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Effects of Water Suspension and Wet-Dry Cycling on Fertility of Douglas-Fir Pollen

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Abstract

Studies were made to determine how long Douglas-fir pollen remains viable after suspension in cool water from 0 to 34 days. Linear regression analysis of in vivo and in vitro tests indicated that filled seed efficiency and pollen viability, respectively, decreased about 3 percent per day. The relation may have been nonlinear the first 6 days, as little decrease occurred during that time. An in vitro test of the effect of none, one, or two drying cycles on previously wetted pollens revealed a great decrease in pollen viability after just one drying cycle. The in vivo test of 1-, 2-, and 3-percent pollen suspensions showed that the 3-percent suspension resulted in 15 percent greater filled seed efficiency than the 2-percent and 57 percent greater than the 1-percent suspension.

Keywords: Supplemental mass pollination, seed orchard, flowering, reproduction, filled seed efficiency, Douglas-fir, *Pseudotsuga menziesii*.

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Introduction

Tree improvement workers use supplemental mass pollination technology (SMP) in seed orchards to increase filled seed efficiency (FSE), increase genetic gain, and decrease loss of adaptation. To be effective, SMP must be done at an early stage of megastrobili receptivity before unacceptable levels of background pollen arrive, and sufficient quantities of SMP pollen must be applied to effectively block entry of the background pollen into the micropyle areas. Current SMP technology differs greatly, and results have not been entirely satisfactory. Unsolved reproductive biology problems, inadequate pollen-application equipment, and high labor costs combine to limit use of the technology. Pollen-application equipment must be capable of rapidly and uniformly delivering precise amounts of valuable pollen during a limited period. Effective management of conventional seed orchards requires affordable and dependable equipment that can be used by several workers to pollinate several hundred large trees in a few hours.

Webber (1995) reports that localized or point applications of pollen directed at individual or clusters of megastrobili produce greater parentage control than do general crown applications (mist treatments). The primary factors limiting use of point applications is the excessive time required to completely pollinate large trees and the associated high cost of the labor-intensive process. A worker using a hand-held wand may need 2 to 4 hours to completely pollinate all receptive flowers on one large tree; thus, it is not possible to effectively use that technology when many large trees require rapid pollination.

In earlier testing of large-scale pollen application procedures and equipment, we noted that pollen drift from wind often is a problem when dry pollen is applied with a tractor-mounted duster (Copes et al. 1995). Even low-velocity winds cause a significant portion of SMP pollen to move away from the trees being pollinated. To reduce pollen drift in this study, we tested liquid-pollination methods. The hypothesis was that pollen suspended in water would be less prone to wind drift. Use of liquid pollination depends on the pollen retaining viability or fertility after suspension in water. A small trial of pollen in water by Allen and Sziklai (1962) indicated that viable Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seeds could be produced, but the results were based on only 15 cones. They state that Douglas-fir pollen remains viable in water for at least 24 hours, but they do not present data supporting that observation. In Monterey pine (*Pinus radiata* D. Don), liquid pollination was effective in producing filled seeds (Sweet et al. 1993).

An effective water pollination program for Douglas-fir orchards requires additional knowledge of when and at what rate pollen viability decreases after water suspension for various times. In this paper, we report the results of four studies examining in vivo ability of Douglas-fir pollen to fertilize ovules after suspension in water for as little as 1 minute to as long as 34 days. An in vitro test of one or two drying cycles on pollen viability also is reported.

Methods

Four in vivo studies of liquid pollination were done between 1993 and 1997. The primary objectives of each study were to determine if and when pollen fertility or viability changes as time in water increases. When time in water treatments encompassed several days, replications in time of each treatment were made at regular intervals before pollination to ensure that the proper solutions were available for 3 to 5 days after megastrobili bud flush. This corresponds to stages B+2 to B+4 stages of megastrobili receptivity (Webber 1987).

All pollen was collected with the pollen vacuum described by Copes et al. (1991). Collection was done from 1990 to 1992. The pollen was air dried to 4 to 7 percent moisture content and stored at -135 °C in a cryogenic freezer. Each pollen source came from multiple tree collections. Different pollen sources were applied each year. Stored pollen was rehydrated overnight in a saturated atmosphere before suspension in distilled water. All pollen tested at 90 percent (or more) viability after rehydration, based on in vitro viability tests using the 20P10B viability solution of Webber and Bonnet-Masimbert (1993). Pollen solutions were stored in a refrigerator at 1 to 3 °C until day of pollination and were transported to the field in a cooler. The times in water before pollination were strictly controlled. The 0- to 40-minute treatments in 1993 were accomplished by adding hydrated pollen to the water after transport to the field. In 1997, the pollen solutions that had been in water for 0 to 12 days were tested for in vitro viability by using the Webber and Bonnet-Masimbert (1993) solution described above.

The megastrobili for evaluating liquid pollination were placed in isolation bags before the reproductive buds opened. Different clones were used as female parents each year. All male catkins were removed from the branch areas enclosed by the bags. Each isolation bag was removed from the branch for 1 to 2 minutes for pollination when megastrobili reached B + 2 to B + 4. The pollen solutions were applied as a fine spray with a hand-held 1.7-liter Spray Doc sprayer.[2] Spraying was continued until liquid ran off the megastrobili. Pollen was kept uniformly distributed in solution during pollination by frequently shaking the sprayer during application. Each isolation bag was immediately reapplied after pollination of each branch.

Conventional pollination with dry pollen (applied directly to each megastrobili from a small, plastic squeeze or dropping bottle) was done in 1994 and 1997. A heavy dusting of pollen was applied to individual megastrobili (about 0.25 cubic centimeter of pollen per isolation bag). The FSE value from the dry pollination of each tree was used as an indication of maximum FSE when pollen was not limited.

Mature cones from the tests were collected in August each year when the cones were brown and fully mature but had not opened their cone scales. Seeds were extracted by hand from each cone, and all the round seeds were X-rayed to identify filled seeds. The FSE was calculated as a percentage of the number of filled seed divided by the number of round seeds per cone. Flat seeds (nonviable) were not included in the seed efficiency calculations because they were not viable at fertilization and not capable of accessing pollen fertility.

For an in vitro test of the effects of wetting and drying cycles on pollen viability, five pollen samples (P1 through P5), each from a different source taken from cryogenic storage, were evaluated. The test consisted of three pollen treatments (control [pollen wetted], cycle 1 [pollen wetted, suspended in water, dried, and rewetted], and cycle 2 [pollen wetted, redried, rewetted, redried and rewetted]), four replications, and five pollen sources. Before each rewetting, the viability of the pollen was tested. The five pollen sources were not the same sources evaluated in the in vivo tests. Moisture content of

[2] The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

samples from the five stored pollen lots ranged from 2.1 to 5.4 percent. About 100 microliters of each pollen sample was placed in a petri dish with approximately 500 microliters of distilled water. The pollen-in-water suspension was air dried for 24 hours and then suspended again in water and dried an additional 24 hours. Aliquots of pollen solution were removed by pipette from each sample immediately after each suspension. Each aliquot was placed into fresh pollen germination solution (Brewbaker and Kwack [1963] modified by Webber and Bonnet-Masimbert [1993]) and incubated for 48 hours on a rotary shaker at low speed. The viable (elongated), damaged, and dead pollen grains were tallied from five randomly selected areas in the microscopic view. The percentage of viable grains (number of viable grains divided by the number of all pollen grains) was calculated for each sample.

Data were analyzed by using GLM procedures (SAS 1989). The statistical model was,

$$FSE = \mu + female_i + pollen_j + b_1 * time + b_2 * time^2 + e_{ijk}.$$

Data from each year were analyzed separately. For the field tests, the FSE per cone was the experimental unit. Each isolation bag was a replication of a female by male (or pollen concentration in 1993) by time in water treatment. The time in water was the fixed effect for the field tests and the 1997 in vitro viability test, and the number of drying cycles was the fixed effect for the in vitro pollen viability test. No transformation of data was required. Level of significance was $\propto = 0.05$. Type III mean squares were used when appropriate.

Results

Time in water was increased from 0 to 40 minutes in 1993 to a maximum of 34 days in 1995. In 1993, 1-, 2-, and 3-percent pollen concentrations in water were examined on five female trees (table 1). The objective was to determine whether Douglas-fir pollen remained highly fertile after suspension in water and to determine which concentration

Table 1—Experimental designs of four liquid pollination tests

Year	Parents		Time in water	Pollen concentration	Bags per treatment	Control pollination with dry pollen
	Females	Males				
	- - - Number - - -				*Percent*	*Number*
1993	5	1	1, 5, 10, 20, and 40 minutes	1 - 3	2	No
1994	1	2	0, 1, 2, 3, 4, 5, and 6 hours	3	2	Yes
1995	3	3	0, 10, 18, and 34 days	3	3	No
1997	1	1	0, 1, 3, 6, 10, and 16 days	3	3	Yes

produced the greatest FSE. The three concentrations of the single pollen source are listed in table 2 as pollen sources A, B, and C. The 1-, 2-, and 3-percent concentrations had mean FSE values of 35.7, 48.7, and 56.2 percent, respectively. The differences were significant. Mean FSE for the five time periods between 1 and 40 minutes in water ranged from 43 to 49 percent (table 2), but no significant decrease in pollen fertility related to time in water was found. Differences in FSE among the three female parents are not shown in table 2 but also were significant.

Table 2—Filled seed efficiency values from 4 pollination tests with pollen suspended in water for various intervals from 1 minute to 34 days

Year	Time in water	Pollen sources[a]			Mean
		A	B	C	
1993	1 minute	33.6	57.0	55.7	49.4
	5 minutes	30.1	43.8	61.8	44.6
	10 minutes	41.8	42.4	57.2	47.1
	20 minutes	36.4	49.2	59.4	48.0
	40 minutes	34.0	51.3	47.0	43.7
	Mean	35.7	48.7	56.2	46.6
1994	0 hour	42.7	39.2		40.0
	1 hour	32.2	55.5		47.8
	2 hours	57.4	37.2		47.3
	3 hours	32.7	55.8		44.3
	4 hours	29.0	56.3		42.7
	5 hours	36.0	39.8		37.9
	6 hours	39.7	40.8		40.2
	Mean	39.3	45.9		42.7
1995	0 day	43.9	42.6	49.7	44.6
	10 days	35.8	24.6	13.4	24.0
	18 days	31.5	34.9	9.1	25.5
	34 days	1.1	4.4	0.6	2.0
	Mean	29.7	25.9	16.2	23.9
1997	0 day	69.2			69.2
	1 day	77.8			77.8
	3 days	55.2			55.2
	6 days	73.2			73.2
	10 days	42.9			42.9
	16 days	24.5			24.5
	Mean	60.1			60.1

[a] Pollen sources A, B, and C in 1993 were not 3 different sources but were 1-, 2-, and 3-percent concentrations, respectively, of a single pollen source.

In 1994, maximum time in water was increased to 6 hours, and two pollen sources were used on one female tree (table 1). In this test it was assumed that there was no female-by-pollen interaction. The FSE values from the two pollen sources were not significantly different, and no significant decrease in FSE was found after 6 hours of suspension in water (table 2). The 76-percent FSE from dry pollination was significantly greater than the 43- to 49-percent FSE from water pollination.

In 1995, three pollen sources were suspended in water for 0, 10, 18, and 34 days (table 1). All three pollen sources were applied to different bags on each female tree. The FSE values for the 0-, 10-, 18-, and 34-day suspensions were 44.6, 24.0, 25.5, and 2.0, respectively (table 2). The differences were significant. An average decrease occurred in FSE of 46 percent from day 0 to day 10 and 43 percent from day 10 to day 18. The negative trend is shown in figure 1. Surprisingly, male C, the poorest performer after 10, 18, and 34 days in water, had the greatest FSE when used immediately after suspension (table 2). The 1995 contrasts, such as females, males, days in water for linear and quadratic effects, and the males by days in water, were significant.

Pollination in 1997 evaluated pollen fertility after water suspension for up to 16 days. The interval between 0 to 10 days also was sampled at days 1, 3, and 6. Results from only one female tree are reported in table 1 because severe frost damage to megastrobili on two other trees made their FSE data worthless. The relation between FSE and number of days in water was negative (fig. 1). A decrease of 3 percent per day in pollen fertility occurred each day the pollen was in water. A similar negative trend was shown in the in vitro pollen viability test (fig. 1). In that test, the same pollen solutions used for field pollination were subjected to the in vitro evaluation. In 1997, the FSE from liquid pollination after 0, 6, and 10 days in water were all greater than the 66 percent FSE from dry pollination.

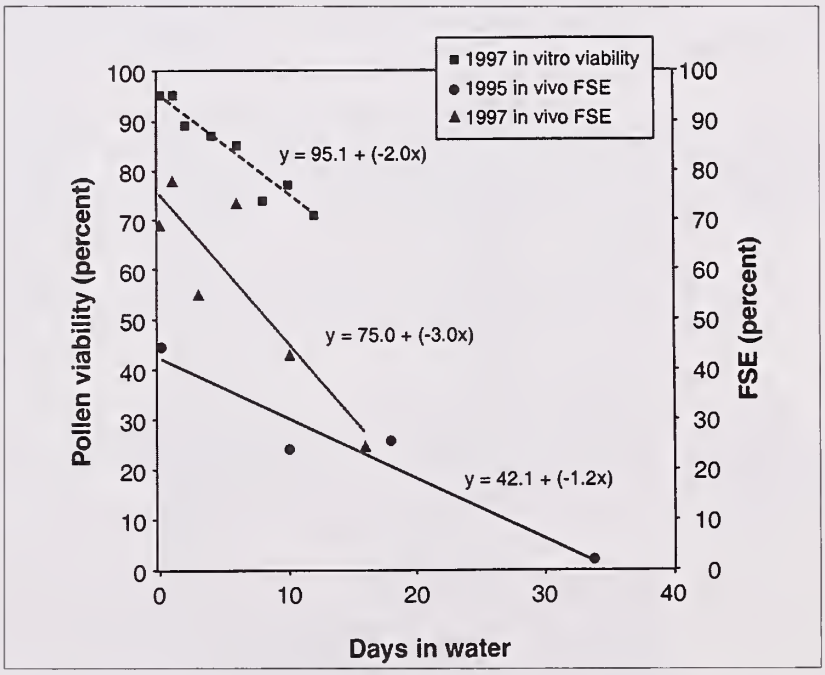

Figure 1—Mean filled seed efficiency (in vivo) and pollen viability (in vitro) percentages resulting after pollen was suspended in water for 0 to 34 days.

The in vitro test of none, one, or two cycles of rewetting and drying on pollen viability shows that viability declined for the five pollen sources (P1 through P5) tested (fig. 2). Mean pollen viability of the five sources after none, one, or two drying cycles was 82.7 (2.0 SE), 25.1 (1.2 SE), and 2.3 percent (0.5 SE), respectively. The first drying cycle after the suspension in water reduced mean viability by more than 57.6 percent; the second drying cycle reduced mean viability by about 80.4 percent.

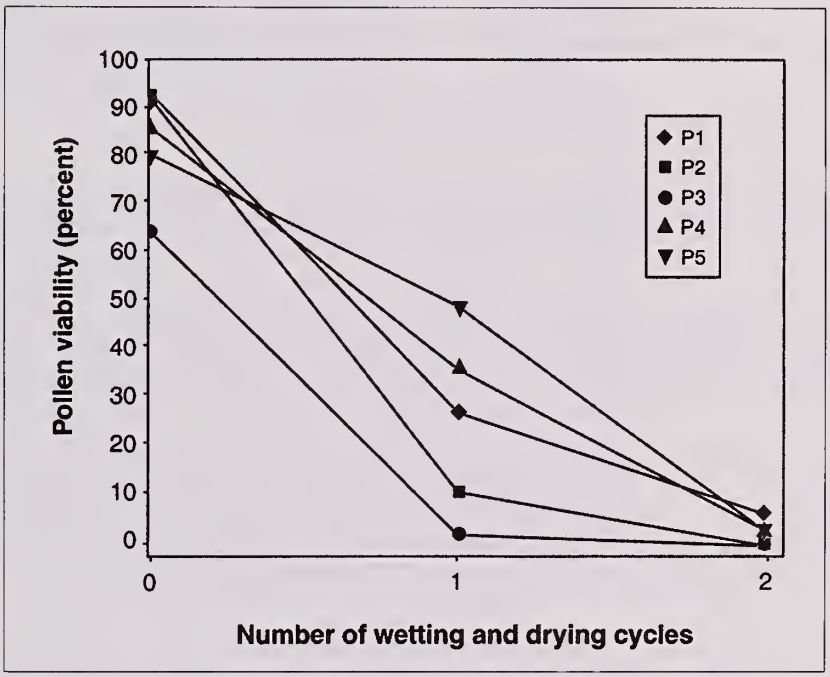

Figure 2—Effects of drying and wetting cycles on mean in vitro pollen viability. The five pollen samples tested are labeled P1 through P5.

Discussion and Conclusions

In our tests, Douglas-fir pollen, following suspension in water, retained the ability to fertilize and promote the formation of viable seed far longer than previously reported. No decrease in fertility was detected in 1993 or 1994 after short-term submersion for ≤ 6 hours. This finding confirmed the results of Allen and Sziklai (1962). The 1995 test of longer submersion indicated that a moderate level of pollen fertility (24 and 25 percent FSE) existed after 10 and 16 days in water, respectively, but pollen fertility was reduced to just 2 percent after 34 days. No sampling was done between 1 and 10 days in 1995, so it was not possible to determine when and at what rate the decrease occurred.

The 1997 test evaluated water suspensions for 0, 1, 3, 6, 10, and 16 days. Fertility averaged 69 percent FSE through day 6 and then decreased to 43 and 24 percent FSE after 10 days and 16 days, respectively. Linear regression analysis indicated a FSE decline of about 3 percent per day, but it is possible that the relation was nonlinear because little decrease occurred in the first 6 days. The mean for the 6-day treatment was 73 percent FSE. The regression lines for 1995 and 1997 FSE differed somewhat, possibly because of the large difference in FSE means for the two years. In both years, long-term submersion significantly reduced pollen fertility. An earlier decline would have occurred if the stored solutions had not been kept refrigerated at 1 to 3 °C.

The in vitro 1997 pollen viability evaluation revealed that a similar decline occurred with time in water. In that test, pollen viability declined from a mean of 95 percent after 1 day to 72 percent after 12 days. The rate of decline approximated the in vivo FSE regression from the same pollen suspensions, although the in vitro values were larger.

Results indicated that water suspensions can be used for operational SMP in Douglas-fir seed orchards. The longevity of Douglas-fir pollen in water suspension will allow orchard workers to prepare pollen-and-water suspensions ahead of pollination and store the suspensions until needed. A storage period of up to 6 days at 1 to 3 °C does not significantly decrease FSE below what can be obtained with suspensions prepared and immediately sprayed on receptive megastrobili.

The in vitro pollen viability tests demonstrated that pollens cannot be dried a second time and be used after being in water solution. Drying of pollen suspended in water resulted in a great decrease in viability after just one drying cycle. The sensitivity to drying of pollen in water suspension raises the question of how pollen retains viability in the Douglas-fir region of the Pacific Northwest, where frequent rains occur throughout the pollination season. One possible answer may be that the pollen grains in the narrow area near the mouth of the micropyle remain dry even in wet weather because the width of the opening is less than the diameter of the drops of rain. If this is so, those pollen grains would remain dry and viable, whereas pollen grain resting on the more exposed areas would be subject to wet and dry cycles and experience reduced viability. Caution should be expressed in extrapolating results of in vitro tests as pollen was suspended in water for 24 hours at room temperature, which could cause physiological changes to occur. This probably is not the scenario in field conditions.

The comparison of FSE results from 1-, 2-, and 3-percent pollen suspensions yielded results showing that the 3-percent suspension gave a 15 percent greater FSE than the 2-percent suspension and 57 percent greater than the FSE from a 1-percent suspension. An unanswered question is whether an operational program would be more effective with several spray dates with a 2-percent suspension versus fewer sprays with a 3-percent suspension. The total amount of pollen used would be similar, but the FSE results might be different. Copes et al. (1995) with Douglas-fir and Sweet et al. (1992) with Monterey pine both report successive pollination gave better FSE results than a single pollination. Sweet et al. (1992) found the optimum pollen concentration to be 2.5 to 3.0 percent.

The comparison of FSE from dry pollination with the FSE from liquid pollination did not yield clear results owing to the weakness of that comparison—too few megastrobili sampled from dry pollination. For example, in 1994 the FSE from dry pollination was greater than the FSE from liquid pollination, but in 1997 the FSEs from the 0, 1, and 6 days of liquid pollination were greater than FSE from the dry pollination.

Completing the pollination studies in four different years was difficult because two or more periods of freezing weather occurred during each pollination season. Freezing weather reduced the number of round seeds per cone, the number of cones of each treatment that survived to maturity, the replication (isolation bags) of some treatments, and the FSE values of cones surviving to maturity. In 1997, two of three trees used as females had to be deleted from the study because of the excessive freeze damage to their megastrobili.

Future Application

Liquid pollination may be used for operational SMP programs in Douglas-fir seed orchards. Pollen suspended in cold water retains high fertility even after 6 days. The FSE results from dry and liquid pollination were comparable. The long storage life of suspended pollen will reduce pollen waste, because solutions not completely used each day can be refrigerated and applied at a later date. The negative impact of attempting to rewet pollen that has been suspended in water and redried clearly indicates that the practice is not a management option.

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