function of AP-A as a marker locus. It parallels the SD-system if AP-A is assigned the role of the Sd-locus and D acts as the Rsp-locus. In this case, A_0 would correspond to one of the Sd-alleles and A_1, A_2, and A_3 would be allelic variants of the other, while D_1 would correspond to the "insensitive" allele Rsp^i and D_2 to the "sensitive" allele Rsp^s. With the help of this analogy, we can explain all of our observations. For one, the reversal in the direction of distortion with respect to A_0 can be reasoned by the fact that, in a male heterozygous for Sd/Sd+ and Rsp^s/Rsp^i, the Sd-allele which happens to be located on the Rsp^s-chromosome is the one to be distorted. In addition, the observation of Rsp^s-alleles of differing sensitivity (corresponding in our case to D_2 and D_3) can be drawn upon to explain the heterogeneity in the amount of distortion observed. In fact, one of the amounts is of the same extreme order of magnitude as that observed in the SD-system. Furthermore, the necessarily two-fold function of the AP-A-enzyme as modifier and marker locus is quite credible, since the Sd-locus is assumed to produce a regulatory protein (perhaps an enzyme?) which could, at least in principle, be made visible by means of electrophoresis. Finally, just as A_0 can be thought to cause internal "stress" conditions for megasporogenesis, as mentioned above, the modifying effect of the allele Sd on Rsp^s can be considered as a similar condition for spermatogenesis.

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Genetic Control and Inheritance of Isoenzymes in Poplars of the Tacamahaca Section and Hybrids

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Abstract

Eleven full sib families were analysed by means of starch gel electrophoresis or isoelectric focussing in order to verify the genetic control and the mode of inheritance of the polymorphism of 15 enzyme systems. The study includes a check on environmental, ontogenetic and tissue specific variation of isoenzymes with inclusion of in vitro stages and also on the localization of certain enzyme systems within cell compartments. Intra-individual modification of isoenzymes can occur in the systems ACP, EST, and PER, which therefore were not included in the genetic analyses.

Segregation within full sib families revealed condominant expression of the studied enzyme types. It is concluded that 12 enzyme systems are controlled genetically by at least 18 polymorphic gene loci. For a set of 39 *Tacamahaca* clones and hybrids the average number of alleles per locus is 3.4. Recombination analyses were performed on the basis of 62 different two-locus-combinations. Highly significant deviations from random segregation are indicated for each of two pairs of loci: GOT-A/GOT-B and NDH-A/PGM-A. The loci IDH-B, 6PGDH-B,

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and PGI-B can be expected to belong to the same linkage group.

Key words: Enzyme gene marker, inheritance, linkage, clones, *Populus*.

Introduction

The genus *Populus* comprises a large number of species which commonly are subdivided into five sections. Particularly species of the sections *Aigeiros* DUBY, *Tacamahaca* SPACH, and also *Leuce* DUBY are the subject of tree breeding programs (for review see e.g. MUHLE LARSEN, 1970; STEENACKERS, 1970; ZSUFFA, 1975; WEISGERBER, 1989). The success of such programs primarily depends on the degree to which the phenotypically selected traits are genetically controlled and are ontogenetically consistent. The identification of genetic markers has become an important tool in forest genetics but is mostly used in coniferous species. The majority of studies apply biochemical genetic methods and identify genotypes at enzyme-coding gene loci (for recent surveys see EL-KASSABY and WHITE, 1985; RUDIN, 1986). In addition to these enzyme gene markers, molecular biological methods were established in forest research in order to utilize DNA restriction fragment length polymorphisms (RFLP's) for various purposes in forest genetics and tree breeding (e.g. NEALE and SEDEROFF, 1988; KRIEBEL, 1988; WAGNER et al., 1988; SZMIDT et al., 1988).

Both enzyme gene loci and RFLP-loci can function well in increasing the efficiency of tree breeding: Genetic markers help to characterize the genome (linkage maps) and to distinguish between nuclear and extra-nuclear genetic information. Correlations between genetic and phenotypic characters can be tested ("quantitative trait loci") and used as criteria for early selection so that long term forest breeding programs can be substantially shortened. More than that, selection based on genetic characters and not exclusively on phenotypic traits will help to ensure the success of breeding programs.

In spite of the long tradition in poplar tree breeding, biochemical and molecular methods are not yet integrated into this field (suggestions for molecular approaches see STETTLER et al., 1989). Generally, there is a surprising deficiency in genetic studies on poplars as compared to other species. Isoenzymes, i.e. enzyme phenotypes, have been assayed electrophoretically in various studies but very little information is available on their genetic control and modes of inheritance. GUZINA (1971, 1978) and BERGMANN (1981, 1987) studied isoenzymes in single clones and multiclonal varieties of *Populus tremula*., MITTON and GRANT (1980), CHELIAK and DANCIK (1982), CHELIAK and PITEL (1984), and HYUN et al. (1987) in numerous clones of *Populus tremuloides* by utilizing a total of 20 different enzyme systems. The last named study is exceptional, because it is the only one which refers to the analyses of offspring from controlled crosses in the *Leuce* section, although the resulting segregations among the offspring were not communicated. On this basis, Mendelian inheritance was observed for isoenzymes at five enzyme coding gene loci and there was evidence that the same holds for five additional loci.

In the *Aigeiros* section, RAJORA (1986a) reported the identification of nine enzyme-coding gene loci by using offspring from one clone each of *Populus deltoides* and *P. nigra*. RAJORA (1986b) used offspring from twelve controlled crosses to evaluate the inheritance of isoenzymes in *Populus deltoides, P. nigra*, and a species of the *Tacamahaca* section (*P. maximowiczii*). The expected number of loci ranged from 36 to 39, but for only seven of them segregation among full sibs could be observed in order to confirm a Mendelian mode of inheritance. Multilocus genotypes were scored for different clone collectives of *Populus deltoides* and *P. nigra* (e.g. RAJORA, 1989a, 1989b). MALVOLTI et al. (1991) studied 33 full sibs from a 6 x 6 cross design of *Populus deltoides* and verified the inheritance of six polymorphic gene loci. CASTILLO and PADRO (1987) used isoenzymes to discriminate between two clones of *Populus* x *euramericana*. In the *Tacamahaca* section. WEBER and STETTLER (1981) studied the isoenzyme variation at twelve systems in ten populations of *Populus trichocarpa*, and FARMER et al. (1988) at eight systems in five populations of *Populus balsamifera* (for *Populus maximowiczii* see RAJORA, 1986).

Summarizing our present information, segregation studies among full sibs which unequivocally verify the genetic control and the inheritance of isoenzymes are very rare. Linkage between gene loci has not yet been observed. These facts are surprising in the case of a tree genus which comprises an extraordinarily large number of species, many of which can be expected to be important for current and future tree breeding purposes. The present study intends to proceed in the verification of genetic markers and will particularly refer to a set of *Tacamahaca* clones.

Materials and Methods

Controlled Crosses

In 1988 and 1989, controlled crosses (*Table 1*) were performed in the greenhouse during March by using flowering branches, which were kept in water with standard nutrition additives. Most of the applied techniques go back to VON WETTSTEIN-WESTERSHEIM (1933). Humidity was kept greater than 50% in order to prevent withering and thus the breakdown of the ripening process of pollen and seeds. Pollination was performed twice. During the three to four weeks period of seed ripening, water and nutrition additives were replaced and branches were cut back every second day. Right after the opening of the capsules, seeds were germinated on vermiculite in germination cabins and three weeks later transferred to soil. If seed capsules did not open, the seeds were transferred to a modified MURASHIGE and SKOOG medium (MURASHIGE and SKOOG, 1962): MS-O supplemented with 2% sucrose and 0.5% agar, SRIVASTAVA (pers. comm.).

Table 1. — Parental clones and their affiliation to poplar species (HSP = Hybrid between species, HSE = Hybrid between sections).

Female clones		Male clones	
Designation	**Species**	**Designation**	**Species**
Muhle-Larsen	P. trichocarpa	Androscoggin	P. maximowiczii x P. trichocarpa (HSP)
Oxford	P. maximowiczii x P. x berolinensis (HSE)	Columbia River	P. trichocarpa
Rochester	P. maximowiczii x P. nigra var. plant. (HSE)	Weser 1	P. trichocarpa
		Weser 3	P. trichocarpa
Weser 4	P. trichocarpa	K. Schn. 3*+	Tacamahaca section
Weser 8	P. trichocarpa		
Danndorf 21*	Tacamahaca section		
Danndorf 50*	Tacamahaca section		

*) Clones of unknown origin classified morphologically to belong to the *Tacamahaca* section.

+) Genotypically identical (18 loci, see *Table 7*) to the clones "Senior".

Electrophoretic methods

Bud and leaf tissues were extracted by means of a 0.08 M tris — 0.02 M HCl buffer pH 7.3 with the addition of selected substances for the inhibition of phenols and tannins (see MÜLLER-STARCK, 1982). The enzymes were separated from crude homogenate by standard horizontal starch gel electrophoresis (SGE, see *Table 2*) or by isoelectric focussing (IEF).

For SGE, gel-concentration was 11%, voltage distribution 20 V/cm to 30 V/cm, bridge distance 12 cm. IEF was performed by using 6% polyacrylamide gels with mixed carrier ampholytes (80% ph 3.5 to 10, 20% pH 3.5 to 5). Desalting of the crude extracts can be recommended but is not indispensable. Buffers were 0.5 M HPO_4 for the anode and 0.5 M NaOH for the cathode. The run was started at 160 V and stopped after approximately 2.5 hours at 2500 V. Because zymograms of IEF and of conventional electrophoresis did not result in contradictory identification of genotypes, SGE was preferred due to its lower health risks and the ease of slicing gels. The high resolution power of IEF is necessary only in the case of two enzyme systems (see *Table 3*).

Buffer formulations for enzyme stains were taken from CHELIAK and PITEL (1984) with slight modifications BERGMANN and MÜLLER-STARCK, in prep.). By omitting enzyme substrates parallel to the regular stains, it was proven for each enzyme system whether or not the isoenzyme variants in the zymogram in fact referred to the enzyme system under consideration.

Table 2. — Composition of buffers for starch gel electrophoresis (SGE).

No.	Electrode buffer/pH	Gel buffer/pH
1[1]	0.05 M LiOH-0.19 M boric acid/8.1	0.05 M tris-0.01 M citric acid/8.1[3]
2[2]	0.06 M NaOH-0.30 M boric acid/8.0	0.07 M tris-1.00 M HCl/8.7
3	0.14 M tris—0.04 M citric acid/7.8	Diluted electrode buffer (2.5 : 1)
4	0.14 M tris-0.04 M citric acid/6.5	Diluted electrode buffer (2.5 : 1)
5	0.14 M tris—0.04 M citric acid/7.0	0.04 M tris 0.001 M EDTA-0.05 M histidinHCl/5.7

[1]) LUNTKVIST (1979)
[2]) POULIK (1957) modified
[3]) Contains 10% electrode buffer

Table 3. — Enzyme systems and electrophoretic methods used for visualization. SGE buffer systems see *table 2*; IEF (1) indicates routine application, IEF (2) facultative applicability with priority in favour of SGE.

Enzyme system	E.C. Ref.	SGE buffer	IEF (1)	IEF (2)
Acid phosphatase (ACP)	3.1.3.2	2		
Aconitase (ACO)	4.2.1.3	3		
Diaphorase (DIA)	1.6.4.3	3	X	
Esterases (EST)	3.1.1.1	1,2		
Glutamate-oxaloacetate transaminase (GOT)[1]	2.6.1.1	2		
Isocitrate dehydrogenase (IDH)	1.1.1.42	3		X
Leucine aminopeptidase (LAP)	3.4.11.1	1		X
Malate dehydrogenase (MDH)	1.1.1.37	3		X
NADH-dehydrogenase (NDH)	1.6.99.3	5		X
Peroxidase (PER)	1.11.1.7	2	X	
Phosphoenolpyruvate carboxylase (PEPCA)	4.1.1.31	5[2]		
6-Phosphogluconate dehydrogenase (6PGDH)	1.1.1.44	3		X
Phosphoglucose isomerase (PGI)	5.3.1.9	1	X	
Phosphoglucomutase (PGM)	2.7.5.1	4		X
Shikimate dehydrogenase (SKDH)	1.1.1.25	4		X

[1]) = Asparate aminotransferase (AAT)
[2]) BERGMANN and SCHOLZ (1989)

Test on variation of enzyme phenotypes within clones

Environmental impacts on the phenotypic expression of enzymes were studied by using bud and leaf tissues from several individuals of the same clone (ramets) which originate from different locations in Lower Saxony and Hessia (the full sib progenies which were used for the genetic analysis were grown under the same greenhouse conditions). For each enzyme system the band patterns in the zymogram were then compared.

To verify the tissue specific and ontogenetic variation within ramets, the following tissues of the parental clones were tested: buds and leaves (up to the age of three months) from juvenile and adult plants, and tissues from in vitro material (callus, roots, leaves) which was regenerated from callus and supplied by C. PASBERG-GAUHL. Leaves from in vitro material were available only for the clones Androscoggin, Muhle Larsen, Oxford and Weser 8. In addition, pollen samples were studied electrophoretically from Androscoggin and from Kl. Schn. 3.

Test on the localization of enzymes

The enzyme activity in different cell compartments was tested by applying a method which aims at the isolation and purification of cell organelles (R. RADETZKY, pers. comm.): Homogenization of leaf tissue in a buffer pH 7.6 (ratio 1:5) with 0.05 M tris, 0.3 M mannit, 1 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA, 0.1% cysteine, 0.01 M diethyldithiocarbamic acid, 0.14% β-ME, 0.5% PVP; filtration through gauze and Miracloth and differential centrifugation at 4°C as follows:

(1) Separation of cytoplasma and organelles: 150 g, 5 min. (supernatant)
(2) Separation of plastids from supernatant: 3,000 g, 5 min (pellet)
(3) First step separation of mitochondria from supernatant: 10,000 g, 20 min (pellet)

These methods will not fully exclude contaminations but the employed electrophoretic methods cannot be expected to monitor low concentrated contaminants.

Genetic analysis

The segregation of parental enzyme phenotypes among the offspring from controlled crosses was analysed by testing its conformity with the Mendelian mode of inheritance (Chi^2-test "Goodness of fit"). By this the genetic control and the mode of inheritance can be verified only with respect to the enzyme phenotypes which are represented in the available full sib families. If the same isoenzyme variant follows Mendelian segregation in different combinations with other enzyme phenotypes, the respective type is considered to represent an allele at one of the enzyme-coding gene loci and thus to be applicable as a species-specific gene marker. Inferences on the inheritance of enzyme phenotypes which are not represented among the parental clones are tentative.

Linkage was tested by utilizing full sibs with at least one double heterozygote parent. The observed segregations were compared statistically with the expected ones (see subsequent hypotheses) by means of the Chi^2-test of goodness of fit and the log likelihood ratio test (G-test) of heterogeneity in contingency tables. Hypotheses (2), (3), and (4) were tested only if (1) had to be rejected. The hypotheses imply regular segregation of

(1) all two locus types,
(2) the gametic types at the first gene locus,
(3) the gametic types at the second gene locus,
(4) the summed "recombinant" and the summed "non-recombinant" genotypes.

If hypothesis (4) had to be rejected and thus linkage can be assumed, the recombination frequency was estimated by means of a maximum likelihood calculation on the basis of the "folded binomial" probability function (NORDHEIM et al., 1983).

Results and Discussion

1. Offspring from controlled crosses

Only full sib families larger than 20 seedlings were included in the genetic analysis in order to reduce the chance for sampling errors (*Table 4*). There were several crosses without any offspring. It can be assumed that most of these losses result from incompatibilities, because certain parental clones are involved and because the dieback of the female capsules started a few days after pollination without any withering of the leaves. This holds for the intersectional hybrid clone Rochester (also Max 2 and Max 3 which are not included in the present study).

2. Characterization of enzyme phenotypes prior to genetic analysis

2.1 Environmental, ontogenetic and tissue specific variation

In the case of even-aged ramets of one and the same clone under field conditions, environmental impacts on the expression of enzyme phenotypes could not be observed for any of the enzyme systems.

The study of enzyme phenotypes in various tissues resulted in a discrimination of three classes of enzyme systems. This classification combines modifications during ontogenesis (seedlings vs. adult plants) and among the tested tissues of one and the same individual:

(1) Enzyme systems which can show substantial variation in the isoenzyme patterns between different ontogenetic stages and among different tissues are acid phosphatases (ACP), esterases (EST), and to certain extent peroxidases (PER). In the case of ACP and EST, alterations occur to such an extent that genotyping on the basis of the tested tissues is questionable. The isoenzyme patterns of PER contain one zone which appears to be tissue unspecific but is faintly visible and one zone which shows modifications.

(2) Among the remaining enzyme systems, alterations can occur in very specific tissues: Only under in vitro conditions, the fastest migrating zone of GOT (=GOT-A) is not expressed during early callus stages. This can be interpreted to indicate regulation of gene activity (switched off status) or may occur due to specific inhibition by substances contained in the in vitro medium (examples for aminopeptidases see MÜLLER-STARCK and HÜTTERMANN, 1981). In the same tissue, the activity of SKDH phenotypes is reduced. These exceptions do not prevent an unequivocal genotyping in the remaining tissues. In the case of PEPCA, faint staining occurred in all in vitro stages prior to shoot regeneration. Furthermore, in pollen tissue IDH, MDH, 6PGDH and PGI show additional bands which can be discriminated from the tissue unspecific ones.

(3) All other enzyme systems revealed neither ontogenetic nor tissue specific effects.

In order to avoid uncertainties in genotyping, the enzyme systems which belong to the first category, i. e. ACP, EST, and PER, are excluded from further analyses.

Table 4. — Offspring from controlled crosses used for the genetic analysis at an age of four to ten months.

Female Parent	x	Male Parent	Number of individuals
Muhle-Larsen		Androscoggin	78
Muhle-Larsen		Columbia River	70
Muhle-Larsen		Weser 1	72
Oxford		Androscoggin	72
Oxford		Columbia River	40
Oxford		Kl.Schn.3	72
Weser 4		Weser 3	68
Weser 8		Androscoggin	71
Danndorf 21		Androscoggin	48
Danndorf 21		Kl.Schn.3	60
Danndorf 50		Kl.Schn.3	28

Table 5. — Electrophoretic study of enzyme activity in different cell fractions.

Material	Enzyme systems	Comments
Crude extract	All *	Normal activity
Cytoplasma & organelles	All *	Reduced activity
Plastids	MDH, PGI, NDH, SKDH	Reduced activity
	6PGDH, PGM,	Weak activity
Mitochondria (crude)	MDH, NDH	Reduced activity
	6PGDH, SKDH	Weak activity

*) see *table 3*

2.2 Preliminary statements on the localization of enzymes

In *table 5*, the results of a comparison of enzyme activity in crude extracts and different cell compartments are surveyed briefly.

The organelle fractions are part of the material which is used in further steps for isolation of DNA. Lacking enzyme activity should therefore not be due to inviable tissues. Evidently the enzyme activity appears to decrease with increasing cell fractioning., i. e. increasing purification. The majority of the monitored enzymes seem to be located primarily in the cytoplasma. In the plastids six systems can be monitored, four of which are also found in the mitochondria fraction. Crude extracts which fully contain the cytoplasma appear to be the most suitable material for electrophoretic studies.

3. Genetic analysis

3.1 Genetic control and mode of inheritance

A total of 118 single locus segregations was studied in order to test their conformity with the expected Mendelian segregations (ACP, EST, PER excluded).

A set of examples is surveyed in *table 6*. The designations anticipate result of the genetic analyses, i. e. letters stand for gene loci and numbers for alleles. In several cases the sample sizes are smaller than those given in *table 4* due to losses of plants during the weeks which were needed for the electrophoretic studies (sample sizes vary between 28 and 78 seedlings).

With only one exception (see *table 4*, ACO-A, cross D 21 x Androscoggin), all observed segregations were in statistical agreement with the expected ones. This exception is interpreted for the present as a consequence of viability selection in early life stages: Among the full sibs which are listed in *table 4*, losses between germination and sampling stage were at a maximum among the offspring

Table 6. — Segregation of parental isoenzymes among the offspring from controlled crosses (f = female, m = female; *= significance level 0.05, ns = not significant); the assumed gene loci are designated by capital letters, the assumed alleles by numbers (for nomenclature see *figure 1*).

Enzyme system	Parental types (clonal designation) f		m	Segregation among the offspring: Types and frequency				X^2 value
ACO	A_3A_3 (Oxf)	×	A_5A_6 (Kl.S.3)	A_3A_5 39	A_3A_6 24			3.57 ns
	A_3A_4 (D21)	×	A_3A_5 (Andr)	A_3A_3 9	A_3A_5 16	A_3A_4 24	A_4A_5 12	8.31 *
	A_3A_4 (D50)	×	A_5A_6 (Kl.S.3)	A_3A_5 10	A_3A_6 18	A_4A_5 16	A_4A_6 12	2.86 ns
	A_4A_4 (ML)	×	A_3A_5 (Andr)	A_3A_4 28	A_4A_5 24			0.31 ns
	B_3B_4 (D23)	×	B_3B_4 (Kl.S.3)	B_3B_3 9	B_3B_4 or B_4B_3 17		B_4B_4 13	1.46 ns
	B_3B_4 (ML)	×	B_4B_4 (W1)	B_3B_4 21	B_4B_4 17			0.42 ns
DIA	A_1A_2 (Oxf)	×	A_1A_2 (Andr)	A_1A_1 18	A_1A_2 or A_2A_1 27		A_2A_2 15	0.90 ns
	A_1A_2 (Oxf)	×	A_2A_2 (CR)	A_1A_2 20	A_2A_2 20			0.00 ns
	A_2A_2 (ML)	×	A_1A_2 (Andr)	A_1A_2 28	A_2A_2 39			1.81 ns
GOT	A_2A_2 (ML)	×	A_1A_2 (CR)	A_1A_2 39	A_2A_2 31			0.91 ns
	A_2A_3 (W4)	×	A_2A_3 (W3)	A_2A_2 11	A_2A_3 or A_3A_2 36		A_3A_3 20	2.79 ns
	B_1B_1 (Oxf)	×	B_1B_2 (CR)	B_1B_1 24	B_1B_2 16			1.60 ns
	B_1B_2 (D21)	×	B_1B_2 (Kl.S.3)	B_1B_1 20	B_1B_2 or B_2B_1 24		B_2B_2 16	2.93 ns
	B_2B_3 (W4)	×	B_1B_2 (W1)	B_1B_2 22	B_2B_2 15	B_1B_3 17	B_2B_3 14	2.24 ns
IDH	B_1B_4 (W4)	×	B_4B_4 (W3)	B_1B_4 34	B_4B_4 26			1.07 ns
	B_4B_4 (D23)	×	B_2B_4 (Kl.S.3)	B_2B_4 27	B_4B_4 33			0.60 ns
	B_4B_4 (D23)	×	B_2B_4 (Andr)	B_2B_4 31	B_4B_4 35			0.24 ns
	B_4B_4 (ML)	×	B_2B_4 (Andr)	B_2B_4 26	B_4B_4 34			1.07 ns
	B_4B_4 (Ox)	×	B_2B_4 (Kl.S.3)	B_2B_4 34	B_4B_4 38			0.22 ns
	B_4B_4 (ML)	×	B_4B_5 (CR)	B_4B_4 36	B_4B_5 34			0.06 ns
LAP	A_1A_4 (D23)	×	A_1A_4 (Andr)	A_1A_1 16	A_1A_4 or A_4A_1 37		A_4A_4 12	1.74 ns
	A_1A_3 (Oxf)	×	A_3A_5 (CR)	A_1A_3 11	A_1A_5 10	A_3A_3 6	A_3A_5 12	2.12 ns
	A_2A_4 (W8)	×	A_1A_4 (Andr)	A_1A_2 17	A_2A_4 15	A_1A_4 20	A_4A_4 17	0.74 ns
	A_3A_4 (ML)	×	A_3A_5 (CR)	A_3A_3 22	A_3A_5 9	A_3A_4 21	A_4A_5 18	6.00 ns
	A_3A_4 (ML)	×	A_1A_4 (Andr)	A_1A_3 14	A_3A_4 16	A_1A_4 19	A_4A_4 8	4.54 ns
	B_1B_2 (D23)	×	B_1B_2 (Andr)	B_1B_1 16	B_1B_2 or B_2B_1 36		B_2B_2 14	0.67 ns
	B_1B_2 (W4)	×	B_2B_2 (W3)	B_1B_2 27	B_2B_2 33			0.60 ns
	B_2B_2 (W8)	×	B_1B_2 (Andr)	B_1B_2 31	B_2B_2 29			0.07 ns
MDH	A_4A_4 (ML)	×	A_2A_4 (CR)	A_2A_4 29	A_4A_4 41			2.06 ns
	A_4A_4 (Oxf)	×	A_2A_4 (CR)	A_2A_4 18	A_4A_4 22			0.40 ns
NDH	A_2A_2 (ML)	×	A_2A_3 (CR)	A_2A_2 35	A_2A_3 35			0.00 ns
	A_2A_3 (W8)	×	A_2A_3 (Andr)	A_2A_2 9	A_2A_3 or A_3A_2 23		A_3A_3 7	1.46 ns
	A_3A_3 (Oxf)	×	A_2A_3 (Andr)	A_2A_3 24	A_3A_3 26			0.08 ns
PEPCA	A_2A_2 (ML)	×	A_2A_3 (CR)	A_2A_2 37	A_2A_3 32			0.36 ns
6PGDH	B_1B_2 (D50)	×	B_5B_5 (Kl.S.3)	B_1B_5 12	B_2B_5 16			0.57 ns
	B_2B_2 (W4)	×	B_2B_5 (W3)	B_2B_2 34	B_2B_5 34			0.00 ns
	B_2B_4 (Oxf)	×	B_2B_5 (Andr)	B_2B_2 15	B_2B_5 12	B_2B_4 20	B_4B_5 13	2.53 ns
	B_5B_5 (ML)	×	B_2B_5 (W1)	B_2B_5 33	B_5B_5 38			0.35 ns
PGI	A_2A_2 (ML)	×	A_1A_2 (CR)	A_1A_2 29	A_2A_2 39			1.47 ns
	A_2A_2 (ML)	×	A_1A_2 (Andr)	A_1A_2 32	A_2A_2 46			2.51 ns
PGM	A_1A_1 (D21)	×	A_1A_2 (Andr)	A_1A_1 39	A_1A_2 27			2.18 ns
	A_1A_3 (Oxf)	×	A_1A_2 (Andr)	A_1A_1 15	A_1A_2 20	A_1A_3 13	A_2A_3 12	2.53 ns
	A_2A_2 (ML)	×	A_1A_2 (Andr)	A_1A_2 33	A_2A_2 34			0.02 ns
	A_2A_2 (W8)	×	A_1A_2 (Andr)	A_1A_2 31	A_2A_2 40			1.14 ns
SKDH	A_1A_2 (D50)	×	A_1A_1 (Kl.S.3)	A_1A_1 10	A_1A_2 18			2.28 ns

from D 21 x Androscoggin (approx. 50%). All other ACO-A segregations did not reveal significant deviations from the expected frequencies. The hypothesis of early selection is supported by the fact that also other segregations reveal large Chi²-values although still below the significance level.

The suggested viability selection implies that the sampling should take place as soon as possible after zygote formation in order to reduce the chance for interferences independent from the meiosis. Additional study of haploid tissue (e. g. from anther cultures) could help to monitor the regularity of segregation during meiosis and thus to accomplish the genetic analysis. Such material was not yet available for the respective poplar clones. The situability of enzyme markers from genotyping anther cultures and for monitoring their ploidy level (haploid or auto-diploid vs. diploid status) has already been proven for decidues tree species (MÜLLER-STARCK and JÖRGENSEN, 1991).

The observed segregations do not refute the hypothesis of one-locus control for those isoenzyme bands which appear within the same zone in the zymograms and which are designated in *table 6* by the same capital letter. These gene loci need not be the only ones which are involved in the genetic control of an entire enzyme system. Furthermore, it cannot be ruled out that other gene loci than each of the identified ones share in the genetic control of the intra-zonal polymorphism. In this sense, the statement of one-locus control of the observed intra-zonal variation is preliminary but can be upheld because the results do not contradict it.

It can be stated that each allele is represented in the zymograms by a single band. Combinations of two different alleles appear as two single bands in the case of monomeric enzyme systems, as three-banded types (one additional hybrid band with intermediate mobility) in dimeric systems, and as five-banded types (three hybrid bands) in tetrameric systems. There is to date no indication for a dominant or recessive status of alleles, so that all will be considered for the present as being codominant.

The gene loci are designated such that the letter A refers to the alleles which are apparent within the

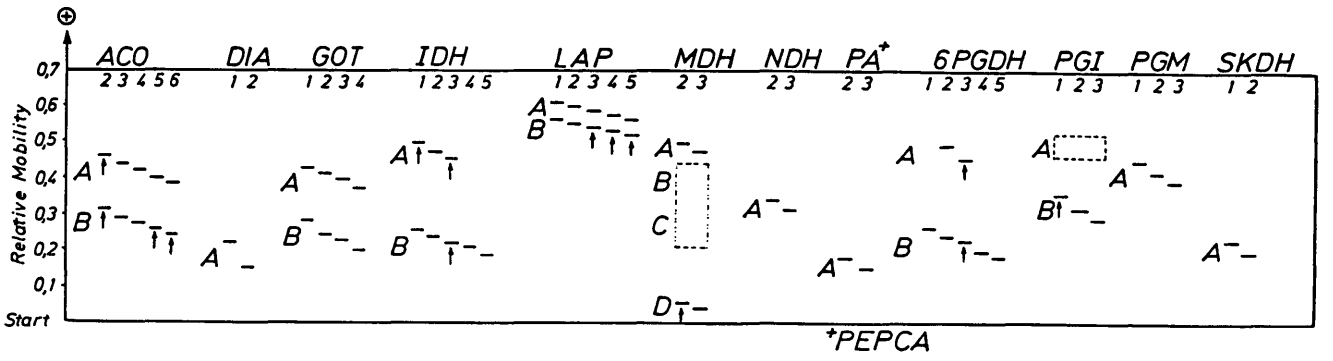

Figure 1. — Electrophoretically monitored enzyme polymorphisms among the offspring from controlled crosses or among other *Tacamahaca* clones (the latter are marked by arrows). Studied tissues are buds, leaves, callus and succeeding in vitro stages. Each band represents a homozygote or a heterozygote if "null" alleles can be expected. Other heterozygotes appear double banded, or with additional hybrid bands according to the enzyme structure (see *Table 7*). For IEF see article.

zone of the zymogram with the largest relative mobility. The succeeding letters designate the following zones in decreasing order of relative mobility. In *figure 1*, two classes of genetic types are surveyed: Firstly, the alleles which are represented in the controlled crosses and, secondly, those isoenzymes which were monitored among a set of 39 *Tacamahaca* clones and hybrids (MÜLLER-STARCK and BERGMANN, in prep.) and are not present as full sibs. There are several observations to support the assumption that the types of the latter category (see markings in *Figure 1*) should represent alleles:

(1) These suggested alleles are intra-zonally located and appear to be independent from the polymorphism among the other zones of the zymogram.
(2) Within each zone, each of the suggested alleles appears in single bands or combinations of bands, all of which cannot be interpreted other than as representing homozygotes or heterozygotes.
(3) Statement (2) also holds for all combinations between the identified alleles and the suggested ones, with no exception.

Figure 1 surveys the results of starch gel electrophoresis. If isoelectric focussing (IEF) is applied for PGI and DIA, bands are evident analogously to the ones illustrated in *figure 1*. In the acrylamide gels the DIA bands are located within the pH range of 5.0 to 6.5 and the PGI bands within 4.5 to 7.0.

The number of polymorphic gene loci is 18 (see *Table 7*). As can be seen from *figure 1*, the total number of genes (alleles) is 61, so that the average number of alleles per locus is 3.4 in the present study. Not included are those loci which control ACP, EST, and PER, because these systems do not provide the required environmental stability or the tissue unspecificity. Also monomorphic zones were excluded. Between MDH-A and MDH-D there are three banded configurations which are considered for the present as heterodimers like in many other tree species and are designated MDH-B and MDH-C.

Table 7. — Enzyme systems, enzyme structure and enzyme coding gene loci (ACP, EST, PER and monomorphic systems or zones excluded).

Enzyme system	Structure	Gene locus
Aconitase	monomeric	ACO-A, ACO-B
Diaphorase	tetrameric	DIA-A
Glutamate-oxaloacetate transaminase	dimeric	GOT-A, GOT-B
Isocitrate dehydrogenase	dimeric	IDH-A*, IDH-B
Leucine aminopeptidase+	monomeric	LAP-A+, LAP-B+
Malate dehydrogenase	dimeric	MDH-A, MDH-D*
NADH-dehydrogenase	dimeric	NDH-A
Phosphoenolpyruvate carboxylase	polymeric	PEPCA-A
6-Phosphogluconate dehydrogenase	dimeric	6PGDH-A*, 6PGDH-B
Phosphoglucose isomerase	dimeric	PGI-B
Phosphoglucomutase	monomeric	PGM-A
Shikimate dehydrogenase	monomeric	SKDH-A

*) Inferred from the study of 39 clones
+) Not independent from alanine aminopeptidase

Species specific information is evident in the systems GOT, IDH, and 6PGDH: Between the GOT-A and GOT-B zones and within the IDH-B and the 6PGDH-B zones, additional bands can be observed in the case of those clones which are hybrids between the Aigeiros and the *Tacamahaca* section (e. g. Oxford, Rochester). It appears that in each system at least one additional gene locus is expressed within the *Aigeiros* section. This hypothesis is also suggested if the results of FARMER et al. (1988) and of RAJORA (1989a, b) are compared.

Lack of activity ("null alleles") could not be observed in any of the systems. Artefacts are expressed particularly in the case of IDH-B. The system of PEPCA appears faintly stained. The PGI-A zone was excluded from further interpretation because of unsufficient substrate specificity. In the case of gene loci "LAP-A" and "LAP-B", the involvement of alanine aminopeptidases (AAP) cannot be ruled out (no independent staining of both systems). For the present, the common terminology is used to facilitate the comparison with other studies but in the long run it would be more appropriate to term these loci AP-A and AP-B.

There are deviations to other authors which mainly concern the number of loci per enzyme system. By applying starch gel electrophoresis and using root tip tissues of *Populus trichocarpa*, RAJORA (1986b) observed for instance 5 loci coding for DIA, 5 loci for MDH, 3 loci for PGM, and 5 loci for 6 PGDH; among the loci which were subject to a genetic analysis, only MDH does not correspond to the present results: One of the zones with medium relative mobility is not specific to MDH (proof by omitting the substrate) and cannot be counted as a MDH-coding gene locus. The same holds for one of the PGM loci. As mentioned above, species specific expression of gene loci can account for deviating numbers of monitored gene loci per enzyme system. For instance, in case of GOT (AAT) the zone with medium relative mobility is controlled genetically by one and not by two gene loci in the case of clones which belong to the *Tacamahaca* section (this corresponds to the results of FARMER et al., 1988). The three-

Table 8. — Survey of tested two-locus combinations and number of available full sib families (upper right half) and results of homogeneity tests (lower left half). Sample sizes vary between 21 and 78.

Gene locus	ACO A	ACO B	DIA A	GOT A	GOT B	IDH B	LAP A	LAP B	MDH A	NDH A	PA* A	6PGD B	PGI B	PGM A	SKD A
ACO-A		1	6	1	4	1	6		-	4		5	4	5	-
ACO-B	ns					1									
DIA-A	ns			1	4	1	6		-	4		5	5	5	-
GOT-A	ns		ns		3		3		1	3	1	3	1	1	-
GOT-B	ns		ns	***			7		1	4	1	6	2	4	1
IDH-B	ns	ns	ns				1		-	1	-	1	1	1	
LAP-A	ns		ns	ns	ns	ns		1	1	7	1	9	4	6	1
LAP-B							ns		-			1	1	-	
MDH-A	-		-	ns	ns	-	ns	-		1		1	1	-	-
NDH-A	ns		ns	ns	ns	ns	ns		ns			7	3	2	-
PA-A*				ns	ns	-	ns					1	1		
6PGD-B	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns		4	7	1
PGI-B	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		-	-
PGM-A	ns		ns	ns	ns	ns	ns	-	-	***		ns	-		1
SKD-A	-		-	-	ns		ns		-	-		ns	-	ns	

*) PEPCA

banded types in the front zone of 6PGDH were interpreted in the past to represent three monomorphic loci (6PGDH-1,2,3) but appear in the present study to be coded by one locus (6PGDH-A) in each of the studied clones.

3.2 Recombination studies

Among the full sib families, 76 heterozygous pairs of loci were available for a testing of the mode of recombination between gene loci. From these, 14 had to be excluded from analyses due to their small sample size (expected frequency per class less than 5). The remaining 62 combinations represent 59% of the 105 possible two-locus combinations which can be formed from 15 gene loci (IDH-A, MDH-D and 6PGDH-A could not be included due to lack of variation among the parental clones). A survey of the realized combinations is given in *table 8.* As can be seen from the upper right half, the missing combinations mostly concern the gene loci ACO-B, LAP-B, PEPCA-A, and SKDH-A.

The results of a testing of uniformity of the two-locus genotypes (hypothesis (1), see relevant topic in Material and Methods) is illustrated in the lower left half of *table 8.* In *table 9* two-locus segregations are listed, which reveal statistically significant deviations from random recombination. In addition, a set of examples is presented, which suggest certain trends in the deviations from a uniform distribution of two-locus types but which do not suffice as statistical proof. These cases were included in the calculation of recombination frequencies and can only function as hints on possibly existing linkage between gene loci. Further studies are needed to verify these assumptions.

The statisticals tests in *table 9* refer to the frequency distribution of all two-locus types and of the recombinant and the recombinant types (hypotheses (1) and (4)). For tests on the hypotheses (2) and (3), both of which deal with single locus segregations, see *table 6.* For each cross, the frequency of the recombinants and of the non-recombinant types is derived by means of addition of the respective frequencies given in *table 9.* These two values were compared statistically with the expected ones. If both parents are doubly heterozygous, statistical tests and recombination values were calculated for each parent separately (see GOT-A/GOT-B). If two classes of two-locus-genotypes could not be distinguished from

Table 9. — Segregation among two-locus genotypes and results of a statistical testing of hypotheses (1) and (4). Alleles are printed in italics, observed numbers in boldface (significance levels: 0.05(*), 0.01 (**), 0.001 (***); ns = not significant).

Gene loci (pair)	Cross fem. male	2-locus segregations among full sib families		χ^2 (1)	χ^2 (4)	Recomb. frequency
		Genotypes	Frequency			
	Oxf x Andr					
ACO-A	*33* *35*	*33 33 35 35*	**3:6:8:6**	4.06 ns	2.47 ns	0.39
6PGDH-B	*24* *25*	*22 25 22 25*				
		33 33 35 35	**5:7:9:5**			
		42 45 42 45				
	ML x Andr					
ACO-A	*44* *35*	*43 43 45 45*	**12:16:16:8**	3.39 ns	2.77 ns	0.38
PGM-A	*22* *12*	*21 22 21 22*				
	ML x CR					
GOT-A	*22* *12*	*21 21 22 22*	**10:29:17:13**	12.10**	7.67**	0.33
GOT-B	*22* *12*	*21 22 21 22*				
	W8 x Andr					
GOT-A	*23* *22*	*22 22 32 32*	**9:20:26:5**	18.80***	17.07***	0.23
GOT-B	*13* *11*	*11 31 11 31*				
	W4 x W3					
GOT-A	*23* *23*	*22 22 23 23*	**0:5:3.5:2.5**	40.93***	8.82**	0.31
GOT-B	*23* *12*	*21 22 21 22*				
		22 22 23 23	**3:6:5:2.5**		4.82*	0.36
		31 32 31 32				
		32 32 33 33	**3.5:2.5:14:2**			
		21 22 21 22				
		32 32 33 33	**5:2.5:3:0**			
		31 32 31 32				
	W8 x Andr					
GOT-A	*13* *11*	*11 11 11 11*	**8:11:11:5**	6.40 ns	2.40 ns	0.40
LAP-A	*24* *14*	*21 24 41 44*				
		31 31 31 31	**5:5:9:6**			
		21 24 41 44				
	W4 x W3					
GOT-A	*23* *12*	*21 21 22 22*	**12:9:4:8**	6.93 ns	2.40 ns	0.40
6PGDH-A	*22* *25*	*22 25 22 25*				
		31 31 32 32	**10:6:5:6**			
		22 25 22 25				
	ML x CR					
GOT-B	*22* *12*	*21 21 22 22*	**17:10:18:25**	6.46 ns	2.80 ns	0.40
NDH-A	*22* *23*	*22 23 22 23*				
	ML x A					
IDH-B	*33* *13*	*31 31 33 33*	**10:12:22:9**	8.06*	4.12*	0.36
6PGDH-B	*55* *25*	*52 55 52 55*				
	ML x A					
IDH-B	*33* *13*	*31 31 33 33*	**11:11:8:23**	10.02*	4.25*	0.36
PGI-B	*22* *12*	*21 22 21 22*				
	D50 x Kl.S3					
LAP-A	*13* *44*	*14 14 34 34*	**3:11:7:7**	4.57 ns	2.29 ns	0.36
SKDH-A	*12* *11*	*11 21 11 21*				
	Oxf x Andr					
NDH-A	*33* *23*	*32 32 33 33*	**0:11:13:4**	31.36***	27.94***	0.12
PGM-A	*13* *12*	*11 12 11 12*				
		32 32 33 33	**2:10:9:0**			
		31 32 31 32				
	ML x Andr					
NDH-A	*22* *23*	*22 22 23 23*	**8:17:20:8**	8.66*	8.32**	0.30
PGM-A	*22* *12*	*21 22 21 22*				
	ML x CR					
6PGDH-B	*55* *25*	*52 52 55 55*	**14:29:13:14**	10.11*	2.80 ns	0.40
PGI-B	*22* *12*	*21 22 21 22*				
	D50 x Kl.S3					
6PGDH-B	*15* *55*	*15 15 55 55*	**8:4:7:10**	2.59 ns	1.69 ns	0.38
PGM-A	*13* *22*	*12 32 12 32*				
	D50 x Kl.S3					
PGM-A	*13* *22*	*12 12 32 32*	**7:7:3:10**	3.67 ns	1.82 ns	0.37
SKDH-A	*12* *11*	*11 21 11 21*				

each other, the respective number of individuals was estimated by dividing the observed number by two.

The estimates for recombination values deviate in several cases considerably from the expected value of 0.50 (*Table 9*). The lowest value is 0.12 for the gene loci NDH -A/PGM-A (offspring from the cross Oxford x Androscoggin). There was only one additional full sib family with which to verify this result (Muhle Larsen x Androscoggin); in this case also the deviations from random recombination were statistically significant but resulted in a greater

recombination value (0.30). Concurrent statistical significance also holds for the three full sib families which allow the study of the combination GOT-A/GOT-B (recombination values vary between 0.23 and 0.36). In the case of the gene loci IGH-B/6PGDH-B and IDH-B/PGI-B no other full sib families were available to verify the observed statistical significancy. Generally, no conflicting trends were evident in the studied material. Summarizing the results, the following statements are suggested:

(1) The majority of the studied gene loci is not associated to a single coupling group. There is evidence for a clustered location of the loci on several chromosomes.
(2) For the present, linkage is clearly indicated for two pairs of loci, namely GOT-A/GOT-B and NDH-A/PGM-A.
(3) More moderate forms of linkage can be expected for the pairs IDH-B/6PGDH-B and IDH-B/PGI-B. For LAP-A/SKDH-A the estimated recombination frequency is the same (= 0.36) but will not be taken into account for the present because of smaller sample size (28 vs. 53) and lacking statistical significance.
(4) Seven loci listed in (2) and (3) could be localized relatively to each other on not more than three coupling groups:
GOT-A —— GOT-B
PGI-B —— IDH-B —— 6PGDH-B
PGM-A —— NDH-A

Due to the information deficiencies on linkage in poplar species, the present results cannot directly be compared with those of otner authors. Rajora (1986) tested the pairs ACO-1/MDH-4, ACO-1/6PGDH-4, MDH-4/6PGDH-4, MDH-6/6PGDH-4 in *Populus nigra* and IDH-2/6PGDH-4 in *Populus maximowiczii* but obtained no evidence for linkage. The same conclusion was drawn by Hyun et al. (1987) who could test recombination between 6PGDH-2 and PGI-2 for *Populus tremuloides*. These loci correspond to 6 PGDH-B and PGI-B in the present study which are considered here to be located in the same coupling group. Lack of information is also evident in other decidous tree species.

The results of linkage studies in coniferous species do not contradict the present ones: Loci which are also the subject of the present study are for instance GOT-A/LAP-B, GOT-A/PGI-B, LAP-A/LAP-B (for review see O'Malley et al., 1986; Strauss and Conkle, 1986) and GOT-A/GOT-B (*Cunninghamia lanceolata*, Müller-Starck and Liu, 1988).

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Controlled Pollination without Isolation – a New Approach to the Management of Radiata Pine Seed Orchards

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Abstract

This paper records a series of experiments, in hedged and meadow seed orchards, aimed at evaluating and improving the techniques of controlled pollination without prior isolation of strobili.

The results indicate that in a New Zealand hedged *Pinus radiata* seed orchard, multiple pollination of strobili with pollen suspended in water is effective at excluding unwanted pollens from entering the ovules. The effect is further improved by the prior use of compressed air to "blow off" any unwanted, naturally dispersed pollen grains from the surface of the strobili. With these methods it has proved possible, without isolating strobili, to ensure that only pollen applied by the orchard manager enters the ovules.

Key words: Seed orchard, meadow orchard, controlled pollination.

Introduction

For many years now there have been concerns expressed about the reduced levels of genetic gains from radiata pine orchard seed in New Zealand, relative to those expected on the basis of progeny trials. The deficiencies of conventional seed orchards in this respect have been reviewed by Sweet and Krugman (1977). They indicated a major problem arising from the lack of control over pollination. Radiata pine seed orchard technology in New Zealand has been progressively modified since that time to improve genetic gains (see e. g. SHELBOURNE et. al., 1989). Thus current practice by the major seed orchard company in New Zealand is to produce a high proportion of its orchard seed by controlled pollination on hedged rows of grafted clones.

Since 1989, new seed orchard plantings by that company (Proseed New Zealand) have been with meadow orchards (SHELBOURNE et al, 1989; SWEET et. al., 1990). These consist of high density (5000 sph) clonal blocks of grafts, which will bear strobili on their leading shoots one or two years after planting, and will produce high yields of seed per hectare at a young age. Their attraction, vis a vis the hedged orchards which they will replace, is economic. In financial terms, the seed they produce is much less costly; and in terms of genetic improvement, they allow improved genetic material to come into production much faster (see SWEET et. al., 1990; ARNOLD, 1990).

Controlling pollination is seen as the key to increased genetic gain from seed orchards but, if done with isolation of strobili, there is a significant cost component to it (see ARNOLD, 1990). The intent of the research reported in this paper was to explore the genetic effectiveness of controlled pollination carried out without isolation. Should sound technique permit this without significant reduction of genetic gain, then seed orchard management would be both cheapened and simplified logistically.

There has been considerable research reported on the process of pollination in *Pinus*. The classical work by SARVAS (1962) with *Pinus sylvestris* has been important in the development of controlled pollination technology. In particular, from the point of view of the questions asked in this paper, it drew attention to the finite capacity of the micropyle to contain pollen, and to the method by which pollen was moved (against the force of gravity) into the micropyle. The pollination droplet (first reportet by DOYLE and O'LEARY, 1935) was seen as the key to pollination. Work by LILL and SWEET (1977) explored for *Pinus radiata* some of the issues explored by SARVAS for *Pinus sylvestris*. A synthesis of the significant parts of the above research, for the problem reported in this paper, would indicate that:

1. The micropyle of *Pinus radiata* has a maximum capacity of 7 or 8 pollen grains, and on average holds fewer than 5 grains.
2. Everything else being equal, the pollen grains in the micropyle reflect in number and constitution the mix found on the micropylar arms; which in turn reflect the mix in the air, over time.
3. Once the micropyle is full of pollen, any subsequent pollen events in the orchard are of no relevance to the manager.

Thus the pollination droplet has for long been seen as critical to controlled pollination technology. If the

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