ENZYME IDENTIFICATION OF AUSTRIAN X JAPANESE RED PINE HYBRIDS IN SEED FROM MIXED POLLEN CONTROLLED CROSSES.

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Abstract.--Mixed pollen samples with 10:90, 50:50, and 90:10 percent of Pinus densiflora and P. nigra pollen respectively were used to control pollinate P. nigra seed trees. The test was undertaken to determine if hybrids were produced in proportion to P. densiflora pollens in the mixes. Offspring embryos were electrophoresed and hybrid offspring were identified using alcohol dehydrogenase alleles that differed between the two species. In all three pollen-mix treatments, hybrid production was significantly reduced over that expected from the ratio of P. densiflora species pollen in the mix. With low and moderate concentrations of P. densiflora low frequencies of hybrids are produced. The pollen mix had to contain high concentrations of P. densiflora pollen before significantly large proportions of hybrids were produced. The data supports the hypothesis that native embryos are highly favored over hybrid embryos as judged by the proportion of hybrids in the resulting seed, The results are discussed briefly with reference to the suggestion that hybrids can be mass produced by natural fertilization of mixed plantings of P. nigra and P. densiflora trees.

Additional keywords: Alcohol dehydrogenase, crossing incompatibility.

Inter-racial and inter-species hybridization as a tool for crop improvement has greatest utility for enriching genetic variability of populations under selection and for transferring desirable traits into populations that lack them. In this sense, the utilization of F-1 hybrids that show outstanding growth traits are rare in forestry. The F-1 hybrids between Austrian and Japanese red pine appears to be one of these rare exceptions.

Nursery and field observations of the hybrid between Austrian pine (Pinus nigra Arn. var. Austrica [Hoess] Aschers and Graebn.) and Japanese red pine (P. densiflora Sieb. and Zucc.) indicate that the tree holds promise for forest and speciality plantings in the Lake State Region, The hybrids have several distinguishing traits (Austin 1927, Wright and Gabriel 1958, Vidakovic 1966, and Wright et al. 1969), the most important from the local standpoint being that they outgrow both parent species in the area of southern Michigan and northern Indiana. In Michigan and Indiana Austrian pine has been found to be an acceptable tree for planting for a long time. The Austrian pines have a moderate growth rate and appear to be tolerant of road salt, but they are susceptible to some forest pests in southern Michigan (Wright, in press). Japanese red pines have a greater rate of height growth than Austrian pines but the trees have small diameters and much less wood volume. The height growth of hybrids generally exceeds the growth of Japanese red pines. Their diameter growth is observed to be significantly greater than both Austrian and Japanese red pines but they too appear to be susceptible to some pests.

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The hybrids characteristically have a deep tap root and seedlings are excellent competitors on sandier sites. If the hybrids continue to excel in growth, they will be preferred over the pure species for planting sites in southern Michigan and northern Indiana.

Spontaneous hybrids have been produced by wind pollinated trees in a southern Michigan plantation where Austrian pines and Japanese red pines occupied adjacent blocks (Wright et al. 1969). Volunteers with good growth rates were confirmed to be hybrids. The earliest hybrids became established when the plantation trees were 15 years old. Open-pollinated seed collections, collected about 10 years later, contained significant proportions of hybrid seed as judged by subsequent nursery and field trials. The proportion of hybrids in open-pollinated seed collections were determined from 1-, 2-, and 4-year observations of nursery-grown seedlings (Wright et al. 1969). In those tests, after 2 years, numerous seedlings could be. classified as hybrids. By the fourth year, 92 percent of total of 629 trees were determined to be hybrids. In another test, 81 percent of 2,000 transplanted 1-1 seedlings were probable hybrids. Why were such high proportions of hybrid seed obtained from natural pollinations in the presence of pollen from the parent species?

Two causal factors were suggested: 1. Differences in phenology may have resulted in disproportionately large quantities of P. densiflora pollen fertilizing P. nigra cones. Local wind patterns in the Michigan plantations favored the transfer of Japanese red pine pollen into the area of the Austrian pines. But the Austrian pines were in full pollen production and the effect of pollen migration alone was not thought to be sufficient to account for the high proportion of hybrids. Or, 2., hybrid embryos may have shown heterosis during seed development. Since the vigor of hybrids at young ages was greater than that of the parental P. nigra trees, it was speculated that embryoselection might favor hybrids in seeds with multiple fertilizations. If selection for hybrids during the polyembronic stages of development was accounting for the high proportion of hybrids produced on P. nigra parents, only 27 percent of P. densiflora pollen in the pollen cloud could result in 80 percent hybrid seed production. Since it is likely that this hybrid will be mass produced in the future, it is desirable to establish which hypothesis is most likely.

Wright had no way of testing the percentage of P. densiflora pollen actually arriving at the P. nigra flowers nor was there a way of testing the initial frequency of hybrids in the seed of open pollinated P. nigra trees.

We tested the hypothesis that hybrid embryos show heterosis during the seed development stage by determining the frequency of hybrids in progeny from controlled crosses using pollen mixtures from the two species. The effect of different phenologies is eliminated. Pollen of both species are delivered to receptive cones at the same time.

TEST CONDITIONS

During 1970 and 1971, three P. nigra trees in Fort Wayne, Indiana', were used as seed parents for a test of polyembronic selection favoring hybrid embryos. Controlled pollinations were carried out with known proportions of P. nigra and P. densiflora in a pollen mix. To minimize the effect of single-tree incompatibilities, equal quantities of fresh pollen from 10 trees

were mixed to create a pollen sample for P. nigra. The P. nigra pollen parents were different trees than those chosen as seed parents. There was minimum opportunity for self incompatibility to be a factor in crosses between P. nigra parents. Nine P. densiflora pollen parents were chosen to represent a P. densiflora sample. The P. nigra pollens were mixed with P. densiflora to make mixed pollen lots with 10, 50, and 90 percent P. densiflora pollen by volume.

Standard control-pollination techniques were used to obtain the seed progeny. Samples of seed from the different pollen treatments were grown in a nursery trial to determine the proportion of hybrids. These trials will be reported at a later date. Another sample was analyzed using starch gel electrophoresis to estimate the initial frequency of hybrids for the various pollen treatments.

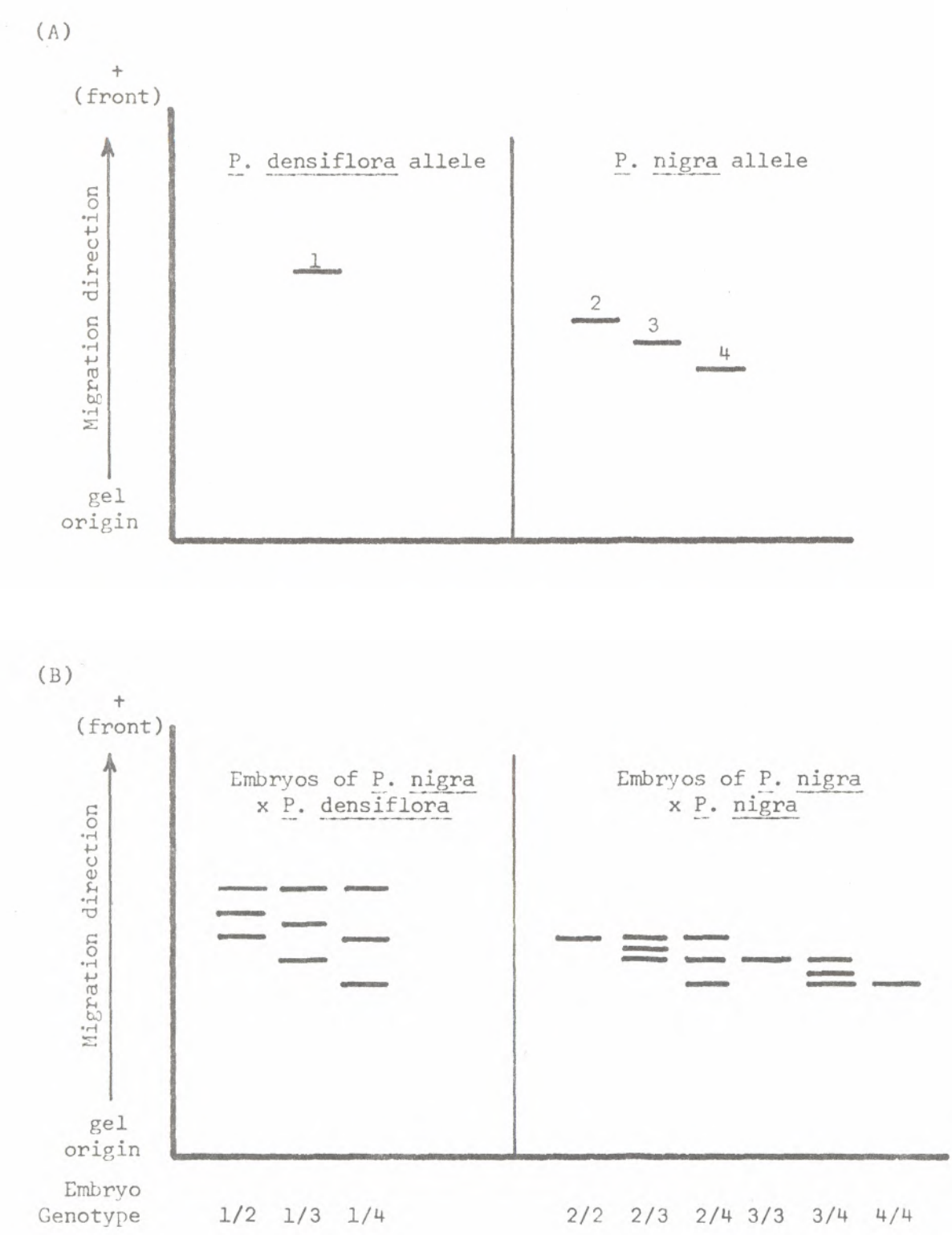
DETERMINATION OF HYBRID PROGENY PROPORTIONS USING ENZYME MARKERS

During the course of a study of the inheritance of various enzymes, we determined that P. nigra and P. densiflora had alleles for glucose-6-phosphate-dehydrogenase and alcohol dehydrogenase (ADH) that were unique for each species. The alleles were unique in the sense that certain forms were always associated with P. nigra seed and different forms were always found for P. densiflora seed. Either or both of the enzyme analyses would have yielded accurate estimates of hybrid proportions in the mixed pollen treatments but ADH was chosen because of the exactness with which alternative alleles could be identified.

Newly germinated embryos (radicles 0-3 mm.) were separated from gametophytes. The embryos were analyzed by technique developed for conifers (Conkle 1972). ADH band patterns on starch gels occur in three zones. The current analysis was concerned only with the bands of the darkest staining zone. Previous genetic studies confirmed that P. densiflora had a single band in this region that equated to a fixed allele in the seeds thus far sampled (fig. 1A-allele 1). Pinus nigra had bands that displayed three different mobilities (fig. 1A-alleles 2, 3, and 4). The mobilities are controlled by alleles that segregate and assort according to strict Mendelian proportions. Furthermore, the active ADH enzyme produced patterns that suggested the molecule consisted of two molecular subunits. In heterozygous seeds, containing alternative alleles, the subunits combined to give three bands. Two bands correspond to the migration distances of the two alternative alleles and a third band was intermediate. The intermediate band had subunits from both alleles. Hybrids also form three banded phenotypes that are expected when they have different alleles of the same active enzyme.

Various types of band combinations were found in the mixed pollen offspring. There were heterozygotes between the P. densiflora allele and the three alternative P. nigra alleles (fig. 1B, embryos of P. nigra x P. densiflora, genotypes 1/2, 1/3, and 1/4). Six types of offspring were found that corresponded to combinations of the three alternative alleles in P. nigra. The enzyme bands from embryos in the mixed pollen treatments were scored according to whether they were P. nigra x P. densiflora combinations or P. nigra x P. nigra combinations.

Figure 1. Alcohol dehydrogenase band patterns on starch gels; (A) ADH alleles in Pinus densiflora and P. nigra, (B) ADH forms and their genotypes found in embryos from mixed pollen controlled crosses



The proportions of hybrids in the mixed pollen families were determined for the three pollen treatments (table 1). When the pollen treatment had 10 percent P. densiflora pollen, 1 percent of the offspring are hybrids. When P. densiflora was 50 percent, 15 percent of the offspring are hybrids. Lastly, when P. densiflora pollen was 90 percent of the mix, 75 percent of the offspring are hybrids. In all cases there is a reduction in the proportion of hybrids produced over that expected if hybrids are produced in the proportions of pollen in the pollen mixes. There is a 9-fold reduction when P. densiflora is 10 percent of the mix. There is a 0.7 reduction when P. densiflora is 1/2 of the mix and there is a 0.17 reduction when P. densiflora is 90 percent of the mix. In other words, when P. densiflora pollen is a small percentage of the mix there is a substantial reduction in the production of hybrids, when a moderate percentage, there is a moderate reduction in hybrid production, and when in high concentration there is a slight but highly statistically significant reduction.

Table 1. Proportion of hybrid progeny in Pinus nigra families when the pollens of controlled crosses contained different concentrations of P. densiflora and P. nigra pollen.

Proportion of P. densiflora pollen in the pollen mix	Total number of seed analyzed	Proportion of hybrids in mixed pollen families
.10	204	.01 1/
.50	312	.15
.90	106	.75

1/ All three proportions of hybrids were highly significantly different ($p<.001$) from the expected proportions based on P. densiflora pollen in the mix.

These results argue against the hypothesis that hybrids are favored during polyembronic selection. Hybrids are at a disadvantage in comparison with pure P. nigra and only when P. densiflora pollen is in high proportions are a significantly high percentage of the offspring hybrids produced.

SIGNIFICANCE OF THE RESULTS

This data indicates that the hybrids are at a selection disadvantage in a pollen mix trial. Our initial expectation was the same as Wright's. Namely since hybrid seedlings show early growth vigor in comparison with pure species offspring, the growth of hybrid embryos during seed development might exceed that of pure species embryos. With multiple embryos and the opportunity for embryo competition within ovules the frequency of hybrids in the final proportion was expected to exceed the proportion of "hybrid" pollen in the pollen mix. Instead, the reverse was true. Native species embryos are strongly favored over hybrid embryos.

These findings imply that factors other than selection favoring hybrid seed production in the presence of mixed pollen are contributing to the high

proportion of hybrids in open-pollinated P. nigra progeny. The Michigan plantation of P. nigra parents might have been subjected to high concentrations of P. densiflora pollens. Pollen ratios of nine P. densiflora to one P. nigra would be required to approach Wright's observation and this would occur if pollen production from P. densiflora swamped that of P. nigra. The high proportion of hybrids in the open-pollinated progeny could also be accounted for by species differences in pollen and cone phenology. Pinus densiflora pollen-shed precedes P. nigra. If P. nigra cones were receptive at the peak of P. densiflora pollen shed a high percentage of the resulting seed could be hybrid. Arguing against this possibility is the field observation that pollen-shed and cone receptivity are usually synchronous within a species.

Wright determined hybrid proportions in the southern Michigan studies after 1 to 4 years of seedling growth in the nursery or transplant beds. Generally hybrids cannot be readily identified until seedlings are 2 to 4 years old since the traits of hybrids are more strongly displayed at older ages. Hybrid seedlings are more vigorous than P. nigra seedlings and thus the high proportion of observed hybrids may be due in part to their higher survival. Isozyme analysis eliminate any bias due to mortality since the pedigree of every filled seed can be accurately and rapidly determined.

Hypotheses to explain the significant reduction in hybrid seed production when pollens are applied in mixtures can be advanced. The non-native species pollen may be less successful than native species pollen in fertilizing ovules. This could be the case if P. densiflora pollen was less viable than P. nigra or if once growing in the nucellar tissue, the P. nigra pollens had a greater rate of growth and fertilized more eggs. Or, the hybrid embryos may be less successful in competition with native species embryos in ovules with both embryo types. The species are somewhat incompatible (Wright and Gabriel 1958); hybrid seed set is lower than that from the pure species combinations. Genetic deaths apparently eliminate a proportion of the hybrid embryos. In the mixed pollen study, the death of hybrids in ovules with mixed multiple embryos would tend to produce the higher observed non-hybrid proportions. Indeed, the actual situation may be a combination of several or all these cases. We have no data that can discern between the different alternatives.

To summarize, high proportions of hybrid seed have been obtained from open-pollinated seed collections of P. nigra which were adjacent to a P. densiflora pollen source. Mass production of hybrid seed in the future may be accomplished by duplicating these conditions. But the results of this study indicate that hybrids will be produced in low proportions in most mixed pollen situations. Only when P. densiflora pollens are in high concentrations will significant numbers of hybrid seed be obtained. It remains to be seen whether mixed plantings of P. nigra and P. densiflora will consistently provide the conditions needed to produce significant quantities of hybrid seed. Vie suggest that mixed species plantings should initially be attempted on a small scale. At present, with high pure P. nigra seed production from mixed pollen crosses, the surest way of obtaining hybrids is by controlled-pollinations.

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