ISOZYME IDENTIFICATION OF COMPETING POLLEN PARENTS IN SEED FROM MIXED POLLEN CROSSES BETWEEN AUSTRIAN PINES AND AUSTRIAN X JAPANESE RED PINES

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ABSTRACT.--Pollen mixes were used to estimate pollen/embryo competition and the effects of timeliness of competing pollens. Pollen parents of offspring were distinguished by electrophoretic banding patterns of alcohol dehydrogenase and glutamic-oxaloacetic transaminase. Pollen/embryo competition was estimated by applying 50:50 percent pollen mixes to several Austrian pine seed trees. Offspring from these mixes are produced in a 1:1 ratio in the absence of competition. The majority of crosses using mixed pollens of Austrian pine produced seedling offspring in the expected proportions. All Austrian and Japanese red pine pollen mixed produced offspring in proportions that deviated markedly from that expected. Native embryos were highly favored over hybrid embryos. The effect of timeliness was estimated by sequential pollen applications of the same conelet. The pollens of two different parents were applied separately one or two days apart. Both intra- and interspecific combinations showed that the first pollen received was substantially more effective than the second. Results are discussed in reference to pollen management and production of hybrids.

The use of isozymes as markers may be useful in a variety of genetic studies. Such markers may be employed to test hypothesis regarding aspects of genetic structure of natural and artificial populations (Muller 1976), trace pollen flow; and to test the relative effectiveness and identity of competing pollen parents (Tobolski and Conkle 1976). The potential for using isozyme markers is high since over 20 polymorphic isozymes are currently being resolved from pine tissue. Further, most isozymes are simply inherited as codominant alleles and are not normally associated with deleterious mutants. On the other hand, morphological markers obtained from selfing may be linked to embryonic lethals which result in disturbed segregation ratios (Sorenson 1967). Further the scarcity of simply inherited dominant mutants limits the usefulness of morphological markers.

The objectives of this study were to estimate pollen/embryo competition and to determine the effects of timeliness of competing pollens. Embryo competition has been reported in Scots pine (*Pinus sylvestris* L.) by Sarvas (1962), by Fowler (1964) for red pine (*Pinus resinosa* Ait.) and in slash pine (*Pinus elliottii* Engelm.) by Franklin (1974). This event is determined by comparisons of seedling offspring after self and cross pollinations of the same individual.

Timeliness of pollination appears to be an important factor in influencing the effectiveness of a pollen in accomplishing fertilization. Franklin (1974) found that the first pollen reaching the female strobilus was much more effective than those arriving later. Pollen effectiveness was determined by making - sequential cross-self and self-cross pollinations on a single slash pine. This individual when selfed produced a regular frequency of albino mutants which were used as a marker.

Could timeliness account for the high yields of natural hybrids between Austrian pine (*P. nigra* Arn. var. Austrica [Hoess]) and Japanese red pine (P. *densiflora* Zieb and Zucc.) observed in a southern Michigan plantation (Wright *et al*. 1969)? Observation during two successive years showed that Japanese red pine produced abundant pollen and that it began shedding pollen 3 to 4 days before Austrian pine. These species occupy adjacent blocks and wind patterns favor the transfer of Japanese red pine pollen into the area of the Austrian pines.

METHODS

Austrian and Japanese red pines of known isozyme genotypes were selected for crossing. The seed parents for all crosses consisted of 3 Austrian pines growing on the Indiana University-Purdue University Campus at Fort Wayne, IN. The Austrian pollen parents included these trees plus one additional individual. Japanese red pine pollen parents were 8 trees whose pollens were pooled. The Japanese red pines were of natural origin and are growing along the edge of an older stand at Michigan State University's W.K. Kellogg Forest in southwestern Michigan. All trees are of unknown origin.

To insure high pollen viability, catkins shedding pollen were collected and the pollen was extracted at room temperature within 48 hours. Pollen was stored in the refrigerator or freezer until pollinations were completed within 8 days after collection.

Crosses performed included: (1) 50:50 percent pollen mixtures of Austrian pines or Austrian-Japanese red pine; and (2) sequential pollinations in which the pollens of two different parents were applied separately one or two days apart.

Seeds resulting from these crosses were stratified in water for 1 to 2 days at 5°C and germinated in petri dishes. Germination varied between 98 and 100 percent. Embryos and/or megagametophyte tissues were separated and analyzed by starch-gel electrophoresis according to techniques developed by Conkle (1972). Isozyme bands of alcohol dehydrogenase (ADH) and glutamic oxaloacetic transaminase (GOT) were used as markers. ADH isozymes were separated and stained using procedures developed by Scandalios (1969). The buffers of Fowler and Morris (1977) and staining techniques of Brewbaker et al. (1968) were utilized for GOT.

DETERMINATION OF POLLEN PARENTS BY ISOZYME MARKERS

Crosses between Austrian pines

Isozymes of GOT were utilized to distinguish the offspring of competiting pollen parents between crosses of Austrian pines. From a previous study, this species was found to exhibit three zones of activity for this enzyme. Each zone is known to be a product of a separate gene and are designated GOT-1, GOT-2 and GOT-3 for the fast, intermediate and slow migrating zones, respectively. GOT-2 bands were used as markers. This enzyme is polymorphic and exhibits either a fast or slow variant (alleles).

All pollen combinations included parent A-6 which was homozygous for the slow allele (2/2 genotype) and parents A-7, A-8 or A-11 which were homozygous for the fast allele (1/1 genotype). Selected cross-pollen combinations were applied to A-8 and A-11 which were also used as seed parents. The pollen parents of the offspring are easily identified by phenotypic banding patterns as illustrated in Figure 1. Embryos homozygous for the fast variant are single banded (1/1) and those heterozygous (1/2) are triple banded.

Crosses with an additional Austrain pine seed parent, A-3, were also made. Since A-3 was heterozygous, distinguishing the pollen parents was not as straight forward. Crosses with combined pollens result in two indistinguishable sets of heterozygous embryos. However, the allelic contribution of both parents of a heterozygote can be deduced if their megagametophytes are also analyzed. The allele of the egg is identical to that found in the megagametophyte. Thus the identity of the pollen parent of each heterozygote can be resolved. In Figure 1, heterozygous offspring are differentiated by designating their genotypes as 1/2 or 2/1.

Crosses between Austrian and Japanese red pine

Competing pollens of Austrian and Japanese red pine are distinguished in the offspring by banding patterns of ADH. From a previous study, Japanese red pine was found to contain a single, species-specific band for the ADH-2 locus (Tobolski and Conkle 1976). This fast-migrating allele produces unique triple-banded phenotypes found only in the hybrids between these species. Thus offspring derived from competing pollens are readily identified.

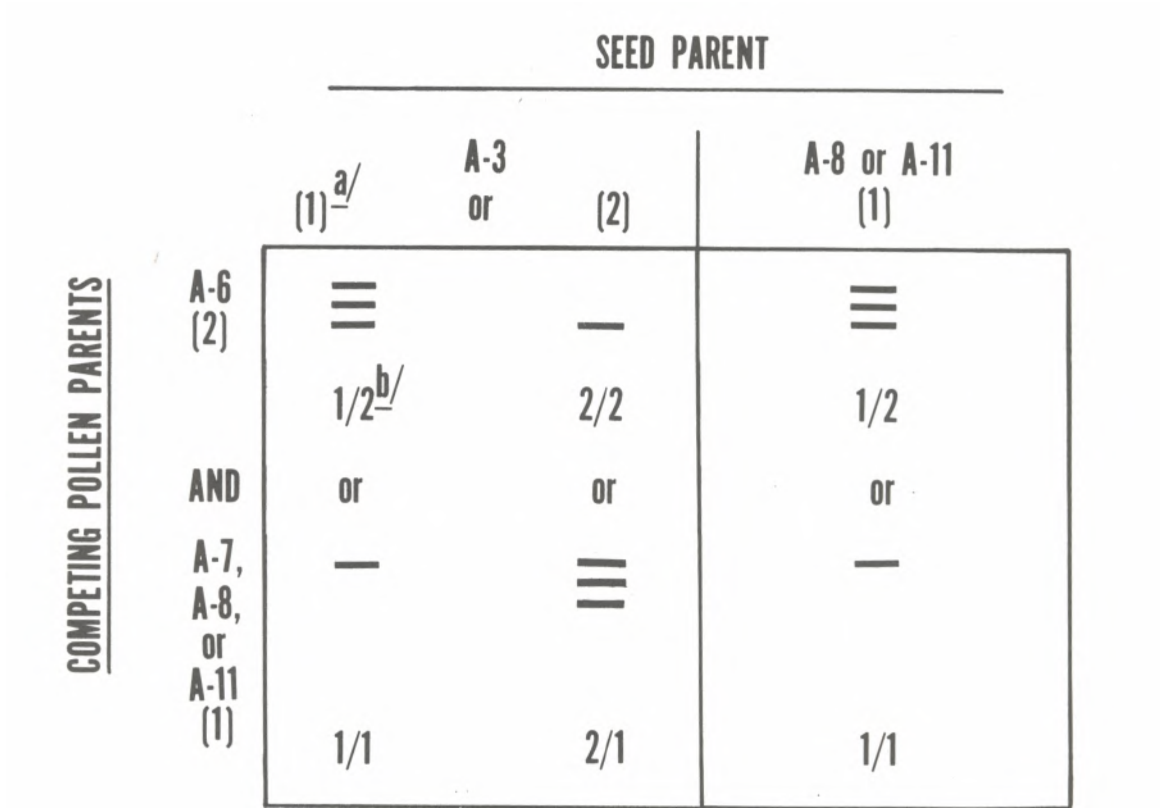
RESULTS AND DISCUSSION

Crosses between Austrian pines

Three of the five 50:50 percent pollen mixtures produced 'offspring in an expected 1:1 ratio (Table 1). Pollen mix A-6 + A-8 applied to parent A-11 and A-6 + A-11 applied to parent A-8 produced offspring which deviated markedly from that expected (X^2=7.3 and X2=7.6, respectively). In both cases pollen parent A-6 produced more offspring than the other parents. However the A-6 + A-8 combination competed equally when applied to parent A-3 as did A-6 + A-7 in crosses with the other parents.

The reasons for these skewed ratios is a matter of conjecture. Results of previous single pollen crosses between these trees showed that seed sets were high and of a similar magnitude. Differences in compatibility were not apparent. However when competing equally with other pollens, A-6 had an advantage in these two cases.

Figure 1. Allelic banding patterns and genotypes of embryo offspring derived from 2-parent pollen mixtures of controlled crosses in Austrian pine. The bands are characterstic of the GOT-2 locus.



a/ numbers in parenthesis designate alleles

b/ fractions designate genotype of embryos

Table 1. Proportions of offspring obtained after control pollinations among Austrian pines with 50:50 percent mixtures and sequential applications.

Austrian Seed Parent No.	Type of Pollination	Number Seedlings Analyzed	Competitive Effectiveness of Pollen No. 6	7	8	11	Increase in Effectiveness of 1st Pollen over mix
			PERCENT				
A-3	6 + 8 a/	170	52	-	48	-	-
	$6 \overset{2}{\rightarrow} 8$ b/	142	100	-	0	-	48
	$8 \overset{2}{\rightarrow} 6$	189	3	-	98	-	49
A-8	6 + 7	157	48	52	-	-	-
	$6 \overset{1}{\rightarrow} 7$	99	96	4	-	-	48
	$7 \overset{1}{\rightarrow} 6$	162	12	88	-	-	36
	$6 \overset{2}{\rightarrow} 7$	137	98	2	-	-	50
	6 + 11	111	63	-	-	37	-
	$11 \overset{2}{\rightarrow} 6$	107	8	-	-	92	55
A-11	6 + 7	169	53	47	-	-	-
	$6 \overset{1}{\rightarrow} 7$	124	63	37	-	-	10
	$7 \overset{1}{\rightarrow} 6$	154	31	69	-	-	22
	6 + 8	198	60	-	40	-	-
	$6 \overset{1}{\rightarrow} 8$	237	78	-	22	-	18
	$8 \overset{1}{\rightarrow} 6$	142	11	-	88	-	48

average effectiveness of first pollen after:
1 day -- 80.3 percent
2 days -- 96.8 percent

a./ 50-50 mix

b./ number denotes days lapsed between application of 1st and 2nd pollens

This may be due to a more vigorous pollen tube growth of A-6 and/or embryos fertilized by A-6 outcompete those of other pollens of multi-fertilized ovules.

Sequential applications of separate pollens on the same cone showed that the first pollen received was much more effective in accomplishing fertilization. When pollinations are separated by one day, the mean effectiveness of the first pollen was 80.3 percent which was an increase of 30.3 percent over 50:50 mixtures. After a two day lapse, effectiveness of the first pollen averaged 96.8 percent. This represents an increase of 50.3 percent over equally competing mixtures. Similar results were found by Franklin (1974) in slash pine. He found that the average effectiveness of the first pollen was 67 percent when the second pollen was followed from 0 to 5 days later.

The advantage of an earlier arriving pollen may be due to physical or biological phenomena associated with the mechanism of pollination. In Scots pine (*Pinus sylvestris* L.) (Doyle and O'Leary 1935) and pine (Franklin 1974) pollen is drawn into the pollen chamber by readsorption of the nucellar fluid. Once this occurs the fluid does not reappear and therefore, later additions of pollen are ineffective in fertilization. In Scots pine the nucellar fluid appears only in fully receptive cones. Immature cones may gather pollen for several days before the fluid is exuded and pollen grains withdrawn to the nucellus. Further not all ovules of a cone probably mature at the same time. Results of sequential pollinations therefore, may vary depending upon the receptive status of the ovules at the time each pollen is applied. Some of the variation among crosses observed in Austrian pine may be a result of such factors.

Sarvas (1962) contends that the nucellar fluid reappears for several successive nights in Scots pine regardless of the presence of pollen. However the limited capacity of the pollen chamber may physically exclude later arriving pollen. In this species, 18 percent of the ovules have room for only 1 pollen grain and 54 percent for only two grains. Further the first few pollen grains entering the chamber are in a better position for growth and eventual fertilization of the ovule. Whether the nucellar fluid is exuded only once or several times has not been determined for Austrian pine. However, similar physical limitations of the pollen chamber undoubtedly exist in Austrian pine which would also contribute to the advantage of early arriving pollen.

Crosses between Austrian and Japanese red pine

A 50:50 percent mixture of Austrian:Japanese red pine pollens applied to cones of Austrian pine yielded an average of 12.5 percent hybrids and 87.5 percent non-hybrids. The percentage of hybrids ranged from 5 to 25 percent among seed parents (Table 2). Similar proportions were observed in a previous study (Tobolski and Conkle 1976). Since these species are somewhat incompatible, these results are not unexpected. The favoring of native embryos over hybrid embryos probably result from a combination of embryonic competition and hybrid embryo deaths.

The relative importance of each of these factors may be determined by estimating the frequency of fertilization of each species. Estimates are based on the proportion of filled and empty seeds from crosses using mixtures of Austrian pine pollens and Austrian-Japanese red pine pollens. These estimates assume that the majority of empty seeds are a result of embryonic deaths (Sarvas 1962) or a breakdown of the ovule just prior to fertilization due to incompatible combinations (McWilliam 1959). The average number of empty seeds from Austrian pine pollen mixes was 9 percent and from Austrian-Japanese red pine mixes 23 percent. Total fertilizations or near fertilizations for both pollen mixes are assumed to be essentially the same since the total number of seeds per cone were similar. Austrian pollen mixtures produced an average of 40.2 seeds per cone and Austrian-Japanese red pine pollen mixtures produced 41.7 seeds per cone. In a mixture where Austrian pine makes up one-half of the pollen, 4.5 percent (9÷2) of the total fertilizations would result in empty seed. The proportion of empty seed due to fertilization by Austrian pine pollen would be approximately 20% (4.5÷23). Approximately 80 percent ([23-4.5]÷23) of the empty seed would be due to incompatible fertilizations with Japanese red pine. Thus the total number of ovules fertilized by Japanese red pine would be equal to the number of hybrid seedlings observed plus 80 percent of the empty seeds. The results of these adjustments indicate that approximately 25 percent of the ovules were fertilized by Japanese red pine and 75 percent by Austrian pine. Without embryonic mortality therefore, the percent of hybrids would double. The remaining proportions, 75 percent:25 percent, are likely a result of embryonic competition.

The disadvantage of the Japanese red pine pollen is dramatically overcome when it is applied first. The

Table 2. The proportion of hybrid and non-hybrid seed obtained after pollination with mixtures and sequential applications. Pollens utilized in the control crosses were Austrian Pine and Japanese Red Pine.

Austrian Seed Parent No.	Type of Pollination	Number Seedlings Analyzed	Hybrids	Non-Hybrids	Advantage of First Pollen Over Mix
			PERCENT		
A-3	A + J a/ 50:50 mix	104	14	86	-
	J30 $\xrightarrow{1}$ A6 b/	97	97	3	83
	J30 $\xrightarrow{2}$ A6	97	70	30	56
	J30 $\xrightarrow{1}$ A8	97	95	5	81
	J30 $\xrightarrow{2}$ A8	100	90	10	76
	J30 $\xrightarrow{1}$ A11	100	99	1	85
	A6 $\xrightarrow{2}$ J30	97	2	98	12
	A8 $\xrightarrow{2}$ J30	98	0	100	14
A-8	A + J 50:50 mix	100	25	75	-
	J30 $\xrightarrow{1}$ A6	101	82	18	57
A-11	A + J 50:50 mix	188	5	95	-
	J30 $\xrightarrow{1}$ A6	184	25	75	20
	J30 $\xrightarrow{2}$ A6	100	18	82	13
	J30 $\xrightarrow{1}$ A7	197	38	62	32
	J30 $\xrightarrow{2}$ A7	168	59	41	54
	A7 $\xrightarrow{1}$ J30	94	0	100	5

a./ A -- Austrian Pine Pollen; J -- Japanese Red Pine Pollen

b./ Number denotes days lapsed between application of 1st and 2nd pollen yield of hybrid seedlings averaged 67.3 percent when Japanese red pine pollen was applied 1 or 2 days before the pollen of Austrian pine. This represents an average increase of 53 percent in the production of hybrids over that obtained from mixed pollens. When Austrian pine pollen was applied first, the yield of native embryos averaged 99.3 percent. The greater effectiveness of Austrian pine pollen further substantiates the incompatibility between these species.

These results support the hypothesis tat abundant yields of natural hybrids in southern Michigan may largely be due to differences in phenology. The earlier shedding of Japanese red pine pollen and its favorable transfer by local winds would :_increase its effectiveness over Austrian pine.

However, even the combination of these events may not entirely account for a natural hybrid production of over 90 percent as reported by Wright et al. (1969). The yields of natural hybrids in the southern Michigan studies were based on nursery trials. Cen pollinated seed collected from Austrian pine were broadcast sown and identity of the hybrids were made after 1 to 4 years of seedling growth. Since hybrid seedlings are much more vigorous than Austrian pine seedlings, differences in mortality may have increased the proportion of hybrids observed. Bias due mortality is virtually eliminated with isozyme analysis.

Another important variable to consider is interspecies compatibility. Compatibility between these species appears to vary considerably among Austrian pine seed parents. This is reflected in the proportion of hybrid seedlings produced from pollen mixes in this study and in the variation in yields of filled seeds from single pollen crosses in previous studies (unpublished). Seed parent A-11 produced only 5 percent hybrids from Austrian-Japanese red pine pollen mixtures and 35 percent hybrids after sequential pollinations using Japanese red pine pollen first. A-3 was apparently more compatible. This seed parent produced 14 percent hybrids with mixtures and 90 percent hybrids after sequential pollinations. Compatibility of the Austrian pines in the southern Michigan plantation is unknown. A relatively high compatibility, of course, would also contribute to the high yield of natural hybrids.

CONCLUSIONS

Results of these studies imply that the production of a random mixture of seeds from a seed orchard is unlikely. Some pollens will be more successful than others in producing seed. Success of a pollen parent will be a consequence of such factors as: timing of pollen arrival, growth rates of pollens, embryonic competition and embryo mortality. Other variables such as the location of a selection, wind patterns, seasonal weather conditions, and phenology of surrounding selections will also influence the effectiveness of a particular pollen parent. Franklin (1974) suggests that mass artificial pollination, just 1 day in advance of natural pollination, could increase seed production by reducing the loss of fertilized seed due to selfing.

The production of natural hybrids may be accomplished by mixed species plantings. The degree of success will depend upon the compatibility of selected clones and differences in phenology. Timeliness however, appears to be the most important variable. This suggests that a one-way crossing pattern should be devised in which the species acting as the male parent releases pollen just prior to the species acting as the female. If phenological differences cannot be attained through clonal selection, chemical or environmental regulation may be possible. For example pollen development can be advanced by warming the catkins through bagging (Boyer and Woods 1973). Perhaps other cost-effective techniques could be devised on a larger scale to advance pollen development. Successful production of natural hybrids will also depend upon the transfer of adequate amounts of pollen to the female strobili. Thus the design of a mixed species planting is critical and will be influenced to a large measure by local wind patterns.

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