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Isoelectric focusing of acid phosphatase and esterase from European larch *(Larix decidua)**

INTRODUCTION

During the last several years the mode of inheritance of various enzyme systems occuring in forest trees has been well documented. However the data concerning genetic polymorphism of enzymes in European larch are very scarce. Most data on larch concern enzyme variation in needles of Siberian and Japanese larch (Larionova 1979, Larionova and Miljutin 1981, Mikami 1973).

Only one study describes the inheritance of enzyme patterns in haploid macrogametophyte tissue of European larch (Mejnartowicz and Bergmann 1975).

Simultaneously, most of the present data concerning genetic polymorphism of forest tree enzymes are based on starch electrophoresis separations and very little attention has been paid to the other methods of enzyme separation. As has been shown recently, it is possible that the use of isoelectric focusing or polyacrylamide electrophoresis can provide additional informations about the nature of variation of forest tree enzymes (McMullan and Colangeli 1982, Szmidt 1982).

In this paper we describe the genetic polymorphism of esterase (E.C. 3.1.1.2) and acid phosphatase (E.C. 3.1.3.2) in haploid macrogametophyte tissue of European larch analysed by means of isoelectric focusing. Attempts have also been made to determine the level of genetic variation in a seed orchard of this species, composed of clones originating from Poland.

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MATERIALS AND METHODS

Open pollinated seeds were collected from 24 clones of European larch growing in the clonal seed orchard near Kórnik and stored at 3°C. Selected clones represented all origins of European larch from Poland including Polish larch (*Larix decidua* subsp. *polonica*).

From 10 to 76 seeds were analysed for each clone. Before separation seeds were germinated on filter paper until 1 - 3 mm long radicles were visible.

EXTRACTION AND ISOELECTRIC FOCUSING

Haploid macrogametophyte tissue was isolated from germinated seeds, and homogenized in double distilled water containing 0,06% 2-mercaptoethanol. Homogenates were soaked into pieces of Whatman 3MM filter paper and placed on 5% acrylamide gel slabs containing 2% Ampholine pH 3 - 10 (LKB Produkter AB, Bromma, Sweden) for esterase, and 2,5% Servalyt pH 3 - 7 (Serva Feinbiochemica, Heidelberg, Germany) for acid phosphatase separation. 1 M NaOH and 1 M H_3PO_4 served as cathode and anode solutions respectively. Separation was conducted for 4 hours at the following voltages: 50, 100, 150, 200, 250, 300, 400 and 500 every 30 min. Cooling temperature was approximately 12°C. Esterase (EST) was stained for 20 min. at room temperature according to Shaw and Prasad (1970). For acid phosphatase (APH) visualization gels were incubated for 15 min. in 0,2 M acetate buffer pH 4,5 and than stained with 70 mg of α-naphtyl acid phosphate and 140 mg of Fast Blue RR salt in 100 ml of acetate buffer pH 4,5. Stained gels were subsequently immersed in 5% acetic acid and fixed in glycerol or dried on a filter paper.

Agreement between the observed segregation patterns of EST and APH variants with 1 : 1 proportions expected for Mendelian characters was checked using the Chi-square goodness-of-fit test (Snedecor 1961). The Chi-square test was also used for the estimation of heterogenity of results over clones analysed (Mather 1963). Expected heterozygosity values were calculated according to Nei (1975).

RESULTS

ACID PHOSPHATASE (APH)

APH patterns comprised 5 to 7 closely located but well resolved bands (Fig. 1). Bands denoted as 1, 2 and 3 were sometimes very faint or did not appear at all especially when homogenates from dry seeds

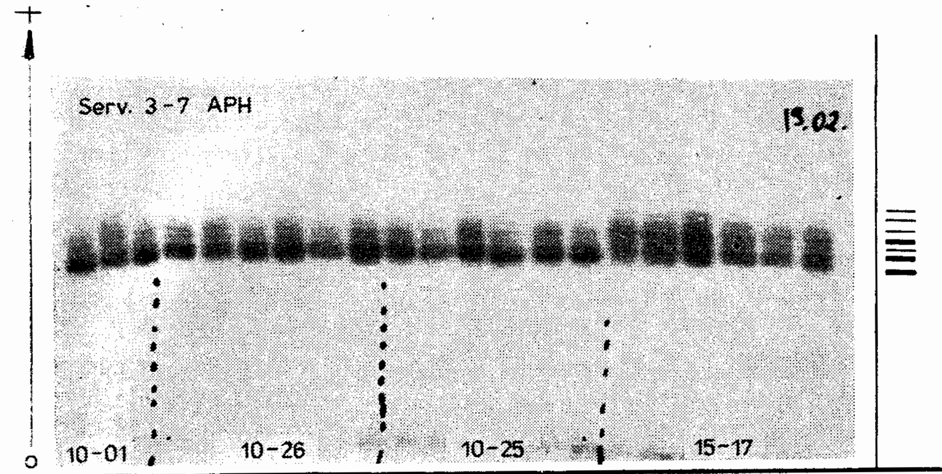

Fig. 1. Acid phosphatase patterns from European larch macrogametophytes. Phot. E. Szubert

were analysed. No variation was found among the European larch clones investigated with regard to mobility of APH bands.

ESTERASE (EST)

From 13 to 15 bands of esterase have been found in our material. They differed both with regard to activity and isoelectric points. However due to faint staining, bands occuring in the middle part of the gel (region C — Fig. 2) were omitted from further analysis. The remaining intensively stained bands occurring in the anodal and cathodal parts of gel were reproducible enough to permit interpretation. They have been grouped in 3 regions designated as EST-A, -B and -D. Regions EST-A and -B occupied very close positions on the gel which made it difficult to distinguish between them. However, two clones

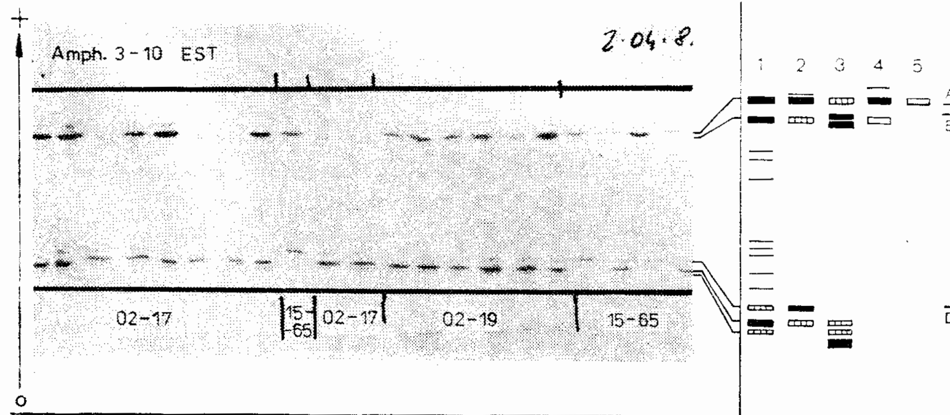

Fig. 2. Esterase patterns from European larch macrogametophytes. Phot. E. Szubert

(10 - 16 and 15 - 88, Tab. 1), possessed segregating variants within each region indicating that regions A and B should be considered separately. In regions EST-A and -B 5 and 4 different variants have been found respectively. They differed with regard to their isoelectric points as well as number of bands (1 or 2) and staining intensity, including so called "silent" variants i.e. variants showing no enzyme activity (Fig. 2).

In the region EST-D 3 segregating variants consisting of 2 or 3 bands were found.

In all three regions only one or two alternative variants occurred simultaneously in one clone. Chi-square tests confirmed that all variants segregated in good agreement with 1 : 1 proportions expected for a Mendelian character (Tab. 1). The lack of significant heterogeneity indicates consistency of data over the clones heteromorphic for EST-A, -B and -D regions (Tab. 1).

Table 1

Segregation EST variants from macrogametophytes of heterozygous larch clones, Chi-square goodness-of-fit test of 1 : 1 segregation ratio and heterogenity

Enzyme region	Clone No.	Segregation pattern					Deviation		Heterogeneity	
		1	2	3	4	5	x^2	P	x^2	P
EST-A	02 - 12	15	–	13	–	–	0.143	0.50 - 0.75	–	–
	10 - 16	38	–	38	–	–	0.000	0.995	–	–
	joint	53	–	51	–	–	0.038	0.75 - 0.90	0.105	0.50 - 0.75
	15 - 88	35	–	–	–	26	1.328	0.10 - 0.25	–	–
	02 - 17	–	–	16	–	21	0.676	0.25 - 0.50	–	–
EST-B	10 - 26	23	–	–	27	–	0.320	0.50 - 0.75	–	–
	15 - 88	26	–	–	35	–	1.328	0.10 - 0.25	–	–
	joint	49	–	–	62	–	1.523	0.10 - 0.25	0.125	0.50 - 0.75
	10 - 16	–	41	35	–	–	0.474	0.25 - 0.50	–	–
	02 - 11	–	14	–	13	–	0.037	0.75 - 0.90	–	–
	10 - 27	–	15	–	13	–	0.143	0.50 - 0.75	–	–
	joint	–	29	–	62	–	0.164	0.50 - 0.75	0.016	0.90 - 0.95
EST-D	15 - 17	17	13	–	–	–	0.800	0.25 - 0.50	–	–
	15 - 65	10	14	–	–	–	0.667	0.25 - 0.50	–	–
	joint	27	27	–	–	–	0.000	0.995	1.467	0.10 - 0.25
	10 - 01	14	–	19	–	–	0.758	0.25 - 0.50	–	–

Table 2

Frequencies of EST and APH variants in European larch seed orchard, values of expected (h_e) and observed (h_0) heterozygosity

Enzyme	Variant					h_e	h_0
	1	2	3	4	5		
EST A	0.813	0.083	0.063	0.021	0.021	0.327	0.167
B	0.042	0.229	0.021	0.708	–	0.444	0.208
D	0.938	0.042	0.021	–	–	0.118	0.125
APH	1.000	–	–	–	–	0.000	0.000
Average						$H_e=0.222$	$H_0=0.125$

VARIATION WITHIN SEED ORCHARD

All investigated clones were monomorphic for one and the same APH variant. Esterase patterns were variable among the clones, however at each of the EST regions one variant occurred with prevailing frequency in the seed orchard analysed (Tab. 2). The expected and observed heterozygosity value was 0,000 for the monomorphic APH. For esterase regions expected heterozygosity value ranged from 0,118 to 0,444 and the observed values were on the average 50% lower (Tab. 2).

DISCUSSION

Genetic polymorphism of acid phosphatase has been extensively studied in recent years and in most cases this enzyme was reported to be highly polymorphic (Lundkvist 1975, Mejnartowicz 1979, Witter and Feret 1979, Szmidt 1982). Only in the case of red pine, lodgepole pine and western red cedar no variation was found with regard to acid phosphatase electrophoretic patterns (Fowler and Morris 1977, Conkle 1979, Copes 1981). Separation of acid phosphatase from European larch female gametophytes by means of starch electrophoresis revealed 4 zones of activity of which one is coded by a single genetic locus (Mejnartowicz and Bergmann 1975). In our study, however, all 24 clones investigated had identical invariant acid phosphatase patterns.

The variation observed in activity of acid phosphatase patterns probably resulted from differences in the stage of development of particular seeds. This suggestion was supported by the fact that separation of macrogametophytes from dry seeds yielded very faint APH patterns and the most anodal bands were not visible at all. On the other hand samples from seeds following prolonged germination showed the most intensive staining. Similar changes in enzyme staining intensity between seeds germinated for different periods of time were reported by Conkle (1971).

Taking into account the limited number of clones studied here the observed monomorphism of acid phosphatase isoelectric focusing patterns need not be representative for natural populations of European larch. However isoelectric focusing of this enzyme from other conifers resulted in highly polymorphic patterns (Szmidt 1982, Szmidt unpublished). Furthermore variability of acid phosphatase electrophoretic patterns from European larch was also rather low (Mejnartowicz and Bergmann 1975).

To our knowledge, esterase polymorphism has not been studied in European larch so far. In other conifers this enzyme was found to be very polymorphic following both electrophoretic and isoelectric focusing separation (Lundkvist 1977, Rudin and Ekberg 1978, Witter and Feret 1979, Szmidt 1982). Our present study shows that in the case of European larch also, esterase can be resolved into a variety of bands differing with regard to isoelectric points and activity.

Unfortunately some bands on our gels were to faint to permit further analysis. According to Rudin and Rasmuson (1973) certain esterase bands from Scots pine needles also stained very poorly when α-naphtyl acetate was used as a substrate. The use of other alternative substrates considerably improved staining of these bands. It is therefore possible that a more complete picture of esterase isoelectric focusing patterns in European larch could be achieved by the use of different staining methods. Segregation patterns of esterase variants occurring in the regions EST-A, -B and -D indicated good accordance with 1 : 1 proportions expected for a one locus/two alleles system. However taking into account the multibanded nature of certain variants the above explanation does not look appropriate here. Similar segregation of complex esterase variants has been reported for two other conifers (Witter and Feret 1979, Szmidt unpublished). As pointed out by the former authors such complex enzyme patterns can hardly be explained as single gene products. Therefore, more detailed biochemical studies are required to understand better the molecular nature of the observed esterase variants. Nevertheless, good reproducibility of our results as well as Mendelian segregation of EST-A, -B and -D variants prove their usefulness as genetic markers for European larch.

The estimates of genetic variation in the seed orchard studied here confirm suggestions of Mejnartowicz and Bergmann (1975) of considerable homozygosity of European larch with regard to biochemical markers. The observed average proportion of heterozygotes ($H = 0,125$) was much smaller than values reported for other conifers (Lundkvist 1979, Mejnartowicz 1979, Szmidt 1980). The same was true for expected heterozygosity values. As pointed out by Mejnartowicz and Bergmann (1975) low genetic polymorphism of European larch can result from its reproductive biology. Natural populations of this species usually consist of small groups of scattered trees which facilitates inbreeding and consequently increased homozygosity. In addition, unlike e.g. Scots pine, larch pollen grains have no air sacks and therefore their migration is limited. It is possible that the above phenomena have still more pronounced effects in seed orchards due to selected clones and limited clone number which could also account for the low genetic diversity found in our material.

SUMMARY

Genetic polymorphism of acid phosphatase (APH) and esterase (EST) was studied in macrogametophyte tissue of 24 European larch clones by isoelectric focusing. One monomorphic zone of APH and 3 polymorphic zones of EST have been found. Segregation patterns of EST variants within each region were in accordance with those expected for Mendelian characters. Estimation of genetic parameters confirm earlier data about low enzyme polymorphism of this species.

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Ogniskowanie w punkcie izoelektrycznym enzymów kwaśnej fosfatazy i esterazy modrzewia europejskiego (Larix decidua)

Streszczenie

Stosując metodę ogniskowania w punkcie izoelektrycznym na żelu poliakrylamidowym, badano polimorfizm genetyczny kwaśnej fosfatazy (APH) i esterazy (EST) w haploidalnej tkance makrogametofitu u 24 klonów modrzewia europejskiego. Wykazano istnienie jednej monomorficznej strefy APH i trzech polimorficznych stref EST. Statystyczna analiza częstości występowania różnych fenotypów EST w próbkach nasion z pojedynczych drzew heteromorficznych, wykazała zgodność z dziedziczeniem w stosunku mendlowskim. Analiza częstości fenotypów i heterozygotyczności potwierdziła wcześniejsze dane o małym polimorfizmie enzymatycznym modrzewia europejskiego.

Фокусирование в изоэлектрической точке ферментов кислой фосфатазы и эстеразы лиственницы европейской (Larix decidua)

Резюме

Применяя метод фокусирования в изоэлектрической точке на полиакриламидном геле исследовали генетический полиморфизм кислой фосфатазы (APH) и эстеразы EST в гаплоидной ткани макрогаметофита 24 клонов лиственницы европейской. Доказано существование одной мономорфной зоны APH и трех полиморфных зон EST. Статистический анализ частоты встречаемости различных фенотипов EST в семенах собранных с отдельных гетероморфных деревьев указывает на соответствие с наследованием по менделевскому соотношению. Ннализ частоты фенотипов и гетерозиготности подтверждает полученные ранее данные о незначительном энзиматическом полиморфизме лиственницы европейской.