EVALUATING POLLEN COMPETITION IN DOUGLAS-FIR USING A CHLOROPLAST DNA MARKER

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Received June 4, 1998; accepted January 23, 1999

ABSTRACT

An experiment was conducted to test pollen competition as reflected in the reproductive success of individual pollen lots in two polymixes in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco). One polymix contained six pollen lots in equal proportion by weight, the other polymix was formulated using the same six pollen lots but in inversely proportional amounts to their viability. Pollen lot viability was measured as respiration (oxygen uptake).

Reproductive success of individual male parents (pollen lots) was determined following control pollinations using both polymixes separately on six female clones. The paternity analysis was carried out using a chloroplast DNA marker amplified by the polymerase chain reaction (PCR) in total DNA extracts of embryos dissected from mature seed, allowing the identification of the male parent in "polycrossed" seed.

Average number of filled seed per cone was not affected by the two polymixes, but the reproductive success of individual males in the polymixes varied greatly. Progeny arrays for both polymixes showed reproductive bias with reproductive success strongly correlated with pollen viability ($r^2 = 0.9$).

Keywords: *Pseudotsuga menziesii*, pollen respiration, reproductive success, seed orchard management, supplemental mass pollination, seed yield.

INTRODUCTION

Pollen competition and reproductive success of individual pollen lots in conifer polymixes have been widely studied (MORAN AND GRIFFIN 1985, SCHOEN & CHELIAK 1987, CHELIAK *et al.* 1987, WISELOGEL &VAN BUIJTENEN 1988, APSIT *et al.* 1989, EL-KASSABY & RITLAND 1992, NAKAMURA AND WHEELER 1992). In general, these studies indicate that polymixes formulated using equal male gamete compositions (*i.e.*, equal pollen volumes or weights), produce biased results. For example, in Norway spruce (*Picea abies* (L.) Karst), up to three times as many gametes from one male donor were found in the progeny as predicted under the assumption of equal male gamete contribution (Cheliak *et al.* 1987), while Apsit *et al.* (1989) detected significant interaction in male-female complementary. In contrast, WISELOGEL and VAN BUIJTENEN (1988) detected equal mating in polymix pollinations of loblolly pine (*Pinus taeda* L.). This latter study corroborates FOWLER's (1987) conclusions that male reproductive bias in polycrosses is within acceptable limits in the Pinaceae (excluding *Tsuga spp.*) provided three or fewer pollen grains are within close proximity to the nucellus and 20 or more males are represented in the polymix.

Differential male reproductive success from polycross pollinations may lead to biases in breeding values due to the increase in the relatedness among progeny (MORAN & GRIFFIN 1985). Furthermore, the genetic diversity of progeny after supplemental mass pollination (SMP) is lower than expected since the effective male population size is smaller than expected (SCHOEN & CHELIAK 1987).

There are several mechanisms that may lead to differential reproductive success using polymix crossings. First, differences in pollen viability and/or differences in pollen tube growth rate could result in pollen competition, especially in conifers where the presence of a pollen chamber (micropylar canal) (OWENS *et al.* 1991) limits the number of pollen grains (EL-KASSABY & RITLAND 1992). Secondly, selective embryo abortion following polyembryony may be implicated in differential male reproductive success (Nakamura and Wheeler 1992). Thirdly, female × male interactions at the time of pollination may be responsible for differential reproductive success (EL-KASSABY & RITLAND 1992).

The objective of this study was to determine if differential reproductive success after polymix crossings in Douglas-fir (*Pseudotsuga menziesii* (Mirb.)

Franco) is caused by pollen lots of different pollen viability. We hypothesize that if pollen lots of known but differing viabilities are used in a polymix of equal proportions, differential reproductive success will result. Further, if our hypothesis is correct, then a polymix that is inversely weighted by individual pollen lot viabilities (*i.e.*, highly viable pollen lots are present at low amounts and poorly viable pollen lots are present in high amounts), will yield a male gamete array in the progeny that is closer to the proportions expected under equal male parent contribution.

To investigate this hypothesis we utilized a recently developed hypervariable chloroplast DNA (cpDNA) marker that enables the efficient, unambiguous identification of the male parent in the resultant progeny of Douglas-fir (STOEHR *et al.* 1998).

MATERIALS AND METHODS

Polymix Composition and Pollinations

Douglas-fir pollen was collected from five clones growing in a hedged clonal-row micro orchard of 20 clones, located north of Victoria, B.C., Canada (48° N, 123° W, 30 m elevation) (Webber *et al.* 1992) and one clone from a Western Forest Products, Saanichton, B.C. (48° N, 123° W, 60 m elevation) seed orchard. Pollen collections were dried and stored for 2 years as described in Webber and Painter (1996). In the spring of 1996, two days prior to seed cone receptivity, the pollen viability of each lot was determined by measuring pollen respiration as the oxygen uptake in mL per mg of pollen (BINDER & BALLANTYNE 1975, WEBBER & BONNET-MASIMBERT 1993). Based on these respiration values, a polymix composed of the inverse of oxygen uptake value and weighted for a total volume of 30 mL was prepared (Table 1). A second polymix consisting of equal volumes (5 mL) of each pollen lot served as control. Both polymixes were stored in sealed containers at 4 °C in the dark until pollinations were made.

Table 1. Pollen lot viabilities (O_2 uptake) and the composition of polymixes used in control pollinations of six female Douglas-fir clones growing in a clonal-row micro orchard.

Paternal clone	O_2 uptake ($\mu L \cdot mg^{-1}$)	Polymix composition (mL)	
		Equal volume	Weighted by viability
3213	24.7	5.0	1.8
3249	18.1	5.0	2.5
3256	21.4	5.0	2.1
3234	3.3	5.0	13.8
3241	23.7	5.0	1.9
100	5.8	5.0	7.8

Approximately one week prior to expected peak seed cone receptivity (stage 3 of WEBBER &PAINTER 1996), at least four pollination bags were placed on a single ramet of six clones each growing in the micro orchard. All developing pollen cones within the bags and all but six seed cone buds were removed at this stage. At peak seed cone receptivity two randomly selected bags per ramet were pollinated with the polymix adjusted for viability and two bags were pollinated with the control polymix. Polymix applications were performed using a nitrogen-gas driven pollinator device ("power hitter") (see WEBBER &PAINTER 1996). Ten days after pollination, pollination bags were replaced by insect exclusion bags.

Cone and Seed Analysis

At the end of August, all fully developed control pollinated seed cones were harvested and kept separate by treatment and clone. An additional four untreated, wind-pollinated cones were also collected from each ramet. Following seed extraction, the number of filled seed per cone was determined by X-ray analysis (WEBBER &PAINTER 1996).

The number of filled seed per cone was analysed by polymix treatment and maternal clone using a fixed linear analysis of variance (ANOVA) model.

Paternal Analysis

For each polymix × female clone treatment combination between 27 and 50 seeds were randomly selected and their embryos excised. Total DNA was extracted from individual excised embryos according to the method of GUILLEMAUT and MARECHAL-DROUARD (1992) and suspended in 15 mL TE buffer. A region of the chloroplast DNA was amplified using polymerase chain reaction (PCR) primers T_f (5'-CTAAATATAAATCTATTGG-3') and T_r (5'-AAAGTATCAATTCATGG-3') (Stoehr *et al.* 1998). The target DNA is a hypervariable region, presumably a mutational hotspot (HIPKINS *et al.* 1995), located between the *trn*D and *trn*E genes (NEWTON, pers. comm.). Each amplification reaction contained 2mL of embryo DNA extract (between 50 and 100 ng total DNA), 0.625 mM of both T_f and T_r, 1.5 mM $MgCl_2$, 50 mM dNTP each, 1X PCR reaction buffer and 1.5 U Taq DNA polymerase (Perkin-Elmer). Amplification cycling conditions were as follows: DNA denaturation at 94 °C for 1 min., followed by 31 cycles of 94 °C (30 sec), 43 °C (60 sec), 72 °C (90 sec), and a final 10 min chase at 72 °C.

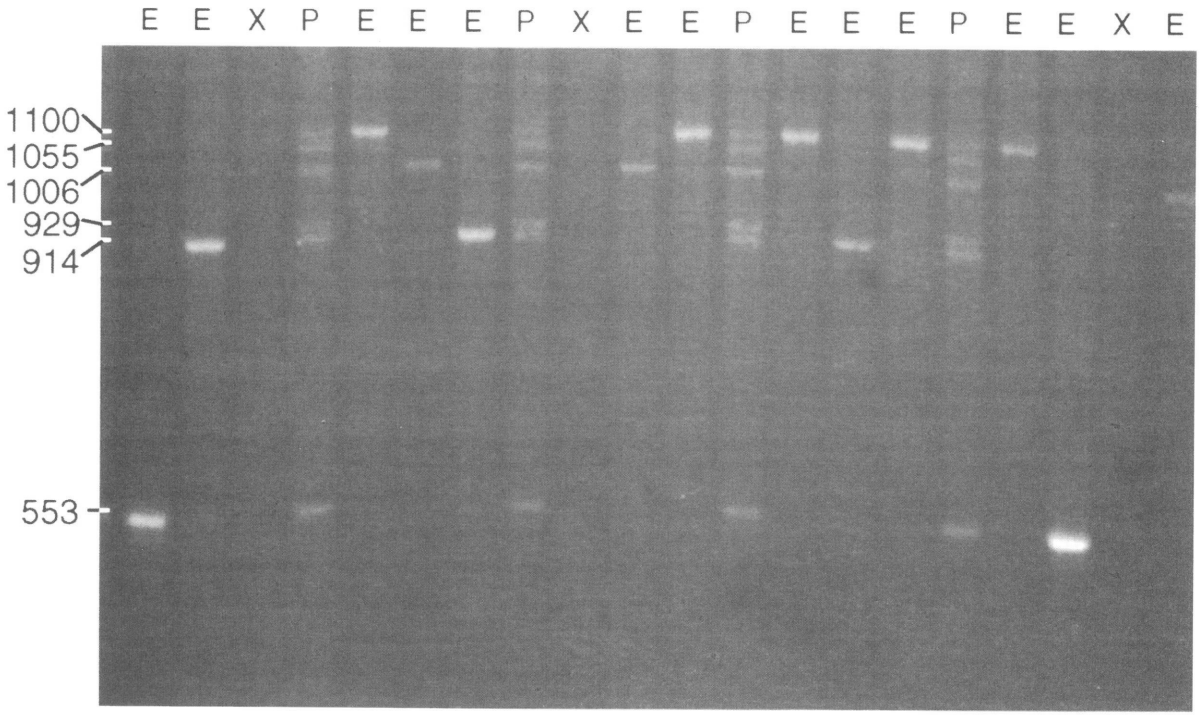

Figure 1. Typical electrophoresis agarose gel stained with ethidium bromide. Paternal PCR products (P) are shown combined as a ladder (with the numbers indicating their size in base pairs), embryo PCR products (E) and reactions that did not yield a detectable PCR product (X).

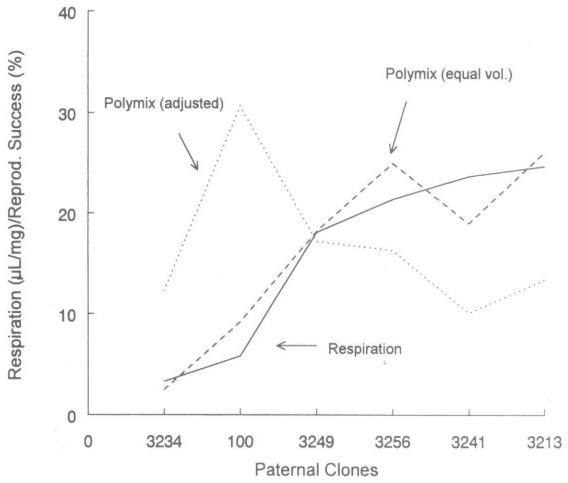

Figure 2. Individual pollen lot respiration and their associated reproductive success either inversely weighted by respiration (adjusted) or of equal proportion in two polymixes.

Pollen parents of the polymix were characterized using DNA extracted from 10 mg of vegetative bud tissue using the above procedure. Hypervariable cpDNA was amplified as above and 2mL of PCR product for each male parent was combined to prepare a ladder of polymix males for paternal scoring after gel electrophoresis. All PCR products were separated on 3:1 (by weight) 2% NuSieve (FMC):agarose for 600 volt hours. After staining with ethidium bromide, PCR products were visualized on a UV transiluminator and documented on Polaroid film for scoring. Amplified embryo PCR products were ascribed to a polymix parent based on comigration of parental PCR products (Figure 1).

RESULTS AND DISCUSSION

Cone abortion soon after pollination was high in this experiment, resulting in one polymix by clone treatment combination having no seed cones available for paternal analysis. For other treatment combinations 6 or fewer cones were available for seed yield analyses.

The average number of filled seed per cone was higher in the control pollinated (polymix) cones compared to wind pollinated cones (41 vs. 22) (Table 2) but no significant difference in the number of filled seed per cone could be attributed to polymix. Variation in the number of filled seed per cone due to maternal parents was highly significant (ANOVA not shown).

In all cases, PCR products of embryo DNA could be matched to one of the male parent's PCR products and male parentage could be unambiguously determined (Figure 1). In the control polymix (equal volume lots) our results show strong evidence for differential male reproductive success (Table 3). In fact, reproductive success was very closely correlated with pollen

Table 2. Number of filled seed per cone and their standard error (SE) as affected by polymix composition in comparison to wind-pollinated seed yield per cone in a clonal-row Douglas-fir micro orchard.

Treatment	N[1]	Filled seed / cone	SE
Polymix			
Equal volume	22	41.5	4.9
Weighted by volume	40	41.0	2.7
Wind-pollinated	24	21.6	3.3

respiration (Figure 2) with an r^2-value of 0.9. Furthermore, assuming equal male gamete contribution in a six-lot polymix, one would expect each male parent to sire 17% of all progeny. The observed values in our study ranged from 2.5% to 26.1%. This unbalance of the observed versus the expected frequencies, resulted in an effective number of males siring offspring () of 4.8. In the $N_e = 1 / \sum p_i^2$ polymix that was adjusted for viability, the observed reproductive success frequencies ranged from 10.1% to 30.7%. The associated effective number of males was 5.2, indicating a slightly broader representation of paternal genotypes in the resultant progeny array, however, the differences in N_e were not significant (not shown).

NAKAMURA and WHEELER (1992) attributed differential paternal success in Douglas-fir to selective embryo abortion, rather than pollen competition. If embryo abortion was responsible for the effect, then both of our polymixes would have produced similar reproductive success rates for each pollen donor. This was not observed in our data (see Table 3). Furthermore, it is also known that pollen that arrives early has the greatest chance of pollination success (WEBBER & YEH 1987) and highly viable pollen will therefore be more competitive, unless there is too much "poor" pollen in the micropylar chamber.

Based on our data, there is some evidence for preferential specific female × male crossing combinations, indicating female × male interactions. If female × male interactions are an important aspect of biased reproductive success, then specific female × male combinations (*i.e.*, their reproductive success after polycrossing) would be apparent as successful in both polymixes used. For example, male 3249 sired most offspring with female 3276 in both polymixes. Similarly, male 3234 combined best with female 3235 and

Table 3. Paternal contribution frequencies as affected by polymix composition in control-pollinations of six female clones in a clonal-row Douglas-fir micro orchard.

Polymix by: [1] Equal volume	Male parents						No of seed sampled
	3213	3249	3256	3234	3241	10	
Females							
3276	22.0	42.0	20.0	0.0	16.0	0.0	50
3265	27.1	12.5	29.2	4.2	14.6	12.5	48
3240	34.0	10.6	14.9	0.0	31.9	8.5	47
3235	25.0	11.1	27.8	8.3	13.9	13.9	36
3202	N/A[2]	N/A	N/A	N/A	N/A	N/A	N/A
3179	22.2	14.8	33.3	0.0	18.5	11.1	27
Mean	26.1	18.2	25.0	2.5	19.0	9.2	
Viability							
3276	14.0	36.0	18.0	6.0	10.0	16.0	50
3265	13.3	13.3	20.0	6.7	6.7	40.0	45
3240	12.2	12.2	12.2	16.3	14.3	32.7	49
3235	6.1	14.3	10.2	30.6	6.1	32.7	49
3202	21.3	8.5	21.3	8.5	12.8	27.7	47
3179	13.5	18.9	16.2	5.4	10.8	35.1	37
Mean	13.4	17.2	16.3	12.3	10.1	30.7	

1) Polymix made either of equal volume of pollen lots of six male parents or weighted by inverse proportion of pollen viability of individual pollen lots (see Table 1).

2) N/A indicates that no seed was available for paternal analysis.

male 3241 with female 3240. The importance of these female × male interactions in Douglas-fir has been shown by EL-KASSABY and RITLAND (1992).

Adjusting the polymix based on its respiration did not eliminate paternal reproductive success bias, but did not reduce seed yield per cone either. Incorporating pollen germination parameters and pollen tube growth rate into a weighting factor for pollen lot adjustments may have reduced differential paternal success. Alternatively, choosing pollen lots with similar respiration rates and/or a higher number of male parents may further improve polycross mating design assumptions of equal male reproductive success.

In this study we have shown that the close relationship between pollen lot respiration and reproductive success of individual pollen lots in a polymix is a driving force of pollen competition resulting in male reproductive bias. The use of the cpDNA marker, paternally inherited in Douglas-fir (NEALE *et al.* 1986), made it possible to identify the male parentage of single embryos with one PCR reaction.

ACKNOWLEDGEMENT

We would like to thank Clint Hollefreund and Doug Hedlin for their technical assistance through out the project. Constructive comments on the manuscript by Drs. Wolf Binder and Alvin Yanchuk, Research Branch, Ministry of Forests, Nick Wheeler, Weyerhaeuser Comp., John Owens, University of Victoria and Yousry El-Kassaby, Western Forest Products are greatly appreciated. The funding for this study came from Forest Renewal BC Project HQ 98099.

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