Scand. J. For. Res. 1: 309–315, 1986

Supplemental Mass Pollination in a Seed Orchard of *Pinus Sylvestris* L. Investigated by Isozyme Analyses

REZA YAZDANI,[1] GUSTAF HADDERS[2] and ALFRED E. SZMIDT[1]

[1]*1Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S-901 83 Umeå, Sweden,* [2]*Institute for Forest Improvement, Central District, Box 7007, S-750 07 Uppsala, Sweden and* **Permanent address: Institute of Dendrology, Polish Academy of Sciences, 62-035 Kornik, Poland*

Scandinavian Journal of Forest Research

Yazdani, R., Hadders, G. and Szmidt, A. E. (The Swedish University of Agricultural Sciences, Department of Forest Genetics and Plant Physiology, S-901 83 Umeå, Sweden and Institute for Forest Improvement, Central District, Box 7007, S-750 07 Uppsala, Sweden). Supplemental mass pollination in a seed orchard of *Pinus Sylvestris* L. investigated by isozyme analyses. Accepted Apr. 4, 1986. Scand. J. For. Res. 309–315, 1986.

Isozyme-analysis techniques have been applied to test the efficiency of supplemental mass pollination in a seed orchard of *Pinus Sylvestris*. Mass pollination was done on one single occasion before the general pollen dispersal. The results of isozyme analyses of seed after mass pollination demonstrate that it is possible to introduce desirable genes into the seed orchard crop. Mass pollination without isolation of female strobili gave an average contribution of 4% to the total fertilizations and after isolation of female strobili 26.5%. The main conclusion is that supplemental mass pollination offers very interesting possibilities for breeding, particularly if some kind of isolation of the female flowers is made. It can increase the seed quality as well as improve the breeding value of the seed orchard. More attention should be paid to increasing the efficiency of the controlled pollination. It is suggested that in the future, more effort is invested in improving the technique of mass pollination. *Key words: Pinus sylvestris, seed orchard, pollination, isozymes.*

INTRODUCTION

The strobilus of pine receives pollen continuously during its receptive period. Of several pollen grains reaching the ovule, only one succeeds in introducing its genes to the seed which is produced after fertilization. A mature, pollinated cone, might contain seeds of various paternal origin.

By isozyme analysis of both the haploid and diploid seed tissues, it is possible to determine the allelic composition of the ovule and the pollen forming the embryo. In this way the pollen gene pool existing in the seed orchard, can be determined. By using a multilocus system it is possible to determine the contribution of each clone to the pollen pool and to determine the amount of pollen coming into the seed orchard from outside (Friedman & Adams, 1982; Smith & Adams, 1983; Nagasaka & Szmidt, 1984; Friedman & Adams, 1985). Another approach is to use so-called rare or unique alleles for identification of pollen sources (Shen et al. 1981).

In this study we have tested the efficiency of a mass pollination method using a model helicopter. Isozyme techniques have been applied to investigate the genetic make up of the embryo and endosperm of seeds, and to determine the effectiveness of artificial mass pollination. Our results clearly show that this method could be successfully applied in seed orchards.

MATERIAL AND METHODS

The male parental clones for the mass pollination study were selected on the basis of the presence of rare isozyme markers. Clone W 1038 was heterozygous for LAP-B with

genotype B1/B2, and with B1 as the rare allele, while clone F2029 was heterozygous at the GOT-B with genotype (B1/B3), and with B1 as the rare allele. Both clones originated from the seed orchard at Långtora in central Sweden.

Male strobili were collected from grafts of these two clones in the seed orchard at Långtora shortly before pollen release and kept indoors. Pollen was extracted and dried under controlled temperature and humidity conditions and then stored at −20°C for one year.

The pollination was done with the help of a radio-controlled model helicopter in June 1981 in the seed orchard at Sollerön in central Sweden. The pollen was distributed over the top of ten sample trees. Mass pollination was carried out with 20 ml pure pollen per graft (equivalent to four litres per ha with 200 grafts) on one single occasion and at a time when most of the female strobili were receptive but before the general pollen shedding in the seed orchard. Seed for examination was collected from the upper and lower parts of the crown. For some clones the female strobili were isolated directly after the mass pollination. Seed from open pollination was also collected from the same clone but in another part of the seed orchard. Seven seeds from each clone were used to study the genotypic structure of clones in the seed orchard at Sollerön. The seed orchard consisted of 25 different clones.

Isozyme separation was carried out by starch gel electrophoresis. Glutamate oxalate transaminase (GOT) and leucine aminopeptidase (LAP) were analysed in haploid macrogametophyte tissue as well as in the diploid embryo. Methods of extraction and staining of enzymes were previously described by Rudin (1977) for LAP and Rudin (1975) for GOT.

RESULTS AND DISCUSSION

Frequency of LAP-B1 in open-pollinated seed

In open-pollinated seed from one graft of the W 1038 clone present in the seed orchard at Långtora, (genotype LAP-B1/B2), 21 out of 46 embryos carried the LAP-B1 allele (Table 1). This is compatible with 1 : 1 distribution from a heterozygous clone.

In the whole seed orchard of Sollerön, after open pollination, 2 out of 1633 embryos carried LAP-B1 allele. This gives a frequency of 0.12%, which is an extremely low background allelic frequency in the seed orchard.

Frequency of GOT-B1 in open-pollinated seed

Clone W 4009 with 55 grafts distributed throughout the seed orchard of Sollerön was found to be a heterozygote for GOT-B1/B2 genotype. In one graft of the W 4009 clone, 72 embryos out of 145 appear to have GOT-B1 (Table 1). This segregation is also compatible with a 1 : 1 distribution pattern. No material was available for test of inheritance in clone F2029 with GOT-B1/B2 genotype from the Långtora seed orchard.

Of 899 embryos from all clones, after open pollination, 46 carried GOT-B1, which corresponds to 5.1% frequency (Table 1). This frequency of background pollen is rather high and is assumed to be partly due to the presence of the same allele in 55 ramets of the clone 4009 and partly due to the general pollen cloud from outside sources.

Pollination with clone W 1038 carrying LAP-B1 as marker

About 700 embryos from five clones, with one graft each, were studied for LAP-B1 marker after mass pollination without isolation. Isozyme analysis has been carried out on seeds collected from two parts of the crown, low (L) and high (H) (Table 2). On an average, 2.1% of the embryos had LAP-B1. This means that 4.2% of the pollen from W 1038 clone had participated in fertilization of the seed analysed, since half of the pollen grains carry

other LAP alleles. There are differences among clones for LAP-B1 frequency in embryos. In some clones no trace is found of this allele. The efficiency of the mass pollination was higher in the upper parts of the crown than in the lower parts, and corresponded to 6.6 and 1.32%, respectively.

Pollination with clone F 2029 carrying GOT-B1 as marker

High allelic frequency for GOT-B1 in Sollerön partly resulted from clone W 4009 within the orchard and partly from pollen coming from outside sources. The average background frequency for GOT-B1 allele is 5.1%. Therefore it is necessary to use a correction factor (GOT-B1% −5.1)×2 for calculating the pollen contribution of clone F2029 to artificial pollination. A similar correction factor can be applied to clone W1038 with LAP-B1 marker. Background pollen correction is a difficult thing to estimate in this sort of experiment. Isolation will certainly decrease it, but not totally. Thus it will not be as high as when no isolation was used but will also differ from 0. In low parts of the crown, 11 out

Table 1. *Isozyme pattern in open-pollinated seed from clones which were used in the mass-pollination study*

Seeds were collected from high (H) and low (L) parts of the crown

Clones	Crown part	Sample size	No. of valid observations with LAP-B	Embryos with LAP-B1	No. of valid observations with GOT-B	Embryos with GOT-B
Z2017	H	10	10	0	8	3
Z2090	L	56	35	0	47	1
Z2090	H	93	87	0	61	4
Z2091	H	10	10	0	10	0
Z2092	L		107	0	39	0
Z2092	H		98	0	51	0
Z3032	L	93	92	0	66	4
Z3032	H	93	93	0	71	3
Z4005	H	10	10	0	0	0
Z4012	L	60	58	0	58	0
Z4012	H	67	67	1	45	0
Z4402	L	96	78	0	52	3
Z4402	H	143	139	0	37	4
Z4404	L		89	0		
Z4404	H		70	0		
W1038	?	67	46	21	61	4
W2000	H	10	10	0	8	1
W2001	L	60	60	0	54	6
W2001	H	61	61	0	50	3
W4009	L	68	68	0	64	32
W4009	H	81	74	0	81	40
W4013	?	7	7	0	6	2
X4209	L	61	57	0	46	5
X4209	H	71	49	0	41	0
X4302	L		90	0	33	1
X4302	H		104	1	47	2
X4303	?	10	10	0	8	0
Sum of all clones	L		728	0	395	20
	H		888	2	429	20
	H+L+ others		1 633	2	899	46

of 217 embryos carried GOT-B1 and in upper parts of the crown 25 out of 293. A total of 36 out of 510 seeds carried GOT-B1, and this corresponds to 7.1% frequency. To compensate for heterozygosity, values for both lower and upper parts of the crown were multiplied by 2, giving a value of efficiency corresponding to 14.2% (Table 2).

Mass pollination with isolation of female strobili

Two clones, 2090 and 2092, were mass pollinated with clone F2029 carrying GOT-B1. Three clones, 2092, 4302 and Z4404, were pollinated with clone W1038 with LAP-B1 as marker. The female strobili were isolated after mass pollination to prevent further introduction of new pollen. Some pollination with background pollen in the seed orchard is likely to have occurred before the isolation of female strobili.

Of 57 embryos analysed, 10 were found to carry GOT-B1 after artificial pollination with pollen from clone F2029. This gives a frequency of 17.5% and efficiency of 35% (Table 3). 26 out of 181 embryos were carrying LAP-B1 allele. This corresponds to 14.4% frequency which gives an efficiency of 28.8% (Table 3).

The total number of embryos with GOT-B1 and LAP-B1 allele frequency in open pollinated seed are presented in Table 4 along with the effects of mass pollination with and without isolation of female strobili. The efficiency of artificial mass pollination is found by subtracting the background pollination from the open pollination. This gives on average a 4% efficiency for both LAP-B1 and GOT-B1. Higher efficiency was found in the upper parts of the crown than in the lower parts. Efficiency of the mass-pollination together with isolation of female strobili and after subtraction for background pollen gives a value of

Table 2. *Effect of artificial mass pollination without isolation of female strobili from high (H) and low (L) parts of the crown*

Crosses	Crown part	Sample size	No. of valid observations with LAP-B	Embryos with LAP-B1	Embryo frequency (%)	No. of valid observations with GOT-B	Embryos with GOT-B1	Embryo frequency (%)
X4302×W1038	L		80	1	1.25	66	1	1.5
X4302×W1038	H	112	4	3.6	51	0		
Z2090×F2029	L	91	58	0	0	30	6	20.0
Z2090×F2029	H	88	60	0	0	74	6	8.1
Z2092×F2029	L	91	91	0	0	56	2	3.7
Z2092×F2029	H	77	77	0	0	54	6	11.1
Z2092×W1038	L		79	0	0	49	1	2.0
Z2092×W1038	H		80	6	7.5	52	4	7.7
Z3032×F2029	L	71	71	0	0	53	3	5.7
Z3032×F2029	H	87	86	0	0	52	5	9.6
Z4010×F2029	L	34	33	0	0	28	0	0
Z4010×F2029	H	59	58	1	1.7	55	1	1.8
Z4012×F2029	L	62	60	0	0	50	0	0
Z4012×F2029	H	97	71	0	0	58	7	12.1
Z4402×W1038	L	73	43	0	0	70	9	12.9
Z4402×W1038	H	67	66	1	1.5	51	1	2.0
Z4404×W1038	L		46	1	2.2	11	6	54.5
Z4404×W1038	H		82	2	2.4	51	25	49.0
W4606×W1038	L	67	54	0	0	63	1	1.6
W4606×W1038	H		58	0	0	53	5	9.4
Total	L		302	2	0.66	217	11	5.1
Total	H		398	13	3.3	293	25	8.5
Total	L+H		700	15	2.1	510	36	7.1

28.6% for LAP-B1 and 24.8% for GOT-B1. The results of mass-pollination for both LAP-B1 and GOT-B1 are rather consistent. In both cases it is clear that the efficiency of mass pollination is increased by isolation of the female strobili.

Earlier experiments with artificial mass pollination

In an investigation with *Pinus elliotti* with controlled crosses, it has been shown that in 73% of fertilizations the first applied pollen was active (Franklin, 1971; Franklin, 1974). According to Sarvas (1962), there is often not room for more than 2–3 pollen grains in the pollen chamber of *Pinus sylvestris*. The optimal time for mass pollination in *Pinus sylvestris* is short—from the time when the female strobili reach the respective stage for pollination to the time when pollen to a large extent begin to disperse (Hadders, 1984). Allen & Sziklai (1962) have succeeded in pollinating Douglas-fir with suspensions of pollen in water, applied as a spray, thereby showing that viability is not necessarily impaired by immersion in water. The experiment with controlled pollination in *Pieca abies* was made on one clone under good weather conditions. The result demonstrates that without isolation of female flowers, no or only very small effects of supplemental mass pollination

Table 3. *Effect of artificial mass pollination with isolation of female strobili from high (H) and low (L) parts of the crown*

Crosses	Crown part	Sample size	No. of valid observations with LAP-B	Embryos with LAP-B1	Embryo frequency (%)	No. of valid observations with GOT-B	Embryos with GOT-B1	Embryo frequency (%)
2090×2029	H	18	18	0	0	5	3	60.0
2092×2029	H	53	50	0	0	52	7	13.5
W1038×2092	L	25	25	0	0			
W1038×2092	H	25	25	3	12.0			
W1038×2092	Unknown	76	16	21				
W1038×4302		13	2	15.4				
W1038×Z4404		42	5	11.9				
Total		181	26	14.4		57	10	17.5

Table 4. *Total effect of artificial mass pollination with and without isolation of female strobili from high (H) and low (L) parts of the crown*

Crosses	Crown part	No. of valid observations with LAP-B	Embryos with LAP-B1	Embryo frequency (%)	Efficiency (%)	No. of valid observations with GOT-B	Embryos with GOT-B1	Embryo frequency (%)	Efficiency (%)
Open pollination	High	882	2	0.23	0.46	429	20	4.7	9.4
	Low	734	0	0.00		395	20	5.1	10.2
	Total	1 633	2	0.12	0.24	899	46	5.1	10.2
Mass pollination	High	398	13	3.30	6.60	293	25	8.5	17
without isolation	Low	302	2	0.66	1.32	217	11	5.1	10.2
	Total	700	15	2.10	4.20	510	36	7.1	14.2
Mass pollination with isolation		181	26	14.40	28.80	57	10	17.5	35

on full-seed production could be detected (Dietrichson et al., 1980). This is also explained by the fact that not all strobili, and not even all ovules within a single strobilus, are receptive to pollination at any one time. Under conditions with a very rich pollen dispersal the pollen-chambers are likely to be filled successively as they open. In this case artificial mass-pollination done all at one time will not be successful. A simple method of mass-production of *Pinus echinata* Mill. × *Pinus elliottii* Engelm. hybrids was attempted using an artificial mass-pollination technique. The progenies from these crosses showed definite evidence of hybridisation both in the nursery and in a field trial, and appeared to be very promising in growth characteristics (Wakeley et al., 1966). It was demonstrated that artificial hand-pollination in some South African orchards more than doubled the yield of filled seeds compared to open-pollination even with abundant pollen production in the orchard (Denison, 1971). Supplemental mass pollination could significantly increase filled-seed production in both very early or late flowering clones (Daniels, 1978). The seed set of hand-pollinated and open-pollinated clones in the orchards of *P. taeda* and *P. elliotti* were compared by van der Sijde (1971). In *P. taeda* hand pollination resulted in a 20% increase in full seed per cone, while in *P. elliotti* the increase was nearly 200%.

According to Hadders (1975), it is possible to decrease the frequency of selfed seed in self-fertile clones of *Pinus sylvestris* and to increase the breeding value of seed from seed orchards with artificial mass pollination.

The utilization of a water spray cooling system to delay bud development within a Douglas-fir seed orchard has proved to be beneficial in minimizing contamination from local pollen (Silen & Keane, 1969; Fashler & Devitt, 1980; El-Kassaby & Ritland, 1985), improving panmixis in the orchard, increasing the out-crossing rate, and eliminating differences in out-crossing rate between different crown segments (El-Kassaby et al. in press). For increasing the out-crossing rate and decreasing the frequency of consanguineous mating in the seedling portion, it is suggested that supplemental mass pollination to be used (El-Kassaby et al., in press).

The isozyme approach was used to estimate the proportion of hybrid seed obtained when unbagged female flowers of pitch pine were mass pollinated with pollen mixes from loblolly pine trees (Adams, 1979). On the basis of the proportion of pollen pools of mass-pollinated clones carrying a certain isozyme marker, the proportion of hybrid seed was estimated to range from 2 to 42% (average 16%) for six clones. Since the isozyme technique determines the extent of hybrid seed set, it is suggested that for nursery establishment only seeds of clones with high hybrid seed percentages should be sown (Adams, 1979).

Since the potential gains to be made by artificial pollination are several, it is suggested by Woessner & Franklin (1973) that more thought and research effort must be given to this particular aspect of seed-orchard management. The results of our study, which are based only on pollination on a single occasion, clearly demonstrate that artificial mass pollination is possible in *Pinus sylvestris* if some kind of isolation of the female flowers is made. It is suggested that more effort is invested in improving the technique of mass-pollination, with the aim of achieving higher efficiency.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Swedish National Board of Forestry. We thank Professor Dag Lindgren for valuable comments on the manuscript. We also thank Gun Lindqvist and Maj-Lene Åman for skillful laboratory work, and Helene Risberg, Lena Törnström for typing the manuscript.

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