Increase the yield of pollen in orchard trees through fertilization or related treatment.
6. Apply pollen artificially from clones collected within the orchard or from other superior clones (for supplemental pollination methods, see Franklin 1971 and chapter 11).
7. Establish orchards in areas outside of the species range, or in areas where the species in question is relatively scarce, or in areas where flower phenology of orchard trees will differ from native ones.
8. Change the flowering period of orchard trees so that it will not be in harmony with background pollen. This change may be impractical in most cases, but it was feasible in a Douglas-fir orchard (Silen 1969).
9. Delay collection of cones from orchards until pollen production is abundant.

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van Buijtenen, J. P.

Wang, C. W., T. O. Perry, and A. G. Johnson.

Wright, Jonathan W.
Lack of pollen is a problem in young orchards, both for breeding and seed production purposes. After the orchard approaches full production, management stresses seed production, but a good pollen supply is also important. Here we discuss constraints on pollen production; methods for inducing pollen on small, sexually immature trees, primarily for breeding purposes; and the management of pollen production in established seed orchards, where maximizing the production of genetically improved seed is the main objective. Because maximizing genetic gain is also important, we briefly discuss suppression of pollen production.

Environmental and Genetic Constraints on Pollen Production

Juvenility. Conelet production in *Pinus* usually begins several years before catkin production. Catkins on *Pinus sylvestris* (L.) do not occur until about age 11, several years after the first conelets have appeared (Wareing 1953). When male and female flowering was observed on a 5-year-old loblolly pine (*P. taeda* L.) plantation near Hot Springs, Arkansas, females were present on 9 percent of the trees and males on only 3 percent (Greenwood and Gladstone 1978). Schmidtling (1971) also found that female flowering was two to three times more frequent than male flowering on 4-year-old loblolly pine grown in southern Mississippi.

Female strobili also occur more frequently than male strobili on young, grafted ramets in loblolly pine seed orchard trees, even when mature scion wood is used on the grafts (Schmidtling 1979, Greenwood 1977). Male strobilus production in *Pinus* is usually confined to slowly growing branches in the lower part of the crown (Eggler 1961, Wareing 1957), and in southern pines occurs mainly on those that have produced only one cycle of growth during the growing season (Eggler 1961, Greenwood 1980). Small trees, whether seed grown or grafted ramets, have relatively few of these branches. Controlled crossing began at Weyerhaeuser's Magnolia and Craig loblolly seed orchards about 4 years after the orchards had been established in 1971. In 1977, however, pollen had to be obtained either from trees in older seed orchards or from the original ortet for 37 clones, 69 percent of the total. In contrast, female strobili were present on 86 percent of the clones in the seed orchards (Ron Campbell and Ray Oram, 1977, personal communication, Weyerhaeuser Company, Magnolia, Arkansas). Pollen is usually limiting for breeding purposes or seed production in young loblolly pine seed orchards.

Inheritance. Male strobilus production is clonally determined, probably at least as strongly as female strobilus production. Counts of male and female strobili in several 7-to 10-year-old seed orchards have yielded broad sense heritability estimates for male strobilus production of 0.4 to 0.7 for shortleaf pine (*P. echinata* Mill.) in Arkansas and North Carolina, 0.4 to 0.6 for loblolly in south Mississippi and South Carolina, 0.7 to 0.9 for Virginia pine (*P. virgiana*) in North Carolina, and 0.3 to 0.6 for slash pine (*P. elliottii* Englem.) in southern Mississippi. These figures are in the same range as female strobilus heritabilities in the same orchards. The genetic correlations between male and female flowering are usually positive but not large, ranging from 0.1 to 0.4 for orchard data mentioned above. In many seed orchards, early thinning plans are based on cone production only, with no consideration given to pollen production.

Though most of the really outstanding pollen producers are also good female strobilus producers, some clones are below average in female strobilus production but above average in male strobilus production. For example, one loblolly clone averaged 50 catkin clusters per ramet, which is twice the average, but only one female strobilus in a south Mississippi seed orchard in 1952. If this clone is eliminated, a valuable pollen producer will be lost.

Evaluation of reproductive potential of clones must be based upon several years' production because year × clone interaction is strong. In an analysis of variance and covariance for 18 south Mississippi loblolly clones for 1976-78, the year effect and clone effects were very strong for both male and female flowering, as was the year × clone interaction (Schmidtling 1980). The covariance between male and female flowering for the clone term was positive, but the year and year × clone terms were negative. This indicates not only that male flowering and female flowering were not synchronized by years overall, but also that the clones were interacting with yearly climatic variation differently for male than for female flowering.

One important consequence of this year × clone interaction in pollen production is that the genetic "worth" of seed-orchard seed may vary considerably from year to year, even if seeds are kept separate by mother tree. The same would apply to various bulk collections from seed production areas, etc., used as check lots. In this case, the variation would be even larger because there is a year × clone interaction in female flowering as well, and it is independent of interaction with male flowering.

Geographic Effects. Much of the literature dealing with geographic effects and fruitfulness considers only female flowering or cones. In Europe, seed orchards are often located in southern areas because it is assumed that cone and seed production will be enhanced. Both loblolly pine and shortleaf pine seem to produce more cones in southerly locations (Schmidtling 1977, 1979). In studies of shortleaf and Virginia pine, several ramets from the same clones were planted in seed orchards near the origin of the ortets (Arkansas and North Carolina) and also in a clone bank in south Mississippi, the southern edge of the shortleaf pine distribution and about 200 miles southwest of the nearest natural Virginia pine.

Shortleaf grafts had more male and female strobili at the south Mississippi location than at either the Arkansas or North Carolina orchards (Schmidtling 1977). Male strobili were about five times more abundant at the southern location.
than at other locations. Similarly, the Virginia pines flowered better in south Mississippi than in North Carolina. For the 3 years the flowers were counted, the southern location averaged from 1½ to 7 times as many male strobili, and from 2 to 10 times as many female strobili as the northern location, though ramets were larger in the northern location (fig. 6). Thus, seed orchards should be located about 100 miles south of the origin of the ortets if possible. Female flowering and probably male flowering will be enhanced.

Figure 6.—Flowering of Virginia pine clones, planted in western North Carolina (north) compared with the same clones planted in south Mississippi (south) for 3 years.

Regulation of Pollen Production for Breeding

Topworking. Topworking involves grafting scions into the crown of a sexually mature, heavily flowering tree in hopes that the sexual competence of the understock will be transmitted to the scion. This method has been used for many years on fruit trees (Hartman and Kester 1968) and has also been tried with varying success on several Pinus species (Mirov 1951, Barnes and Bingham 1963, McDaniel and Einert 1976, Greenwood and Gladstone 1978). Greenwood and Gladstone (1978) is the only report of effects of topworking on male flowering of southern pines. Scions from 1-year-old loblolly pine seedlings were grafted into the middle half of the crowns of mature seed-orchard trees in the spring of 1973, 1974, and 1976 (the grafts were established in 1957). Surviving grafts were examined for male and female strobili in 1975, 1976, 1977, and 1978 (table 4). Of the 10 half-sib families originally grafted, eight were represented by living scions in 1977 and well over 50 percent produced catkins. Graft mortality, which includes some losses due to removal of scion-containing trees during roguing of the orchard, was between 60 and 70 percent over the duration of the experiment. Flowering between grafts made in 1973 and 1974 did not differ significantly. Additional grafts made in 1976 and 1977 had begun to produce males in 1978, and appear to be following a course of male flowering similar to that of the earlier grafts. Both male and female strobili have been observed in 3 successive years on scions that ranged from 3 to 5 years old from seed. Catkin production by the topworked grafts was about 20-fold greater than for field grown trees of the same age (5 years) and families. Except for the presence of reproductive structures, the topworked scions still resemble juvenile branches. So, topworking may cause scions that appear juvenile to flower by modifying the growth behavior of their buds. An influence or influences—either physical, nutritional, or hormonal—apparently can be transmitted to the scion from the interstock. The result is precocious flowering, probably partly caused by a “mature” type of growth behavior imposed on the otherwise juvenile buds. The result appears to be similar to that achieved with out-of-phase dormancy, which also causes early bud set on juvenile material thus allowing more time during conditions favorable for vegetative growth for strobilus differentiation to occur (Greenwood 1978).

Despite considerable mortality, which could be lessened by more careful grafting and some aftercare of the scions, most of the half-sib families yielded some pollen. So, we feel topworking might have some utility for pollen production from very young scion wood. Yet, we have not evaluated pollen produced through this method in controlled crosses. Also, there is a lag of 2 to 3 years after grafting before much pollen is produced. Other methods described here produce responses sooner.

Girdling. Like topworking, girdling is another ancient method for inducing flowering in trees and has probably been more widely investigated than any other method except fertilization (Noel 1970, Puritch 1972). The success of the technique is variable, because the time of application, the type of girdle employed, and the condition of the tree are probably crucial (contrast Greenwood 1977 with Ross and Greenwood 1979). Unfortunately, rigorous studies to optimize the method and timing treatment on the southern pines are lacking. However, there are numerous reports on girdling southern
pines, with generally promotive effects (Puritch 1972) on strobilus production. Varnell (1970), Hare (1979), and Hare and others (1979) observed promotive effects on male strobilus production for longleaf, slash, and loblolly pine. They girdled only lateral branches, removing overlapping 1/2-inch bands of bark, with 1 inch separating the partial girdles. Hare (1979) girdled loblolly and slash pine branches on May 22, 1977, and treated the branches with a mixture of naphthalene acetic acid and gibberellins (NAA-GA$_4$/7). This treatment combination increased frequency of male production eightfold, with males occurring on 30 to 50 percent of the treated branches of 7-year-old trees. NAA-GA$_4$/7 alone brought about a sixfold increase, but girdling alone was not evaluated. However, longleaf pine girdled in late May 1976, responded well to girdling only (Hare and others 1979). Varnell (1970) chose primary branches in the upper two-thirds of the crowns of 29-year-old longleaf pines, and girdled them as described above in the spring (the exact date is not given). He also observed an eightfold increase in the number of branches bearing male flowers, with about 20 percent of the surviving treated branches responding.

A complete stem girdle with 17-gauge steel wire (electric fence wire) applied in early spring to branches in the lower half of the crown has yielded excellent results on male flowering (Greenwood, unpublished data, table 5). The grafts used in tests A and B (see table 5) resulted from scions from 8- to 12-year-old trees growing in progeny tests in North Carolina, while the field-grown grafts in test C resulted from scions from mature, wild trees from Arkansas to Oklahoma. In both experiments, wire girdling caused catkin production on 80 percent of the clones versus none for the controls.

The wire was wrapped twice around the branch 6 to 12 inches from the trunk, and tightened by twisting so that it fit snugly without digging into the bark. Tywraps, small self-locking plastic straps normally used for bundling electrical wires, were also used because they can be installed quickly. The wire girdle was very effective on young grafts grown outdoors, whether potted or in the ground (table 5). Tywraps were ineffective, possibly because they are more flexible than wire and because the girdling process may not have started soon enough.

The indoor-grown trees in B, table 5, were producing heavy crops of males in response to water stress. The trees were kept in a large cover-house year-round, under natural photoperiod. The house was evaporatively cooled in the summer, and heat was applied in the winter only when the temperature fell below 0° C. Wire girdling did not promote male flowering in the greenhouse, even though swelling distal to the girdle was observed. Apparently, whatever girdling provides for the promotion of male flowering was not limiting in the greenhouse. Marino (1979, personal communication, International Paper Company, Bainbridge, Georgia) also found that wire girdling did not promote flowering on 7-year-old trees that were already producing males abundantly. Although the foliage of the branches girdled outdoors in 1977 was somewhat yellowish during the winter of 1977-78, all branches that survived with males shed pollen in the spring. Some of the girdled branches were pruned off during the summer of 1978, but 11 were spared, 7 of which had another crop of male strobilus buds in December 1978. The method is effective on small grafted ramets that have not yet begun to produce catkins.

<table>
<thead>
<tr>
<th>Table 5.—Effect of complete branch girdling on male strobilus production by loblolly pine grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Effect of wire girdling (on 3/14/77) on male strobilus production by 1974-grafted loblolly pine growing in 45-gallon pots outdoors in Hot Springs, Arkansas.</strong> Equal-size control and girdled branches were located in the lower 1/3 of the crown on a total of 10 clones, each represented by 3 to 4 ramets.</td>
</tr>
<tr>
<td>Branch survival Girdled 36 of 41 Control 35 of 35</td>
</tr>
<tr>
<td>Percent of trees with male strobili Girdled 58 Control 0</td>
</tr>
<tr>
<td>Percent of clones with male strobili Girdled 80 Control 0</td>
</tr>
<tr>
<td><strong>B. Effect of wire girdling (on about 4/15/77) on male strobilus production by 1974-grafted loblolly pine growing in 45-gallon pots indoors near Hot Springs, Arkansas.</strong> Equal-size control and girdled branches were located in the lower 1/3 of the crown on a total of 20 clones, each represented by 1 or 2 ramets.</td>
</tr>
<tr>
<td>Branch survival Girdled 23 of 23 Control 23 of 23</td>
</tr>
<tr>
<td>Percent of trees with male strobili Girdled 61 Control 48</td>
</tr>
<tr>
<td>Percent of clones with male strobili Girdled 75 Control 55</td>
</tr>
<tr>
<td><strong>C. Effect of wire girdling (on 4/14/78) on male strobilus production by 1974- and 1975-grafted loblolly pine growing in a seed orchard near Magnolia, Arkansas.</strong> Equal-size control and girdled branches were located in the lower 1/3 of the crown on a total of 20 clones, each represented by 4 to 5 ramets. Both Tywraps and steel wire were used for girdling.</td>
</tr>
<tr>
<td>Branch survival Girdled, Tywrap 21 of 22 Girdled, wire 12 of 18 Control 22 of 22</td>
</tr>
<tr>
<td>Percent of trees with male strobili Girdled 0 Tywrap 0 Control 0</td>
</tr>
<tr>
<td>Percent of clones with male strobili Girdled 0 Tywrap 80 Control 0</td>
</tr>
</tbody>
</table>
**Greenhouse Culture.** Greenhouse-grown conifers flower better than similar trees grown out-of-doors (Tompsett and Fletcher 1977, Young and Hanover 1976, Rudolph 1965). We have been growing grafted loblolly pine indoors in 45-gallon (170 liters) pots (as described in the previous section) since early 1976, providing only water stress to stimulate male flowering (Greenwood, O'Gwynn, and Wallace, unpublished data). In the spring of 1978, the grafts ranged in age from 2 to 6 years, and 83 to 110 clones (75 percent) produced male catkins. Each clone was represented by one to three ramets each. The nonflowering clones were mainly represented by younger grafts, 25 percent of which produced males. Outdoors, only 2 of 14 (14 percent) clones, each represented by several 4-year-old grafts, produced male strobili, even with water stress. Field-grown grafts of the same age produced almost no pollen (when Weyerhaeuser's Craig and Magnolia seed orchards had been established for 3 years, male strobili could only be collected from 1 of about 70 clones).

Water stress is essential to stop active vegetative growth, because initiation and differentiation of strobili occur when the bud is quiescent (Greenwood 1978, 1980). Bud quiescence is a misnomer because the bud is actively initiating new primordia and slowly increasing in size. In the absence of stem elongation, however, these primordia undergo the time-consuming process of differentiation into strobili. The resting buds of greenhouse-grown loblolly pine are significantly larger than the buds of ramets of the same clones grown outdoors (Greenwood 1977). The increased size resulted from a greenhouse environment where extremely hot daytime temperatures are avoided. On large, mature, seed-orchard trees that are flowering heavily, the resting buds are also large. Consequently, the greenhouse environment may permit smaller trees to mimic closely the bud behavior of large, fecund trees growing outside.

**Out-of-Phase Dormancy.** Out-of-phase dormancy, first described by Greenwood (1978), may be the fastest and most reliable way to generate male strobili on small, sexually immature grafts. The technique consists of prolonging vegetative growth through the winter then forcing bud set during the spring with lowered temperature and photoperiod. This delays elongation by about 2 months and apparently allows time for differentiation of strobili buds to occur in very young loblolly pines. Sixty percent of 1-year-old grafts produced male strobili, and the scions at the time of flowering were only 3 years old from seed.

Ramets representing all families and clones used in the experiment produced males in response to this method, which imposes bud quiescence by drastically shortening photoperiod and lowering temperatures in the early spring. After quiescence, the buds appear to go into a state of physiological dormancy (Romberger 1963) requiring a cold treatment for prompt resumption of vegetative growth. Garber (1978) has demonstrated that loblolly pine seedlings have a partial cold requirement. Resumption of stem elongation is significantly delayed if the cold requirement is not satisfied. The photoperiod and temperature shock provided by the out-of-phase dormancy treatment induces dormancy for long enough during early spring for male strobili buds to develop. Although the males form earlier than normal, most of them do not complete their development until pollen is shed at the usual period for loblolly pine the next spring.

Another trial of this method has recently been completed on 15 North Carolina clones grafted in the spring of 1977. This trial compares two dormancy treatments begun February 21, 1978, and March 21, 1978 (table 6). The treatment begun in March was significantly more effective than that begun in February. The successful 1977 trial was started in mid-February, but there were many more cold days in the early spring of 1978. For a 28-day period starting on February 21, there were 12 days in 1977 and 22 days in 1978 when the minimum daily temperature was between 0° and 5° C. This colder period in 1978 caused the trees whose treatment began February 21, 1978, to resume vegetative growth much earlier than where treatment started a month later. The early growth resumption apparently decreased male strobili production. Although further trials are needed to specify the best date for starting this treatment, the March date is recommended, because the likelihood of enough cold weather after that time to affect the cold requirement is small.

### Table 6.—Male strobilus production as of September 1978 on ramets representing 15 clones treated by out-of-phase dormancy (OPD)

<table>
<thead>
<tr>
<th>Date OPD was begun</th>
<th>Percent of trees with male strobili</th>
<th>Percent of clones with male strobili</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/21/78</td>
<td>36 (12 of 33)</td>
<td>53 (8 of 15)</td>
</tr>
<tr>
<td>3/21/78</td>
<td>80 (12 of 15)</td>
<td>80 (12 of 15)</td>
</tr>
</tbody>
</table>

**Regulation of Pollen Production for Seed Production**

**Fertilization and Subsoiling.** Although there has been much research on the effects of silvicultural treatments such as fertilization on cone production, little has been done with pollen. Pollen production is more difficult to assess than cone production, because catkins do not persist on the tree and can be counted for only a limited part of the year.

Webster (1974) found that N and P fertilization inconsistently increased pollen production in loblolly pines. Fertilizers showed no effect on male flowering in young loblolly pine grafts, while substantial effects were observed on female flowering (Schmidtling 1975). On older trees the same treatments that affect female flowering will probably affect male flowering, but the timing may be critical.

Preliminary results of recent experiments involving the timing of fertilizer application on four species of southern pines are summarized in table 7. Complete results will be published later and will include a 3d year's flowering data as well as cone and seed yield. Optimum times for fertilization were earlier for male than for female flowering (table 7). The earlier response for male flowering agrees with the
observation that male strobili initiate and differentiate first (Goddard 1960, Greenwood 1980).

Table 7.—Optimum timing of fertilizer application for male and female flower induction for several southern pines as indicated by recent fertilizer timing experiments

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Optimum Time</th>
<th>Male flowering</th>
<th>Female flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loblolly</td>
<td>S. Mississippi</td>
<td>Late June-</td>
<td>August</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>early July</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loblolly</td>
<td>E. South Carolina</td>
<td>Late June</td>
<td>August</td>
<td></td>
</tr>
<tr>
<td>Virginia</td>
<td>W. North Carolina</td>
<td>Early June</td>
<td>Late July-</td>
<td>August</td>
</tr>
<tr>
<td>Slash</td>
<td>S. Mississippi</td>
<td>Late June-</td>
<td>August</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>early July</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shortleaf</td>
<td>Arkansas</td>
<td>Late May-</td>
<td>July</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>early June</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In all species, timing was much more critical for male than for female flowering. Response curves for female flowering generally follow those observed for loblolly pines (Schmidtling 1975), i.e., a gradual rise in response until August, and a dropping off thereafter. Male flowering, on the other hand, was characterized by a negligible response more than 1 month before or after the optimum times listed in table 7. Fortunately, both male and female flowering will be enhanced if fertilizers are applied at the optimum time for male flowering, as female flowering levels will be 75 to 85 percent of the maximum obtainable.

Other treatments that affect female flowering will probably affect male flowering too if timing is proper. In a recent subsoiling and fertilization study in Virginia pine, male flowering was enhanced by the same treatments as female flowering. Subsoiling in early June increased male and female flowering over controls. Fertilization, also done in early June in the same experiment, enhanced male and female flowering, and the combination of subsoiling and fertilization was best. In this experiment, the treatments were applied at about the critical time for male strobilus induction, early June (table 7).

Irrigation. Although water stress promotes female flowering in southern pines, data on male flowering are only available for slash pine. Barnes and Bengtson (1968) showed that some clones responded to irrigation by producing more pollen while others did not. Water stress, however, may promote male flowering if started early enough (see section on Greenhouse Culture, this chapter), but inhibit it if applied later in the season (Greenwood 1977). Associations between rainfall records and the next year’s flower crop in south Mississippi indicate that the combination of abundant rainfall in spring and early summer plus late summer moisture stress favors heavy female flower crops, but the combination of late spring and early summer drought and abundant late summer rain favors heavy pollen crops. This pattern probably results to stopping growth in buds that produce male and female reproductive structures at critical times. Early drought stops bud growth in buds in the lower crown. These buds normally produce male strobili and do not resume growth with late summer rains. Buds in the upper crown, however, normally produce female strobili and will resume growth if conditions are favorable. These buds in the lower crown have the necessary time to differentiate male structures and produce a good male crop. Female crops are poor under these same conditions, perhaps because less photosynthate is available later at the critical time for female bud differentiation. The opposite rainfall pattern, abundant early rain and late summer drought, stops growth early enough for the differentiation of female strobili, but too late for male strobili, resulting in a good female flower crop and a poor pollen crop. Therefore, it may be difficult to optimize both male and female flowering through irrigation.

Site Effects. Little information is available on site effects for either male or female flowering. Gallegos (1979) concluded in a survey of loblolly orchards that the sites most suited to produce many female cones were the well-drained sites with a shallow clay layer, or droughty sites that would produce moisture stress in early summer. Data from a south Mississippi seed orchard support this observation, and sites favoring female flowering probably will also favor male flowering (Schmidtling 1980). In the 3-year data on 18 loblolly clones mentioned in section II A, the analysis of covariance yielded a correlation between male and female flowering of r = 0.8 for the ramet in a clone component (or environment effect). The correlation indicated that, over the 3 years of the study, sites that favored female flowering also favored male flowering.

If an orchard is located on a well-drained to excessively well-drained site and supplied with irrigation during the critical period of establishment, both pollen production and cone production probably will be optimum.

Pollen Suppression. The suppression of pollen production, which might be desirable under some conditions, could be accomplished either by preventing male strobili initiation or by using gametocides. Spaces does not permit a treatment of gametocides, because we have hardly been able to locate new or promising work since their potential was stated by Dorman (1976). The only substance that induced male sterility was 2,4-dichloro-phenoxyacetic acid, but it caused foliar damage to pitch pine. More basic research is needed before chemical induction of male sterility can be used to produce genetic gains.

The only practical method for pollen suppression appears to be through regulation of moisture stress to favor female strobilus initiation at the expense of male strobilus initiation.

Conclusions

Male flowering capability is highly heritable, but catkin production by a single clone varies greatly from year to year. The correlation between male and female flowering by clone is positive but weak. So, the amount of pollen produced by a given clone will not always be related to its female
production, and therefore the genetic gains from open-pollinated seed may vary from year to year.

The lack of relationship between male and female flowering is not surprising because catkin buds initiate and differentiate well before cone buds do (Goddard 1960, Eggler 1961, Greenwood 1980). Because environment has a large impact on flowering in pines, and climatic conditions are not necessarily similar during male and female development, there is no reason to expect such a relationship.

Some researchers (e.g., Giertych 1967) have reported that such treatments as fertilization and hormone application may have opposite effects on catkin and conelet production. We feel that subsoiling, fertilization, and water stress can promote both male and female formation probably by the same mechanisms, but must be timed so that they affect the development of both sexes. In general, earlier treatment is required to stimulate catkin production, so delays in these treatments can promote female development at the expense of male development. Therefore, some clones, or some portions of an orchard, can be managed to stress the development of either sex.

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Chapter 5
Diseases and Insects of Catkins and Pollen
by Thomas Miller and G. L. DeBarr

Diseases

Relatively little has been published on the diseases of pollen. In the phytopathological literature, the only reported
diseases are caused by viruses. Virus particles have been found
inside the pollen grains of barley and pear, and those viruses
have been transmitted by the pollen to healthy plants (Gold
and others 1954, Tomlinson and others 1968). Most of the
other information about micro-organisms and pollen either is
found in paleobotanical literature or else in medical research
on allergic reactions to pollen or its associated micro-
organisms.

Pollen of various tree species, particularly conifers, are
reportedly susceptible to degradation by Phycomycetes,
especially the Chytrids (Goldstein 1960; Skvarla and Anderegg
1972). Spores of certain species of fungi have been found
consistently on pollen of certain tree genera (Colldahl and
Carlsson 1968), but whether these fungi caused any damage to
the pollen was not investigated.

A basic problem in the collection and handling of conifer
pollen is its contamination by micro-organisms (Stanley and
Linskens 1974). Fresh pollen that is collected and stored
under improper conditions of moisture and temperature will
invariably nurture growth of many different fungi and
bacteria that will render it inviable. These contaminating
micro-organisms are either on or in the pollen before the
opening of the catkins, or else they are airborne contaminants
that alight on the pollen during its collection and hand-
ling. Because a technique has been reported for obtaining
supposedly contaminant-free conifer pollen by collecting and
decommiting catkins before they open (LaRue 1953,
Tulecke 1954), most of the damage is presumably caused by
airborne microflora encountered during collection and hand-
ling of the pollen.

Therefore, it is recommended that to reduce or eliminate
the damage caused by airborne microbial contaminants,
proper temperature and moisture content should be
maintained during the collection, processing, and storage of
pollen. A method for disinfecting contaminated pollen is to
treat it with a suspension of an appropriate fungicide or
antibiotic and redry the pollen to the proper moisture content
(Stanley and Linskens 1974).

Insects

Insects often are the major obstacle to seed production in
southern pine seed orchards. Many different species attack the
female flowers, conelets, cones, and seed from bud stage to
cone maturity (Ebel and others 1975). In contrast, an
abundance of pollen rarely is disrupted by insects. However,
in pollen management where the goal is to maximize pollen
supplies from particular genetic selections, the control of
insect pests may be especially important.

The most common insects associated with the catkins of
pines are the small, white larvae to the xyelid sawflies (Xyela
spp.). Pollen is consumed within the microsporangia (pollen
sacs) of the developing catkin. Outward symptoms of
infestation are resin droplets and distortion of the catkin (fig.
7). Larvae emerge when the pollen sacs rupture and are
frequently encountered when pollen is extracted (fig. 8).
Xyelid larvae can easily be separated from the pollen by using
screen sieves. Xyela minor Norton and X. bakeri Konow
occur in catkins from both slash (Pinus elliottii Engelm.) and
longleaf pines (P. palustris Mill.) (Ebel 1963). Xyela pini
Rohwer has been reared from slash (Ebel 1963) and loblolly
pines (P. taeda L.) (Ebel 1966). Large swarms of X. minor
and X. bakeri wasps also have been observed feeding on slash
pine pollen (Ebel 1966).

Figure 7.—Distorted catkins, sunken areas, and
patches of dried resin characterize feeding damage
by xyelid sawflies or leaffooted pine seed bugs.

Several important cone and seed insects also feed upon
male catkins. Larvae of the coneworms, Dioryctria amatella
Hult., D. clarioralis (Wlk.), and more rarely D. abietella
(D&S) infest the catkin buds and catkins of longleaf and slash
pines (Ebel 1963). Overwintering adults of the leaffooted pine
seed bug, Leptoglossus corculus (Say), feed upon pollen. The
insects puncture the pollen sacs, causing necrotic areas (fig.
9). Damaged catkins are often stunted and deformed, and
pollen production is reduced. Conophthorus coniperda

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Service, School of Forest Resources and Conservation, University of Florida,
Gainesville, Fla. and Entomologist, U.S. Department of Agriculture, Forest
Service, Forestry Sciences Laboratory, Athens, Ga.
Schwarz is primarily a cone-infesting beetle, but it attacks catkin buds when the cone crops of eastern white pine (P. strobus L.) are limited and beetle populations reach epidemic levels (Godwin and O'Dell 1965).

Little is known about the other insects that commonly infest catkins of the southern pines. Large blister beetles strip the microsporophylls down to the central axes. Allen and Coyne (1956) observed one such beetle, Pomphoepa polita (Say), destroying shortleaf pine (P. echinata Mill.) catkins in Mississippi, while Ebel (1963) reported that Lytta sp. caused similar damage to the catkins of slash pine in north Florida.

Ebel (1963) also listed several other insects associated with catkins of slash and longleaf pines. Frass and silk webbing among catkin buds indicated feeding by Holocera lepidophaga Clarke larvae. The small larvae of Satronia tantilla Heinrich destroyed male catkin flower buds. Orange-colored fly larvae of the family Cecidomyiidae and thrips, Frankliniella tritici (Fitch), fed upon pollen. Because of their small size, thrips were difficult to remove from extracted pollen. Nymphs and adults of a mirid, Lepidopsallus australis Blatchley, were found on the catkins of slash pines. No obvious injury was evident, but it was presumed that the bugs were feeding upon the buds.

To control these and other pests, chemicals are applied in seed orchards during the critical period of pollen shedding and female receptivity. However, the chemicals may produce severe phytotoxic effects that ultimately reduce catkin survival, seed set, and seed viability.

Seed set of slash pines sprayed with malathion, heptachlor, or with the fungicide, ferbam, before receptivity was not reduced (DeBarr and Matthews 1971). However, Ritter and Miething (1967) showed that germination of both spruce (Picea) and pine pollens was inhibited by fungicides and insecticides at concentrations below the amounts that are normally used in seed orchards. To avoid damage to pollen and seed set, applications of pesticides should not coincide with pollen shedding or receptivity of the female flowers.

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The procedures described in the following text on timing and methods of pollen collection evolved over a period of several years in producing loblolly (Pinus taeda L.) and slash (P.elliottii Engelm.) clonal seed orchards.

Timing of Collection

Simple Tests of Maturity. The ideal time to collect catkins is at the first sign of pollen shedding from basal scales; however, this stage is dangerously late because a tree will often shed all of its pollen during a 1- or 2-day period or even during several hours, if weather conditions are right. Therefore, unless a tree can be continuously watched, it is desirable to collect at a slightly earlier stage of pollen development. The best timing is to collect catkins after they turn yellow and produce very little liquid when squeezed between the fingers. Do not collect catkins wetted by rain or dew because moisture prolongs the extraction period and may cause molding.

Forcing Pollen to Maturity. Collection of unripe catkins can result in decreased pollen yields and pollen of low viability (Snyder and Clausen 1974). To hasten pollen maturation, it is advantageous to enclose branch tips bearing clusters of catkins with sausage casings. This method usually forces pollen to mature several days early, especially in cold weather, making it available when conelets are receptive. During cold weather, pollen development is delayed, but conelets are less affected. (N.C. State Industry Cooperative Program. N.C. State Tree Improvement Handbook, p. 7.)

Dormán (1976) suggests painting the outside of the sausage casings a reflective color to avoid heat buildup and subsequent loss of pollen viability.

As an alternative to bagging, pollen may be forced to maturity by placing catkin-bearing branches in a pail of water (Dormán 1976). It is equally effective to place defoliated, catkin-bearing branches in 2-quart canning jars and hold them in a heated greenhouse for several days.

Methods of Collection

Small Lots. Collect catkins from the individual ramets only after some pollen sheds from the basal ends of most of the catkins upon tapping. Catkins should not be rinsed in water before they are picked. If contamination is feared, bag the catkins before harvesting.

Pluck catkins from the tree and place them in sausage casings or paper bags for transport to the drying facility. Do not fill sausage casings more than one-third to one-half full. Exclude all needles, twigs, and other trash from the bags. Transparency of the sausage casings makes it easy to check the condition of the catkins.

Wash hands in rubbing alcohol before collecting catkins from another clone. If repeated alcohol rinsing is too uncomfortable, a thorough wash in soapy water is recommended. Disposable gloves may be worn as an alternative to sanitizing with alcohol or water (fig. 10).

Figure 10.—Harvesting a cluster of slash pine catkins. (Photo courtesy of W. L. Beers, Jr., Buckeye Cellulose Corporation, Perry, Fla.)

Individual ramets from which catkins are not to be collected are flagged in advance, with consideration given to the degree of pollen ripeness and the volume of catkins needed.

An aerial-lift unit (fig. 11) or truck-mounted ladder should be used for large-scale collections. Branch tips bearing a cluster of catkins and a whorl of needles are dropped to the ground. The needles parachute the catkins to the ground and soften their impact. Ground crews gather the fallen tips and remove adhering needles. The associated vegetative bud should not be removed because removal results in excessive gum flow, which partially coats and seals the catkins.

Catkin clusters are loaded into 5-quart pails by ground crews and are transferred into a large foam chest. The chest should be kept in the shade, without ice or top until about five pails of catkins are collected. The catkins should be taken to a heated building and spread out to dry in paper-lined racks or trays (fig. 12). The drying room should be ventilated but free of air turbulence or strong drafts. An open flame should be excluded from pollen extraction rooms because it increases the chances of a dust (pollen) explosion. The above procedure requires a three-man crew. With an additional crewman, another procedure can be used for large collections.

An aerial lift or ladder could be used as above except the lift operator cuts needle-free clusters of catkins from the tree.
Two members of the ground crew catch falling catkin clusters in a net made by attaching a large cloth (bedsheet) to two cone poles. The cloth is kept in a spread formation by attaching full-length cane poles to opposite sides. Two ground crew members spread the cloth to form a catch net. Net men catch the catkins and tilt the net, which causes the catkins to roll to the edge, where a third member of the crew scoops them up and places them in a pail. The remainder of the operation is the same as for a three-man crew.

Advantages of this technique are that catkin clusters stay intact better, and two steps are eliminated: Picking up clusters from the ground and reclipping them to remove needles. This technique achieved a production rate of one-half quart (0.6 liter) of catkin clusters per minute, which included the harvesting, catching, and defoliation of catkin clusters. (Setup time for the lift unit and other preparations were not included.)

**Yields and Labor Requirements.** Pollen yields and labor requirements were recorded during a 3-year period, during which about 50 quarts (55 liters) of catkin clusters were collected annually in a loblolly pine seed orchard. Laborer hours were totalled for the complete operation, which included setting up drying racks, determining ripeness, selecting ramets, collecting catkins, and transporting catkins to the extraction facility. As shown in table 8, the yields of loblolly pine pollen ranged from 26 cc to 86 cc per quart (1.1 liters) of catkin clusters; pollen yield per laborer hour ranged from 105.7 cc to 214.3 cc. Similar pollen yield data for a slash pine seed orchard are shown in table 9. Pollen yield averaged $121.3 \pm 17.7$ cc per quart (1.1 liters) of catkins and $3.61 \pm 1.06$ cc per cluster. Pollen yield per catkin averaged $0.35 \pm 0.08$ cc.

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Table 8.—Yields and labor requirements to collect pollen in a loblolly pine (Pinus taeda L.) seed orchard

<table>
<thead>
<tr>
<th>Year</th>
<th>Cone</th>
<th>Date of collection</th>
<th>Catkin clusters collected</th>
<th>Pollen yield per quart (1.1 liter)</th>
<th>Pollen yield per cone</th>
<th>Laborer hours</th>
<th>Collection time per quart (1.1 liter) of catkins</th>
<th>Pollen yield per laborer hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1976</td>
<td>A</td>
<td>3/6/76</td>
<td>75</td>
<td>53</td>
<td>3,975</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3/6/76</td>
<td>65</td>
<td>48</td>
<td>3,120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>140</td>
<td>7,095</td>
<td>36</td>
<td>0.26</td>
<td>197.1</td>
<td></td>
</tr>
<tr>
<td>1977</td>
<td>A</td>
<td>3/17-18/77</td>
<td>75</td>
<td>36</td>
<td>2,700</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3/17-18/77</td>
<td>75</td>
<td>26</td>
<td>1,950</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>150</td>
<td>4,650</td>
<td>44</td>
<td>0.29</td>
<td>105.7</td>
<td></td>
</tr>
<tr>
<td>1978</td>
<td>A</td>
<td>4/1-2/78</td>
<td>75</td>
<td>86</td>
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<td>75</td>
<td>54</td>
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<tr>
<td></td>
<td>Total</td>
<td></td>
<td>150</td>
<td>10,500</td>
<td>49</td>
<td>0.33</td>
<td>214.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 9.—Pollen yield in a clonal slash pine (Pinus elliottii Engelm.) seed orchard, Taylor County, Florida, 1979

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number of clusters</th>
<th>Number of catkins</th>
<th>Catkins per cluster</th>
<th>Total pollen yield</th>
<th>Catkins per cc of pollen</th>
<th>Pollen yield per catkin</th>
<th>Pollen yield per cluster</th>
<th>Pollen yield per quart (1.1 liter)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>cc</td>
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<td>A</td>
<td>28</td>
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<td>33</td>
<td>348</td>
<td>10.6</td>
<td>104</td>
<td>3.35</td>
<td>0.30</td>
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<td>C</td>
<td>40</td>
<td>434</td>
<td>10.8</td>
<td>107</td>
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<td>0.27</td>
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<td>123</td>
<td>3.01</td>
<td>0.33</td>
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<td>E</td>
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<td>401</td>
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<td>131</td>
<td>3.06</td>
<td>0.33</td>
<td>3.20</td>
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<tr>
<td>F</td>
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<td>130</td>
<td>10.8</td>
<td>69</td>
<td>1.88</td>
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<td>324</td>
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<td>105</td>
<td>3.09</td>
<td>0.32</td>
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<td>498</td>
<td>13.1</td>
<td>163</td>
<td>3.06</td>
<td>0.33</td>
<td>4.29</td>
<td>-</td>
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<tr>
<td>I</td>
<td>56</td>
<td>374</td>
<td>6.7</td>
<td>128</td>
<td>2.92</td>
<td>0.34</td>
<td>2.28</td>
<td>-</td>
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<tr>
<td>J'</td>
<td>—</td>
<td>242</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>—</td>
<td>115.0</td>
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<td>K</td>
<td>—</td>
<td>412</td>
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<td>—</td>
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<td>—</td>
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<tr>
<td>L</td>
<td>—</td>
<td>358</td>
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<td>M</td>
<td>—</td>
<td>552</td>
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<td>—</td>
<td>139.0</td>
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<tr>
<td>N</td>
<td>—</td>
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<td>O</td>
<td>—</td>
<td>380</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>137.0</td>
</tr>
</tbody>
</table>

Mean 10.4 3.00 0.35 3.61 121.3
S.D. 2.0 0.58 0.08 1.06 17.7

1One quart (1.1 liter) of catkins collected from clones J through O.
Chapter 7
Extracting and Drying Pine Pollen
by J. R. Sprague and E. B. Snyder

The processing of pine pollen for use in a breeding program is a delicate procedure consisting of collection, extraction, and storage. The viability of the pollen that is ultimately stored depends upon the care exercised during each of these steps. Here, we describe procedures for shipping, extracting, and drying loblolly (Pinus taeda L.) and slash (P. elliottii Engelm.) pine pollen.

Care and Shipment of Unopened Catkins
The combination of high temperature and high moisture content is the greatest deterrent to successful pollen storage. High moisture content is probably the most important single cause because pollen can tolerate fairly high temperatures (80°F, 27°C) for several weeks (Sprague and Johnson 1977) if it is dry. If the pollen moisture content is above 20 percent, pollen may mold at a wide range of temperatures, even under refrigeration and especially in sealed containers (Snyder 1961). So all preextraction and extraction procedures should be predicated on keeping the catkins and pollen dry.

It cannot be overstressed that, to insure minimal extraction time, stroboli should be collected as near to shedding time as possible. Catkins collected too early take several days to dry; if they do yield pollen, they yield little.

Unopened catkins must sometimes be shipped before pollen extraction. Green catkins that are closed up in a container create a warm and wet environment that causes molding within a few hours. Temperatures within a closed box can rise in excess of 100°F (38°C). Pollen is ruined in a short time if proper packaging procedures are not followed.

Packaging Procedures. 1. Collect catkins by clusters or individually. Collection by clusters allows more aeration of the individual catkin when it is packed for shipping, but the catkin stem is a source of high moisture and mold. If collected individually, catkins pack together, impeding air flow and drying.

2. If clusters are still green, put them in paper bags. Do not use sausage casings or plastic bags. Also, do not use more than two layers deep of clusters or 1 inch deep of individual catkins. Hang catkins in a warm (70°F to 80°F, 21°C to 27°C), dry (20 to 40 percent relative humidity) room where air is circulated around the bags with a fan. Air movement drives moisture away from the pollen. As with packaging and shipping, paper bags should be used, and catkin clusters should be no more than two layers deep (or 1 inch deep for individual catkins). When the catkins open fully, the bags can be shaken to extract the pollen. If the paper bags are not tight at the seams, tape should be applied to prevent leakage.

Other methods are described by Snyder and Clausen (1974) and Beaver (1976). For example, sausage casings can be used for extracting single clusters of catkins. This is sometimes necessary to force development of late-flowering trees or trees whose pollen is needed immediately in the field. Sausage casings can be recommended only in special circumstances where a few pollen lots are needed. Drying pollen under uncontrolled conditions is apt to give high pollen moistures and lead to disastrous results. Also, the pollen will have to be routinely cleaned of insects and other debris and supplementally dried. For cleaning flower parts, insects, and other debris from pollen extracted under more primitive methods a 60- to 100-mesh screen (as used for soil testing) can be used. Also, for small lots of pine pollen, voile cloth can be used to sift the pollen. To prevent scattering, the voile cloth can be used to form a pouch at the top of a fruit jar. The flowers are placed on the pouch, and the lid is screwed on to hold the pouch in position before the jar is shaken (Snyder and Clausen 1974).

A more satisfactory system for extracting pollen consists of a temperature-humidity controlled pollen chamber where the pollen can be extracted and dried to the proper moisture content in one process. A room about 8 by 8 feet (2.4 by 2.4 m) will hold 24 to 48 extraction units, which are large funnels with air blown directly into each lot of pollen. For small programs, the same principles can be applied with fewer units and a smaller room. Twelve-inch (30.5 cm) top diameter, 8-quart (9 liter) gasoline funnels (Brookins No. 55, Belcrank, Division of Wheelabrator, Mishawaka, Indiana) are mounted on shelves built around the walls of the room (fig. 13). At Gulfport, slightly smaller funnels have been satisfactory.

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Figure 13.—A temperature-humidity controlled pollen room showing funnels mounted on shelves. (Photo courtesy of Westvaco Corporation, Summerville, South Carolina)

Screen wire and hardware cloth filters, 0.25-inch (0.64 cm) mesh, are placed inside the funnel to support the catkins and partially filter the pollen. Another fine mesh wire (about 50 mesh) is located where the funnel constricts near the bottom to filter smaller particles such as larvae (fig. 14). Widemouth, 8-ounce (227 ml) bottles (available from scientific supply houses) are attached to the bottom of the funnel by gluing the caps permanently onto the funnel with epoxy or fiberglass putty compound. Forced air is generated by a squirrel cage blower, 3450-rpm, 4-inch (10.2 cm) fan attached to a 3-inch (7.6 cm) diameter manifold made of galvanized steel, stovepipe, or plastic. The manifold has several T-outlets from which Tygon tubing carries the air to each funnel (fig. 15). The tubing is connected to a 0.25-inch (0.64 cm) copper tube, which leads into the bottle where the pollen collects. This copper tube is about 7 inches (17.8 cm) long. It is bent into an L-shape and soldered into the funnel (fig. 14). The proper temperature of the room can be maintained by a thermostatically controlled heater and the humidity by a commercial dehumidifier. Contamination is avoided by placing tightly woven cloth bonnets (usually a cotton-polyester blend or 10-ounce [283 g] canvas) over the funnels and securing them by drawstrings or elastic bands. To avoid contamination, one should either have the fan ducted to the dehumidifier outlet or have incoming air blown through silica gel (fig. 13).

**Procedures for Operating Forced-Air Extractors**

1. Maintain the temperature of the extractor between 70° and 80° F (21° to 27° C) and the relative humidity between 20 and 40 percent.

2. Place the pollen clusters no more than two layers deep in the funnels (or no more than 1 inch (2.54 cm) deep if individual catkins are collected). Air in the transfer room may be decontaminated by mist, and the processor must wash his or her hands between lots.

3. Observe pollen flow daily. When pollen appears, vibrate it down into the bottle where it can be air-dried. An electric sander with a piece of rubber inner tubing stretched over the sandpaper part and held against the side of the funnel makes a good vibrator (fig. 16). The air flow into the bottle will eventually blow the pollen back into the bottom of the funnel, so it must be vibrated down again. The more often it is vibrated, the quicker the pollen will dry, thus minimizing the extraction time. The funnels should be vibrated at least three times daily.
4. Decontaminate funnels after each use by washing thoroughly with hot soapy water. Then wash the funnels in rubbing alcohol or sterilize them in an oven at 176°F (80°C) for 12 hours. Cloth covers must also be laundered or sterilized between lots. Alternate methods of decontamination are given by Snyder and Clausen (1974).

5. Leave catkins in the funnels from 2 to 7 days, depending upon their moisture content when placed in the extractor. Remove pollen from the extractory after it has been dried to the proper moisture level. (For determination of moisture content, see chapter 9.) If pollen is not dry enough for storage, return it to the extractory for a few more hours of drying.

Figure 16.—An electric sander does a good job of vibrating pollen into the bottles. (Photo courtesy of North Carolina State University, Industry Tree Improvement Program)

An extractor between the primitive and sophisticated systems has been constructed by the North Carolina Forest Service (Summerville and Turner 1973). The extractor consists of a 4- by 4- by 8-foot (1.2 by 1.2 by 2.4-m) plywood chamber (fig. 17) equipped with a commercial dehumidifier (Model 4H311, Dayton Electric Manufacturing company, Chicago, Illinois) for controlling the relative humidity. An exhaust fan is located in the ceiling of the chamber and is thermostatically controlled (White Roger 201-8 space thermostat). A 6-inch square (15.2 cm) vent in the lower portion of the door activates when the fan turns on, allowing cool air from the outside to enter and maintain 72°F (22°C). A 75-watt incandescent light bulb and the motor of the dehumidifier provide enough heat to make a heater unnecessary. An individual extraction unit itself consists of a 2.5-inch (6.4 cm) galvanized pipe nipple, a sausage casing or paper bag, a Nalgene plastic funnel, and a 12-gram size vial plus cap (fig. 18). The main disadvantage of this system is that air is not directly blown onto the pollen, so the drying process is slow and supplemental drying may even be necessary.

Figure 17.—A small plywood chamber may be suitable for small lots of pollen. (Photo courtesy of North Carolina Forest Service)

Figure 18.—This extraction unit consists of galvanized pipe, sausage casing, plastic funnel, and a small vial. (Photo courtesy of North Carolina Forest Service)
Supplemental Drying

Pollen extracted in paper bags or in the type of extractory used by the North Carolina Forest Service may not be dry enough and may require supplemental drying. One way to dry pollen is to place it in a desiccator over silica gel. Pollen can also be dried to a moisture content of 11 to 14 percent after 15 minutes over silica gel at a vacuum pressure equivalent to 5 mm of mercury. The 11- to 14-percent moisture content would be safe for refrigerator storage, but the pollen would need to be dried about 30 minutes for freezer storage (5 to 10 percent moisture content). Reducing very high moisture contents is efficient only for small lots of pollen.

Freeze-drying (lyophilization) pollen has given good short-term results. King (1965) successfully freeze-dried pollens of several tree species and sealed them in glass capsules under vacuum or nitrogen. Viability was maintained for up to 3 years at room temperatures. Storage at low temperatures offers more promise. The pollens of jack pine (P. banksiana Lamb.), white spruce (Picea glauca [Moench] Voss), and Norway spruce (P. abies [L.] Karst) were successfully germinated after being freeze-dried and held for 5 years at 4° C (Schoenike and Stewart 1963). Success with Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco) after 2 years of storage has also been reported (Livingston and Ching 1967).

Attempts to deep-freeze pollen in liquid nitrogen (-196° C) have recently proved successful. Two Japanese workers stored 30 angiosperm pollens in liquid N of 5 to 7 years and found many that retained germinability even at moisture contents of 10 to 23 percent. For Cryptomeria, Larix, and several Pinus pollens, the critical moisture content when such deep-freezing is used is about 10 percent, above which pollens will be injured (Ichikawa and Shidei 1971).

Vacuum drying for storage in sealed ampules is described in chapter 8.

Summary and Recommendations

1. Besides the proper timing of catkin collection, the most important factors in successful pollen extraction are temperature and humidity control. A good extractory should control the relative humidity at 20 to 40 percent and the temperature at 70° to 90° F (21° to 27° C).

2. Investment in a forced-air system insures best results. This system allows pollen to be dried to the proper moisture content during extraction, thereby eliminating the need for supplemental drying. Final moisture content should be 11 to 14 percent for refrigerator storage and 5 to 10 percent for freezer storage.

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Snyder, E. B.

Snyder, E. B., and K. E. Clausen.

Sprague, J. R., and V. W. Johnson.

Chapter 8
Pollen Storage

By Fred R. Matthews and John F. Kraus

Despite the intensity of tree improvement and breeding work with the southern pines, surprisingly little has been published on methods of pollen storage. Mergen and others (1955) recommended that slash (Pinus elliottii Engelm.) and longleaf (P. palustris Mill.) pine pollen be stored in desiccator with a drying agent at 39°F (4°C). They suggested a relative humidity over the drying agent of 30 percent, which can be obtained by using a sulfuric acid solution with specific gravity of 1.5. Under these conditions, the moisture content of the pollen is probably about 15 percent. For many years, pollen has been successfully stored in this way at the Naval Stores Tree Improvement Project of the Southeastern Forest Experiment Station.

Successful storage of pollen at an initial moisture content of 12 percent in unsealed vials at about 45°F (7°C) was reported by Snyder (1957). At this moisture content, the amount of pollen stored per vial apparently had no effect on germination. Pollen stored at a higher initial moisture content (51 percent) decreased in germinability, especially when large quantities per vial were stored. A moisture content of about 14 percent at the time of storage was recommended. Subsequently, Snyder (1961) reported successful storage of slash and longleaf pine pollen for up to 32 months and recommended storage at about 45°F (7°C) in sealed or unsealed tubes. The prerequisite for success was the moisture content of the pollen at the time of storage.

Successful freeze-drying of loblolly (P. taeda L.) pine pollen was achieved by King (1959) using a Vir-Tis® freeze-dryer with dry ice and acetone at a temperature of about -76°F (-60°C) and a vacuum of 2 to 10 inches of Hg (50 to 250 mm). Pollen prepared in this manner was successfully shipped to and from New Zealand and Brazil with no apparent loss of viability.

Research on pollen storage by the North Carolina State University-Industry Cooperative Tree Improvement Program has shown that vacuum storage of pollen is better than storage with no vacuum. Initial moisture was the most important factor for successful storage (Sprague and Johnson 1977). The research indicated that, if the moisture content is low (8 to 10 percent), pollen will store well regardless of the method used.

Although eastern white pine (P. strobus L.) is not generally regarded as one of the southern pines, it is used in reforestation of northern or western sections of several Southern States. Duffield and Snow (1941) obtained their best results with pollen stored over sulphuric acid at a relative humidity of 50 percent at 35°F (2°C) for 1 year. Under these conditions, the moisture content of the pollen was probably about 20 percent. Worsley (1959) reported successful storage of eastern white pine pollen at 32°F (0°C) and 10 to 25 percent relative humidity. Ahlgren and Ahlgren (1978) successfully stored white pine pollen for up to 8 years after desiccation over silica gel in a deep freeze for 48 hours followed by evacuation at 0.08 inches of Hg (2 mm) for 20 to 30 minutes before sealing and storing ampules at 41°F (5°C). They reported no reduction in viable seed from control pollinations using 8-year-old pollen.

Longevity of stored pollen increases with decreasing moisture content. High moisture content allows greater metabolic activity and also promotes the destructive activities of fungal and bacterial contaminants. All storage methods are designed to reduce quickly the moisture content of fresh pollen (25 to 55 percent) and to insure a minimum of fluctuation of humidity during the storage period.

Pollen grains are living, breathing (respiring), single-celled organisms. Before going into storage, these organisms should be fully developed, mature, and viable. For long-term storage (from one pollination season to the next or longer), the pollen must be kept in a dormant state with the capability of being brought out of dormancy back to a fully active and viable condition.

**Recommendations**

**Short-Term Storage.** Short-term refers to the day-to-day storage of pollen that will be used during the season in which it is collected ("fresh pollen"). Here is how the pollen can best be stored:

1. Place fresh pollen in glass containers with a volume of up to 3.5 oz (100 ml), but only fill to half capacity. Plug the container with cotton so that it is tight enough to prevent spillage and contamination but loose enough to permit the exchange of gases.
2. Place the containers in a sealed desiccator. Use a drying agent such as lithium chloride or calcium chloride under the containers to prevent fluctuations of relative humidities that reduce pollen viability. (Granular anhydrous lithium chloride or calcium chloride is recommended for desiccators because of easy handling, low cost, and the capacity of maintaining a relative humidity of 0 percent in a closed space.)
3. If pollen is used daily, keep the desiccator in a cool room removed from heaters and sunlight. If pollen will not be used for several days, store the desiccator in a refrigerator or other cold storage facility at about 39°F (4°C).
4. If refrigerated, the desiccator and its contents must be brought back to room temperature before the pollen is removed. This can be done by removing the desiccator from the refrigerator the night before the pollen will be used.

Day-to-day or short-term storage of fresh pollen is not difficult and should present no problem if the causes of damage are avoided. These are high temperatures and high moisture content of the pollen caused either by condensation on cold containers or undue movement between moist and dry atmospheres.

**Long-Term Storage.** Long-term storage should be designed to prevent deterioration of the living pollen by creating...
conditions that will diminish physiological activity without decreasing viability.

At present, there are three commonly used methods of storing southern pine pollen. These methods are based on the vacuum and refrigeration equipment that is available.

Desiccator Storage. The simplest method of long-term storage is essentially the same as that for short-term storage, and the same precautions apply. Pine pollen has successfully been stored for 3 years in this way:

1. Fresh, viable pollen is cleaned and dried to a moisture content of about 9 percent. Moisture content is best determined by means of an Ohaus® Moisture Determination Balance. If this balance is not available, the ovendry method can be used (see chapter 9).
2. Place pollen in 3.5-oz (100 ml) glass containers. Fill to half capacity to prevent the trapping of toxic respiratory gases at the bottom of the container. Loosely plug with cotton, or if using screwcap bottles, leave caps loose.
3. Place containers over lithium chlorides or calcium chlorides in a desiccator.
4. Store the desiccator in a refrigerator or other refrigerated storage facility that will maintain constant temperature of about 39°F (4°C). Avoid refrigerators that cycle and frequently change temperature (self-defrosting) and those that are frequently used for short-term storage of other supplies or food.
5. Remove the desiccator from the refrigerator in time to permit it and its contents to reach room temperature before the pollen is removed. After the pollen is removed, the desiccator should be immediately resealed and returned to cold storage.

Freezer Storage. Pine pollen can successfully be stored in freezers that maintain temperatures of about -4°F (-20°C) (Duffield and Callaham 1959). Successful storage at these temperatures requires proper drying of pollen before freezing. If pollen contains too much moisture, freezing causes formation of intracellular ice crystals that pierce cell walls. For storage at these temperatures:

1. Put the pollen into half-full, cotton-stoppered containers. Place containers inside a desiccator over calcium chloride for at least 1 week at refrigerator temperatures in order to lower the moisture content to about 9 percent.
2. The pollen can either be left in the desiccator for freezing or removed from the desiccator and quickly placed into small, tightly capped vials or bottles and then frozen.
3. To prevent moisture condensation from ruining container-stored pollen when it is removed from the freezer, immediately place containers over calcium chloride in a desiccator for rewar ming.
4. Rewarming in two stages is best. First, place the frozen pollen in a desiccator, and put the desiccator into a refrigerator for 24 hours. After the pollen has reached refrigerator temperature, the desiccator is transferred to normal room temperature for a final warming period of several hours.
5. Repeated freezing and thawing is detrimental to pine pollen and should be avoided. If there is a possibility that a particular pollen lot is to be stored and used for several years, it is advisable to divide it into smaller lots and remove only as much pollen as need in a particular pollination season.

Vacuum Drying and Storage. Vacuum drying and storage of pine pollen in evacuated ampules is probably the best method for long-term storage. Vacuum drying prolongs longevity of the pollen by creating conditions that reduce metabolic activity to a minimum during storage. Moisture content is reduced to a low level. Storage ampules are sealed under vacuum to preclude fluctuation of humidity and gas exchange. Ampule-stored pollen also will better maintain viability during shipment when control of temperature and humidity is not feasible. Necessary equipment is expensive and not readily available, but centralized facilities for handling pollen can be equipped to use vacuum drying as a general method for storage.

1. Place small amounts of pollen in cotton-stoppered containers over calcium chloride in a desiccator at about 39°F (4°C) for at least 1 week for initial moisture content reduction.
2. Transfer up to 0.17 fl oz (5 ml) of a desiccator-dried pollen to 5-ml vacuum-dry ampules (10-ml ampules also may be used) and dry for 2 hours under a vacuum of 0.002 inches of Hg (0.05 mm) with a freeze-dry apparatus. Flame-seal the ampules while they are under vacuum. Pollen undergoing this method of drying usually has a moisture content of about 3 to 4 percent. A freeze-dry system that uses either mechanical refrigeration or a dry ice/solvent method of vapor trapping is essential because of the high vapor content of pine pollen.
3. Properly evacuated and sealed ampules can be stored at about 39°F (4°C) in a refrigerator or at -4°F (-20°C) in a freezer for several years with minimal loss of viability. So far as we know, pollen quantities exceeding 0.34 fl oz (10 mls) have not been vacuum-dried; however, it may be feasible to process much larger quantities with a drying apparatus similar to those used in food industries.
4. All pollen stored with low moisture requires rehydration before testing (see chapter 9).

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Snyder, E. B.

Snyder, E. B.

Sprague, J. R., and V. W. Johnson.

Worsley, R. G. F.
Chapter 9
Pollen Testing
by Ray E. Goddard and Fred R. Matthews

Pollen must be tested for viability before its use if seed production is to be successful. Testing is also useful to check procedures for the extraction and storage of pollen so that its viability is maintained. Procedures for testing pollen, particularly pine pollen, are presented in this chapter.

Germination Tests
Pollen usually germinates well if it is collected at or near the time of normal shedding and is extracted properly and applied the same season. The time between pollen collection and the receptivity of female flowers is frequently so short that the testing of the viability of fresh pollen is rarely needed or done. However, because of natural aging and accidents in processing and storage, the germination capacity of pollen that is stored for 1 or more years is highly variable. All pollen that has been stored should be tested to determine its viability.

Several procedures for the testing of germination have been developed. Pine pollen will germinate in distilled water and on agar with or without additives. The most frequently suggested additives are sucrose, honey, or boron.

Additives. Natural in vivo germination of pollen takes place in a humid atmosphere on rich, vital medium. Many in vitro procedures are attempts to duplicate partly the conditions of natural germination. Stanley (1967) listed principal factors affecting germination:

<table>
<thead>
<tr>
<th>Primary factors</th>
<th>Secondary factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>Growth hormones</td>
</tr>
<tr>
<td>pH</td>
<td>CO₂</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Micronutrients</td>
</tr>
<tr>
<td>Osmotic pressure</td>
<td>Irradiation</td>
</tr>
<tr>
<td>Moisture</td>
<td></td>
</tr>
<tr>
<td>Cations: Ca⁺⁺, K⁺, heavy metals</td>
<td></td>
</tr>
<tr>
<td>Anions: BO₃⁻⁻⁻⁻⁻⁻⁻, PO₄⁻⁻⁻</td>
<td></td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
</tr>
</tbody>
</table>

Of these, carbohydrates in the form of sucrose or honey are most often added to germination media. Pollen of several species of pine has a higher percentage of germination, more rapid germination, and longer pollen tubes in a sucrose medium than in distilled water (Stanley 1967). Sucrose supplies energy to germinating pollen. A sucrose concentration of 10 percent is the average recommendation for use, but some stored pine and spruce pollens give highest germination counts at 20 percent (Snyder and Clausen 1974). Of the cations, hydrogen ion concentration (pH) is the most important, and the optimum pH range is 5.5 to 6.5 for pine pollens. Of the anions, boron trioxide (BO₃) usually has the most pronounced effect. Trace amounts of boron (0.001 to 0.01 percent) often help the germination of hardwood pollens. Other additives are not commonly used in germination tests of pine pollen, but low concentrations of citric acid often improve the germination of hardwood pollens.

Pollen Density. There appears to be a "mutual stimulation effect" of germinating pollen (Kirby and Stanley 1976). When pollen grains are unevenly distributed over the medium, localized groups may have higher germination than widely spread single grains. Because of intertwined pollen tubes, this distribution causes difficulty in the counting of an adequate sample of the grains and in the determination of actual germination in the groups. Care should be taken to disperse pollen grains evenly.

Germination Methods. Pine pollen that is sprinkled on a dry glass slide will germinate if the slide is placed in high humidity at room temperature so that moisture condenses on the slide. Pollen may also be germinated in small vials of distilled water at about 85° F (29° C). Continuous light shaking during germination helps to prevent clumping of grains. Containers and media must be sterilized to reduce bacterial and fungal contaminations. These contaminants reduce germination or make its tallying difficult. Pine pollen is often contaminated with fungi; and, after 72 hours of hyphal development, determination of pollen germination may be difficult. The fungistat mycostatin can be used at low concentration (0.01 percent) without reduction of pollen germination, but this substance adds considerably to the expense of germination testing. Avoidance of sugar or other carbohydrates in the germination medium reduces hyphal growth.

As stated above, many variables affect the germination of pollen. Results from one method of germination testing are often not directly comparable with results of another. Therefore, a standard method is needed. Matthews' procedures (unpublished data) for southern pine pollen give consistently repeatable results and can serve as a standard method:

1. Rehydration of dried pollen is required before its testing or use. Spread a small amount of pollen evenly in a 60-mm plastic petri dish. Place it in a 150-mm dish lined with filter paper that is saturated with water. Cover the large dish and place it in an incubator at 70° F (21° C) for 16 hours, or leave it in the open at room temperature no greater than 78° F (26° C). Under these conditions the moisture content should approximate that of fresh pollen.
2. Put 1.25 g of a dry Difco Bacto Agar in a 500-ml flask with 250 ml of distilled or deionized water (0.5 percent agar). Loosely cap and sterilize it at 15 pounds pressure for 20 minutes. Allow it to cool until it is barely liquid, and pour it into small petri dishes to one-quarter full. Poured agar dishes may be stored 3 to 5 days under refrigeration.
3. With one camel's-hair brush for each pollen lot, place pollen on the agar surface. The brush is dipped into rehydrated pollen, the excess is flicked off, and the remainder is flicked lightly and evenly on the agar. Prepare two dishes for each pollen lot. Place petri dishes in a large covered dish or plastic container to prevent moisture loss, and incubate the pollen at 82° F (28° C) for 48 to 72 hours.
4. For determinations of percentage of germination, place petri plates on a microscope stage at 100X magnification.

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with illumination from below. Pollen grains with tube lengths equal to or exceeding grain diameters are tallied as germinated. Stanley and Linskens (1974) suggest the following sample sizes for 0.05 levels of statistical significance:

<table>
<thead>
<tr>
<th>Percent germination</th>
<th>No. of grains to count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 or 99</td>
<td>15</td>
</tr>
<tr>
<td>5 or 95</td>
<td>73</td>
</tr>
<tr>
<td>10 or 90</td>
<td>138</td>
</tr>
<tr>
<td>15 or 85</td>
<td>196</td>
</tr>
<tr>
<td>25 or 75</td>
<td>288</td>
</tr>
<tr>
<td>30 or 70</td>
<td>323</td>
</tr>
<tr>
<td>40 or 60</td>
<td>369</td>
</tr>
</tbody>
</table>

With two agar plates per lot, a count of 200 grains per plate should satisfactorily rate percentage of germination over the normally encountered ranges.

Other Viability Indicators

As pollen ages, higher amounts of material defuse into an aqueous medium. Dead pollen very rapidly yields water-soluble components. The viability of pine pollen tends to decrease with time, partly because of chemical changes that take place during storage. These changes provide a basis for the determination of the relative viability of pollen without a germination test.

Staining. Color reaction to stains facilitates assessment of pollen viability. Enzymes of pollen that are involved in pollen respiration cause changes in the color of dyes when they are absorbed. The most frequently suggested vital stain is 2,3,5 triphenyltetrazolium chloride. Reduction of this chemical by pollen enzymes produces a red color in viable grains. However, the reaction is influenced by temperature and oxygen, and the intensity of color that is exhibited can be affected by variations of application techniques. Also, other chemical reactions can result in a false color reaction. For these reasons, despite its speed of assessment, the staining technique has not been widely accepted for the testing of pollen viability.

Electrical Conductance. Ching and Ching (1976) described tests of pollen constituents and membrane integrity that provide rapid assays of pollen viability. Working with pollen of Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco), noble fir (Abies procera Rehd.), and western hemlock (Tsuga heterophylla [Raf.] Sarg.), these researchers leached samples in distilled water and tested the leachate for ultraviolet absorption, sugar and amino acid content, and electrical conductance. High values for all of these analyses were closely related to in vitro germination tests of the same pollen lots and indicate loss of substances from the pollen grains. In addition, Foster and Bridgewater (1979) working with loblolly pine (Pinus taeda L.) found that ultraviolet absorbance and conductivity of pollen leachate were both significantly and negatively correlated with in vitro germinability of stored pollen.

Tests of these procedures were conducted with several lots of slash pine (P. elliottii Engelm.) at the University of Florida with comparable results. Because of the simplicity and rapidity of the procedure and its high correlation with germination tests, the electrical conductance test has been extensively used for testing pollen viability in Florida's slash pine breeding program:

1. Place 100 mg of pollen to be tested in 30 ml of distilled water. Agitate for 30 minutes at 86° F (30° C).
2. Filter through standard (No. 1 or No. 2) filter paper and save the filtrate.
3. Determine conductivity of the solution with a conductance meter.

A dozen or more pollen lots can be tested simultaneously. Arrange each lot in a separate small beaker, and place it on a shaker. After agitation, all lots can be quickly filtered and their conductivity tested in a few minutes. In these tests, pollen with a conductivity reading greater than 90 µmho and a cell constant of k = 1.0 usually has corresponding germination of less than 30 percent.

From a number of germination and conductivity tests of pollens over the entire range of viability, a predictive equation can be derived to indicate the percentage of germination of slash pine pollen for particular conductance readings. Pollen deterioration is accompanied by loss of membrane integrity. Because electrical conductance provides a good indication of this deterioration, relative usefulness of pollen can be expressed without conversion to percentage of germination. The primary advantage of electrical conductance and similar viability indicators is that several pollen lots can be tested in 1 to 2 hours in contrast with 2 to 4 days for germination tests. In many instances, time is not available for germination tests when conelets are at or near maximum receptivity.

Determination of Moisture Content

The moisture content of pollen is of critical importance for the maintenance of viability during long periods of storage. Moisture content should be reduced to 9 to 10 percent for storage under refrigeration or frozen conditions. Moisture reduction is normally accomplished by placement of extracted pollen over a desiccant such as calcium chloride or silica gel inside a sealed container. Tests of moisture content indicate when the pollen is adequately but not excessively dried.

In laboratories equipped with a drying oven and a balance accurate to at least 0.01 g, moisture content can be determined by: Drawing a 1-g sample of pollen; weighing the sample immediately; overdrying the sample; reweighing it; and using the weight loss and oven dry weight to calculate percentage of moisture. A reasonably priced piece of equipment, the Ohaus® Moisture Determination Balance, accomplishes these functions in a single operation. A pollen sample, 1.0 ml in volume, is placed on a disposable aluminum pan. With the heat lamp set at 4.5 watts for the preset time of 7 minutes, the percentage of pollen moisture can be read directly from the optical scale at the end of the drying time. Users emphasize the need to calibrate this equipment with standard weights when it comes from the manufacturer. A similar unit can be purchased to convert a Mettler top-loading balance for moisture determination.
Because heating destroys the viability of a pollen sample, it is most useful for determining the moisture of large pollen lots. If only a few grams of pollen from an individual tree are collected, one is normally reluctant to destroy the sample through such testing.

Procedures are available that regulate the moisture content of pollen without the need of a direct reading of each lot. This regulation is accomplished by establishing atmospheres of known relative humidity in a closed space. In a container such as a desiccator, the desired humidity depends upon the equilibrium vapor pressure of water with a chemical dissolved in it. The relative humidity, for example, of air above an aqueous solution of sulfuric acid at 68-percent concentration is about 8 percent. Pollen in a desiccator above this concentration of sulfuric acid will come to equilibrium at 8 percent moisture content. Sulfuric acid, however, is somewhat volatile and emits SO₂ and SO₃, which may adversely affect pollen germination. Similar moisture stabilization in pollen can be achieved with desiccants less hazardous to handle than sulfuric acid. A 96-percent concentration by weight of glycerine in a desiccator will develop an atmospheric relative humidity of 8 percent at equilibrium.

It is recommended for pollen drying that the desiccator containing vials of pollen and the desiccant be refrigerated for several days while coming to equilibrium. A pollen lot of ample size should be sampled to determine the moisture content as described above. This sampling provides assurance that the desiccant concentration is correct and that equilibrium has been achieved.

Jensen (1970) has described a manometric method of moisture determination that allows nondestructive determination of moisture in small quantities of pollen. Accuracy is satisfactory, and the pollen can be used for pollination or further studies. The apparatus measures absolute moisture content of a sample that is exposed to high vacuum. Water vapor that is liberated is condensed as ice. The specimen is isolated from the system, and the ice is allowed to evaporate, causing a pressure rise in a system of known volume. The resultant rise of pressure is proportional to the mass of water that is liberated, and it is measured by an oil manometer.

Pollen Viability and Seed Yield

In vitro germination tests and other indicators of relative viability of pollen do not precisely predict the yield of sound seed that will result from the use of a pollen lot. A few pollen lots will germinate in vitro but fail to yield sound seed; conversely, some pollen with apparently low germination gives satisfactory seed set. The situation is further complicated, particularly with pines, by the long period between pollination and seed maturity. During the approximately 1.5 years required for seed development in pines, many accidents, particularly insect attacks and conelet abortion, can occur to confound the relationship between pollen viability and seed yield.

Studies of pine pollen stored for up to 15 years indicate that normal or near-normal seed yield can result (Stanley 1962). Callaham (1965) reported that dilution of pollen with dead pollen caused no reduction in the yield of sound seed except with dilutions having less than 30 percent viable pollen. Kraus and Hunt (1970) compared stored pollen of at least 60 percent germination with fresh pollen and reported no consistent superiority in seed yield from fresh pollen in 8 years of observation. In some of their individual comparisons, however, average seed per cone was less with stored pollen. Feret and Stairs (1970), studying storage procedures for white spruce (Picea glauca [Moench] Voss) pollen, found that some storage conditions significantly reduced in vitro germination but did not change seed set and seed germination.

These results suggest that precise determinations of pollen viability provide only rough indications of seed-setting ability. Viability tests will show whether or not extraction and storage procedures are resulting in the delivery of living pollen and suggest minimum viability levels for its use.

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Chapter 10
Controlled Pollination
by David L. Bramlett and Claude H. O'Gwynn

Controlled pollination of forest trees as needed to produce families of known parentage for progeny test selections and populations for advanced-generation breeding. It began more than 40 years ago when Schreiner (1937) initiated a breeding program to improve future tree planting stock. Cumming and Righter (1948) described procedures for pollinating pines in California; and, simultaneously, controlled pollinations were being done by tree breeders and geneticists in the South. Several references chronicle the state of the art of controlled pollinations of pines and other conifers (Cumming and Righter 1948, Wakeley and Campbell 1954, Goddard and Allen 1955, Mergen and others 1955, Nienstaedt and Kriebel 1955, and Orr-Ewing 1956). Many aspects of this past research are still applicable to current pine breeding programs, and readers who want historical accounts of controlled pollinations in southern pines should consult these references.

In the last 2 decades, the rapid development and expansion of southern pine forest tree improvement programs have resulted in thousands of controlled pollinations that are completed each year by State, Federal, and industrial programs. This chapter presents some of the different pollination procedures that are available, reports yields from published data, and shows ways to maximize seed yields from controlled pollinations of southern pines.

Methods of Controlled Pollination
Selection of Sample Branches. At the beginning of southern pine breeding programs, the selection of large trees in forest stands was based on phenotypic criteria. Scions of these original selections were grafted into clone banks and production orchards. But, because it took several years before grafted ramets begin conelet production, the first pollinations were completed by climbing the ornets. These pollinations were completed on conelets that could be reached from the interior of the crown by bending branches upward toward the trunk (Perry 1954). Once conelet production began on grafted material, access to receptive conelets was greatly increased. Young grafts were bagged from stepladders or trunk-mounted platforms. Now, larger trees are bagged from hydraulic bucket trucks. With these trucks the entire crown is accessible, and usually the best branches are selected for pollination.

The primary branch tips of strongly vigorous shoots in the upper crown are preferred because these primary shoots have a large stem diameter, and they can withstand the wind resistance that is increased by the isolation bag. In general, branches with isolation bags that stand as close to the vertical as possible have the greatest probability of survival. If possible, avoid any branches with a small stem diameter, branches at right angles to larger branches, or branches where the tip approaches a parallel with the ground. If conelets must be used wherever they occur, use special supports for bags that are placed on the weaker branches.

Developmental Stages of the Conelet. Conelet primordia, which are enclosed by protective bud scales, develop slowly during the winter months. As the conelet develops, it emerges from the bud scales to the stage where it can effectively trap windborne pollen. Stages of conelet development have been described by several researchers and tree breeders. For comparison, two systems of classification are listed in table 10.

This chapter uses the development stages of conelets that are described by Cumming and Righter (1948) and illustrated in figure 19. In stage 1, the bud is relatively small and still tightly enclosed within the bud scales. In stage 2, the buds are enlarged, but the conelet primordia are still enclosed within the scales. Light-colored scales are noticeable at the tip of the bud. To prevent contamination by outside pollen sources, place isolation bags on branches with buds in stage 2. In stage 3, the conelets begin to emerge through the tip of the scales. Tests have shown that the pollen reaching the conelet in stage 3 usually is not effective. However, to avoid seed formation from unknown parentage, bag conelets before stage 3 begins.

By stage 4, the conelet elongates and extends beyond the bud scales. The lower one-third to one-half of the conelet is enclosed by the bud scales. Stage 4 is too early to apply pollen, but conelets that receive pollen at stage 4 will produce some seeds.

At stage 5, the conelet has completely emerged from the bud scales. The conelet scales approach a right angle with the axis of the conelet. At this stage, the conelet has maximum receptivity, and it is here that the opening between the scales and bracts offers the greatest access to pollen. Recognition of stage 5 is the key to successful pollinations. This stage may last from 1 day to 1 week depending upon the weather and air temperature. Individual clones in the seed orchard may have receptive flowers that are different in appearance from the classic description of a conelet with the scales at right angles. Also, not all the conelets within the same bag are at stage 5 at the same time. Many breeders prefer to pollinate a bag several times, but excellent results can also be obtained by pollinating at only the one time that coincides with the greatest number of flowers in stage 5. It is not necessarily true that it is better to be too early than too late. Flowers in late stage 5 may appear almost closed; yet, if space is adequate for pollen penetration, successful pollinations can be completed. Some reports indicate that in stage 5, the flowers will remain longer inside a bag if the pollen is not applied (Snow and others 1943).

In stage 6, the openings between the conelet scales begin to close as a result of growth and thickening of the scales. Therefore, receptivity is over because the pollen cannot enter the spaces between the scales, and controlled pollinations will not yield any seed in this stage.

Selection and Preparation of Flowering Branches.
Installation of the pollination bag resembles grafting because each worker has the same general objective but uses
Table 10.—Stages of pine conelet development

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>System of Snow and others (1943)</th>
<th>System of Cumming and Righter (1948)</th>
<th>Description</th>
<th>Tree breeding significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>Small flower buds are covered by tight bud scales</td>
<td>Identify potential branches to pollinate</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>Large flower buds; flower is still covered by bud scales. Top of female bud is light colored</td>
<td>Place pollination bags on branches</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>Flower is emerging from bud scales; rudimentary cone scales are observable</td>
<td>Avoid bagging flowers; contamination by unwanted pollen may have occurred</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>Flower extends beyond the bud scales; cone scales are small</td>
<td>Too late to install bags; some flowers can be successfully pollinated, but seed yields will be reduced</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>Maximum flower receptivity; large opening between the cone scales; cone scales form right angles with cone axis</td>
<td>Pollinate flowers until stage 6 is reached</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>Spaces between cone scales are closed by the enlargement and growth of the scales</td>
<td>Too late to pollinate; remove pollination bags</td>
<td></td>
</tr>
</tbody>
</table>
Figure 19.—Developmental stages of conelets: A, Stage 1, small flower buds; B, Stage 2, large flower buds; C, Stage 3, flower begins to emerge through the tip of the bud scales; D, Stage 4, flower extends beyond the bud scales; E, Late stage 4, the flower is most of the way out of the bud scales but is not yet fully receptive; F, Stage 5, flower is fully extended from bud scales, and scales are at stage 5; G, Later stage 5, the opening between the scales and the bracts is closing; H, Stage 6, the opening between the scales is closed by the scale enlargement.
somewhat different procedures or materials. Here is a typical procedure that is used in most tree improvement programs:

1. Select the location where the bag will be attached to the branch. This location may vary for different species or even different branches within the same tree. If possible, choose a natural "node" on the branch where a portion of the stem is without needles. If no natural zone exists, strip off a zone of needles about 6 to 8 inches (15 to 20 cm) long. Do not remove all the needles on the branch because they are needed to protect the flowers and to provide carbohydrates for the branch.

2. Hold the remaining needles that extend past the tip of the bud. Trimming is more important for slash pines (*Pinus elliottii* Engelm.) than for other pines because needles hide the developing bud and flower. With loblolly pine (*P. taeda* L.), however, the tips frequently do not have to be trimmed because the elongated shoot will have flowers beyond the needle tips at the time of pollination.

3. Support the bags to prevent their collapsing and damaging the enclosed shoots and conelets. Floral canes 24 to 48 inches (70 to 120 cm) long are frequently used as supports. Or you can bend aluminum wire into various shapes for bag support and conelet protection. One or two circular loops that are slightly smaller than the bag diameter are effective. Adjust or bend the supporting wire as required to insure that the loops provide a protective frame around the centered buds. Regardless of the type of branch, it is important to attach the support securely to the conelet-bearing branch in at least two places. One location should be near the lower end of the support, and the other at the place where the bag is secured.

4. Wrap nonabsorbent cotton or polyester fiberfill padding around both the support and the conelet-bearing branch. The padding can be precut into strips about 2 to 4 inches (5 to 10 cm) long to reduce field application time.

**Installation of Pollination Bags.** Pollination bags made of plastics, paper, and cloth have been evaluated for use in the isolation of conelets for the controlled breeding of pines. Each kind has some advantages and disadvantages, which are listed in table 11. Viscose sausage casing bags, the most frequently used in the breeding of southern pines, are made into extruded flattened cylinders of tough and transparent cellulose film available in various lengths and widths. For loblolly pine, tubing of this kind with a flattened width of about 5-3/4 inches (14.6 cm) is cut to lengths of 24 to 30 inches (60 to 75 cm). Pollination bags for longleaf pine (*P. palustris* Mill.) and slash pine are sometimes wider than those for loblolly pine, but the length can be reduced. Shortleaf (*P. echinata* Mill.) and Virginia pine (*P. virginiana* Mill.) can accommodate bags with a flattened width of 3-3/8 inches (8.5 cm) and a length of 15 inches (38 cm). One end of each tubing section is closed securely to prevent pollen entrance or escape. This closure is made by machine-installed metal clamps, and this service is commercially available. Closures can also be made satisfactorily by folding and stapling one end of the tubing (Mergen and others 1955).

<table>
<thead>
<tr>
<th>Bag types</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulosic sausage casing</td>
<td>1. Transparent</td>
<td>1. Relatively large increase and decrease in temperature¹</td>
</tr>
<tr>
<td></td>
<td>2. Inexpensive</td>
<td>2. Stiff when dry</td>
</tr>
<tr>
<td></td>
<td>3. Injury resistant</td>
<td>3. Often collapses when wet unless it is supported</td>
</tr>
<tr>
<td>Paper with plastic panel</td>
<td>1. Inexpensive</td>
<td>1. Slightly more susceptible to injury</td>
</tr>
<tr>
<td></td>
<td>2. Relatively small increase in temperature²</td>
<td>2. Conelets can be viewed only through one side (plastic panel)</td>
</tr>
<tr>
<td>Cloth (Placerville)</td>
<td>1. Injury resistant</td>
<td>1. Expensive</td>
</tr>
<tr>
<td></td>
<td>2. Relatively small increase or decrease in temperature³</td>
<td>2. Conelets are visible only through the window</td>
</tr>
<tr>
<td></td>
<td>3. Reusable</td>
<td>3. Heavy, especially when wet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Requires support</td>
</tr>
</tbody>
</table>

¹ McWilliam (1959a) found that the temperature of conelets enclosed in unshaded sausage casing bags increased as much as 30°F (16.5°C) above ambient shade temperatures. Temperatures of 115°F (46°C) were lethal to germinating pollen.

² In a 1978 comparison, peak air temperatures were: ambient 95°F (35°C); inside sausage casing bags, 121°F (49°C); inside bleached paper bags, 106°F (41°C). Data on file, Weyerhaeuser Company, Hot Springs, Ark.

Isolation bags made of paper and transparent plastic film are used in England, Canada, and the western region of the United States. Bags of this kind have also shown promise in some trials in the southern pine breeding region. Those used on loblolly pine are made of bleached porous paper with a clear plastic window that extends the full length of the bag on one side. The bag is 20 inches (50.8 cm) long and 6 inches (15.2 cm) wide when it is folded flat, and it has a 5-inch (12.7 cm) gusset when it is opened; the window width is 3-1/2 inches (8.9 cm). The glue used in the manufacture of these bags is effectively water proofed, and the only failures have occurred when the bags were placed in positions that allowed wind damage by other branches. There is danger of bag puncture, however, if cane or other sharp-pointed stakes are used for internal support. For paper bags, looped-wire supports without protruding ends are recommended for the prevention of damage.
Pollination bags that are made of closely woven cotton fabric are described by Cumming and Righter (1948). These bags are used in the breeding of western pine species, but they are not used to any extent in the breeding of southern pines.

To install pollination bags, stretch them open before placing them over the conelet-bearing branch tip and support. Once the bag is placed over the branch tip, push the cane or support wire to the top of the bag. Leave space for the shoot to grow toward the top of the bag without excessive crowding. The decision about the amount of space to be left is a judgment that comes with experience; but, generally, slash pine needs only 6 to 10 inches (15 to 25 cm), while loblolly pine requires 12 to 18 inches (30 to 45 cm) on vigorous shoots in the upper crown of young grafts.

Once the bag is placed over the branch tip, position the support for maximum protection to the conelets. Extend the canes to the top of the bag, and bend wires to prevent conelets from rubbing the bag. For the final step in the installation, secure the bag and support to the base of the stem. Gather the bag around the padding-wrapped stem, and support and secure it tightly with string or some other binding material. Recently, several organizations have used plastic ties that have notches cut in a narrow plastic strip that tightens a loop when pulled. If necessary, secure the support to one or more locations below the point of the bag attachment.

**Pollen Application.** Pollen may be applied to the isolated female flower by several devices that introduce pollen into the bag and onto the female strobilus. Pollen that is dispersed into the bag lands on the bracts or cone scales and slides to the central axis. The pollen is absorbed by a pollen droplet that is produced by the ovule during periods of high relative humidity (Doyle and O’Leary 1935). The pollen is carried by the pollen droplet into the pollen chamber and is sealed off by the expansion of the micropyle arms (McWilliam 1959b). Tree breeders must accurately determine the flowering phenology for each clone to insure that pollinations are completed at the maximum receptivity of the conelet. Conelet receptivity may vary slightly within a clone, and often there is considerable variation within a tree crown. The duration of conelet stages may also depend on the temperature, and the span of time can vary considerably from year to year. Tree breeders rarely can pollinate all of the bags on one tree at the same time but must visit a tree several times before every one is pollinated. From experience, tree breeders recognize the conelet stages, and they can quickly evaluate the receptiveness of flowers without removing the bag. Some tree breeders prefer the early morning for controlled pollinations before pollen flight begins. Others prefer to let the bags thoroughly dry in order to prevent the introduced pollen from adhering to the wet bags or from being released from surrounding trees before the bagged conelets are pollinated. A general guide is that controlled pollinations should begin when pollen is released from nearby unbagged pollen catkins.

There is a standard procedure for pollination in bags. First, hold the bag in an upright position and dispense the pollen with some type of air-delivery system. Then, inject the pollen toward the top of the bag and allow it to settle by gravity toward the flower. Shake the bag during this process and then “thump” it to dislodge pollen that adheres to the sides of the bag.

If it is necessary to pollinate in bags during a prolonged rainy period of several days, it may be necessary to inject the pollen directly onto the flower rather than disperse the pollen into the wet or damp bag.

**Pollinators.** Numerous devices for controlled pollinations have been used over the past 30 years. Most tree breeders use some form of air-delivery system to dispense pollen from a reservoir into the bag and subsequently to the conelet. The basic devices are described below.

**Syringe With Rubber Bulb.** The syringe and rubber bulb is perhaps the most widely used pollinator in the South, and although different components may be used in different orchards, the basic system is essentially the same (Mergen and others 1955). The major components are a syringe with a capacity of 10 cm³ or larger and a No. 15 to No. 18 veterinary needle. A rubber bulb with an air-intake valve is attached to the base with a rubber stopper and a glass tube. When the bulb is compressed, air is forced through the syringe, and pollen is discharged through the needle. Only the needle is inserted into the bag, and pollen is dispersed toward the top of the bag.

**Ear Syringe.** The ear syringe is a modification of the bulb and syringe pollinator. A small ear syringe is cut so that a veterinary needle fits directly into the bulb. Pollen is supplied to the bulb and expelled into the pollination bag. The advantages of this system are its simplicity and ease of cleaning. Its disadvantages are the relatively greater difficulty of loading pollen and the problems associated with the orientation of the bulb pollinator. Also, the quantities of pollen discharged into the bag may vary considerably depending on the amount of pollen in the bulb (Wakeley and Campbell 1954).

**Cyclone Pollinator.** This pollinator was developed by the Forest Service at Athens, Georgia. A rust-spore collector that was used to collect wheat-rust spores was modified into a pollinator by reversing the air flow. This pollinator expels air from a small vial through a sharpened port that extends into the pollination bag. The air is supplied by a relatively large rubber bulb that delivers a large volume of air into the pollination bag. The large volume of air results in a swirling cloud of pollen throughout the bag. The swirling pollen greatly enhances pollen distribution. In a recent experiment, the cyclone pollinator resulted in significantly greater seed yields than did the syringe pollinator (Matthews and Bramlett, unpublished data).

**Wash Bottle.** Small polyethylene wash bottles of about 30 ml capacity are used in some breeding programs. Pollen is placed in the bottle and expelled through the nozzle when the bottle is squeezed. The major advantages of this system are its simplicity (because no modifications are required) and its low.
cost. Its disadvantages are the necessity of puncturing the pollination bag with a relatively large hole and the varying quantities of pollen that are discharged into the bag, depending on the amount of pollen in the bottle.

**Camel's-Hair Brush.** A frequent problem of controlled pollination is that only small quantities of pollen may be available. These small lots of pollen may be effectively used by applying pollen to the flowers with a camel's-hair brush. The brush is dipped into the pollen container, and the pollen is "flicked" or lightly brushed onto the flower. The difficulty of using the brush is that a large hole must be cut into the bag, or the bag must be removed. Either practice increases the probability of pollen contamination. Another disadvantage is that, if many flowers are present in each bag, each flower must be individually pollinated, whereas air-delivery systems will pollinate the whole bag with one application. However, in one test with Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco), the percentage of filled seed was significantly greater when pollen was applied by brush than when it was applied by syringe (Carlson and Hsin 1976).

**Bag Removal.** Remove bags as soon as all the conelets in the bag have reached stage 6, and the openings have completely closed between the scales. Closure usually occurs 7 to 10 days after stage 5 begins, so bags are usually removed 2 weeks after pollination. Cloth and paper bags are easily removed, but bags of sausage casing must be cut loose at the base and carefully removed to avoid damage to the succulent conelets. Only the tag identification should be left. All cones beyond the tag should come from the same pollination bag. The lower portion of the bag is sometimes left to facilitate location of the pollinated branches, or else flagging may be used to identify pollinated branches for cone harvest.

**Development and Yield of Pollinated Cones.**

The successful completion of the pollination of receptive flowers does not necessarily guarantee the success of good seed yields. The cones must develop for the next 18 months and reach maturity without damage from either biotic or abiotic causes before the pollinations are successfully completed.

**Protection.** Losses of cones or seeds before maturity can seriously impede the production of seeds from controlled pollinations. Because controlled pollinations are expensive, take extra precautions to prevent insect and fungal losses. Most losses are caused by insects. Therefore, primarily direct preventative measures at the most serious insect pest, such as *Diorctria* (DeBarr and others 1975, Bramlett and others 1977). Some losses are also attributable to injury caused by severe weather.

**Seed Yields From Controlled Pollinations.** Despite the many pollinations that are completed each year, published reports of actual yields from controlled pollinations are sparse. Snyder and Squillace (1966) summarized their results from several years of controlled pollinations of southern pines (table 12), and reports by Kraus and Hunt (1970), Franklin (1969), and Bramlett (1977) also give data for seed yields.

Most organizations have an estimated value for seed yields, but they more often describe seed yields in terms such as "low," "good," "failing," or "poor." In addition, most reports of seed yields are from controlled pollinations that were completed before intensive insect protection was available for seed orchard use. Therefore, a large portion of seed losses from early results may reflect insect damage to developing ovules and seeds as well as variations in techniques or pollen supplies.

When DeBarr and others (1975) compared seed losses from wind and controlled pollinations with and without screen cages, seed yields were not as dramatically improved with insect protection in the controlled pollinations as in wind pollinations. These results indicated that wind pollination was effective in the slash pine seed orchard but that insects were reducing the seed yield. The controlled pollinations, however, were not effective, and the increased insect protection did not substantially raise the seed yields. These results indicated that inadequate pollen was supplied to the pine ovules. Bramlett (1977) tested this hypothesis by applying large quantities of pollen to individual bags to evaluate how quantity of pollen affected seed yield. Results with both slash and loblolly pine indicated that larger quantities of pollen greatly increased seed per cone up to an average of 100 filled seeds per cone for both species. These cones were protected for 2 years of development with screen-wire cages. Cone survival was not reduced by the heavy pollen applications, and both cone length and width increased with larger quantities of pollen.

**Recommendations.**

High seed yields per pollinated flower may be produced by following these basic guidelines. Select strong conelet-bearing branches in the upper crown that are growing in a relatively upright position. Install pollination bags when conelets are at stage 2, and support the bag with a cane or wire. Periodically check bags for conelet receptivity, and apply about 0.5 cm$^2$ of pollen when the majority of conelets are maximally receptive at stage 5. Carefully remove the bag 2 weeks after pollination, and protect conelets, cones, and seed until maturity.

These precautions should be taken to insure seed yields and reduce losses:

- Do not bag too early because the bag will speed flower development, and flower receptivity will occur before fresh pollen is available.
- Avoid bagging weak branches.
- Use pollen of high viability, and use fresh pollen instead of stored pollen if it is available.
- Use adequate quantities of pollen.
- Try to achieve good distribution of pollen within the bag.
- Do not pollinate too early. Late stage 5 is preferred for pollination instead of stage 4 because the cone scales have not completely emerged from the bud scales at stage 4.
- Do not remove the bag too early. Be sure that scales are closed on all conelets before the bag is removed.
- Check tags for proper identification and location.

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**CameFs-Hair Brush.** A frequent problem of controlled pollination is that only small quantities of pollen may be available. These small lots of pollen may be effectively used by applying pollen to the flowers with a camel's-hair brush. The brush is dipped into the pollen container, and the pollen is "flicked" or lightly brushed onto the flower. The difficulty of using the brush is that a large hole must be cut into the bag, or the bag must be removed. Either practice increases the probability of pollen contamination. Another disadvantage is that, if many flowers are present in each bag, each flower must be individually pollinated, whereas air-delivery systems will pollinate the whole bag with one application. However, in one test with Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco), the percentage of filled seed was significantly greater when pollen was applied by brush than when it was applied by syringe (Carlson and Hsin 1976).

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**Seed Yields From Controlled Pollinations.** Despite the many pollinations that are completed each year, published reports of actual yields from controlled pollinations are sparse. Snyder and Squillace (1966) summarized their results from several years of controlled pollinations of southern pines (table 12), and reports by Kraus and Hunt (1970), Franklin (1969), and Bramlett (1977) also give data for seed yields.

Most organizations have an estimated value for seed yields, but they more often describe seed yields in terms such as "low," "good," "failing," or "poor." In addition, most reports of seed yields are from controlled pollinations that were completed before intensive insect protection was available for seed orchard use. Therefore, a large portion of seed losses from early results may reflect insect damage to developing ovules and seeds as well as variations in techniques or pollen supplies.

When DeBarr and others (1975) compared seed losses from wind and controlled pollinations with and without screen cages, seed yields were not as dramatically improved with insect protection in the controlled pollinations as in wind pollinations. These results indicated that wind pollination was effective in the slash pine seed orchard but that insects were reducing the seed yield. The controlled pollinations, however, were not effective, and the increased insect protection did not substantially raise the seed yields. These results indicated that inadequate pollen was supplied to the pine ovules. Bramlett (1977) tested this hypothesis by applying large quantities of pollen to individual bags to evaluate how quantity of pollen affected seed yield. Results with both slash and loblolly pine indicated that larger quantities of pollen greatly increased seed per cone up to an average of 100 filled seeds per cone for both species. These cones were protected for 2 years of development with screen-wire cages. Cone survival was not reduced by the heavy pollen applications, and both cone length and width increased with larger quantities of pollen.

**Recommendations.**

High seed yields per pollinated flower may be produced by following these basic guidelines. Select strong conelet-bearing branches in the upper crown that are growing in a relatively upright position. Install pollination bags when conelets are at stage 2, and support the bag with a cane or wire. Periodically check bags for conelet receptivity, and apply about 0.5 cm$^2$ of pollen when the majority of conelets are maximally receptive at stage 5. Carefully remove the bag 2 weeks after pollination, and protect conelets, cones, and seed until maturity.

These precautions should be taken to insure seed yields and reduce losses:

- Do not bag too early because the bag will speed flower development, and flower receptivity will occur before fresh pollen is available.
- Avoid bagging weak branches.
- Use pollen of high viability, and use fresh pollen instead of stored pollen if it is available.
- Use adequate quantities of pollen.
- Try to achieve good distribution of pollen within the bag.
- Do not pollinate too early. Late stage 5 is preferred for pollination instead of stage 4 because the cone scales have not completely emerged from the bud scales at stage 4.
- Do not remove the bag too early. Be sure that scales are closed on all conelets before the bag is removed.
- Check tags for proper identification and location.
Table 12.—Seed yields from controlled pollination of southern pines

<table>
<thead>
<tr>
<th>Pine species</th>
<th>Seeds per cone</th>
<th>Pollination description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slash</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>Breeding program</td>
<td>Snyder and Squillace (1966)</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>Outcrosses and polycross</td>
<td>Squillace and Krauss (1963)</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>Polycross</td>
<td>Kraus and Squillace (1964)</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>Polycross, fresh pollen</td>
<td>Kraus and Hunt (1970)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>Polycross, stored pollen</td>
<td>do.</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Polycross without cage</td>
<td>DeBarr and others (1975)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Polycross with cage</td>
<td>do.</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Flowers at stage 4</td>
<td>Campbell and Wakeley (1961)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Flowers at stage 5</td>
<td>do.</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>Polycross (0.12 cm³ pollen/bag)</td>
<td>Bramlett (1977)</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>Polycross (0.25 cm³ pollen/bag)</td>
<td>do.</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>Polycross (0.50 cm³ pollen/bag)</td>
<td>do.</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>Polycross (1.00 cm³ pollen/bag)</td>
<td>do.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>Polycross (2.00 cm³ pollen/bag)</td>
<td>do.</td>
</tr>
<tr>
<td>Loblolly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Breeding program</td>
<td>Snyder and Squillace (1966)</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>Polycross, fresh pollen</td>
<td>Kraus and Hunt (1970)</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>Polycross, stored pollen</td>
<td>do.</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>Inbreeding study</td>
<td>Franklin (1969)</td>
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<td></td>
<td>42</td>
<td>Polycross (0.12 cm³ pollen/bag)</td>
<td>Bramlett</td>
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<td>64</td>
<td>Polycross (0.25 cm³ pollen/bag)</td>
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<td>73</td>
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<td>Polycross (2.00 cm³ pollen/bag)</td>
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<td>Longleaf</td>
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<td></td>
<td>42</td>
<td>Polycross</td>
<td>Campbell and Wakeley (1961)</td>
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<tr>
<td></td>
<td>58</td>
<td>Flowers at stage 4</td>
<td>do.</td>
</tr>
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<td>Shortleaf</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>19</td>
<td>Breeding program</td>
<td>Snyder and Squillace (1966)</td>
</tr>
<tr>
<td>Virginia</td>
<td>69</td>
<td>Diallel cross</td>
<td>Bramlett and Pepper (1974)</td>
</tr>
</tbody>
</table>

*Data on file at Macon, Ga.*

- Allow cones to mature on the tree, and do not collect them before the specific gravity of the cones is 0.89.

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Supplemental mass pollination (SMP) is the broadcast application of pollen to conelets that are not isolated from airborne pollen. Potential benefits from SMP are the increases in yields of sound seed and the realized genetic gains from conventional, intraspecific forest tree improvement. SMP may also be required to produce large numbers of hybrid seeds economically.

Uses of SMP

Improving Yields of Sound Seeds. The yields of sound seeds may be improved by SMP when not enough viable, incident pollen is present to guarantee the pollination of receptive conelets. In southern pines, an ovule that is not pollinated aborts early in its first year of development (Bramlett and others 1977). If not enough ovules are pollinated, conelets will abort (Sarvas 1962), and cone production will be reduced (Boyer 1974).

Pollens may be limited for a variety of reasons. The production of pollen in small lobolly pine (Pinus taeda L.) orchards characteristically lags behind conelet production for several years. Also, rain during the shedding of pollen may reduce airborne pollen density and pollination (Dorman 1976). Freezing weather during meiosis prior to the shedding of pollen may render pollen impotent (Dillon and Zobel 1957). Genetic or physiological mechanisms, such as self-fertilization, reduce filled-seed percentages and germinability (Franklin 1970).

Another serious obstacle to adequate pollination is a lack of synchronization between pollen shedding and the receptivity of female conelets. Adequate pollination depends upon high pollen density in the immediate vicinity of receptive female conelets. The initiation and duration of pollen shedding and female receptivity may be markedly and even differentially affected by weather conditions (Wasser 1967). Cool, wet, spring weather retards the development of the conelets of young slash pine (P. elliottii Engelm.) but not that of cattines (Bengston 1965).

Improved yields of sound seeds will result from supplementing an inadequate supply of pollen or preventing self-pollination by the delivery of enough viable, outcross pollen to conelets when they are receptive.

Increasing Genetic Gains by SMP. The realization of expected genetic gains from seed orchards depends upon panmixia among orchard clones. Panmixia does not happen when: pollination occurs by unselected trees outside the orchard; self-fertilization or related mating is significant; or orchard trees do not contribute equally (pollen and seeds) to the seed crop. Because isolation zones around seed orchards do not guarantee insignificant pollination from without, and self-fertilization in seed orchards may be significant, potential genetic gains may not be achieved. Other impediments to realized genetic gains include: Differential impact of season climatic changes upon plant development (floral phenology) and selective fertilization.

SMP offers solutions to these problems of seed quality as well as the opportunity to improve expected genetic gains by using a pollen mix of the best parents (Franklin 1971, Woessner and Franklin 1973).

Successful application of SMP to seed orchards can be accomplished by methods that exclude incident pollen, such as emasculation, isolation, and the use of gametocides, or the proper timing of applications. The isolation of receptive females is a familiar technique for the production of controlled matings in genetic testing. The difficulty and expense of bagging, pollinating, and unbagging conelets are also well known to orchardists. These procedures have precluded the use of controlled pollinations to produce large numbers of propagules, except in certain cases of interspecific hybridization. The emasculation of less desirable pollen parents in seed orchards likewise seems economically impractical, and tests of gametocides have not yet resulted in usable techniques (Dorman 1976). If any methods are developed that render less desirable trees in seed orchards ineffective as pollen parents, orchardists must still contend with levels of background pollen that may be sufficient for complete pollination (Squillace 1967).

To increase genetic gain, SMP must use methods that increase the probability by selected parents.

Production of Interspecific Hybrids. Interspecific hybridization of forest trees is not a widely used breeding strategy. There are several reasons for the lack of interest in this method, but the basic reason is that tree breeders have determined that the same goals can often be accomplished more economically by intraspecific improvement (Fowler 1977). The most promising use of interspecific hybridization is that of combining favorable traits of one species with those of another (Brown 1972).

One deterrent to the use of hybridization for tree improvement is the lack of reliable methods for producing many hybrid seeds economically. The production of hybrid seeds is often difficult, because the crossing of species with widely separated ranges may result not only in inferior growing stock but also in reduced seed set (Fowler 1977). When species ranges overlap, as with the southern pines, seed yields from intermatings are reduced because of isolating mechanisms that have evolved and that keep the species distinct. These may be species incompatibility mechanisms that inhibit fertilization or differences in flowering phenology that limit pollination (Dorman 1976). In the latter case, SMP may be useful for producing hybrids.

To accomplish any of the above objectives, SMP depends upon successful pollination by selected pollen in ovules that are receptive at different times. If supplemental pollen is delivered to ovules before pollination by incident pollen, the probability is increased that selected pollen will fertilize the ovules (Franklin 1974).
Results of SMP Trials

SMP has been used to increase filled-seed yields and the genetic gains of several species of conifers, but the genetic gains resulting from it have not been demonstrated.

Hand SMP of 9- and 10-year-old grafts of loblolly and slash pines in South Africa, after they were pruned and staked down to form low, flat-surfaced crowns, increased loblolly filled-seed yields from 54 to 62.6 seeds per cone (a 16-percent increase) and slash pine yields from 15.6 to 60.1 filled seeds per cone (a 285-percent increase) over comparable control ramets (van der Sijde 1971). SMP was discontinued when the increases in filled-seed yields were offset by the losses from the pruning of female buds to maintain low crowns.

SMP of grafted Scots pine (P. sylvestris, L.) increased the average percentage of conelets that developed into 1-year-old cones from 59 for wind-pollination to 72; and it increased filled seeds per cone from 17.5 to 20.5. A 1:1 mix of stored pollen and talc was blown from the ground onto receptive conelets with a motorized, backpack duster four times before there was general pollen dispersal in the seed orchard; and, it was applied when wind strength could not alter the pattern of pollen distribution (Hadders 1977).

Similar results were obtained in a young, loblolly pine seed orchard that was not producing significant numbers of catkins. Percentage of conelets that were retained after pollination was 66 for wind-pollinated and 76 for SMP in a 1975 trial, and 28 for a wind-pollinated and 51 for SMP in a 1976 trial. The number of 1st-year aborted ovules was reduced from 60 to 50 per cone by SMP in the 1976 trial (Bridgwater, unpublished data). While female conelets were receptive, ramets were pollinated three times with a pollen mix of four male parents whose viability averaged more than 50 percent. The pollination of clusters of female conelets was done with a mechanical insecticide sprayer. However, in a 15-year-old loblolly pine seed orchard that produced large quantities of pollen in a single year, SMP did not result in an increased percentage of conelets that were retained after 1 year.

Filled-seed yields have been increased by SMP in a 15-year-old (40-50 feet tall) slash pine seed orchard in northeastern Florida (E. C. Franklin, 1977, personal communication). Three applications of SMP during 1973 with a backpack, motorized duster (the better of two application methods in Franklin's study) increased filled-seed percentages of five clones from 69 (wind-pollination) to 78. Two applications to seven clones during 1974 increased the percentage of filled seeds from 57 to 59 for the wind- and backpack-pollinations, respectively. This meant an increase in the number of filled seeds per cone from 26 to 30—an increase of about 15 percent for the 1974 trial.

SMP was conducted in another slash pine seed orchard during 1974. Three applications of about 50 cm² of stored pollen per tree were made on receptive conelets. Half was applied to each of the crowns on separate passes by a swivelling, truck-mounted, motorized backpack duster (fig. 20). Filled-seed percentages were not significantly greater for SMP than for wind-pollinated trees. However, inadequacies in sampling procedures were suspected and may have masked differences between treatments (W. L. Beers, 1978, Personal communication, The Buckeye Cellulose Corporation, Perry, Fla.).

A single application of supplemental pollen to unbagged conelets of Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco) did not appreciably increase the percentage of filled seeds per cone over wind-pollination (Daniels 1978). However, it was concluded that SMP can increase production of filled seeds in early- or late-flowering clones or in seed orchards where wind-pollination is inhibited.

SMP has been used to produce interspecific hybrids. Twenty years of experience in producing pitch pine x loblolly pine (P. rigida x P. taeda) hybrids for use in Korea was reviewed by Hyun (1976). Initially, mass-controlled pollinations were made in 8- to 10-year-old stands of pitch pine with imported loblolly pine pollen. Each pollination bag received 1 g (0.3 cm³) of pollen that was split among two to three syringe applications. The 267 staff-days required to complete all phases of the controlled-crossing procedure for 10,000 bags produced 58 fertile seeds per bag (Hyun 1961). This process was economical during the 1950's, but rising labor costs made its continuance impractical.

Ten-year-old pitch pines received four to five applications of highly viable (50 to 64 percent) loblolly pine pollen mixed with talc (1:20) by a mechanical insecticide duster. The result was an average of only 2.5 hybrid seeds out of 88 sound seeds
per cone, significantly fewer than the 13 seeds per cone from controlled pollinations (Hyun 1969). These results indicated that SMP was not economically feasible.

Artificial pollination was necessary to produce hybrids because of loblolly pine pollen was shed 7 to 10 days before pitch pine ovules were receptive. Because mass-controlled pollination was not economical, several two-clonal orchards were established with clones of the two species being selected for synchronous flowering.

In an attempt to produce the same hybrid for use in the Northeastern United States, the U.S. Department of Agriculture Forest Service and the Westvaco Corporation are jointly evaluating SMP. During 1976, 2,670 cm$^3$ of a loblolly pine pollen mix of four male parents (whose average viability was 42 percent) was applied to 65 ramets, which represented 10 clones of pitch pine. Pollinations were made between April 29, 1976, and May 7, 1976, with a motorized backpack duster. Ramets were treated while they were apparently receptive. Individual clones were visited three to six times during the 8-day period between 6:30 and 8 a.m. Alternate halves of each crown were treated, and only the north sides of some crowns were pollinated once or twice because they apparently differed in their times of receptivity from the south sides of the same crowns. Individual ramets received an average of 5.6 to 13.3 cm$^3$ of pollen mix per treatment. The average amount for the entire 8 days was 9.6 cm$^3$ per ramet.

A problem encountered in this effort to mass produce the pitch x loblolly hybrid was much shedding on pitch ramets being dusted with loblolly pollen. The air blast from backpack duster visibly dislodged the pitch pollen from the catkins into the airstream resulting in contamination of the loblolly pollen.

The average percentage of hybrid seedlings for six clones was estimated to be 16 by subjective grading based on the stem and foliage characteristics of 1-year-old nursery stock (Trew, unpublished data). Estimates of the percentage of hybrids ranged from 0 to 52 among the six clones that were studied. Isozyme analyses of seeds from the same clones also placed the percentage of hybrid seeds at 16 while estimates of percentages of hybrids ranged from 2 to 42 among clones. However, estimates from the two methods agreed well for only three of six clones. Seeds from one ramet were used in the isozyme analysis, while seedlings from all the ramets that received loblolly pollen were used in the morphological determination. Because the success of the SMP may have varied among the ramets, a sampling of all the ramets for the isozyme analysis might have resulted in closer agreement (W. T. Adams, 1978, personal communication, Oregon State University, Corvallis, Oreg.).

These results are compatible with those of Wakeley and others (1966), who used SMP to produce shortleaf pine x slash pine (P. echinata x P. elliottii) hybrids.

About 150 cm$^3$ of slash pine pollen mix was applied to the receptive conelets of shortleaf pine on each of one to four visits that began when one-third of the conelets appeared to be receptive. More visits were made to trees whose ovules were receptive over a longer period. Applications were made with a mechanical insecticide duster from a location within the tree's crown. Pollinations were made between 6 and 9 a.m. in order to avoid the wind.

Hybrid percentages were determined by morphological examination of the saplings after they were in the field 2.5 years. Putative hybrids averaged an overall 10.7 percent, but the percentages ranged from 1.2 to 21.5 among 10 female parent trees. SMP cones averaged 13.2 filled seeds per cone, which implies 1.4 (10.7% x 13.2) hybrid seeds per cone.

**Methods of SMP**

SMP has been done with brushes, commercially produced mechanical applicators, mechanical applicators that were adapted from insecticide dusters, and compressed-air paint sprayer. All of these devices require the application of pollen to individual or clustered conelets, and they are likely to be practical only in young, small trees or in trees that have low, managed crowns. Pollination of larger trees has been done with motorized backpack dusters. Devices, like bucket-lift trucks, may be required to pollinate larger trees adequately, even with powered dusters. Equipment, such as that used in a mature slash pine seed orchard, may improve the efficiency of SMP (fig. 20).

The devices for SMP in southern pine seed orchards have been adapted from other uses, and they may not be the most efficient or effective instruments. In one comparison that was made between devices for SMP, E. C. Franklin (1977, personal communication) compared a mechanical fruit-tree pollinator (that had a long extension designed to reach into tree crowns) with a motorized backpack duster. He found no difference in the percentages of filled seeds between the cones that were pollinated with the two devices.

Hadders (1977) studied the distribution of pollen that was applied with a motorized backpack duster from the ground. He concluded that, under calm conditions, the distribution of 20 ml of equal parts of pollen and talc gave satisfactory pollen density at a distance of 5 to 10 ml from the dispersal point.

To be economically feasible, SMP must use efficient methods. The collection, processing, and storage of relatively large amounts of pollen is costly, and it may be unavailable in certain situations, e.g., in young seed orchards. The method of pollinating individual or clustered conelets conserves expensive pollen, but it is labor-intensive. Motorized dusters require more pollen than does pollinating individual clusters, perhaps more than is needed for successful pollination, because the pollen density in the airstream that leaves the duster must be great enough to be visible so that it can be directed.

The development of more efficient methods for SMP will promote its wider use.

**Pollen Quantities**

The amount of pollen needed for successful SMP depends on the efficiency of its application, its viability, and the number of pollinations to be used.
A trial of SMP with 4-inch paint brushes was initiated in a 5-year-old loblolly pine seed orchard in south Arkansas when a single clone became receptive several days after the general pollen flight (R. V. Welch, 1978, Weyerhaeuser Company, Hot Springs, Ark., personal communication). While the results of this trial are unavailable, the methodology was well documented. About 89 to 118 cm$^3$ of pollen was used to pollinate 277 conelets on 25 ramets, an average of 4 cm$^3$ per conelet. A similar trial on 6-year-old grafts, which used a motorized backpack duster from a bucket-lift truck, spent 37 cm$^3$ of pollen per ramet, or about nine times more than the brush application.

The amounts of pollen dusted on larger trees in the above trials vary greatly. Trew (unpublished study) used about 10 cm$^3$ per ramet and applied an average of 41 cm$^3$ to each ramet during repeated visits. Hadders (1977) applied 20 ml of pollen diluted with talc (1:1), and Hyun (1969) used four or five pollinations with pollen and t alc diluted 1:20.

The dilution of expensive pollen reduces costs, but it also reduces effective viability, which may lessen seed yields. Hadders (1977) cites work by Brown (1970) in which the dilution of pollen with 50, 60, or 70 percent talc did not decrease the percentage of female strobili that survived 1 year. Callaham and Duffield (1961) determined that the dilution of viable pollen to 50 percent with dead pollen did not reduce the proportion of viable seeds from controlled pollinations of P. jeffreyi × P. ponderosa.

The dilution of pollen of the female species to 10, 20, 30, 40, 50, 70, and 100 percent live pollen, with dead pollen as the diluent, did not affect the cone set of three interspecific hybrids; but 10 and 20 percent of live pollen produced fewer seeds per cone and decreased the percentage of sound seed (Callaham 1967). Thirty to forty percent of viable pollen—where the viability of pollen that was mixed ranged from 50 to 81 percent—was recommended for high percentage yields of sound seeds per cone.

Pollen with a high percentage of viability can be diluted to a greater degree than that with low viability and still retain its effectiveness. However, because effective viabilities have not been related to seed set for SMP, perhaps a minimum of 40 percent of viable pollen grains should be taken as the lower limit for SMP when the objective is to increase yield of filled seeds.

Here is the rationale for the percentage of viability that is recommended. Sarvas (1962) usually found one or two, and frequently three or four, pollen grains in Scots pine pollen chambers. (This amount varies with the species, the size of the pollen chamber, and the amount of pollen that is available.) If each pollen chamber holds two grains of pollen, whose average viability is 40 percent, the probability that at least one grain is viable is 64 percent. Therefore, 64 percent of the pollen chambers contain at least one viable pollen grain. If the average number of pollen grains per pollen chamber is increased to three grains under optimum conditions for pollination, as in SMP, 78 percent of the pollen chambers would hold at least one viable pollen grain even if incident pollen were lacking or nonviable. Using 40 percent viable pollen should, therefore, result in reasonably good seed yields with SMP. Use of pollen with higher viability will increase the numbers of pollen chambers with at least one viable pollen grain and, therefore, insure filled-seed yields.

If the goal of SMP is to increase the proportion of fertilizations by selected pollen, higher viability may be even more desirable to insure pollination by selected pollen.

**Pollination Timing and Frequency**

This chapter has stressed the importance of programming SMP in order to insure the adequate pollination of receptive conelets and the increased probability that ovules will be fertilized by selected pollen. The optimal opportunities for successful SMP are those occasions when incident pollen is minimal or ineffective, or when applications can be timed to favor fertilization by supplemental (selected) pollen.

For applications to be effective, the timing of applications must coincide with the receptivity of ovules. Daniels (1978) demonstrated that a single application of supplemental pollen did not increase filled-seed percentage over wind-pollination in a 16-year-old Douglas-fir (Pseudotsuga menziesii) seed orchard clone. However, his analysis indicated that SMP can increase filled-seed yields in early- or late-flowering clones, or in young orchards where pollination is inhibited. He also demonstrated the cumulative effect of wind-pollination. Each additional day of wind-pollination resulted in an average increase of 8 percent in filled-seed yields. The cumulative effect of wind-pollination suggests that daily SMP may be the most effective method, particularly if the objective is to insure pollination by selected pollen. Correct timing of pollination is critical, and it requires a knowledge of flowering phenology (Beers 1974).

Bagged, longleaf pine conelets can be successfully pollinated not only before the opening of their ovulate scales is apparent, but also through the period of their maximum receptivity until just before the ovulate scales close. Pollination when the conelet first begins its opening (stage 3) produces some seeds; but pollination when the ovulate scales are partly (stage 4) and fully (stage 5) opened produced the most sound seeds (Campbell and Wakeley 1961).

Most SMP trials that are reported here used three or four applications that were initiated on each orchard clone when one-third to one-half of female conelets were apparently approaching the stage of full extension of their ovulate scales (stage 4 to their maximum receptivity at stage 5). Subsequent applications were timed to pollinate the remaining two-thirds or one-half of the conelets as they reached stages 4 or 5.

**Recommendations**

Here are recommendations based on the authors' personal experiences and those of others who have done SMP. Because there is little data to support these recommendations, future studies will result in changes. Meanwhile, our recommendations are intended as guidelines for those who wish to install trials.
Pollen Viability. Supplemental pollen should contain at least 40 percent of viable pollen grains.

Pollen Amounts. Hand pollination with brushes requires about 4 cm³ per ramet per visit for loblolly pines that are about 6 years from grafting. The dusting of trees of the same size with a motorized duster (as described in the text) will require about 37 cm³ of pollen per ramet per visit. Larger trees that require extensions on motorized dusters or applications from bucket-trucks will need 50 to 100 cm³ of pollen per ramet per visit. These amounts have not been critically tested for their efficacy in pollination, but they are quantities that have been used in previous trials. Tentative amounts of pollen are quantified here as guides in planning the pollination collection and supplemental pollination.

Equipment. The same camel’s-hair brush that is commonly used in controlled pollination can also be used in SMP. Compressed-air paint sprayers, manually-operated insecticide dusters, and commercially available fruit-tree pollinators can be adapted for use with forest trees. Motorized backpack sprayers with dusting attachments are commercially available. Because none of the above equipment was designed for SMP, modifications may be required. Manually-operated pollinators may require the addition of a wind screen that can be placed over the conelets to insure that the pollen contacts them before it is dispersed by the wind. Funnels have been adapted for this purpose. Motorized dusters may require extensions to reach into the crowns of intermediate-size trees. These devices also may require the addition of a positive shutoff device to prevent the pollen from entering the airstream while it is moving from tree to tree. For quantity control, the rate of application of supplemental pollen needs to be calibrated in advance by using dead pollen.

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Chapter 12
Genetic Mutants as Pollen Markers
by E. C. Franklin

Numerous genetic abnormalities (mutants) affecting pigmentation, development, and form have been described for southern pines. Many of these traits behave as simply inherited Mendelian traits in seedlings produced by selfing and crossing. Such traits can often be used as genetic markers to trace pollen distribution, to test relative effectiveness of various pollens, or to make inferences about the genetic structure of stands or other populations such as seed orchards.

Population Genetics

Study of population genetics is directed toward descriptions of (1) defined biological populations in their present genetic structure and (2) trends in gene frequencies, means, variances, and distributions of quantitative traits through time. A quantitative trait is influenced by the combined action of many genes, each with small effects relative to the observed variability of the trait. The analysis of such a continuously variable trait—height growth, for example—requires the use of statistically descriptive units, such as means and variances. Such traits are subject to the laws of quantitative inheritance. Because the effects of individual genes cannot be detected, gene frequencies and distributions cannot be determined.

Qualitative traits are those that can be uniquely described by color, texture, size, chemical properties, etc. Such traits are subject to the laws of particulate (qualitative rather than quantitative) inheritance because each variant can be related to a particular gene. This is Mendelian inheritance after Gregor Mendel, who first described such inheritance from his classical experiment with garden peas. The utility of rare mutant genes in studies of population genetics of trees is based on the exactness of Mendelian inheritance, which permits prediction and interpretation of patterns and frequencies of mutants under specified hypothetical conditions.

Allelic Basis of Mendelian Inheritance

Chromosomes are the physical structures within the nucleus of a cell which contain most of the chemically encoded genetic information necessary for development and maintenance of a tree. Chromosomes occur in pairs; for example, pines have 12 pairs or 24 chromosomes (Mirov 1967). A gene is a genetic regulator located at a particular locus (place) on the chromosome. The chromosome consists of a long series of such loci arranged in definite linear order. Each member of a pair of chromosomes contains the same number and unique order of chemically encoded genes, each at its own locus. Thus, it is proper to say that the gene for a Mendelian trait is carried on a particular chromosome and that each ordinary cell of the tree will carry two genes for that trait.

The chemical arrangement (base pairing) of four simple organic molecules, called nucleic acids, determines the genetic information contained by a gene. This chemically coded genetic information is an allele. The average gene contains 1,500 base pairs. By a change in base pairing, an allele can be altered chemically and changed from one code to another. Thus, the average gene could have $4^{1,500}$ different alleles (Watson 1965). When the result of one such chemical change is seen in a tree, that result is a mutation, i.e., a change in the chemical code of the gene forms a new allele, which causes a distinct difference in the tree. The allele that causes the organism to appear "normal" is called the "wild type" and has the highest frequency in a natural population. Other alleles at the same locus are called mutants and by definition are lower in frequency (rare) and do not produce the "normal" tree.

If a tree contains a wild-type allele on one chromosome and a mutant type on the other, it will usually appear to be normal, just as though it had wild-type alleles on both chromosomes. This property of the wild-type allele is called complete dominance. Mutant alleles which are hidden by the wild type through dominance are recessive alleles, and they appear in differing but predictable ratios (segregation ratios) when trees are crossed or selfed. In this way, mutants can be discovered, described, and evaluated for future use as marker genes in forest research (Franklin 1970).

Description of Mutants

Morphological Description. Some of the most striking mutants in pine seedlings and trees are chlorophyll deficiencies of various types (Snyder and others 1966, Franklin 1969). Color of the cotyledons in these mutants ranges from pure white, yellow, and yellow-green to pale green. Numerous other foliar variations are found, including forms that change color through development (Kraus and Squillace 1964), repeat a color change annually, or show special coloration only under certain environments. Hypocotyls, primary and secondary needles, and even conelets and pollen catkins (Johnson and Critchfield 1974) may show color variants because of recessive alleles. Distinct variations in seedlings are tree size and form, length of needles, and other morphological abnormalities have been described (Franklin 1970). There are so many possible alleles for each trait that the possibilities for variation are almost infinite.

Statistical Description. Statistical data such as segregation ratios and yields of filled seed after selfing and crossing, as well as frequency of the mutant alleles in the population, are necessary to assess the potential usefulness of a particular marker gene. A marker gene is one that has one or more mutant alleles that are suitable for interpreting patterns of gene transfer and assortment through a sexual generation within and between trees or within and between populations.

Self-fertilization is one of the most efficient ways to discover and describe mutations. In loblolly pine (Pinus taeda L.), about one tree in four will show a distinct segregation pattern for at least one readily discernible recessive mutant

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1 Originally published under a slightly different title (Franklin 1977) while the author was Plant Geneticist, U.S. Department of Agriculture, Forest Service, Southeastern Forest Experiment Station, Charleston, S.C.

2 Professor of Forestry and Director, Small Woodlot Forestry Research and Development Program, School of Forest Resources, N.C. State University, Raleigh, N.C.
alleles that are lethal to the organism. The procedure is to
self- and cross-pollinate several trees in a population of interest—for example, a seed orchard. When seeds are
collected and extracted, careful records must be kept on the
numbers that are filled and empty. One effect of selfing is a
large reduction in yield of filled seeds in comparison with
yields after crossing.

Seeds should then be sown in a greenhouse or other area of
relatively moderate and controllable environment. Harsher
environments, such as a nursery, result in fewer mutants
being discovered and reliably described. Careful observations
and counts must be made on all seedlings, especially those
that appear abnormal in any way. Once a mutant type can be
confidently identified, frequency data can be used to estimate
segregation ratios of mutant to normal types.

Under ideal circumstances, a tree containing one dominant
wild-type allele and one recessive mutant allele will yield 25
percent mutant offspring and 75 percent normal offspring
when selfed. Because half of the pollen and half of the eggs
contain the mutant allele, the probability that an individual
has two normal alleles or two mutant alleles or one of each
type is the product of the frequencies of those alleles (fig. 21).
Chromosomal linkages frequently exist between mutant alleles
under study and embryonic lethals (alleles that cause death of
the embryo). In this case, segregation ratios may be distorted
from the expected 3 to 1 ratio of normal to mutant types
(Sorensen 1967). Therefore, the segregation ratio for each tree
under selfing or crossing with another tree must be
determined and confirmed before those alleles can be used as
genetic markers. When chromosomal linkages cause simple
Mendelian mutants to show up more frequently in a family
than the expected 25 percent, the statistical precision of tests
for a given number of offspring surveyed is increased
(Franklin 1974). If very few mutants appear in a family, on
the other hand, too many seedlings must be surveyed, and
that particular mutant allele is not a useful marker.

Uses of Marker Genes

Although most mutants are deleterious, some have little
noticeable effect on subsequent growth. This type of mutant
can be very advantageous if the abnormality can be observed
early in seedling development. An example of such a mutant
was described by Franklin (1969) as "green hypocotyl." As
early as 1 day after germination, seedlings were distinguished
as having either a normal (reddish-brown) or a mutant (green)
hypocotyl. Beyond the cotyledonal stage, both types seemed
to develop normally, including cone and seed production at
ages 6 to 7 years. The significance of this type of mutant is
that pure lines for the mutant type can be established and
maintained by controlled crossing because all mutant types
have only the mutant allele. An application of the green
hypocotyl mutant for study of pollen contamination was
made by Weyerhaeuser Company scientists, who established a
small experimental seed orchard of loblolly pine consisting
entirely of the green hypocotyl strain. When the orchard

Figure 21.—When a tree with one normal allele (N) and one mutant allele (M) is selfed, the result
under ideal circumstances is equal frequencies of
four types of offspring (genotypes), 75 percent of
which are normal because of dominance of the
normal allele and 25 percent of which are the
mutant form.
of 67 percent. Results were consistent with the hypothesis that the first pollen to reach a receptive strobilus was the most effective in accomplishing fertilization (table 13). Differences between self- and cross-pollens were consistent with a second hypothesis that, when two or more fertilizations take place within the same ovule, cross-pollinated embryos usually out-compete self-pollinated embryos and result in higher yields of cross-fertilized seedlings. Thus, cross-pollen applied first averaged 78 percent effectiveness, while self-pollen applied first averaged 56 percent (table 13).

Summary

These two examples of the application of mutant alleles as genetic markers differ in complexity as well as kind of information sought. There are many other possible applications, all of which rely on the same basic principles stated here. The advantages of using genetic markers are:

1. No expensive or complex analytical equipment is needed.
2. Skills and procedures for crossing and selfing trees, and for collecting, processing, and germinating seeds are readily available.
3. Useful mutants for markers are readily available in wild and selected populations.
4. Large populations of seedlings can be screened and scored easily.

Some disadvantages are:

1. Reliable physical and statistical descriptions of the mutant forms are necessary.
2. The relative rarity of most of these alleles restricts application.
3. Relatively low yields of seed from selfing often prohibit use of otherwise acceptable marker-bearing trees.
4. An 18-month wait is necessary to get control-pollinated seeds.

Genetic mutants as marker genes offer attractive opportunities for research and assessment of patterns of pollen distribution in individual trees (Squillace and Kraus 1963) and seed orchards (Franklin 1971, Kraus 1975). Even certain inferences about wild stands can be made if population samples are large.

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Table 13.—Effectiveness of pollens applied at various intervals after strobilus receptivity in a controlled-pollination experiment with slash pine (From Franklin 1974)

<table>
<thead>
<tr>
<th>Type of pollination</th>
<th>Number seedlings screened</th>
<th>Percent albinos</th>
<th>Estimated percent selfing</th>
<th>Percent effectiveness of 1st pollen</th>
<th>Mean percent effectiveness of 1st pollen</th>
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</thead>
<tbody>
<tr>
<td>Self</td>
<td>264</td>
<td>32.2</td>
<td>100.0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cross</td>
<td>456</td>
<td>0</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>50-50 mixture</td>
<td>159</td>
<td>15.1</td>
<td>46.9</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Self-cross 0'</td>
<td>172</td>
<td>13.4</td>
<td>41.5</td>
<td>41.5</td>
<td>55.9</td>
</tr>
<tr>
<td>Cross-self 0</td>
<td>177</td>
<td>9.6</td>
<td>29.8</td>
<td>70.2</td>
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</tr>
<tr>
<td>Self-cross 1</td>
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<td>22.3</td>
<td>77.7</td>
<td></td>
</tr>
<tr>
<td>Self-cross 2</td>
<td>59</td>
<td>20.3</td>
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<td>74.7</td>
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<td>4.4</td>
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<tr>
<td>Self-cross 5</td>
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<td>21.8</td>
<td>67.7</td>
<td>67.7</td>
<td>76.1</td>
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<td>5.0</td>
<td>15.5</td>
<td>84.5</td>
<td></td>
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</tbody>
</table>

Means:                       Self-cross 56.2   67.2
                        Cross-self 78.3
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Chapter 13
Biochemical Gene Markers
by Ralph A. Lewis

Gene movements within and between forest tree populations are difficult to determine. While a method such as labeled pollen tracing can define pollen movement, it does not show actual gene transmission. Numerous studies of chemical extracts from seeds and various organs of the tree have revealed both quantitative and qualitative variations, some of which appear to reflect the activity of specific genes. These biochemical compounds can be used to mark gene presence, variation, and transmission. Often, reliable results can be obtained without control-pollination or lengthy field tests. This chapter presents some applications of biochemical gene markers.

**Gene Marker Criteria**

Regardless of the identity of the compound, a marker should meet the following qualifications:

1. The substance must exhibit reproducible qualitative or quantitative variation. (Qualitative variation is preferred for protein studies.)
2. Environmental and developmental influences should have no effect on marker variation, be kept constant, or else be sufficiently understood for valid adjustments.
3. Genetic inheritance should be as simple as possible. An allelic relationship is ideal in qualitative markers if alleles at a single gene locus control different forms of the markers.

**Chemical Constituents**

In theory, almost any gene product or metabolite that exhibits quantitative or qualitative variation and can be extracted from plant tissue is potentially a marker. In practice, the number of substances that have been investigated is limited. Of these, four types will be presented: Amino acids, phenolics, monoterpenes, and proteins (particularly enzymes). (This selection does not imply that other biochemical substances cannot be markers.)

**Amino Acids**

As basic components of protein synthesis, amino acids are subject to direct gene action. Although they are always present in living plant tissue, both qualitative and quantitative variation have been found in certain tree species. Much of this variation has been related to nongenetic influences such as seasonal changes (Durzan 1968a), time of day (Durzan 1968b, 1968c), mineral nutrition (Durzan and Steward 1967, van den Driessche and Webber 1977), age of leaf (Journet and Cochrane 1979), and climate (Durzan and Chalupa 1968).

Nevertheless, at least three studies suggest that amino acids have useful gene-related variation. Dickson and Larson (1976) found quantitative differences in soluble amino acids among 21 *Populus* hybrid clones. Both qualitative variation and quantitative variation were found between local races of Norway spruce, *Picea abies* (L.) Karst., (Löyttyniemi and Tulisalo 1972). Although climatic factors exerted strong influence, bound and free amino acids from jack pine, *Pinus banksiana* Lamb., seed varied quantitatively with geographic seed source (Durzan and Chalupa 1968). While such research is rare in forest tree species, Lunderstadt (1976a) shows that other branches of the biological sciences have long used amino acid analysis as a screening tool. He concisely examines quantitative methods of amino acid extraction and identification in Norway spruce needles. Specific methods are given for paper chromatography and amino acid analysis.

**Phenolics**

Phenolics comprise a large and varied group of acid organic compounds that have been widely studied for their roles in plant defense mechanisms. Although some are formed in response to attacks by insects or disease organisms, others are normally present in healthy tissue (Rohringer and Samborski 1976).

Aside from their apparent relationship with pest resistance in plants, phenolic variations are associated with other genetic and environmental influences. Hillis (1967) found that certain quantitative and qualitative variations in the leaf polyphenols of *Eucalyptus* are potentially taxonomic markers. In both *Pinus* (Thielges 1972a) and *Picea* (Hanover and Wilkinson 1970), hybridization was indicated by phenolic variations in the foliage. On the other hand, a study of intraspecific variation in the foliage polyphenols of *Pinus* failed to reveal any pattern of geographic variation, and it was speculated that some of the variation was due to interaction between seed source and environment (Thielges 1972b). Differences in season (Feeny and Bostock 1968, Thielges 1972a), site, and tree vigor (Forrest 1974) are among the environmental conditions that influence phenolic variation.

Lunderstadt (1976b) briefly describes a few of the extraction and analytical methods for phenolics. He outlines and references qualitative methods (thin-layer and paper chromatography, spectrophotometric scanning) and quantitative methods (photometric and gas chromatography). Choice of methods will depend upon the type of phenols to be studied. A survey of phenolic studies in forest trees reveals that most attention has been given to analysis of polyphenols by thin-layer or paper chromatography.

**Monoterpenes**

Of the many chemical components present in the tissue of conifers, few have more potential as gene markers than the monoterpenes. Typically, these are volatile hydrocarbons extracted from cortical or xylem oleoresins or leaf oils. Early studies (Bannister and others 1962; Hanover 1966a, 1966b, 1966c; Squillace and Fisher 1966) indicated substantial genetic regulation of the compounds. Subsequent studies of both quantitative and qualitative variation have included variation within trees (Franklin 1976) and clones (Thorin and Nommik 1974) and within populations (Hunt and von Rudloff 1977, von Rudloff and Lapp 1979), species (Stairs 1968), and interspecific hybrids (Ogilvie and von Rudloff 1968, Hanover and Wilkinson 1969). Collectively, these studies show both the complexity and broad potential for genetic applications.

Squillace (1976) condenses many studies and years of experience into his guidelines for monoterpene sampling and
analysis by gas chromatography (GC). Also, he gives an extensive list of references published between 1958 and 1976 and cross-references them by species. His paper is an excellent starting point for anyone interested in using monoterpenes as marker compounds.

While GC is the standard method for analysis, some workers are using other methods to supplement and confirm component identification. Mass spectrometry (von Rudloff and Lapp 1979) was used in tandem with GC, and at least one terpene was identified by infrared and proton magnetic resonance spectra (Hunt and von Rudloff 1977).

**Proteins.** During the past 10 years, proteins have been intensely studied as gene markers in forest trees. All proteins are made up of one or more chains of amino acids called polypeptides. The exact sequence and identity of each amino acid in a chain is coded by a single gene. Genetic variation is often expressed by alterations in sequence or composition of the polypeptide chain, which affects the electrical properties and molecular structure of the protein. Although attention has been concentrated on catalytically active proteins called enzymes, other kinds of proteins such as histones and nonhistone chromosomal proteins may also be useful (Pitel and Durzan 1978).

Electrophoresis is a standard analytical method for proteins. Differential migration of molecules in a polarized electrical field through a gel matrix separates the different forms of a given enzyme (isozymes) into zones or bands. These bands are revealed by allowing the enzyme to catalyze a chemical reaction that produces a visible product (fig. 22). Feret and Bergmann (1976) provide a well-referenced and detailed description of protein extraction, electrophoretic techniques, and evaluation. Numerous refinements of their techniques can be applied for better extraction, band separation, and resolution, depending upon the tree species, type of tissue, and several other variables. Under a given set of study conditions, the investigator is advised to be flexible in the formulation of a test procedure but to maintain rigid quality controls after the procedure is defined.

### Potential Applications

The use of gene markers to help solve or clarify practical problems will probably be limited more by user interest than by technology. A few potential uses are:

1. To define gene flow patterns between individual trees and within and between stands or seed orchards.
2. To measure pollen contamination in seed orchards:
   a. From external sources.
   b. In control-pollinations.
3. To certify seeds of questionable identity.
4. To measure self-pollination frequencies:
   a. In isolated trees, such as seed trees.
   b. In wild stands.
   c. Of clones in a seed orchards.
5. To define pollen sources for out-of-phase flowering trees in seed orchards and isolated seed trees.

6. To compare pollen production with actual pollination effectiveness of individual clones.
7. To verify hybrids and evaluate pollination methods for hybrid seed production.

Obviously, many other applications that were primarily research related in the past will become more important as applied programs unfold in advanced generation breeding. For example, Rudin (1976) suggests that potential problems with uncontrolled coancestry and inbreeding can be avoided by having gene markers for specific male parents. If the pollen parent is also homozgyous for the marker allele, it should be possible to trace that parent’s gene contribution among progeny.

### An Example

Here is an example of the test procedures required for a common seed orchard problem. This is not an actual study but a composite of several studies (as referenced).

**Problem conditions:** A large wild stand of a pine species is located just outside of the isolation zone of a seed orchard of the same species. Because of prevailing winds and topography, a high incidence of pollen contamination is suspected. The seed orchard manager must decide whether the amount of contamination warrants removal of the stand.

**Study Sequence.**

1. Determine which combination of materials, methods, and marker compound is most appropriate.

   **Choice of material.**—Seed are readily available from both the orchard and the wild stand.

   **Choice of method.**—Procedures for starch gel electrophoresis of several seed enzyme systems have been tested for this species. Gymnosperm seed has unique properties as a test material. The embryo tissue is diploid. It carries two sets of genes, one from each parent. The female

![Figure 22.—Example of electrophoretic isozyme pattern. A protein extract is placed in or on the gel (origin) and subjected to an electric current with appropriate buffer solution. A dye is used to mark the leading edge of the migration zone (front marker). After staining, isozymes appear as colored bands that may vary in intensity from very faint to very dark. Band identity is established by rate of migration in relation to that of the front marker.](image-url)
gametophyte or "endosperm" is haploid. It has only one set of genes, which comes from the female parent. Thus, electrophoresis of endosperm protein will produce isozyme patterns (zymograms) that indicate the enzymatic phenotype of the mother tree.

Rudin (1976) has listed several advantages of using enzyme extracts of seed tissue:

a. The isozyme patterns are often very distinct. This is especially important when working with complex banding patterns.

b. Recessive alleles are clearly manifested. When the recessive allele is present, the isozyme for that allele can be detected in the analysis of the haploid tissue. (When a direct relationship is inferred between a given allele and an isozyme, the term "allozyme" is used.) Recessive alleles would be masked in heterozygous, diploid tissue (fig. 23).

c. If an allele produces no detectable isozyme ("silent allele"), the heterozygote can be distinguished from the true homozygote because analysis of haploid tissues will reveal no allozyme on about half of the samples (a 1:1 segregation of the alleles is assumed) (fig. 24).

Correct identification of a phenotype also requires an unbiased gene assortment in the functional megaspore that forms the female gametophyte in each seed. If alleles are not segregating equally (1:1) or if an allele gives a competitive advantage or disadvantage to its megaspore in comparison with megaspores containing the other allele, heterozygotes are more likely to be misclassified as homozygotes. This misclassification can cause serious errors in estimates of gene frequencies. It is also assumed that control of a given enzyme activity in the embryo and female gametophyte resides at the same gene locus. Thus, the gene contribution of the pollen parent can be defined in the allozyme pattern of the embryo extract.

Choice of marker compound.—A valid marker for this type of study requires isozyme patterns that can be interpreted on a Mendelian basis as reported by Tigerstedt (1973), Bergmann (1974), Bartels (1971), and Witter and Feret (1978). For this example, just one enzyme system is used: leucine aminopeptidase (LAP) as described for *Pinus sylvestris* by Müller (1977). The LAP system was shown to have two separate polymorphic gene loci (LAP-A and LAP-B), both with codominant alleles. Each allele is represented by a single isozyme band.

2. Determine the most efficient sampling scheme for the problem.

In order to determine the LAP-A and LAP-B genotype for a tree, at least two seeds must be analyzed. If the zymograms from each seed of a given tree contain different allozymes for each LAP locus, the genotype is heterozygous for both loci. However, if only one allozyme is detected at either locus, additional seed should be analyzed to insure that the genotype is really homozygous at the locus in question. A study by Morris and Spieth (1978) indicates that acceptable accuracy for population studies can be achieved with a three- or four-seed sample. Nevertheless, a larger sample is recommended when it is necessary to determine accurately each individual genotype. Adams and Joly (1980) estimate the probability of genotype identification error to be less than 0.002 for a 10-seed sample.

Because our concern is focused on contamination of the orchard from outside sources, two separate groups of test trees will be required.

a. The LAP genotypes of the orchard clones must be determined. For this test, it is necessary to make an individual assessment on every clone that could be a pollen parent. When an allele is a gene marker for external pollen, an undetected source of an allele within the seed orchard (regardless of its rarity) can bias the data to indicate a much greater degree of contamination than is really present.

b. The wild stand must be sampled to find unique allozymes that are not represented in the orchard. To assess the total impact of contamination, estimates of allelic frequencies in the stand can be made from the data. A
complete inventory of the stand is not needed, but sampling should be well distributed to pick up any localized gene distribution patterns.

3. Make analyses and interpret the results.

For this example, we assume that analyses of 10 female gametophytes from each clone in the seed orchard reveal much allelic variation at both LAP loci. Four alleles are indicated at the LAP-A locus (A1 thru A4) (fig. 25) and three at the LAP-B locus (B1, B2, B3) (fig. 26).

In samples from the wild stand, only two alleles, A2 and A3, are found at the LAP-A locus (fig. 25). At the LAP-B locus, two alleles (B1 and B3) are found to be common to both the orchard and the wild stand. In addition, an allele, B4, is found which is not present in the orchard (fig. 26). Data indicate that this allele is present in about 10 percent of the wild trees, and all are probably heterozygous. Assuming a 1:1 allelic segregation and that pollen production and viability are average in trees with the B4 allele, about 5 percent of the stand’s pollen production should contain B4.

Embryos of orchard seed are then assayed for the B4 allozyme. All B4 alleles are assumed to be from pollen of the adjacent wild stand. Because only a small portion of the wild pollen is estimated to carry the B4 alleles, significant contamination would be indicated if more than a fraction of 1 percent of the embryos contain the B4 allele.

In this simple example, only a few details and assumptions are presented. Almost any practical application will be more complex and less convenient. The mathematics in the example were also avoided: experimental design and statistical analysis could range from simple chi-square tests to multivariate analyses, depending on the investigator’s objectives and test conditions.

Other Strategies

As pointed out by Rudin (1976) the above example represents only one way of handling isozymes in forest genetics. There are at least two other strategies, and these can also apply to compounds besides isozymes.

1. Use isozyme variation in diploid tissue as it is found. The primary criterion for use is repeatability of the isozyme pattern. This approach requires the use of statistical methods for testing the significance of different isozyme patterns. Although less accurate, it lends itself more readily to large-scale surveys of populations than do the other two strategies.

2. Analyze isozymes in diploid tissue when genetic relevance can be checked by controlled crosses. While results are better defined, recessives and silent alleles defy analysis in diploid tissue.

Summary

Adequate research has been done to facilitate the use of gene markers in many practical problems. Nevertheless, all studies must be carefully designed and executed from the sampling techniques to the analyses and interpretation of data (Squillace 1976). Biochemical gene markers make it feasible to examine quickly a population down to the allelic level for insight into gene activity and transmission.

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Processing

Although the methods of pollen processing and storage have been covered elsewhere (chapters 7 and 8), a brief amendment is needed on the processing of pollen that will be sent to a foreign country. All countries do not have ideal facilities; in fact, refrigeration is often scarce or nonexistent. Most pollen is best shipped when it is vacuum-dried (see chapter 8). Pollen that requires high humidity to maintain viability can be transported with dry ice in an insulated bottle (Snyder and Clausen 1974). A label on the outside container should state, “Perishable material, do not exceed 38° C.” It should be written both in English and the language of the country to which it is being sent.

Postal Union Mail

Exchange of all mail except parcel post between the United States and other countries is governed by provisions of the Convention of the Universal Postal Union (U.S. Postal Service 1971). Postal union mail has two categories, surface mail and airmail. These have size and weight limits that are the same for the material being sent from or to the United States. No material and container may exceed 4 pounds (2.0 kg) except shipments to Canada where the limit is 60 pounds (30.5 kg). Maximum length is 24 inches (61 cm) and the combined maximum length, breadth, and thickness is 36 inches (91 cm). The package cannot be less than 5.5 by 3.5 inches (14 by 9 cm). A green customs label (Form 2976) must be completed and placed on the address side of the package (U.S. Postal Service 1974). Labels can be obtained from post offices. If contents have a value over $100, U.S. customs declaration (Form 2976A) must be attached. Surface mail may take as long as 6 months for delivery, while airmail will usually be delivered within 4 weeks. If the package exceeds the weight and size limit, it will be sent parcel post.

Perishable biological materials may be sent in an official laboratory package with violet labels that facilitate careful handling and prompt delivery. If requests arise for such handling, the National Tree Seed Laboratory in Macon, Ga., will apply for it as a special service.

Postal union mail can be registered to most countries. Registration of postal union mail with a return receipt requested establishes a record of that shipment and helps to insure entry into a country. (Nonregistered packages have been known to disappear in some countries.) Unless the return receipt has a Par Avion (label 19) sticker on it, it will be returned by surface mail.

If there is a loss of mail in international shipments, notify the Chief Postal Inspector, U.S. Postal Service, Washington, D.C. 20260.

Special delivery is available. The package should bear the Express sticker (label 57). Delivery is made in the various countries according to the special delivery regulations in force in the country of destination.

Parcel Post

Parcel post is a separate category from postal union mail and the required forms are not interchangeable. Exchange of parcel post with other countries is governed by provisions of individual bilateral agreements that vary from country to country. Any country may return or confiscate articles that are prohibited from circulation within that country, whether or not notice of such prohibition or restriction has been published. Specific requirements and quarantines of individual countries are given in chapter 15.

Parcel post will not handle packages smaller than 5.5 by 3.5 inches (14 by 9 cm). Packages cannot be longer than 3.5 feet (1.1 m), and the combined length and girth cannot exceed 6 feet (1.8 m). The weight limit for most countries is 44 pounds (22.4 kg). Customs declaration (Form 2966) must be attached to the package along with the International Parcel Post sticker (Form 2922). Some countries also require a dispatch note (Form 2972) to be attached. The post office will provide the appropriate forms that are required by each country.

Parcel post is registered to the few countries that have formal agreements with the United States. This restriction also applies to insurance of parcel post. Special delivery is not available for parcel post.

Air Freight

The fastest means of transportation for shipments in excess of 44 pounds (22.4 kg) is air freight. No special labels are required. Shipment is usually made within 5 to 7 days and often much sooner. Some delays are experienced at the point of entry because of backlogs in customs inspections and notification of receiver. Special handling (such as express) that is optional in the United States is not available for international shipments. Therefore, the cargo may be delayed by higher priority shipments. Delays at customs can be minimized if flight information is airmailed or telegraphed ahead so that the receiver can meet the shipment or initiate a followup.

Recommendations

Whenever possible, make shipments of 4 pounds (2.0 kg.) or less, even if a particular shipment must be divided into several packages. Fast service will be obtained when the shipment is sent first class (postal union mail), registered airmail with a return receipt requested. If slower routes are chosen, refrigeration should be considered. Refrigerated materials should be sent in vacuum-sealed ampules to maintain control of moisture.

Specific information for any country can be obtained from the Director, National Tree Seed Laboratory, U.S. Department of Agriculture, Forest Service, P.O. Box 819, Macon, Ga. 31202.

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Microbial Contamination

Although contamination by micro-organisms is considered one of the major problems in the collection and manipulation of pollen (Stanley and Linskens 1974), there is no documentation of the organisms that cause the problems. A variety of fungi and bacteria will develop and grow profusely on pine pollen under high moisture and cause a rapid reduction in pollen viability. But whether these micro-organisms are only airborne contaminates or represent a normal microflora of the pollen is not known.

Pollen from different species of forest trees, including pines, has been used to trap certain phycomycetous fungi, and the damage caused by these fungi to individual pollen grains has been documented (Goldstein 1960). Also, there is some evidence that certain fungi may be host specific on pollen of different tree species (Collodahl and Carlsson 1968).

Microbiological isolation, growth, and identification procedures can be used to specify the types and quantities of micro-organisms that are associated with pollen and also whether the organisms are causal or represent a normally occurring, indigenous microflora. Samples of pollen can be taken at different stages of collection and processing and placed on agar media to determine the kinds and quantities of micro-organisms that are present.

Techniques for obtaining supposedly contaminate-free pollen of conifers and for decontaminating infested pollen have been reported. LaRue (1953) obtained contaminate-free pollen by placing mature but unopened catkins of conifers in a 0.525 to 1.050 percent aqueous solution of sodium hypochlorite (10 to 20 percent Clorox® ) for 20 minutes. This technique was altered by Tulecke (1954), who found that swirling the catkins 10 to 15 times in 70 to 95 percent ethyl alcohol before placing them in the sodium hypochlorite solution was more effective. After being rinsed in sterile water, the treated catkins were blotted dry on sterile paper towels, placed in open aluminum containers, and sealed in glass jars over ovendried calcium chloride. The pollen was shed after 12 to 30 hours at 41° F (5° C).

The method for disinfesting contaminated pollen consists of placing a layer of pollen onto a sterile, sintered glass filter with a slight vacuum. The pollen is then washed with sterile water, dried, and stored over calcium chloride (Stanley and Linskens 1974). Antibiotics and fungicides that are non-toxic to the pollen can be used to reduce or eliminate the micro-organisms by pouring the pesticides over the pollen on a filter or suspending the pollen in a solution or suspension of the pesticides.

Quarantine Restrictions

There are no customs regulations on the exportation or importation of pollen from forest trees. These regulations would require a microscopic examination which, by international agreement, is not required in any country with the exception of one agricultural import into Japan. The Animal and Plant Health Inspection Service (APHIS) has concluded that, although pine pollen may carry a virus, most of the viruses are probably already universal.

Certain countries have restrictions on the importation of pollen. The documentation that is required and the restrictions that are imposed on pollen shipments into countries that responded to a request for this information are shown in table 14. The certificates and the permits that are required by the different countries are relatively simple.

Assistance in obtaining phytosanitary certificates is available from the U.S. National Tree Seed Laboratory, Macon, Ga. The certificate of origin is a document provided by the exporter that identifies when the pollen was collected and its country, state, and longitude and latitude or origin to the nearest degree. Before pollen is shipped, an import permit must be provided by the person to whom it is being sent. Some of the miscellaneous restrictions that are imposed are more difficult to meet, especially because a microscopic examination is not required.

Jamaica, for instance, requires that pollen be free of the spores of the coffee leaf-rust fungus. Coffee rust is not known to occur in the United States, but the only way to be sure is to require the inoculation of coffee plants with a sample of the pollen or a microscopic examination of the pollen.

Mauritius prohibits the import of pollen from areas where Cronartium occurs. Because aeciospores of the fusiform rust fungus and pine pollen are often released at about the same time in much of the South, this mixture can easily occur. Pollen can be determined to be free of aeciospores by a combination of microscopic examination and inoculation of oak leaf with samples of the pollen in order to observe whether or not aeciospores and teliospores develop. This inoculation technique, however, would not be effective with the pine-to-pine Cronartium rusts that occur in regions of the United States outside the South. If these rusts are involved, the susceptible pines would need to be inoculated in order to detect the fungus.

Kenya requires that pollen be certified as free of all plant pathogens; but, even if it were possible, this procedure would be prohibitively expensive.

There is a need for the detection of plant-pathogenic micro-organisms that might be on the pollen that is shipped to other parts of the world. A high percentage can be detected by placing a sample of each pollen collection on standard culture media and identifying and recording the organisms that develop.

Although it is a more difficult technique, another screening procedure for detecting pollenborne viruses can be developed by inoculating appropriate indicator plants with homogenates of pine pollen or with a serum.

Methods for the detection of rust contaminates have been proposed for coffee rust and Cronartium rusts in which susceptible plants are inoculated with pollen that is suspected
to be contaminated, and the plants are microscopically examined.

Conclusions

There are three types of contaminates that may cause problems in a pollen sample: (1) micro-organisms (2) pollen from a different species and (3) pollen of the same species but from an unwanted source. Contaminating pollen from the same species cannot be recognized in routine testing. Purity depends on careful collection and processing procedures. Pollen from a different genera can be recognized and counted during testing procedures. Results can be incorporated into the test records. Contaminating micro-organisms can be microscopically detected.

Table 14.—Documents required by different countries for the shipment of pollen into that country: PC = Phytosanitary Certificate; CO = Certificate of Origin; IP = Import Permit

<table>
<thead>
<tr>
<th>Country</th>
<th>PC</th>
<th>CO</th>
<th>IP</th>
<th>Restrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>x</td>
<td>x</td>
<td></td>
<td>Pollen must not be supplied by beekeepers.</td>
</tr>
<tr>
<td>Barbados</td>
<td>x</td>
<td></td>
<td>x</td>
<td>Imports prohibited from areas outside Canada with destructive pests not indigenous to Canada, e.g., where <em>Gremmeniella abietina</em> (scheroderris canker) occurs.</td>
</tr>
<tr>
<td>Canada</td>
<td>x</td>
<td>x</td>
<td></td>
<td>Pollen must be sealed in glass.</td>
</tr>
<tr>
<td>Jamaica</td>
<td>x</td>
<td></td>
<td>x</td>
<td>Phytosanitary certificate must state: “Pollen was collected in U.S.A. and is free of coffee leaf rust.”</td>
</tr>
<tr>
<td>Kenya</td>
<td>x</td>
<td></td>
<td></td>
<td>Must be certified as free of plant pathogens.</td>
</tr>
<tr>
<td>Korea</td>
<td>x</td>
<td>x</td>
<td></td>
<td>Accept airmail only.</td>
</tr>
<tr>
<td>Mauritius</td>
<td>x</td>
<td></td>
<td></td>
<td>Cannot be collected from areas where <em>Cronartium</em> is present.</td>
</tr>
<tr>
<td>New Zealand</td>
<td>x</td>
<td></td>
<td></td>
<td>Must not be supplied by beekeepers.</td>
</tr>
<tr>
<td>Norway</td>
<td>x</td>
<td></td>
<td></td>
<td>Restricted to scientific use only.</td>
</tr>
<tr>
<td>Solomon Islands</td>
<td></td>
<td></td>
<td></td>
<td>Transport difficult within the islands; no storage facilities available.</td>
</tr>
<tr>
<td>Sweden</td>
<td>x</td>
<td></td>
<td></td>
<td>Laboratory research only.</td>
</tr>
</tbody>
</table>
Chapter 16
Conserving Genes Through Pollen Storage
by Roland E. Schoenike and Calvin F. Bey

Gene conservation undertakes to maintain valuable stocks of germ plasm for future use including both naturally regenerated gene pools (wild stands) and artificially regenerated gene pools of planted stands and storage banks.

As long as wild populations were not extensively modified by man, natural evolutionary processes maintained fitness in them. Now, man has overridden the natural scene so completely that there is scarcely an undisturbed ecosystem left in the world. Even with the best of plans, trees become the victims of roads, parking lots, lightning strikes, insects, and other manmade and natural catastrophies. It is no longer certain that trees in natural stands, arboreta, clone banks, seed orchards, and other living collections are completely adequate for gene conservation purposes.

The methodology for gene conservation has received considerable attention in recent years (Frankel 1970, Frankel and Hawkes 1975, Roche 1975, Stern and Roche 1974, Yeatman 1971, Schoenike 1976, Mohammed and others 1977). Gene conservation can be accomplished in two ways: By perpetuating natural stands (in situ conservation) and by collecting and storing valuable genetic material including whole trees or plant parts such as pollen (ex situ conservation). In this chapter, we consider the special advantages and disadvantages of pollen storage as a means of conserving forest tree germ plasm and suggest long-term strategies for the renewal or revitalization of pollen gene resources.

Advantages and Limitations of Pollen Storage in Gene Conservation

Long-term storage of pollen, in pollen banks or stores, is a useful tool for gene conservation. A storage program is based on the premise that in the cutting of natural and planted stands of trees, we are losing or might lose genes that will be needed in the future. Also, storage is a convenient way to package material compactly and economically.

Recordkeeping and maintenance become problems as trees age and living collections are expanded. Long-term storage of pollen is one way of buffering against natural losses of genetic material. In comparison with tree collections, pollen banks offer these advantages:

1. Storage space is minimized. Up to 1,000 vials of pollen can be stored in the space of a small room, but 1,000 living trees require 10 acres or more.
2. Storage is economical. Although it might entail a high initial expense, a refrigerated storage facility can be run for only a few dollars per month. Maintaining a large living collection costs many thousands of dollars each year.
3. Storage is efficient. One control facility can keep pollen stores that preserve samples of the gene pool of a species throughout its natural range, which for some southern tree species may cover as much as 200,000 square miles.
4. Recordkeeping is simplified. A single register suffices to provide all necessary information on the stored pollen.

The disadvantages of using pollen banks for conserving germ plasm are mainly the retention of viability in storage and the renewal of pollen stored at stated intervals.

Pollen Storage

At present, gene conservation by pollen storage is applicable only to those species that both retain pollen viability over a considerable time and effect an acceptable level of fertilization after removal from storage (Wang 1975). Retention of viability in tree pollen depends on temperature, moisture content, and oxygen pressure (Roberts 1975, Snyder and Clausen 1974).

Long-term storage of tree pollen by freeze-drying or in liquid N is feasible and may be entirely practical (chapter 8). Now, we need more sensitive tests, particularly in relating in vitro to in vivo conditions, over a wide range of storage times and methods and with many tree species (chapter 9).

Long-Term Strategy for Renewal

If we assume that pollen storage problems will be solved in the near future, planning a long-range strategy for renewal will need to take into account these considerations:

1. Selecting the type of material to be stored.
2. Replacing pollen that has declined in viability.
3. Adding new material to the pollen store.

Sampling problems are discussed in chapter 17. We assume that each agency using pollen banks for gene conservation will want to assure itself that the sample obtained is representative of what is desired. Here are the kinds of pollen stores that should be considered.

1. A wide range of current material derived from seed orchards, seed production areas, clone and seedling banks, progeny tests, and a variety of test plantings (special gene pools).
2. Representative samples from natural stands throughout the range of the species (rangewide, provenance gene pools).
3. Mixes of pollens from natural stands throughout an area of interest (limited range provenance gene pools).
4. Exchange material with which to make wide crosses.
5. Material derived from secondary gene pools in foreign countries (such as southern pine pollen from Queensland).
6. Special material such as pollens carrying marker genes, inbreds, or dwarving stock.

The needs of each agency will determine which types of pollen material they wish to store. We suggest that a public agency or large cooperative undertake at least the second of these stores, and possibly also the fifth, because they require a much larger collection base than the others. It would also be more convenient and more economical for one agency to assume the rangewide store because all other agencies could obtain material from this store to meet their own special needs.
Stored pollen needs to be updated both to replace that which shows a decline in viability and to add new material for enlarging the gene pool. Assuming that pollen can be safely stored without loss of viability for 50 years, we suggest that new selections for renewal be added at 10-year intervals. Special gene pools (No. 1 in the list above) might require a complete turnover of material as old seed orchards and clone banks are phased out in recurrent selection programs (Franklin 1976, Weir and Zobel 1975) and new material, termed enrichment, is introduced into genetic base populations. For rangewide gene pools (No. 2) and possibly for secondary gene pools (No. 5), we suggest that the number of samples for revitalization of the gene pool be 20 percent of the original number. Assuming that we start with 1,000 samples per species, we would add 200 samples every 10 years, in essence, adding 2 percent to the original collections each year. At the end of 50 years, the cycle would begin anew, going back to original gene pools to capture its current gene complex.

We do not know how large a sample must be to adequately represent a gene pool. Gene pool management revolves around the risk or probability that a gene complex will be missed. For any given trait, the completeness of the gene pool is the ratio of the number of trees from which pollen is stored to the number needed to get an optimal distribution. For specified traits, the ratio should approach 100. Geneticists, foresters, and managers need to be involved with decisions concerning the material to be included in their own and in public or cooperative pollen storage programs. Major emphasis should be on adequate sampling and broadening the gene pool over time.

In no way do we advocate pollen storage as a substitute for living collections. We strongly support the setting aside of natural areas or genetic reserves where native ecosystems are maintained intact and natural evolutionary processes are allowed to act freely. Such natural reserves should remain for centuries to provide a continuing gene pool for our forest trees, and from which material can be drawn both to enrich current tree improvement programs and to maintain a broad genetic base within them.

Likewise, we support such living collections as clone banks, seedling banks, and arboreta where material can be observed for long periods of time, and from which renewal, both of the plants themselves or of such plant parts as pollen and seed, can be made.

Summary

Gene conservation is needed in forestry. It should involve the establishment of natural reserves to protect existing gene pools and the establishment of pollen banks that could safely and conveniently store much genic material at relatively low costs. Storage problems remain, but new techniques such as lyophilization and deep-freeze chambers give promise of maintaining some pollens in storage for 15 years or longer. In the near future, a storage life of 50 years may be possible for many tree pollens. Long-term renewal of pollen gene pools calls for continual replacement of present stores with new material that is genetically superior and that broadens the gene pool. A systematic approach to pollen storage and renewal by all agencies concerned with gene conservation is much needed.

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Yeatman, C. W.
Chapter 17
Methods of Pollen Sampling for Gene Conservation
by Gene Namkoong

Sampling for gene conservation depends on a program’s objectives, which fall between two extremes:

1. To preserve the unique allelic combination that presently exists in individuals or germ cells, or
2. To save the alleles in a gene pool that can be recombined for future purposes.

The first may be impossible to satisfy, while the second can be easy to accomplish in any reasonable sample of pollen.

Preservation is impossible if each possible family or individual is considered uniquely important, or if the mating of remotely related individuals is prohibited. On the assumption that 5,000 gene loci are active in a species and that 30 percent of the loci have two alleles each to save, there are $2^{1500}$ of $10^{451.5}$ combinations of alleles to consider. To save rare combinations of low-frequency alleles, samples much larger than $10^{451}$ are required, and unique combinations would probably not be recognizable in these huge samples. Therefore, no sampling system can save all allelic combinations in any useful way.

A realistic objective is to save almost all alleles in some unknown combination with alleles at other loci (Roche 1975). Alleles can be recombined in future breeding generations as long as they are present in the conserved pool. The sampling problem is to insure, with minimum probability, that even rare alleles will be included in the chosen sample.

Population Sampling

Within-Tree Sampling. Because the frequency of alleles within any tree either will be 0.0, 0.5, or 1.0, the number of pollen grains that is necessary to insure obtaining potential alleles is trivially small. If the frequency is either 0 or 1.0, one pollen grain is as good as a million for saving the available alleles. Therefore, no sampling system can save all allelic combinations in any useful way.

On a population basis, however, sampling problems are different; and, if the concern is to save alleles with multiple-tree samples, it may not be necessary to sample many seedlings per parent tree.

Within-Population Sampling. Within single, homogeneously distributed populations, intensive sampling among trees may be required to save low-frequency alleles. A large number of trees may have to be selected to prevent the loss of those alleles. At a single locus, for an allele at frequency $q$, the probability of losing the allele in a sample of $t$ trees is: $Pr(\text{loss}) = (1-q)^t$. For $q = 0.05$, $Pr(\text{loss}) = 0.95^t$. To keep at $q = 0.05$, a $t \geq 45$ is necessary. If the objective is to hold $Pr(\text{loss}) \leq 0.05$ for alleles at $q = 0.01$ frequency, $t \geq 150$ is needed; and, if the concern is to save alleles at $q = 0.01$ with $Pr(\text{loss}) \leq 0.01$, $t \geq 230$ is required.

If the objective is to save alleles at multiple loci, the sampling strategy would have to focus on those loci (say $s$ of them) having relatively rare alleles that are not regarded as useful at the time. If it is assumed that 1,500 genetic loci have worthwhile allelic variants, and that 10 percent of them have average frequencies around 0.05, a model of 150 loci with alleles at 0.05 average gene frequency is reasonable. About one-third of these loci may not be seen as useful, and the concern is to sample enough trees to reduce the $Pr(\text{loss})$ at any of these $s = 50$ loci to a selected value of, say, 0.05. For such cases, the probability of not sampling rare alleles at each of the $s$ loci is: $Pr(\text{loss at any locus}) = 1 - Pr(\text{not losing at every locus})$. If the average case is treated as if it were uniformly the same at all loci, and if the $s$ loci segregate independently, the probability of not losing a rare allele at every locus = the $s$th power of the probability of not losing the rare allele at each locus. Thus:

$$Pr(\text{not losing at every locus}) = 1 - \left(\frac{9}{10}\right)^s$$

where $\pi$ denotes the continued product of the probabilities, multiplied over all $s$ loci. In a sample of $t$ trees, the probability of not losing the allele is $1 - Pr(\text{losing the allele}) = 1 - (1-q)^t$. Therefore, the $Pr(\text{not losing at every locus}) = (1-q)^t$ and the $Pr(\text{loss at any locus locus}) = 1 - (1-q)^t$. This probability must be held to a minimally acceptable level by an appropriate choice of $t$ for any given $q$ and $s$ values.

In the situation where $q = 0.05$, $s = 50$, and the risk level of critical level of $Pr(\text{loss}) = c = 0.05$, $t$ is solved by:

$$t = \frac{\ln(1-(1-c)^{1/5})}{\ln(1-q)}$$

In this case, $t = 67.09$.

Thus, a sample of 68 trees would keep the probability of loss below $0.05$ for 50 loci, each with an allele of interest at $q = 0.05$. On the average, less than 5 percent or 2.5 of these alleles will be lost if 68 trees are sampled. For the same genetic situation but for a safer risk level, say of 0.01, $t = 83$ is required to have an expected average loss of 0.5 alleles. For an average loss of one allele, $t = 77$ is required.

In order to save even rarer alleles, say when $q = 0.01$, $s$ might also be smaller. Hence, if 2 percent of the variable loci
have alleles at \( q = 0.01 \) and of these, say one-third, are of no present use but have future value, then \( s \) is near 10. For a loss risk of 0.05, \( t \) must be 263; for a loss risk of 0.01, \( t \) is 344. For an average loss of one allele, \( t = 227 \) is needed. Many trees must be sampled to avoid the loss of low-frequency alleles, even if the number of alleles to be saved is small.

For broadly distributed populations or species over which alleles are dispersed at random, a few hundred trees may be sufficient for most purposes. For the rare alleles of \( q = 0.01 \), even if there are 50 loci, 400 trees are enough to reduce expected loss to only one allele. For a single locus with \( q = 0.05 \), 30 trees are sufficient to reduce the loss probability to less than 5 percent.

It is important that the above derivations are based on the sampling of whole trees, as if the chosen trees were clonally propagated. More often, however, it turns out to be the seeds or seedlings of those parent trees that are sampled. Nevertheless, the derivations are exactly the same regardless of whether one samples the trees in the parent or the progeny generation. The \( Pr(\text{loss}) \) is derived from the assumption that the loss occurred when the homozygote of the undesired allele was sampled: \((1 - q)^2\). If seeds are sampled instead, the loss can occur from both the undesired homozygote parent and the heterozygote parent. From the \((1 - q)^2\) homozygotes, if the other parent for the undesired allele at \((1 - q)\) frequency was sampled, the expected frequency of loss is \((1 - q)^2\). From the \(2q(1 - q)\) heterozygotes, half the gametes are of the undesired type, and again \((1 - q)\) of the other parents' gametes are undesired, for an expected frequency of \(2q(1 - q)^2/2\). The loss rate is therefore \((1 - q)^3 + q(1 - q)^2 = (1 - q)^3\), as before. If \( t \) trees are sampled with one seedling per tree, the expected frequency or probability of loss is \((1 - q)^t\). As long as the gametes are randomly and independently sampled, the derivations are identical and either \( t \) parent trees or \( r \) seedlings can be sampled. One seedling per parent \((r = 1)\) can be used, or let \( r = 2 \) and reduce the number of parent trees by one-half. For all of the above solutions for \( t \) in which the number of parents to be sampled is sought, \( r \times t \) can be substituted as the total number of seedlings to sample.

Thus, for \( q = 0.05 \), \( c = 0.05 \), \( s = 50 \), \( r \times t = 68 \),

for \( q = 0.05 \), \( c = 0.01 \), \( s = 50 \), \( r \times t = 83 \), etc.

These derivations are based on the assumption of a random and independent distribution of alleles and gametes. Because this assumption is critical, appropriate sampling among populations must be treated.

**Among-Population Sampling.** The dispersion of genes in widely distributed populations is seldom random. Climatic and ecotypic variations are common, and clines or discontinuities in allelic frequencies can exist. In such cases, the most critical problem is to sample from all populations because the number of trees sampled per population can be greatly reduced if the gene frequency in the appropriate population is high. For example, if the species-wide average gene frequency is 0.05, but the frequency among 10 subpopulations is 0.5 in one and zero in all others, only three trees need to be sampled to have less than a 5-percent chance of losing the allele if the correct population were luckily chosen. In such cases, it is obviously better to select three trees from all 10 populations than to select 30 trees from one population. However, the best sampling scheme will depend on the distribution of gene frequencies among the populations and our ability to increase \( a \) priori the chances of selecting appropriate populations.

A simple population structure to consider is a cline in gene frequency in which the rarer allele is present at one extreme of the range in relatively high frequency and correspondingly lower frequency further from its selective optimum (Hanson 1966). In such cases, it is best to sample all population extremities. Such extremes may be defined by distinctive environmental factors or physiography. As many extremities as can be defined should be sampled because they may contain higher frequencies of the alleles that have a low species-wide frequency.

All subpopulations should be sampled; and, for alleles than can be characterized by \( q = 0.01 \), a sample of 400 trees is sufficient to have an expected loss of less than 1 out of 50 alleles. This sample is adequate because a frequency of less than 0.01 in any subdivision increases the likelihood of having higher frequencies in other populations. Thus, if there are 10 populations, of which there are 9 with \( q = 0 \) and one with \( q = 0.1 \), a sample of 37 trees in each or a total of 370 trees is sufficient for our requirement of having less than 1 expected loss out of 50 such alleles. If eight of them have \( q = 0 \) and two have \( q = 0.05 \), a \( t \) of 38 in each is sufficient. Similarly, if there are 25 subpopulations with 24 having \( q = 0 \) and 1 having a \( q \) of 0.25, a \( t \) of 14 in each or a total of 350 trees is sufficient. A dispersal of the alleles into more of the subpopulations at lower frequencies than 0.25 requires high levels of \( t \), but \( t \) approaches 16 in subpopulations and the total approaches 400. Obviously, up to 400 subpopulations can be adequately handled by the rule of 400 for \( q = 0.01 \) and \( s = 50 \). If future plantation security is of any concern, replicate population samples can be considered but it is generally preferable to increase the number of sampled populations.

A high number of subpopulations requires a sampling among them. Also, in the extreme case in which there is an infinite number of potential ecotypes or other subpopulations, the probabilities of missing an important allele at any locus can be assessed by sampling among populations. This replicates the earlier situation for which formula (1) was developed; and, therefore, for a general rule, the total tree sample sizes that are required for all of the possible distributions of gene frequencies can be specified. In table 15, tree samples that are required to have an expected loss of one allele for various levels of \( q \) and \( s \) are listed.

From table 15, it is evident that the low frequency of the alleles of interest is the basis of the sampling problems. Also, the requirement of a large number of alleles does not greatly expand the sampling that is required. A change in \( s \) from 10 to 100 merely doubles the sample size that is needed. On the other hand, a decrease in the frequency of the alleles of
Table 15.—Minimum total tree samples required for an expected loss of one allele

<table>
<thead>
<tr>
<th>Average allele frequency (q̄)</th>
<th>Number of obscure alleles</th>
<th>0.05</th>
<th>0.01</th>
<th>0.0005</th>
<th>0.0001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>45</td>
<td>227</td>
<td>455</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>77</td>
<td>389</td>
<td>780</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>90</td>
<td>458</td>
<td>919</td>
</tr>
</tbody>
</table>

concern by a factor of 10 from 0.05 to 0.005, or from 0.01 to 0.001, increases the sample size by a factor of 10. For such low-frequency alleles, the most feasible alternative to saving alleles is to sample extreme populations in which those rarities may be exceptionally frequent.

For major taxonomically recognized groupings of races, species, etc., it would be desirable to sample all of them at high intensity. If this is not feasible, the cost can be reduced by bulking all collections to insure equal chances for all trees to be genetically represented. The principle of dispersing collection points to the range's extremes is valid even if the sample sizes cannot be very high. Because related populations would carry mostly the same alleles, though at different frequencies, it is also desirable to disperse collections among taxonomically different groups to increase the chances of sampling unique alleles. Therefore, the same principles are true for taxon sampling as for intraspecies sampling, but they hold at a higher level of taxonomic order.

Future Losses

After alleles are initially preserved in a gene conservation pool, thought must be given to their survival in future generations. If the conservation pools are constant in size over repeated generations, the frequency of alleles will shift, and useful alleles can be accidentally lost. Without any selection, gene sampling variations alone will eventually lose alleles in exact complementary proportion to their initial frequency. Thus, 95 percent of the alleles at q = 0.05 can be expected to be lost eventually. Even selection for those alleles cannot guarantee that they will be saved. Accidental selection against them because of linkage can diminish any chances that they might otherwise have. Because future values of alleles are uncertain and the aims of breeding can change, it is better to keep subpopulations distinct. Breeding to maintain different gene pools at different gene frequencies can make rare alleles more readily accessible because they occur at higher frequencies in some populations. Controlled random mating or the directing of distinctive populations toward diverse objectives can increase allele frequencies in some populations.

A multidirected set of populations would be as feasible for conserving genes as for improving traits (Namkoong 1976).

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Chapter 4
Pollen Handling for Southern Hardwoods
by Robert E. Farmer, Jr., and John A. Pitcher

In the past 20 years, research on the improvement of southern hardwoods has produced much information on the genetics and biology of about six species; however, pollen management is presently used with only one or two species of hardwoods. Large-scale pollen handling will probably never be used in some hardwoods because of their reproductive characteristics. Nevertheless, continuing research on tree improvement requires some pollen handling, and significant progress has been made in the development of methodologies. This chapter presents the techniques of pollen handling in the southern and Appalachian hardwood species that are currently being used in breeding programs and makes specific recommendations about their utilization.

Ash (Fraxinus L.)

Five species of ash are native to the South (Schopmeyer 1974), but only two of them are commercially important. White ash (F. americana) and green ash (F. pennsylvanica) occur widely throughout the Appalachian Mountains and southern Coastal Plain.

Flower Structure and Development. Both white and green ash are dioecious (Harlow and Harrar 1950, Fowells 1965, Schopmeyer 1974). Because male flowers bloom and ripen a few days ahead of females, pollen is already in the air when the females are receptive. Both types of flowers are borne in panicles near the branch tips and appear before leaf buds start to enlarge (Fowells 1965) or after leaves have begun to unfold (Harlow and Harrar 1950).

Pollen shedding of a single tree occurs over a period of 3 to 4 days and that of a stand over 5 to 7 days (Wright 1953a). Most pollen, which is wind disseminated, travels only 200 to 300 feet (61 to 91 m) from the point of origin. Wright (1952) found that the highest daily pollen counts for white ash occurred on the first day of the blooming period. Pollen counts were high near the source but fell off rapidly to frequencies of less than 10 percent beyond 150 feet (46 m).

The stigmas of the female flowers are receptive as soon as they emerge from the bud, and they remain receptive for 2 to 5 days (Wright 1962). Receptivity appears to end just before the stigmas start to wither (Fowells 1965).

In a study of white ash in New York, Hunt (1964) concluded that the sex ratio of wild trees was about 1:1. He also found that the sex of white ash did not change from year to year and that its sex could not be identified by any morphological characteristic other than its flowering. Male white ash can be recognized in the early spring by the larger flower buds that begin to swell conspicuously, while the female buds are still apparently dormant.

Open-grown vigorous trees may start blooming when they are only 3 to 4 inches (8 to 10 cm) in diameter, but abundant flowering is not attained until the trees are 8 to 10 inches (20 to 25 cm) in diameter. The male trees flower nearly every year, but the females flower only 2 or 3 years out of 5 (Fowells 1965).

Pollen Collection, Extraction, and Storage. Ash pollen can be collected by bringing flower-bearing branches inside, placing the cut ends in water, and allowing the pollen to mature and shed onto paper (Wright 1953b). This technique requires frequent changes of water and repeated cutting of the branch butt to maintain it in a fresh condition. Otherwise, branches dry out before pollen is shed. An alternative procedure is to graft rootstocks in midwinter and force them under greenhouse conditions (Wright 1962). Large quantities of ash pollen are difficult to obtain, but the pollen may be extended by dilution with talc or lycopodium spores without reduction in seed set (Wright 1953b).

Pollen of various ash species has been stored for a few weeks at 25 to 50 percent relative humidity and 40° F (4.5° C) (Wright 1953b). Anderson and Wheldon (1936) reported the storing of white ash pollen for at least 10 months, but conditions of storage were not specified. Nikolaeva (1962a) reported that green ash pollen that was kept in paper packets over calcium chloride inside a desiccator at room temperature remained viable for 2 months.

White ash pollen germinates best on a medium of distilled water with 2 percent agar and 15 percent sugar. Results for green ash were best on a 5-percent sugar concentration and declined as sugar percentages increased (Nikolaeva 1962b). The germination medium was smeared on glass slides, dusted with pollen, and placed in a moist chamber at 68° F (20° to 21° C). Germination commenced in 8 to 12 hours and was complete in 20 to 24 hours (Nikolaeva 1962b). The most viable pollen was obtained from fresh anthers.

Fertilization. Little detailed information is available on the fertilization process in Fraxinus. Wright (1953b) noted that many female trees were unable to mature fruit after pollination. In some instances, very heavy crops of completely empty samaras were produced.

According to Nikolaeva (1962a), mature ash pollen contains two cells: Vegetative and generative. With the formation and extension of the pollen tube at germination, the vegetative cell moves into the tube, followed by the generative cell. Meiosis occurs within the pollen tube and results in the formation of sperm cells, which migrate to the distal portion of the pollen tube. Nikolaeva's description ends here, but presumably fertilization proceeds normally by release of the sperm cells when contact is made with the ovum.

Pollination Techniques. Wright (1953b) used an eye dropper to apply fresh pollen to isolated white and green ash female flowers. The seed sets did not differ between the earliest pollinations (made when the panicles were just issuing from the bud and the pistils were 0.04 inch [1 mm] long) and later pollinations (made when the panicles were 2 to 3 inches [5 to 8 cm] and the styles were up to 0.3 inch [8 mm]) as long as the stigmas were in a fresh condition. Pollinations attempted after the stigmas started to wither were nearly complete failures. In a later publication, Wright (1962)
recommended small camel's-hair brushes or swabs of cotton as pollen applicators for *Fraxinus*.

In nearly all cases, intraspecific pollination gave somewhat higher seed sets than were obtained from open pollination. Incompatibility within species was not evident. Attempted crosses between white and green ash were unsuccessful (Wright 1953b). Problems and Prospects. In white ash, only about 1 in 10 mature trees is reliable as a female parent (Wright 1962). Therefore, progeny tests that may later serve as breeding arboretae should include 10 times more trees of each variety than might be included in a crossing program. With dioecious species, consideration must be given to establishing enough trees of each sex.

Because the pistils of ash are known to be receptive upon emergence, pollination bags must be applied 1 to 2 weeks before anthesis. At this stage, female buds are not readily distinguishable from vegetative buds. Pollination bags may be applied to barren branches thought to contain female flowers with the result that the desired number of flowers is not available for crossing. However, ash bears many flowered panicles, two or three of which may be included in each isolation bag (Wright 1962).

Insect injury to developing fruit is common in *Fraxinus* (Wright 1962). Continuous bagging appears beneficial and results in a greater seed set and higher yields of viable seed (Wright 1953b, 1951). The continuous bagging technique has been reported as only having been used on ash in the vicinity of Philadelphia, Penn. Before this technique is used on a large scale, one or more small trials should be conducted to determine whether or not the procedure is suited to local conditions.

**Black Cherry** (*Prunus serotina* Ehrh.)

There is uncertainty about the degree of selfing in black cherry. Also, the difficulty of emasculating this small-flowered species suggests that more research will be needed before reliable large-scale crossing can be done. Forbes' studies (1969, 1973) showed that, while the crossing procedure is relatively straightforward and productivity is adequate for breeding research, the uncertainty of parentage is a problem in black cherry. He observed no selfing in a Florida population, but about 20 percent of the self-pollinations in a Tennessee population set fruit with normal embryos, though all fruit subsequently dropped before maturity.

Though crossing requires further investigation, the successful collection, testing, and long-term storage of pollen is relatively simple. Small amounts of pollen can be obtained by the gentle agitation of floral racemes over a 40-mesh wire screen during the period of anther dehiscence. Forbes noted that the anthers break off and drop into the receptacle where pollen shedding is completed. Pollen is then separated from the anthers by sieving.

Farmer and Hall (1975) noted that fresh black cherry pollen germinated quickly (tubes elongate within 6 hours) on several media, but they recommend a medium containing 1 percent agar, 10 percent sucrose, and 0.01 percent boric acid. Pollen that is sealed directly in vials or vacuum dried can be stored at 37° F (3° C) for up to 3 years, but storage of pollen at −22° F (−30° C) retains up to 50 percent viability for over 4 years.

Black cherry is pollinated by wide-ranging insects, which suggests that wide isolation strips for seed orchards may be needed, and that orchard beehives would probably be desirable.

**Black Walnut** (*Juglans nigra* L.)

Several major genetic improvement programs are underway with black walnut, but controlled crossing may never be used on a production scale because full-sib seed is too costly. So, pollen handling will probably be restricted to breeding research. A successful crossing technique has been developed by Forbes (1974) and modified by Beineke and Masters (1976). Briefly, it consists in first bagging branches with pistillate flowers immediately after their anthesis but before their receptivity and then pollinating them by the syringe technique commonly used in conifer breeding. Walnut is believed to be technically self-fertile. But because male and female anthesis occurs at different times, selfs are rare. The terylene or Pollentector bags, which are suitable, are removed after about 2 weeks, and marked fruit is harvested after natural abscission. Beineke and Masters report that about 24 percent of the flowers pollinated in this way develop into fruit.

Fresh pollen for this technique can be easily obtained by collecting catkins when they begin to turn yellow and extracting them in soil sieves, or the like. If the cut-branch procedure is used, branches should be collected and placed in water at the first yellowing of catkins. Pollen obtained by these techniques should be free flowing and have a moisture content of 10 to 15 percent.

Viability of black walnut pollen can be tested *in vitro* at 80° F (27° C) with either liquid or agar media containing 10 percent sucrose (Hall and Farmer 1971). Adding boric acid (100 ppm) to the medium enhances germination. Too much pollen has a bad effect. About 100 grains per mm² is required for optimum germination. Under these conditions, germination takes place within 4 hours after pollen is placed on media. Beineke and others (1977) noted that maximum germination counts are obtained in 6 to 8 hours. Wide clonal variation in the germination percentage of fresh pollen has been reported by those investigating walnut pollen viability with the above medium. Beineke and others (1977) also noted major differences within clones from year to year.

Pollen can be stored several weeks at 39° F (4° C), but the most successful long-term storage (over 1 year) has been in liquid nitrogen at −385° F (−196° C) (Farmer and Barnett 1974, Beineke and others 1977). Pollen stored at this low temperature has exhibited slightly higher germination percentages and seed set (29 vs. 25 percent) than fresh pollen. Clones vary widely in time of anthesis, so the use of stored pollen is required to cross early flowering clones conveniently with late ones.
The distribution characteristics of black walnut pollen which might be useful in seed orchard design are unknown. In J. regia, however, most pollen falls 200 to 400 feet (61 to 122 m) from the parent tree (Funk 1970).

**Eastern Cottonwood (Populus deltoides [Bartr.])**
Cottonwood improvement has relied upon the development of selected clonal lines. Because the species roots easily from stem cuttings, propagation for production plantations will probably continue along these lines. Cottonwood seed orchards will probably not be developed because a single female tree produces millions of seeds. However, controlled crossings among selected parents will be required for improved performance in growth and resistance to insects and diseases.

**Flower Structure and Development.** Cottonwood is dioecious with a sex ratio of about 1:1 (Farmer 1964b). Flowering is apparently related to tree vigor. Most forest trees begin flowering between 10 and 15 years of age (Schreiner 1971), but flowering may occur as early as the 4th year. Dhir and Mohn (1974) reported that 75 percent of the trees in a Minnesota test plantation were flowering at the age of 6 years. Provenance differences that are related to flowering have not been established.

Sexually mature cottonwood trees begin to form flower buds in early to late spring. By midsummer, the larger staminate flower buds can be readily distinguished from vegetative buds. Pistillate flowers are not as easily recognized, and verification requires removal of the bud scales and examination of the internal structure (Farmer 1976). Occasionally, both types of flowers are found on the same tree. Flower buds become dormant in the fall, and a period of chilling is required for further development. The exact chilling requirements are not known, but by mid-December in the lower Mississippi Valley, chilling is sufficient to allow the development of flowers (Farmer 1964a). The megaspore mother cells differentiate in the spring. Microsporogenesis immediately precedes anthesis. Flower buds can be forced to open early under greenhouse conditions and will still produce viable seed (Miller 1972).

**Anthesis, Pollination, and Fertilization.** Male flowers ripen and shed pollen a few days before females, thus insuring that pollen is in the air when the first female trees flower. Both types of flowers appear before the first leaves of spring. Most variation in flowering time is due to differences among trees within stands. Relative flowering date is probably a highly heritable trait in cottonwood (Farmer 1976). This variation in pollen shed and receptivity results in a longer pollination period of from 2 to 3 weeks.

Pollen germinates within a few hours after pollination. Fertilization takes place several days later and is usually complete within 10 to 14 days after pollination. Open-pollinated eastern cottonwood produces about 30 seeds per capsule with about 27 capsules per catkin (Schopmeyer 1974). A mature open-grown tree yields several million seeds annually. Intraspecific controlled pollination produces yields comparable to open pollinations in most cases (Miller 1972). However, interspecific crosses have frequently had low seed yields. Mixing viable pollen of the donor species with killed “recognition” pollen of the receptor species helps overcome pollen incompatibility (Stettler 1968).

Pollen storage is generally unnecessary because pollen-forcing techniques are quite successful. For short periods of 2 to 3 months, eastern cottonwood has been satisfactorily stored at low humidity at 37° to 41° F (3° to 5° C) (Farmer 1976). Knox and others (1972) report the storing of cottonwood pollen for several years at 0° F (−18° C) without loss of viability.

**Oaks (Quercus spp.)**
Ledig's work (Ledig and others 1971) with white oak (Q. alba L.) is the only American research on pollen handling in oaks. Ledig's study established that controlled pollination with fresh pollen can result in higher seed set than open pollination (24 vs. 7 percent) and that pollen stored for 1 year is capable of fertilization. The in vitro germination media used were aqueous solutions of 0, 5, and 10 percent sucrose. Stairs (1964) used an aqueous medium of 20 ppm boron and the hanging drop technique to germinate pollen successfully of Q. alba, Q. coccinea, and Q. ilicifolia. Ledig and others stored white oak pollen (viability 34 percent) for 295 days at 36° F (2° C) and 23.5 percent relative humidity with reduction of viability to 20 percent, but, at higher relative humidity, complete loss of germinability within 70 days was noted. Freeze-dried pollen retained viability of 9 percent at the end of 300 days at 23° F (−5° C). These results suggest that the development of techniques for other southern oaks should be relatively simple and that, at least for white oak, controlled crossing should be feasible as a breeding procedure. Pollen production and dispersal patterns are not well documented for American species of oak, but it is known that most oak pollens are wind-pollinated and that mature oaks have high levels of pollen production.

**Sweetgum (Liquidambar styraciflua L.)**
Sweetgum is a wide-ranging and commercially important species throughout the South. Genetic improvement programs are currently underway and should lead to the development of seed orchards in several locations.

**Flower Structure and Development.** Sweetgum is monoecious with both male and female flowers arising from the same bud. The flowers are imperfect with both types appearing in heads. Staminate heads occur as racemes with the flowers lacking a calyx and corolla. Stamens are numerous, and pistillate heads are terminal and solitary. Female flowers have from two to four nonfunctional stamens, a two- to four-celled ovary, and no calyx or corolla (Schmitt 1964).

Flower buds of sweetgum are larger than vegetative buds and can be recognized several months before flowering (Schmitt 1964). Floral buds flush before the vegetative buds. Meiosis and subsequent maturation of pollen grains begins.
soon after bud break, and anthesis follows in 2 to 3 weeks. The greenish-yellow male glomerules are crowded at the summit of a stout, erect floral axis. About 2 days before anthesis, the terminal anthers develop a distinctive red color. Pollen ripens sequentially from the tip of the rachis to the base (basipetally) and is usually shed over a period of 1 to 2 days, although it may be extended for as long as 9 days for a single tree (Schmitt 1964). During pollen shed, the staminate rachis becomes flaccid and dehisces upon the completion of anthesis. The pistillate heads continue their development and assume a drooping position after pollination.

Occasionally, the nonfunctional stamens associated with the female flower produce viable pollen. The pollen is released 3 to 4 weeks after the staminate flower clusters have shed their pollen. In the interim, the pistillate ovules have been fertilized or aborted. The stamens in the pistillate flowers could play some role in cross-pollinations (Schmitt 1964).

**Pollen Collection, Testing, and Storage.** The collection of viable sweetgum pollen presents no difficulties to tree breeders: fresh flower-bearing branches have been collected and inserted in water-filled bottles in the laboratory, and pollen has been collected from dehiscing anthers (Beland 1973). Clusters of anthers have been stripped from the flower buds as they turn red and placed in nylon net bags suspended in Kraft paper or viscose isolation bags. Mature pollen was shed in about 24 hours at room temperature of 68° to 77° F (20° to 25° C), and it was transferred from the outer bags, where it had fallen, to clean, dry, 16-ml glass vials (Schmitt 1964).

After testing the forced-air technique described by Snyder (1961) for pine pollen, Beland (1973) concluded that the viability of sweetgum pollen was greatly reduced by this procedure. Schmitt (1964) reported that pollen germination tests with several liquid and solid media gave poor results. Beland (1973) found a modified hanging-drop technique to be quick, easy, and accurate. Germination of fresh pollen ranged from 90 to 100 percent and was complete in 4 hours.

Extracted and stored pollen has a slightly lower germination and requires more time on the test media to complete germination. After 40 hours of drying and 30 hours of refrigerated storage (39° F or 4° C at 50 percent relative humidity) germination ranged from 80 to 97 percent, but germination extended over 18 hours (Beland 1973).

**Fertilization.** Sweetgum is wind-pollinated and under natural conditions produces seed every year with bumper crops about every 3 years. Late spring freezes can completely wipe out a seed crop (Schompeyer 1974). According to Schmitt (1964), maximum pollen germination usually occurs within 36 hours after pollination. Pollen tube growth normally proceeds along canals that develop in the style, but pollen tubes have been observed in all tissues of the style. At the base of the style, the pollen tube turns sharply upward and enters the placental tissue, continuing growth until it reaches the base of an ovule. Here, the pollen tube rests until the embryo sac is formed in the ovule, then enters the ovule where fertilization is consummated. Pollen tube growth reaches the ovary within 4 days, but Schmitt did not observe pollen tubes in the ovules until 18 or 19 days after pollination.

Sweetgum is self-sterile (Schmitt 1964). The stigmatic surface remains receptive for 2 to 3 weeks, and ovules mature sequentially in the ovary. Staggered anthesis maintains pollen over an area for 2 weeks or more, resulting in maximum opportunities for outcrossing in natural stands or orchards. The sweetgum “ball” has a potential of 1,500 or more seeds, but it never produces that many. Usually only one or two seeds mature per carpel, and each head yields about 56 viable seeds (Schompeyer 1974). Santamour (1972a) reported 81.2 seeds per head in open-pollinated sweetgum.

**Crossing Techniques.** Even though sweetgum is self-sterile, flowers must be emasculated. Failure to emasculate them greatly reduces seed yields. Emasculation is best accomplished when leaves are emerging from bud scales and the bracts of both staminate and pistillate heads are slightly parted. When the pistillate head droops out of the inflorescence, it is too late to emasculate (Schmitt 1964).

Once the staminate flowers and young leaves are removed, the pistillate flowers are covered with an isolation bag. Pollen is introduced through a pollen injection needle and directed to the flowers. The puncture is sealed upon withdrawal of the applicator. Isolation bags should remain in place for 30 days after pollination (Schmitt 1964).

Santamour (1972a) followed a similar procedure to accomplish interspecific crosses in *Liquidambar*. All interspecific crosses gave good yields of hybrid seed and seedlings. Apomictic seedlings were obtained in *L. styraciflua* and *L. orientalis*. Schmitt (1964) also obtained apomictic seed.

**Problems and Prospects.** Sweetgum pollen can be collected and extracted easily for the current year’s controlled pollinations. An adequate method for pollen storage is not available.

Late spring frosts can severely damage emerging flowers. Seed orchards and breeding arboretae should be located where late frost is not likely to occur or where amelioration measures can be applied if needed.

**Sycamore (Platanus occidentalis L.)**

Sycamore has been included in tree improvement programs for over 10 years, but only a few studies have used controlled crosses (Beland and Jones 1967, Santamour 1972b). These studies indicate that controlled pollinations can be accomplished fairly easily by bagging pistillate flowers with either terylene or viscose bags as they emerge and by pollinating the flowers with freshly extracted pollen. Filled-seed yields of up to 80 percent have been obtained from controlled pollinations.

Sycamore is self-incompatible. Less than 2 percent of its seed that results from self pollination germinates normally (Beland and Jones 1967). Self-pollen germinates but does not penetrate the stigmatic surface, while pollen tubes have been
found in or near the micropyle within 30 hours after cross-pollination.\(^2\) Pollen can be obtained by collecting staminate flowers just before normal pollen shed and placing them in a warm air extractor. One hundred normal inflorescences will yield about 8 cc of pollen. Female flowers remain receptive 15 or 16 days after bud break, with pollen shed beginning on the same tree 4 to 7 days after the female flowers have broken bud (Beland and Jones 1967).

Sycamore pollen germinates on a variety of media, but the hanging-drop method and Nygaard's (1969) medium have provided best results. Pollen that was refrigerated for 3 days after extraction by drying flower heads in a single layer in Kraft bags gave 60 percent germination. No difference was found between the germination counts made after 4 hours and those made after 24 hours. Germination rates in distilled water and 0.5, 1.0, 5.0, and 10 percent sucrose solution were much lower (see footnote 2). Methods for long-term storage of sycamore pollen have not yet been developed.

When open grown, sycamore produces many male and female flowers, although male flower crops may be light on young trees. In many cases, two or three female inflorescences can be isolated in a single bag. Because each inflorescence will yield more than 1,000 seeds, the production of enough control-pollinated seed for breeding work should be easier for sycamore than for most southern hardwoods.

**Yellow-Poplar (Liriodendron tulipifera L.)**

Yellow-poplar has been the subject of several breeding programs in the Southeast for over 15 years, and successful crossing techniques were developed early. However, pollen physiology and handling research has been limited by the peculiar reproductive characteristics of this species. For example, yellow-poplars have perfect flowers that bloom over a period of several weeks. Thus, within-tree variance in time of anthesis is usually greater than among trees in an orchard of common origin clones. Also, the species is almost totally pollinated by insects. The most commonly used crossing procedure, first developed by Taft (1962), consists in emasculation and pollination immediately before anthesis. Fresh, uncontaminated pollen is easily obtained by placing screen-protected floral branches in water a few days before anthesis. Corollae are removed from these flowers, and their dehiscing anthers are used directly in pollination. Details of the procedure are given by Taft (1962) and Cox (1973). This predominant use of fresh pollen, which is available from individual trees throughout the flowering season, has resulted in little investigative attention being given to pollen viability testing and storage.

Low seed set (5 to 30 percent depending upon parentage) is characteristic of controlled crosses with fresh pollen, though several studies have demonstrated that open-pollinated and selfed flowers have even lower average percentages of filled seed. This difference in seed set and the generally low fertilization rate of yellow-poplar is mainly caused by inefficient insect pollination (Boyce and Kaeiser 1961, Thor and others 1976). While the fresh pollen used in crossing programs has usually not been tested in vitro for viability, results of diallel crossing schemes and other data (Thor and others 1976) suggest that variation in viability may also account for observed differences in the percentage of seed set.

The work of Wilcox (1966) is the only published information on pollen storage and in vitro testing of viability. He found that freshly extracted pollen from excised dehiscing anthers had a moisture content of 27 percent and exhibited 83 percent germination on 1 percent agar that contained 10 percent sucrose. Vacuum drying for 30 minutes reduced this germination percentage to 20 percent, and 8 hours of drying reduced it to 7 percent. During storage of vacuum-dried pollen for 1 to 12 months, these germination percentages were reduced further. The most successful long-term storage (12 months) was in cotton-stoppered vials at 41°F (5°C), where viability was 7 percent, though 0.5 to 1 percent of vacuum-dried pollen germinated after this period.

**Summary**

The reproductive processes in southern hardwoods present unique problems that require special approaches in pollen handling. Because hardwood pollen rapidly loses viability unless it is extracted and stored under refrigeration, fresh pollen should be used wherever possible.

Seed orchards have been established in several species of southern hardwoods by grafting and with seedling stock. Management techniques are under development. Some early results with sweetgum indicate that ammonium nitrate and diammonium phosphate stimulated flowering but a hydrazide growth retardant had no effect (Jett and Finger 1973).

That some hardwood species are dioecious can be of immense value in the design of seed orchards for the production of hybrid lines. Self-sterility in some of the monoecious species would also help in developing hybrid lines in breeding orchards; however, some species are apparently self-fertile, and require an entirely different approach to pollen management. Many hardwood species are wind-pollinated, but a few commercially important ones are insect-pollinated. Pollen management in these species may require a novel approach to insure adequate production of improved seed.

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Wright, J. W.
Of the more than 400 *Eucalyptus* species plus some 100 subspecies, most are adapted to insect or even bird pollination. According to Pryor (1976), only one or two species are wind-pollinated, one being the forest redgum, *E. tereticornis* Sm. Because eucalypt flowers are bisexual, their pollen management differs widely from the methods that are normally used for conifers. Eucalypt pollen is heavy and sticky and not easy to collect and handle in large quantities; therefore, large-scale hand pollination is not feasible. Rather, seed orchards should be planned and managed so as to encourage maximum production of viable seed.

One of the few comprehensive studies on the reproductive behavior of eucalypts was done by Hodgson (1975, 1976a, 1976b, 1976c). Although his research was restricted to flooded gum, *E. Grandis* Hill ex Maid., it is representative of many insect-pollinated eucalypt species. A wide variety of insects has been observed on *E. grandis* flowers; however, the honeybee (genus *Apis*) is the main pollinating agent in African eucalypt plantations. Because of the foraging behavior of honeybees, the contamination of seed orchards from foreign pollen sources can be reduced through proper orchard placement and management.

The eucalypt inflorescence is a cyme that is considerably reduced but still has the branching structure that resembles the regular cymose dichasium (Pryor 1976). The inflorescence is commonly referred to as an umbel, which may bear several flowers (often consistent in number) on short pedicels.

The flower bud is a cuplike receptacle that carries an operculum—a distinctive feature of the eucalypt flower. In most eucalypt species, the operculum is a combination of two caps—an inner cap which covers the pistil and an outer cap that covers the stamens of the bisexual flower before anthesis. The outer cap may fall away several days or weeks before the flower blooms. The stamens are carried in a staminal ring on the rim of the floral cup. At anthesis, the inner cap falls away, enabling the stamens to unfold. The ovary is situated at the bottom of the floral cup, and the style and stigma commonly extend beyond the rim. These features facilitate emasculation of the flower bud.

Because the open eucalypt flower is without calyx or corolla, the coloration (often quite brilliant) of the stamens is a basic attractant of insects. This was confirmed by Pryor (1957) and Hodgson (1975), who showed that pollination and seed set are negligible in the emasculated flowers of *E. grandis*.

Another feature that encourages insect pollination of most eucalypt species is the production of abundant nectar in the flowers. The nectar flow yields a high-quality honey, which is an incentive to include apiaries in the management plan for seed orchards.

The structure of the anthers is so distinctive that it was adopted for the first classification of *Eucalyptus* by Bentham (1867) (see Pryor 1976). Even though recent classifications have adopted other physical features, the antherial classification was important for many years: for Blakely (1955), the shape of the anthers still formed the basis for classifying the eucalypts into sections and subsections. Three main anther shapes are illustrated by Pryor (1976).

Dehiscence, either by longitudinal slits or terminal pores in the anther, will release pollen grains. In most eucalypt species, the pollen grains are relatively heavy and sticky when compared with pine pollen. Although flowers on any individual tree may occur in abundance, the pollen that is shed from single flowers is sparse compared with *Pinus* or *Cupressus*. These features affect the collection and handling of pollen.

**Flower Production and Pollen Yield**

Most eucalypt species flower abundantly. And, although pollen from individual flowers may be sparse, the availability of flowers should not cause a shortage of pollen. In *E. grandis*, for instance, single ramets in the seed orchard produced up to 30 lb. (13.6 kg) of capsules at 7 years, yielding about 2 lb. (1 kg) of seed and chaff.

A distinct clonal variation was found in the flowering period of *E. grandis*, but generally enough overlap occurred among clones that a cross-pollination program would not be impeded through lack of pollen. There is also a clear difference in the flowering season for *E. grandis* that is planted at different altitudes. In South Africa, at an altitude of 3,940 ft (1,200 m) with about 67 inches (1,700 mm) mean annual rainfall, the main flowering season is from April to June. At 2,490 ft (760 m) and a lower rainfall of about 37 inches (930 mm) per year, the corresponding season is February to March.

The first flowering in *E. grandis* occurs at 2 to 3 years of age. However, this is not so for all eucalypt species; for example, shining gum (*E. nitens* [Deane & Maid.] Maid.) would very rarely produce flowers before the age of 10 years in South Africa. Grafted ramets in the *E. grandis* orchard produce seed 2 years after planting.

**Pollinating Agents**

Pollination in almost all eucalypt species is done by insects. Diverse insects visiting eucalypt flowers have been observed by Pryor (1976) in Australia. These insects include flies, beetles, syrphids, calliphorids, and bees. To some species, especially those with larger flowers, birds such as honeyeaters and brush-tongued parrots pay regular visits to the flowers.

In *E. grandis*, Hodgson (1975) also observed a variety of insects from at least five orders that were active on the flowers; however, most insects have relatively sluggish habits or no set patterns of visiting flowers. Therefore, the honeybee is the most likely pollinator because of its active foraging behavior.

Honeybees are methodical, and their foraging generally follows set patterns that promote a large amount of pollination. A colony of honeybees is highly organized, and different members of the colony may perform different tasks (Grout

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1 The author is indebted to L. M. Hodgson and W. K. Darrow for their critical reviews of the manuscript.

2 Officer in Charge, D. R. De Wet Forestry Research Station, Department of Forestry, Sabie, Republic of South Africa.
Once bees have started working on a certain species they will return to the same species and most often to the same foraging area constantly (Free 1970). Free (1970) states that bees usually alight near the same point in their foraging areas, especially if there are colored marker points in uniform areas. Bees can distinguish among yellow, bluish green, blue, and ultraviolet.

Honeybees may travel long distances if food is not readily available, but most of them usually work in the vicinity of the hive. When food is available, an individual bee will return to a single tree for several visits, but it may “jump” from tree to tree within a restricted area. Competition by bees from other colonies will also cause bees to “wander.” In fruit orchards, trees closest to pollinator individuals (i.e., single trees planted for pollen supply) set fruit much better than trees several rows away (Free 1970). The foraging behavior of the honeybees, their constancy in visiting the same species, the same areas, and the same trees during consecutive trips, can be used for indirect pollen control through planned seed orchard management.

Pollen Management and Procedures

Pollen Collection, Extraction, and Application. The comparative sparsity of pollen and its sticky nature affect its collection and handling. The collection of pollen is a tedious task because of the bisexual nature of the flowers. The pollen is inclined to stick to certain kinds of storage containers. (Glass, for instance, should not be used.) Methods described here were applied to E. grandis (Van Wyk 1977) and may not be applicable to all eucalypt species.

It is necessary to refer to two different methods of applying pollen because they affect collection and storage procedures: “by anther” and “by brush.” Collection for the latter is more tedious, but it is necessary to use this method whenever it is desired to mix pollens from different sources (e.g., when investigating selective fertilization).

Pollination by Anther. Isolate flowering branches before anthesis by using a loosely woven cotton bag or tube. Cover the upper part of the bag with a sheet of plastic as protection against rain. About 2 days after anthesis, cut a flowering branch (flowers still in isolation) and carry it around to do pollination. Open the bag, pick a single flower with tweezers, reclose the bag, and apply the pollen by rubbing the anthers on the stigma of the flower to be pollinated. The pollen flower must be handled carefully to avoid nectar contamination of the pollen, which reduces the efficiency of pollination. Alternatively, a section of staminal ring with a few stamens attached can be broken off the flower cup and used in a similar way.

When the pollen grains are released freely by the anthers, the anther method is quick and simple, and it saves time. The main disadvantage is the need for more pollen flowers than with the brush method. Furthermore, because the anthers are contaminated by stigma secretions, it is difficult to determine how much pollen is available on a pollen flower after it has been used for a few pollinations. If enough flowers are available, it is best to use a flower as a pollinator only once or twice. It is possible by chance to pick a flower producing sterile or low variability pollen, which results in little or no seedset. The disadvantages can be reduced through experimentation and experience.

Pollination by Brush. Some pollen is frequently shed inside mature flower buds before anthesis. This pollen, when it is used for cross-pollination, is viable and can be present in sufficient quantities to give appreciable amounts of fertilized seed in E. grandis.

Therefore, there are two possible procedures for collecting pollen. First, mature flower buds may be collected, the anthers removed, dried, and crushed to release pollen grains. This method is useful under rainy conditions. Second, if mature flower buds are isolated, uncontaminated pollen will be available as a result of maximum pollen shedding that occurs within 2 days after the fall of the operculum. It is important that flowers are isolated before anthesis because insect visits will contaminate and also reduce the amount of available pollen. After the mature flowers are collected, excess nectar is removed from them with a piece of blotting paper. The rim of the receptacle is severed, and the staminal ring with the stamens still intact is obtained. A cut in this ring will enable the stamens to be flattened out on a smooth surface. The anthers can then be scraped off the filaments with a sharp object, e.g., a razor blade. The anthers are placed in a container (a matchbox is suitable), dried in a desiccator with silica gel for a few hours, and crushed to get maximum release of pollen. After being misted, the pollen is brushed onto the stigmas with a small camel’s-hair paint brush. Stigma secretions will contaminate the brush, which should be cleaned regularly with water. (More than one brush is advisable.)

Drying and Storage of Pollen. Pollen should be kept as fresh as possible. Drying pollen together with another tissue or free from it over silica gel is adequate. Pollen can be stored for a few days under low temperature of about 2° to 4° C, but for E. grandis it was found that the germination of pollen drops considerably after 10 days of storage. Because flowers and pollen are always available in abundance, no investigations have been made on the storage of E. grandis pollen over a prolonged time. However, Pryor (1976) reports that dry pollen that is sealed in a tube and placed in a deep freeze will retain germinative capacity for more than 1 year. When it is kept at -50° C, it has been found to be still functional after 3 years and might remain so for much longer.

Pollen Germinability and Maturity

According to Pryor (1976), pollen viability can be assessed readily in vitro by placing a sample on an agar plate with a suitable sucrose content and incubating at 23 °C. Brune finds that 1½ percent agar, 35 percent sucrose, and 100 to 250
p/m boric acid is the most suitable medium.\textsuperscript{1} Germination is observed at 6 hours and will generally be complete in 24 hours. Boden (1958) showed that 6 hours of desiccation improved the percentage of germination of manna gum (\textit{E. viminalis} Labill.).

The hanging-drop method (Boden 1958) involving a 20-percent sucrose solution was adopted by Hodgson (1975). \textit{In vivo} germination on the stigma is relatively rapid. A method that is used by Hodgson to investigate receptivity in \textit{E. grandis} can also be used for testing pollen germination \textit{in vivo}. After pollination, styles are placed in a large drop of xylol on a slide, and stigmas are scraped with a needle that deposits pollen grains into the xylol. The shortest time for the commencement of germination is 4 hours, but 8 to 10 hours is more desirable for this kind of investigation. Longer periods cause the pollen grains to collapse, and the pollen tubes become attenuated and harder to observe.

\textit{E. grandis} pollen on mature flower buds are viable before anthesis (Hodgson 1975). Self-pollination may occur, but generally the stigma is not receptive at anthesis. Hodgson has also shown that pollen will remain viable on a stigma for several days, which results in effective fertilization even though stigmas are not receptive at the time of pollen deposit. Selective fertilization also occurs. Therefore, “foreign” pollen will be favored where a mixture of self- and cross-pollination occurs on immature flowers. Overall viability of pollen is low. Hodgson (1975) found an average of only 58 germinations per stigma compared with 1,183 ungerminated grains per stigma 4 days after anthesis.

In pollen testing of \textit{E. grandis}, one out of some 45 clones was found by \textit{in vitro} testing to produce pollen that did not always germinate. It was thought that this condition might be related to the difference between two types of (long and short) stamens, but negative results were obtained from tests of this theory. Apparently, certain clones are liable at times to produce sterile pollen. One such clone was found in which virtually no seed was obtained when this clone was crossed with 14 other clones (Van Wyk 1975). Considerable variation in the percentage of pollen germination among trees has also been shown (Boden 1958).

### Controlled Pollination Procedures

Van Wyk’s methods (1977) for \textit{E. grandis} are restated here because they should be applicable to most eucalypt species.

Mature flower buds are selected, and open flowers and a number of leaves surrounding the buds are removed to avoid contamination. All flowers on a twig do not normally mature at the same time; therefore, immature flower buds are also removed, leaving up to a dozen buds to be enclosed in the isolation bag. A large number of flower buds and umbels are sometimes removed in this process, but there is no point in emasculating immature flowers because their receptivity is low (if there is any receptivity at all), and emasculation will only exhaust the pollen supply. When studies are done on the number of seeds obtained per pollinated flower, it is important that the flowers be at maximum receptivity.

The selected flower buds are emasculated with maximum precautions being taken to avoid pollen contamination. Emasculation involves the cutting through of the tissue of the lower cup slightly below the staminal ring with an emasculating tool. This tool is an improved version of the kind described by Meskimen (1965).

During this operation, the flower is supported between the thumb and the forefinger to minimize the twisting of the pedicel. The first cut usually removes the whole staminal ring with the stamens and operculum attached, but some anthers may remain at the base of the floral cup and they need to be removed with a pointed instrument. A jet of water or blowing on the flower will remove residual pollen grains that are likely to be deposited on the stigma during emasculation.

After emasculation, a wire spiral is drawn over the branch to enclose the flowers and to support a loosely woven, white cotton tube 27 cm by 10 cm (usually referred to as a bag). The spiral is kept in place by tying its ends to the branch, using cotton wool to limit abrasion. The bag or the wire should not touch the stigmas of the flowers. If a branch is too short to support the wire spiral, it must be extended with a twig to enable the fixing of both ends of the spiral. The bag is drawn over the spiral and closed at both ends. The bags are then labeled. Flowers reach maximum receptivity 4 to 6 days after the fall of the inner operculum. At this stage, the stigma is swollen and secretes a sticky substance that facilitates the adherence of pollen grains and may act as a medium for pollen germination. Because flowers are very receptive, special precautions against contamination should be taken by removing open flowers in the vicinity of the bag and by keeping hands and instruments clean. Bags are opened at one end, and the pollen is applied either by brush or by anther.

After being closed, the bags are covered on the upper side with a transparent sheet of plastic material for 1 or 2 days as protection against the possibility of rain, which will wash pollen off freshly pollinated stigmas. The bags are kept in place until styles have fallen, which may be 2 to 4 weeks after pollination.

The controlled pollination operation requires careful planning and recordkeeping. Emasculation and bagging are done before pollen flowers are isolated to insure that stigma receptivity and pollen availability coincide. A convenient schedule is emasculation and bagging on Wednesday and Thursday and pollen flower isolation on Friday, leaving the weekend for the development of the pollen and the emasculated flower. The recording is done on field sheets and includes the dates of emasculation, bagging, harvesting; the number of flowers that were emasculated and pollinated; the number of capsules that were harvested; and other information depending on the requirements of the project.

One should observe developing flowers and capsules so as to record losses because of mechanical damage, and delete those flowers from the record. One should also note flowers

\textsuperscript{1}Brune, A. 1978. Personal communication. Universidade Federal De Vicoas, Escola Superior De Florestas, 36570 Vicoa, Minas, Gerais, Brazil.
that were lost through abscission, because the losses may result in underestimates in tests of the numbers of seeds per capsule. Hygroscopic tissue is cut in the emasculation process, which might cause valves to open and seeds to be lost.

It is also important that, because branches with emasculated flowers can be suppressed and cause the death and loss of seed, competing branches around the bag be cut back 1 month after controlled pollination.

Effects of Isolation Procedures

It is very important to take maximum precautions against contamination during pollination operations. “Foreign” pollen will stick to isolation material, the surrounding flower buds and leaves, and may also drop onto emasculated flowers from other flowers immediately above.

In testing the contamination that might occur during controlled pollination procedures, Hodgson (1975) emasculated flower buds at various stages of maturity, then placed isolation bags, some of which were opened as if for pollination. Although 1,846 flowers were emasculated for this purpose in the course of several years, only 648 capsules were recovered, from which only 10 seeds were obtained. Comparatively, at an average of 30 seeds per capsule, the potential seed yield after pollinating 1,846 flowers at maximum receptivity is 44,304 seeds, assuming a fruit set (confirmed in tests) of 80 percent after emasculation and pollination. Hence, contamination is less than 0.03 percent.

Seed Orchard Management and Mass Pollination

The work of Hodgson (1975) and that of Krug and Alves (1949) suggest that most eucalypt species are outcrossing. Mass pollination will not be possible in eucalypt seed orchards, except perhaps for the isolated species that are wind-pollinated. Although tests for selective fertilization in E. grandis showed that emasculation is not necessary to obtain a majority of cross-fertilized seed, mass pollination is still such a labor-intensive task that it will not be considered in any seed orchard management plan.

If it is established that the bulk of the pollination in the seed orchard is done by a methodical insect like the honeybee, some indirect control over pollen management may be possible. First, seed orchards can be provided with beehives to prevent bees from carrying in foreign pollen in those cases where orchards are not well isolated. Care should be taken that enough food is available inside the orchard for all bee colonies so that they will not be forced to go outside the seed orchard area. Second, the ramets within a clonal orchard can be planted so that cross-pollination is promoted. The foraging behavior of the honeybee within a restricted area and its constancy in visiting the same area suggest that the trees should be placed close to each other, perhaps in clusters or with close spacing within rows.

If information is available from controlled pollinated progeny tests, it can be used to promote pollination for outstanding specific combinations. Parents with high specific combining ability can be planted in pairs, or even in groups of three or four ramets from the different parents in juxtaposition. Branches may intermingle, and the pairs or clusters will provide a restricted area over which bees can “wander.”

The above idea has not yet been tested in E. grandis seed orchards in South Africa. All orchards still consist of ramets planted at 27- by 27-foot spacing. There is some evidence that the number of germinations per kilogram of seed from plantation collections is higher than that for seed orchard collection. Perhaps this is attributable to more self-pollination in the seed orchards with a wide spacing compared with that of plantations.

Isolation distance of the seed orchards will depend on the management of bee colonies. An isolation distance of 200 m will be adequate. In South Africa, one E. grandis seed orchard is screened off with a plantation strip of 100 m. The trees in this plantation come from seed produced in another orchard (with the same clones), which is completely isolated because of its location within surrounding pine plantations. E. tereticornis is partly wind-pollinated and is also known to hybridize with other species, e.g., with E. grandis and Sidney blue gum (E. saligna Sm.) (Shelbourne and Danks 1963). In such cases, isolation distance will naturally be increased, maybe up to 2 km if true-to-type seed orchard offspring is desired.

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Van Wyk, G.
Numerous plantings of European black alder (Alnus glutinosa, L.) throughout the Southeastern United States have stimulated considerable interest in this species. These plantings have exhibited rapid juvenile growth over a wide variety of sites (Kellison and White 1979). The continued diversion of productive hardwood sites to higher priority uses and the seasonal inaccessibility of wetland site hardwoods make necessary the selection or development of a species that will be available for year-round harvesting. These year-round sites are usually occupied by pines and slow-growing upland hardwoods. Alder, as an alternative species on these sites, can provide earlier and greater gains than would be possible from recurrent selection breeding and movement of indigenous hardwoods to nonalluvial sites. Alder itself is an alluvial sites species, but its early adaptability to a wide variety of soil types and pH's makes this species a prime candidate for study.

Alder is capable of considerable nitrogen fixation (Tarrant and Trappe 1971), and, under appropriate situations, it may be a viable alternative to energy-intensive commercial fertilizer for the production of hardwood and softwood fiber. Black walnut (Juglans nigra, L.) interplanted with alder was taller than walnut grown alone at the same spacing and was as large or larger in diameter than when grown alone (Funk and Krajicek 1975).

There is a need to locate a correct seed source and develop a genetic improvement program for black alder. This species can be genetically improved for fiber and nitrogen production and site adaptability. (An excellent overview of alder improvement is given by Hall and Maynard 1979.)

A method for the controlled pollination of European black alder on selected 5-year-old trees in northern Alabama is presented in this chapter together with procedures for the collection, extraction, testing, and storage of black alder pollen.

**Flower Morphology and Phenology**

European black alder is precocious. Trees have been observed to flower at the end of the first growing season. This precocity presents an opportunity for rapid generation turnover and therefore genetic improvement for various situations, although Verweij (1971) cautions against selections that are too early.

Black alder is monoecious with flowers of both sexes developing from buds that begin to appear in the spring of the year before pollination. It is wind-pollinated (Funk 1965). Two to five male catkins are borne at the ends of the branches. Catkins are long, drooping, and green or purplish in color. Two to 12 female catkins are formed on lateral shoots of the same branches bearing the male flowers. These buds are oval, erect, and dark crimson. The buds enlarge from 1.0 to 2.5 cm in length and become green as the fruit matures (McVean 1953). As the male flower matures, it elongates and becomes flexible. At anthesis, the flower is fully extended, and is yellow-brown. The male catkin can withstand a temperature as low as -6° C; but, once elongation starts, high winds cause desiccation and low temperatures result in the loss of viable pollen.

In northern Alabama, warm weather in late January and early February speeds flower development. Extreme fluctuations in temperature result in obvious damage to the pollen catkin and subtle damage to the pollen grains. Pollen dispersal from one tree will extend over several days. In a population, it will extend over a span of 2 to 3 weeks.

The female flowers begin to exhibit receptivity in unison with anthesis, with considerable variation by parent. The bud scales begin to expand and, when fully expanded, the inner scale surface shows a pink coloration. Two stigmatic surfaces protrude from the inside of each scale opening (R. Hall, 1979, personal communication), which is the stage of maximum receptivity. Schopmeyer (1974) cites flowering dates for the Eastern United States of March through May, and February through March in England. Funk (1965) found that pollination is sometimes delayed until April in Ohio. These dates contrast sharply with those in northern Alabama, where flowering can occur by mid-January.

**Controlled Pollination Procedures**

In early to mid-January, the female flowers are isolated with dialysis tubing (available from scientific supply sources) that is 28 mm in diameter. The flatrolled tubing is opened by an airstream and cut into 4-inch lengths, folded twice against the foldseam and stapled on one end. These isolation sheaths are made up before going to the field to expedite the pollination process. The sheath is slipped over several female flowers after removal of the male catkins. In the selection of female clusters, caution must be exercised because some of them are bisexual and produce a few pollen sacs at the base of an otherwise pistillate stalk. There bisexual flowers resemble a “Turk’s-cap turban” configuration, which is usually a typical male flower formation at the base with a protruding female flower. Such catkins can be used for selfing. The dialysis sheath is stoppered with polyurethane test-tube stoppers. These stoppers are slightly larger in diameter than the sheath opening and, upon release, expand and seal the flowers from outside contamination. The stoppers are slit longitudinally to accommodate the conelet branch, which extends through the stopper into the dialysis sheath. Stoppers of various colors are available to identify the pollen parent. The dialysis tubing and stopper provide adequate isolation and negligible weight on the small and fragile conelet stem. This combination has withstood winds up to 50 mph with minimal losses.

When the male flowers start to elongate, several clusters are covered with a single 7.1- by 27.9-cm sausage casing, which is stapled at the top and stoppered at the base with cotton. The casing is secured with an aluminum-wired identification tag around the cotton stopper. Ten such cluster bags per male parent provided more than adequate pollen to complete about 1,400 crosses of a half-diallel, 17-parent mating scheme.
Barner and Christiansen (1958) indicate that 100 male catkins will provide 4 ml of pollen.

The trees are checked daily, and the pollen bags are collected for each male parent when pollen is shedding heavily. Additional pollen is extracted by vigorously shaking each bag. Pollen is strained through voile cloth to remove debris and then placed in plastic hypodermic syringes with proper identification.

If the female flower is receptive, the pollen is used immediately; otherwise, it is stored at 3° C. Fresh pollen should always be used in controlled pollination if it is available.

Pollination is done by syringe with a No. 16- by 1 1/2-inch hypodermic needle. Each syringe can be color-coded to correspond to the color-coded test-tube stopper on the female or otherwise be given proper identification by pollen parent. Many syringes can be carried to the field at one time using a carpenter's apron or shotgun bandolier. The availability of several syringes permits a person to make several crosses without dismounting and moving the ladder from tree to tree. Needle points are protected and loss of pollen is prevented with small corks. The hypodermic needle is inserted through the polyurethane test-tube stopper into the isolation sheath, and the pollen is injected onto the flowers. The stoppers automatically seal when the needle is withdrawn. If color-coded stoppers are not used, embossed aluminum identification tags can be quickly clamped to the base of each stopper with small hog nose rings and special pliers (available at farm supply stores).

Other Methods of Extraction, Testing, and Storage

Male catkins collected before anthesis and put into a pollen extractor as described by Summerville and Turner (1973) resulted in unsuccessful extraction. Barner and Christiansen (1958) proposed that pollen be collected in a manner similar to that used for collecting pollen from Populus species, i.e., by harvesting branches 4 to 5 weeks before pollen dispersal. Cut branches with their basal ends placed in water will shed pollen if they are maintained at 10° to 16° C with extended daylight for 18 to 22 hours. Pollen can be forced on cut branches and stored for short periods of time under refrigeration (Pirags 1961, Saito 1970, Robison and other 1979) but it is not known whether pollen can be stored long enough to attempt intersectional crosses (Hall and Maynard 1979). Black alder pollen has been stored for periods of 50 to 116 days resulting in unsuccessful extraction. Earner and Christiansen (1958) proposed that pollen be collected in a manner similar to that used for collecting pollen from Populus species, i.e., by harvesting branches 4 to 5 weeks before pollen dispersal. Additional pollen is extracted by vigorously shaking each bag. Pollen is strained through voile cloth to remove debris and then placed in plastic hypodermic syringes with proper identification.

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Suggested methods of testing pollen viability include the use of a 1-percent agar concentration or a 10- to 30-percent sucrose concentration with 0.01 percent citric acid. The recommended incubation period is 24 hours at 20° C (Pfundt 1910). These procedures failed when used to test black alder pollen (J. Sprague, personal communication, North Carolina State University). The author has been unsuccessful in locating other references for testing viability of black alder pollen.

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### Specific Names of Forest Trees

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<td>P. jeffreyi Grev. and Balf.</td>
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<td>P. ponderosa Laws.</td>
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<td>P. radiata D. Don</td>
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<td>P. resinosa Ait.</td>
<td>Red pine</td>
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<td>P. rigida Mill.</td>
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<td>P. strobus L.</td>
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<tr>
<td>P. sylvestris L.</td>
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<tr>
<td>P. taeda L.</td>
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<tr>
<td>P. virginiana Mill.</td>
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<tr>
<td>Platanus occidentalis L.</td>
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<tr>
<td>Populus deltoides Bartr.</td>
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<tr>
<td>Pseudotsuga menziesii (Mirb.) Franco</td>
<td>Douglas-fir</td>
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<tr>
<td>Quercus L.</td>
<td>Oak</td>
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<tr>
<td>Q. alba L.</td>
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<tr>
<td>Q. coccinea Muenchh.</td>
<td>Scarlet oak</td>
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<td>Q. ilicifolia Wangenh.</td>
<td>Bear oak</td>
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### Common Names of Forest Trees

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<th>Common Name</th>
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<td>Yellow-poplar (Liriodendron tulipifera L.)</td>
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Appendix 3

Specific Names of Forest Insects

**Conophthorus coniperda** Schwarz (White pine cone beetle)
**Dioryctria abietella** D.& S. (South coastal coneworm)
**D. amatella** Hulst (Southern pine coneworm)
**D. clarioralis** Wlk. (Blister coneworm)
**Frankliniella tritici** Fitch (Flower thrips)
**Holocera lepidophaga** Clarke (Blister beetle)
**Lepidopsallus australis** Blatchley (Flower thrips)
**Leptoglossus corculus** Say (Leaffooted pine seed bug)
**Lytta sp.** (Blister beetle)
**Pomphoepa polita** Say (White pine cone beetle)
**Satronia tantilla** Heinrich (Southern catkinworm)
**Xyela bakeri** Konow (Pine catkin sawfly)
**X. minor** Norton (Pine catkin sawfly)
**X. obscura** Strobl (Pine catkin sawfly)
**X. pini** Rohwer (Pine catkin sawfly)
**X. spp.** (Pine catkin sawfly)

Appendix 4

Common Names of Forest Insects

Blister coneworm (**Dioryctria clarioralis** Wlk.)
Blister beetle (**Holocera lepidophaga** Clarke)
Blister beetle (**Lytta** sp.)
Flower thrips (**Frankliniella tritici** Fitch)
Flower thrips (**Lepidopsallus australis** Blatchley)
Leaffooted pine seed bug (**Leptoglossus corculus** Say)
Pine catkin sawfly (**Xyela bakeri** Konow)
Pine catkin sawfly (**Xyela minor** Norton)
Pine catkin sawfly (**Xyela obscura** Strobl)
Pine catkin sawfly (**Xyela pini** Rohwer)
Pine catkin sawfly (**Xyela spp.**)
Southern catkinworm (**Satronia tantilla** Heinrich)
South coastal coneworm (**Dioryctria abietella** D.& S.)
Southern pine coneworm (**Dioryctria amatella** Hulst)
White pine cone beetle (**Conophthorus coniperda** Schwarz)
White pine cone beetle (**Pomphoepa polita** Say)
Appendix 5

Glossary

Aborted ovule—a female reproductive cell and surrounding cells that cease to develop before fertilization.

Abscission—natural separation of leaves, flowers, and fruit from plants, generally associated with deterioration of a specialized layer of thin-walled cells.

Agar—a gelatinous product derived from certain Asiatic seaweeds, used as a medium in bacterial culture.

Allele—either of a pair of contrasting characters inherited alternatively according to Mendelian law.

Ampule—small glass storage container with a relatively long narrow neck; prevents contents from exposure to air and moisture when flame-sealed under vacuum.

Anemophilous—fertilized by pollen conveyed by the wind; wind-pollinated. (Cf. entomophilous.)

Angiosperm—a plant that produce seeds enclosed in an ovary.

Anhydrous—free of water; moisture is readily absorbed on exposure to air.

Anion—a small, negatively charged particle of matter.

Anther—pollen-bearing part of a stamen.

Anthesis—1. stage of full flower expansion or period when fertilization occurs. 2. bursting of anthers with release of pollen.

Apomixis—reproduction from seeds or seedlike organs but without fertilization, giving rise to apomicts usually genetically identical with their source plant.

Apparent pollination effectiveness—ratio of total developed seed per cone to the seed potential of the cone.

Aqueous—of, like, or containing water.

Background pollen—pollen produced from natural stands outside the orchard boundary; in general, pollen from outside an area of limited size.

Basipetalous—inflorences that open serially from the apex toward the base.

Blister beetle—long, narrow beetle that feeds on the male strobili of pines; its body fluids cause skin blisters.

Boron—an element essential in small quantities for the nutrition of most forest trees; a trace element.

Bract—1. modified leaf subtending a flower or floral axis. 2. modified leaf subtending a scale in female cones.

Bud scales—outer covering that protects the developing vegetative or reproductive bud.

Calyx—outermost group of floral parts; whorl of sepals.

Capsule—dry, usually many-seeded fruit composed of two or more fused carpels that split at maturity to release their seeds in species such as Kalmia, Koelreuteria, and Populus.

Carpel—simple pistil, or single member of a compound pistil.

Casual micro-organisms—micro-organisms associated with pollen purely by chance.

Cation—a small, positively charged particle of matter.

Catkin—spike of unisexual flowers or fruits with imbricated scaly bracts as in Alnus and Betula. In common usage, this refers to the male or staminate strobilus of conifers. Technically, pine trees do not have catkins.

Chytrids—an order of fungi in the class Phycomycetes, generally aquatic with little or no mycelium.

Clone—a group of genetically identical plants (ramets) derived asexually from a single individual (ortet).

Conelet scale—a modified shoot on which seeds develop, which is attached to the central cone axis in the axil of a bract.

Coneworms—insect larvae of the genus Dioryctria that infest the female strobili and other parts of pines.

Contaminants—impurities; specifically spores, cells, or pieces of micro-organisms present on pollen or catkins that can, under proper conditions, grow, spread, and cause damage.

Controlled pollination—transfer of pollen from a known source to receptive flower parts of a known seed parent, all other pollen being excluded (as by covering flowers with isolation bags before pollination).

Corolla—inner set of floral leaves consisting of separate or fused petals that surround the carpels.

Cross-pollination—pollination with pollen from a tree genetically unrelated to the female parent for several generations.

Cyme—inflorescence in which the main and secondary axes always terminate in a single flower.

Dehiscence—splitting open at maturity to discharge contents, as a capsule discharging seeds or an anther discharging pollen.

Desiccator—vessel used to achieve and maintain an atmosphere of low humidity by use of a chemical agent.

Determinate flowering—terminal flowers blooming slightly in advance of their nearest associates.

Dioecious—having staminate (male) flowers and pistillate (female) flowers borne on different individual plants as in Acer, Fraxinus, Ilex.
Disinfest—to free an object of surface contaminates, most specifically to clear away infesting pests such as weeds, insects, diseases.

Distal—farthest removed; situated at the extremity; most distant.

Electrical conductance—movement of electrical energy through or on a medium.

Emasculate—to castrate; to remove the anthers, catkins, and associated male organs.

Embryo sac—a multinucleate oval cell in the ovule of seed-bearing plants in which fertilization of egg and development of embryo occur.

Emasculate—pollinated by insect-borne pollen; insect pollinated. (Cf. anemophilous.)

Enzymes—complex organic molecules that function as biochemical catalysts.

Fertilization—the process of penetration of a pollen tube through the embryo sac into the ovule (egg cell), discharge of the male nucleus into the ovule, and union of the male and female nuclei.

Gametophyte—in the alternation of generations in plants, the individual or generation that bears sex organs.

Gene conservation (ex situ)—maintaining germ plasm for future use by preserving, collecting, and storing away from the natural stand environment.

Gene conservation (in situ)—maintaining germ plasm for future use by preserving and perpetuating natural forest stands.

Gene pool—sum total of genes in a breeding population at a given time.

Genotype—(1) the entire genetic constitution, expressed or latent, of an organism. (2) the genetic constitution of an individual with respect to a few genes under consideration. (3) a group of organisms having similar genetic constitution.

Glomerule—a cluster of flower heads in a common involucre; a cyme condensed into a headlike cluster.

Gymnosperm—seed plants that produce naked seeds not enclosed in an ovary.

Heterozygous—possessing genes for two members of one or more pair of allelomorphic characters.

Homozygous—possessing genes for only one member of at least one pair of allelomorphic characters.

Hyphal growth—proliferation of filamentous elements of microscopic size that form the mycelium of a fungus.

Imperfect flower—incomplete; having either stamens or pistils but not both. (Cf. dioecious.)

Inbreeding coefficient—a number between 0 and 1, calculated from the number of mating individuals and their genetic relationship to each other, showing the homozygosity remaining in an individual or population as a fraction of the loci that were originally heterozygous.

Indigenous microflora—micro-organisms normally occurring on a plant or part of a plant.

Inflorescence—floral axis with its appendages; flower cluster.

Intercellular—among and between cells.

Intracellular—within a single cell.

In vitro—occurring outside a living organism, in an artificial environment, as tissues cultivated in a test tube; literally, in glass.

In vivo—occurring in a living, functioning cell.

Involucre—a ring of bracts surrounding a cluster of flowers; one or more whorls of leaflike bracts that surround the base of a flower or cluster of flowers below the calyx or calyces.

Isolation bag—a bag made of plastic, cloth, or paper that excludes wind-borne pollen from receptive female structures.

Isozyme analysis—the study of enzymes and isozymes, which are primary gene products and which reflect the segregation and recombination of alleles.

Leachate—material obtained by fluid extraction; the product of leaching.

Locus—the point in a chromosome associated with a particular hereditary character.

Lycopodium—genus (about 180 species) containing the club mosses and ground pines.

Lyophilization—freeze drying.

Manometer—a gauge for measuring changes in pressures of gases.

Marker gene—a gene with demonstratable effects used to identify one parent of offspring resulting from open or controlled pollination.

Megasporo—a spore that develops into the female gametophyte; the progenitor of the ovum.

Meiosis—reduction division resulting in the production of haploid gametes; a process consisting of two consecutive specialized nuclear divisions ultimately leading to the formation of eggs or sperm.
Micronutrient—nutritional element necessary in very small quantities for normal plant growth, such as boron, manganese, sulphur; trace element.

Micro-organism—an organism of microscopic size, such as a bacterium, virus, or fungus.

Micropyle—minute opening in the integument of an ovule through which the pollen tube normally passes to reach the embryo sac; usually closed in the mature seed to form a superficial scar.

Microspore—the male cell produced by sexual cell division (meiosis) and maturing to become pollen grains.

Monococious—having staminate and pistillate flowers on the same plant.

Mycelium—vegetative mass of fungi composed of interwoven filaments or hyphae.

Ortet—original plant from which a clone has been derived.

Osmosis—the diffusion of water through a differentially permeable membrane from a region of low concentration of solutes to a region of high concentration of solutes.

Ovary—basal portion of a pistil that bears the ovules.

Ovulate scale—leaflike structures borne on the central axis of a strobilus, which hold the ovules.

Ovule—rounded outgrowth of the ovary in seed plants that develops into a seed.

Panicle—compound raceme as in Chionanthus, Fraxinus, and Aesculus.

Panmixia—random mating without restriction which results in genotypic frequencies proportional to gene frequencies.

Perfect flower—a flower that bears both stamens and carpels. (Cf. monoecious.)

Phenology—relations between plant functions and seasonal climatic changes such as temperature or day length, especially as such changes affect periodic phenomena like leafing, flowering, and dormancy.

Phenotype—demonstrable characteristics of a plant; the product of genotype and environment.

Phycomycetous fungi—fungi of the class Phycomycetes, closely resembling the algae.

Phytosanitary certificate—document provided by an authority for certification of insect- and disease-free material for export; in the United States, issued by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture.

Pistil—ovule-bearing organ of an angiosperm composed of ovary, style, and stigma.

Pistillate—having pistils but no stamens; female.

Plant pathogens—micro-organisms capable of causing plant diseases.

Pollen—multinuclear cells that carry the male germ cells of most higher plants.

Pollen bank—collection of trees set aside for the long-term production of pollen; a facility where pollen is stored for long periods.

Pollen germination test (in vitro)—test in which the viability of pollen is determined by germinating pollen under controlled laboratory conditions.

Pollen germination test (in vivo)—test in which the fertility of pollen is determined by applying pollen to the receptive female structure, then counting resulting germination.

Pollen trap—a device that captures pollen grains by adhesion, entrapment, or other suitable means.

Pollen tube—an outgrowth of a germinating pollen grain through which the sperm passes to fertilize the egg.

Pollination—(1) deposition of pollen on the receptive part of the female flower. In angiosperms this is the stigmatic surface, in gymnosperms the area inside the integuments. (2) the directed transfer of pollen from a stamen to a stigma or ovule.

Pollination bag—see isolation bag.

Pollination effectiveness—the percentage of ovules in a cone that have at least one viable pollen grain.

Precocious pollen producer—trees or grafts that begin pollen production at an earlier than average age.

Prolific pollen producer—trees or grafts that produce abundant quantities of pollen.

Raceme—elongated inflorescence with flowers on stalks of equal length arising from a main axis as in Prunus, Amelanchier, and Crataegus.

Rachis—axis of an inflorescence.

Ramet—an individual member of a clone, derived from an ortet.

Recurrent selection—a breeding system whereby the frequency of genes for specific traits is increased by repeated generations of selection.

Rehydration—humidifying process by which moisture is restored to dried material.

Samara—dry, indehiscent, winged fruit, one-seeded as in Fraxinus and Ulmus or two-seeded as in Acer.
**Seed orchard**—plantation consisting of clones or seedlings from selected trees, isolated to reduce pollination from outside sources, rogued of undesirable trees, and cultivated for early and abundant production of seed.

**Seed potential**—the maximum number of seeds a plant is capable of producing; in pines this equals two times the number of fertile scales.

**Self-incompatability**—inability to accomplish self-fertilization

**Self-pollination**—pollination of a biotype with its own pollen; in forestry, the pollination of an individual tree with its own pollen.

**Sibs (siblings)**—offspring irrespective of sex, from the same parents but from separate fertilizations. Full sibs have both parents in common; half-sibs, only one in common.

**Stamen**—pollen-bearing organ of a flower in angiosperms consisting of a filament and an anther.

**Staminate**—having stamens but no pistils; male.

**Stigma**—part of the pistil, usually the tip, often sticky, which receives the pollen and on which the pollen germinates (angiosperms only).

**Strobilus**—the male and female inflorescences of most conifers, consisting of imbricated scales developed from a central axis; conelet.

**Style**—stalk of a pistil, connects the stigma with the ovary (angiosperms only).

**Supplemental mass pollination (SMP)**—broadcast application of pollen to conelets which are not isolated from other pollen.

**Talc**—magnesium silicate, a soft mineral available in refined powdered form.

**Thrips**—minute insects with rasping, sucking mouthparts; both male and female pine strobili may be damaged by these insects.

**Ultraviolet absorption**—the selective uptake of high-frequency light energy beyond the visible range.

**Wind pollination**—pollination by wind-borne pollen.

**Xyelid sawflies**—small, white, grublike insects commonly found in the male strobili of pines.