NR	6PGB1	6PGB2	6PGB3	6PGB4	6PGB5	6PGC1	6PGC2	6PGC3	6PGC4	6PGC5	PGIB2	PGIB3	PGMA1	PGMA2	PGMA3	PGMA4	SKDA1	SKDA2	SKDA3	SKDA4	SKDA5
2	-	1.000	-	-	-	0.760	0.005	0.020	0.130	0.085	1.000	-	-	0.360	0.640	-	-	-	0.995	-	0.005
3	0.123	0.867	0.010	-	-	0.716	-	0.005	0.142	0.137	1.000	-	-	0.360	0.640	-	-	-	1.000	-	-
5	-	0.990	-	0.010	-	0.699	0.030	0.084	0.181	0.006	0.995	0.005	-	0.318	0.682	-	-	-	0.990	-	0.010
6	-	1.000	-	-	-	0.721	-	0.049	0.147	0.083	0.995	0.005	-	0.377	0.623	-	0.020	0.025	0.955	-	-
7	0.022	0.974	-	0.004	-	0.684	0.009	0.021	0.263	0.026	0.975	0.025	0.004	0.297	0.699	-	-	0.004	0.992	-	0.004
8	0.089	0.911	-	-	-	0.787	0.005	0.005	0.158	0.035	1.000	-	-	0.308	0.692	-	-	-	1.000	-	-
9	0.059	0.941	-	-	-	0.728	-	0.022	0.154	0.096	0.993	0.007	-	0.287	0.706	0.007	-	-	1.000	-	-
10	0.075	0.925	-	-	-	0.759	0.005	0.005	0.156	0.075	1.000	-	-	0.524	0.476	-	-	-	0.981	-	0.019
11	0.062	0.923	0.015	-	-	0.686	0.010	0.052	0.196	0.057	1.000	-	-	0.292	0.708	-	-	-	0.990	-	0.010
12	0.065	0.915	0.010	0.010	-	0.720	0.005	0.005	0.170	0.100	1.000	-	-	0.295	0.705	-	-	-	1.000	-	-
16	0.058	0.937	0.005	-	-	0.666	-	0.020	0.240	0.074	1.000	-	-	0.431	0.569	-	-	0.005	0.970	0.005	0.020
19	0.048	0.952	-	-	-	0.697	0.014	-	0.183	0.106	1.000	-	-	0.385	0.615	-	-	-	1.000	-	-
20	0.005	0.990	-	0.005	-	0.783	0.010	0.025	0.141	0.041	0.975	0.025	-	0.439	0.561	-	0.020	0.005	0.950	0.005	0.020
22	0.064	0.936	-	-	-	0.728	0.015	0.010	0.153	0.094	1.000	-	-	0.376	0.624	-	-	-	0.980	-	0.020
23	0.080	0.920	-	-	-	0.645	-	0.025	0.190	0.140	0.995	0.005	-	0.330	0.670	-	-	-	0.995	-	0.005
24	0.061	0.939	-	-	-	0.601	0.005	0.010	0.227	0.157	0.980	0.020	-	0.298	0.702	-	-	0.005	0.995	-	-
25	0.015	0.985	-	-	-	0.809	-	0.005	0.176	0.010	0.990	0.010	-	0.282	0.718	-	-	0.019	0.976	-	0.005
26	0.158	0.842	-	-	-	0.797	0.005	-	0.086	0.112	1.000	-	-	0.423	0.577	-	-	-	0.995	-	0.005
28	0.055	0.945	-	-	-	0.710	-	0.010	0.210	0.070	1.000	-	-	0.245	0.755	-	-	-	1.000	-	-
29	0.095	0.900	-	-	0.005	0.750	-	0.045	0.135	0.070	0.970	0.030	-	0.270	0.730	-	-	-	0.995	-	0.005

A Methodical Study to Improve the Isozyme Analysis
for Identification of Clones of Tilia (Linden syn. Lime Tree)[1]

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Abstract

The methodical results obtained for a first isozyme study on linden are presented. These include the choice of plant material, the preparation of enzyme extracts as well as their efficient storage over longer periods of time. Tried and tested buffer systems are given for a variety of enzymes to separate isozymic forms by using starch gel electrophoresis. Moreover, an efficient microwave method for the preparation of starch gels is described.

In first practical applications, the combined zymograms of the enzyme systems tested were used to individually define the *Tilia cordata* clones in a seed orchard as well as to detect the clonal character of linden trees growing in an avenue along a country road.

Key words: Tilia cordata, small-leaved linden, isozyme analysis, starch gel electrophoresis, microwave method, clone identification.

FDC: 165.3; 165.441; 176.1 *Tilia cordata.*

Zusammenfassung

Die methodischen Ergebnisse einer erstmalig an Linden durchgeführten isoenzymatischen Untersuchung werden bezüglich der Wahl des Pflanzenmaterials, der Herstellung von Enzymextrakten sowie deren aktivitätserhaltenden Lagerung über längere Zeit dargestellt. Zur elektrophoretischen Trennung der Isoenzyme mittels Stärkegelelektrophorese werden erprobte Puffersysteme für eine Reihe von Enzymen angegeben. Zudem wird eine effiziente Mikrowellenmethode für die Herstellung von Stärkegelen beschrieben.

In ersten prazisbezogenen Anwendungen kamen Kombinationen von Zymogrammen der untersuchten Enzymsysteme zur individuellen Bestimmung von *Tilia cordata*-Klonen einer Samenplantage sowie zur Aufdeckung des Kloncharakters von Bäumen einer Lindenallee zum Einsatz.

Schlagwörter: Tilia cordata, Winterlinde, Isoenzymanalyse, Stärkegelelektrophorese, Mikrowellenmethode, Klonidentifizierung.

Introduction

Among the estimated 400 tree and shrub species of the *Tiliaceae* family, taxonomically classified in 30 to 40 genera and mostly restricted to the tropics, 10 monoecious deciduous tree species have their natural range in the temperate zones of the northern hemisphere. Only 4 of these species occur naturally in Europe, *i.e.* Caucasian linden (*Tilia dasystyla* Stev.), silver linden (*Tilia tomentosa* Moench.), small-leaved linden (*Tilia cordata* Mill.) and broad-leaved linden (*Tilia platyphyllos* Scop.) (Krüssmann, 1978). The latter 2 species are indigenous

[1] Dedicated to Dr. G. H. Melchior on his 70th birthday.

to Germany where they were found to form all naturally inter-specific hybrids when individuals of either of them exist in the close vicinity (KEIPER, 1916); these forms are called "common linden" (*Tilia x europaea* L. *syn. Tilia x vulgaris* HAYNE).

Despite their enormous importance in the history of European – and in particular of German civilization, the indigenous linden species have become extremely rare as forest trees. For the reduced occurrence of linden in the woodlands, the promotion of beech is mainly considered to be responsible because of different kinds of exploitation during the past 2 centuries (KLÖCK, 1958). However, due to their specific biological and ecological properties (*e.g.* blossoming which occurs in almost every year; regeneration by stump/root sprouting) as well as their silvicultural merits [*e.g.* maintenance of soil fertility because of the very rapid decomposition of the foliage, shading tolerance which brings about the function of serving (mixed) tree species], there has been an ever-increasing interest over the past 30 to 40 years in the domestic linden tree species both by tree breeders and silviculturists (FRICKE, 1982).

In the framework of the programme "Conservation of forest genetic resources in the Federal Republic of Germany" (Bund-Länder-Arbeitsgruppe, 1989), a variety of *in situ* and *ex situ* measures are presently performed in Rheinland-Pfalz for the indigenous linden species (MAURER and TABEL, 1995a and b) which are accompanied both by morphological and biochemical-genetic investigations (MAURER, 1995). The latter studies are performed by applying the method of isozyme analysis in order to ascertain the extent of genetic differentiation in the linden present, since no relevant data are available so far. Moreover, it is intended, by applying tried and tested enzyme systems to reliably examine the origin of linden seed and propagation materials.

In the following methodical aspects concerning the choice of plant material, the preparation of enzyme extracts as well as the electrophoretic separation of isozymic variants are presented. An efficient microwave method for the preparation of starch gels developed and tested in the course of the isozyme study on linden is also described here.

Material and Methods

Origin of plant material

The linden material used was collected (*i*) in a *Tilia cordata* clonal seed orchard established in 1985 with 80 different clones (in 9-fold replication) which originate from mostly 100-year-old and older plus-trees growing in the southernmost part of the Pfälzerwald (Palatinate Forest), and (*ii*) from a country road avenue (K 53 in the Palatinate Forest) founded in the 1920's with nearly 300 linden trees. For isozyme analysis twigs with dormant buds were harvested single tree-wise. They were transferred to a green-house where they were placed in containers with tap water and kept at room temperature. After budburst the very young leaves were allowed to develop until they were completely unfolded, and then they were homogenized for enzyme extraction as described in the following.

Extraction of enzymes

All steps of the enzyme extraction procedure including the clarification of the resulting dark green mushy suspension by centrifugation were performed at 4 °C throughout. Homogenization was carried out by using mortar and pestle. Per sample tree 1 g leaf material previously cut coarsely was ground for 2 min in the presence of 1 g white quartz sand and 4 ml extraction medium [0.1 M TRIS-HCl buffer pH 7.5 containing 0.5 %(w/v) TWEEN 80, 1 mM dithiothreitol (DTT)] and 2 g insoluble polyvinylpolypyrrolidone (PVPP) (WEIMAR *et al.,*

1986). The resulting suspension was completely transferred, by rinsing the mortar with another 2 ml extraction medium, in centrifuge tubes. After centrifuging for 10 min at 20,000 x g, the clear supernatant was taken as enzyme solution.

Storage of enzyme solutions

Immediately after centrifugation, the enzyme solutions obtained were stored at –86 °C in a freezer. Storage of the extracts was performed in 2 ways: (*i*) by using 1.5 ml-Eppendorf microtubes and filling in 0.5 ml to 1.0 ml-aliquots of each enzyme solution, and (*ii*) by applying 20 μl to 30 μl of the extracts onto paper wicks (size: 4 mm x 12 mm, cut from Whatman chromatography paper type 3 MM CHR). Each wick with absorbed enzyme solution was placed individually into a dip of a microwell plate. After filling up the plate with different samples to be electrophorezed, another microwell plate was set onto this upside down, and the whole storing unit was sealed by wrapping a strip of tape around it. In this manner series of samples were prepared and conserved over longer periods of time. Following thawing them for a few minutes at room temperature, the samples could be used instantly for electrophoresis.

Preparation of starch gels

For preparing starch gels, the microwave device type R-10R50 distributed by the Sharp Co. was employed. It is equipped with a turntable and can be operated variably in 100 watt-steps from 100 up to 1,000 watts.

The detailed program for the preparation of the starch gel slabs (dimensions: 27 cm wide, 12 cm long, 1 cm thick) includes eight subsequent steps as given in the following. Each two gel slabs are cast simultaneously in an open-top plexiglass gel casting mould (dimensions: 28 cm wide, 25 cm long, 1 cm high) by subdividing the mould with a plexiglass bar of adequate size.

Step 1: 77 g hydrolyzed (Toronto) starch (purchased from the Biomol Co., Hamburg) is weighed out for 11% starch gel slabs.

Step 2: 700 ml gel buffer to be used is subdivided into three fractions by pouring (*i*) 200 ml into a 500 ml-beaker; (*ii*) 50 ml into a 250 ml-beaker, and (*iii*) the remaining 450 ml into a 1,000 ml-Erlenmeyer flask; this solution is degassed by ultrasonication.

Step 3: Then the flask is transferred to the microwave and placed onto the turntable, the flask opening is covered with a watch glass, and the gel buffer is heated at 1,000 watts for 2 min.

Step 4: Meanwhile the 500 ml-beaker containing 200 ml gel buffer is placed on a magnetic stirrer, and a stirring bar is added. While stirring at high speed, the dry starch is mixed in by pressing it through a (tea) strainer with the aid of a porcelain pestle to obtain a fine powder free of lumps. The resulting suspension is degassed by ultrasonication.

Step 5: The flask containing the heated 450 ml gel buffer is removed from the microwave and placed, after adding a stirring bar, onto a magnetic stirrer. In the 500 ml-beaker the starch suspension is briefly stirred up. The starch suspension is poured along a glass rod into the heated gel buffer while stirring vigorously to avoid any lump formation. The remaining starch is rinsed out of the 500 ml-beaker with the 50 ml-buffer fraction, and the washings are poured into the flask to obtain a final starch-buffer volume of 700 ml.

Step 6: The flask containing the starch-buffer suspension is placed onto the turntable in the microwave and covered with a watch glass. First the suspension is heated at 1,000 watts for 4 min and then at 500 watts for 2 min. Heating is continued at

500 watts for 3 x 1 min with intermissions of 10 sec to 30 sec to avoid any surging. The boiling process is completed when large bubbles form at the bottom of the flask and ascend in the transparent mixture.

Step 7: The flask is removed from the microwave very carefully. It is slanted slightly to allow any bubbles to escape without splashing. The very hot mixture is poured into the prepared gel casting mould with extreme care, The plexiglass bar is inserted in the mould at its premarked position and fixed with cloth tape.

Step 8: After 15 sec to 30 sec of cooling down, the 2 gel slabs are covered separately with heat-resistant (copy) foil. Starting from the inserted plexiglass bar, the foil is smoothed slowly -and evenly on the gel surface avoiding the inclusion of any air bubbles. The gels are cooled down with the foils on top to obtain an even gel surface. Prior to sample application and electrophoresis, the gels are removed from the mould.

Electrophoretical procedure

Standard horizontal electrophoresis was performed in an electrophoresis apparatus equipped with a water-tight cooling plate as distributed by the Krannich Co. (Göttingen, Germany). The gels were loaded, by using a stencil, with 28 paper wicks containing the absorbed enzyme solutions to be separated. For controlling the electrophoretical run, each one paper wick with adsorbed bromophenolblue solution [0.04% (w/v) aqueous solution] was applied at the outmost lanes at the left and right margin of each gel, respectively. Moreover, one distinct sample of enzyme solution was chosen as an (arbitrary) internal standard which was used for all gels; it was applied on the gel both on the right and left side as well as in the middle.

Separation systems

The buffer systems tested to resolve the isozymic forms of a variety of enzyme systems as well as the separation conditions applied for electrophoresis are given in *table 1.*

Staining

For histochemical staining the protocols given by CHELIAK and PITEL (1984) were followed. Parallel to regular enzyme staining, the unequivocal affiliation of isozyme variants in the zymograms to the enzyme system under consideration was made sure by omitting the enzyme substrate specific for each enzyme system tested.

Results and Discussion

Plant material

For this first isozyme study on linden, dormant buds (without their brown scales) were tested as enzyme sources initially. This developmental stage of leaves has been successfully used in the isozyme analysis of broadleaves, *e.g.* oak (LÖCHELT and FRANKE, 1993). They are extremely low in phenolic contents and can be stored refrigerated over longer periods of time without significant losses in the enzyme activities present. Using linden dormant buds, however, it was not possible to prepare enzyme extracts suitable for electrophoresis, since for all the enzyme systems checked by using the separation systems 3 or 5, respectively (*cf. Table 1*) at the very beginning of this study, lanes on the gels were obtained which were diffusely stained and did not show any clear bands. From the very slimy consistency of the enzyme extracts which were obtained after centrifugation, we concluded that high amounts of substances must be present in the buds (due to the developmental stage most probably frost protecting substances like there are in particular carbohydrates and glycoproteins) which

Table 1. – Composition of the buffer systems tested and separation conditions applied in electrophoresis.

No.	electrode buffer, pH	gel buffer, pH	ref.	separation conditions
1	0.18 M TRIS / 0.1M boric acid / 0.004 M EDTA, pH 8.6	electrode buffer diluted 1:10 with aqua dest.	MARKERT and FAULHABER (1965)	200V, 5-6 h
2	0.3 M boric acid / 0.1 M sodium hydroxide, pH 8.2	0.076 M TRIS / 0.005 M citric acid, pH 8.6	POULIK (1957)	95 mA, 5-6 h
3	0.3 M boric acid / 0.06 M lithium hydroxide, pH 8.1	0.03 M TRIS, 0.005 M citric acid *plus* 1% electrode buffer, pH 8.5	RIDGWAY *et al.* (1970)	250 V, 6 h
4	0.3 M boric acid / 0.05 M sodium hydroxide, pH 8.0	0.1 M TRIS adjusted to pH 8.8 with citric acid	FOWLER and MORRIS (1977)	230 V, 6-7 h
5	0.15 M TRIS adjusted to pH 7.5 with citric acid	electrode buffer diluted 1:7.5 with aqua dest.	MEIZEL and MARKERT (1967)	95 mA, 6-7 h
6	0.065 M L-histidinium-hydrochloride adjusted to pH 5.7; pH 6.0; pH 6.5 with citric acid	electrode buffer diluted 1:5 with aqua dest.	CARDY *et al.* (1981) mod.	80 mA, 5 h
7	0.04 M citric acid adjusted to pH 6.1 with N-(3-amino-propyl)-morpholine	electrode buffer diluted 1:20 with aqua dest.	CLAYTON and TRETIAK (1972)	200 V, 5-6 h
8	0.04 M citric acid adjusted to pH 8.1 with N-(3-amino-propyl)-morpholine	electrode buffer diluted 1:20 with aqua dest.	CLAYTON and TRETIAK (1972) mod.	200 V, 5-6 h

strongly interfered with the electrophoretical procedure and led to the diffusely stained lanes on the gels. We overcame this problem by using very young leaves which had developed during one week after budbreak. In extracts originating from this material, all enzyme systems except for the enzyme sorbitol dehydrogenase could be identified unequivocally and isozyme variants could be separated electrophoretically.

Extraction medium

As is well known, the isolation of functional enzymes from plant tissues, in particular of woody plants, is not at all easy to perform mainly due to (*i*) the generally low enzyme contents, (*ii*) subcellular compartmentation of enzymes under consideration and (*iii*) a variety of substances such as phenolics that interact with the enzymes by inactivating them irreversibly (RHODES, 1977). To overcome these problems, the extraction medium as described by BOUSQUET *et al.* (1987) for the extraction of enzymes from green alder (*Alnus crispa*) leaves was used initially. Since this extraction medium is rather instable due to its components and contains several expensive ingredients, we formulated a less complex and costly but equally efficient extraction medium containing (*i*) DTT as an agent which protects sulfhydryl groups of enzymes; (*ii*) TWEEN 80 as solubilizing agent which is highly effective in breaking down cellular membranes at 0.5% [at this concentration, it does not enclose enzyme molecules by forming micelles and most importantly, it does not interfere with electrophoresis (ROTHE and MAURER, 1986)], and (*iii*) insoluble PVPP as a scavenger for phenolic compounds which acts by complexing them through very strong hydrogen bonds and can be easily removed from the enzyme extract by centrifugation.

Storage of enzyme solutions

If large numbers of samples have to be analyzed by electrophoretical separation, it is highly time-saving to have the samples ready-to-use by storing them as described here. After removal from the freezer, the samples will thaw within a few minutes at room temperature, and they can be applicated on the gels without further treating them. A recent check of such linden samples that had been stored for more than 2 years in

this way revealed that all different enzyme activities were still present, and most important no loss in the number of isozyme bands per enzyme system was observed. Presumably, besides the low storing temperature, the cellulose structure with its large number of hydrogen bonds being contained in the paper wicks also exhibits a stabilizing effect on the absorbed enzyme molecules, in particular when considering thawing of the samples where reorganization of enzyme plus water molecules must occur for regaining the native functional structure.

Preparation of starch gels

Solubilizing starch for the production of gels has been reported in literature mostly by using bunsen burners, heating plates or hot water baths. The microwave method described here has been developed in our lab for routine isozyme analysis as an efficient alternative procedure. It is an easy-to-handle and in particular a very safe method for the lab personnel which produces, within a reasonable period of time, highly reproducible gels.

Separation of enzyme systems

In a preliminary contribution of isozyme analysis for *Tilia cordata* enzyme systems, we reported on the first results obtained for a variety of enzymes (MAURER and TABEL, 1995b). In this first study we obtained mostly satisfying results concerning the resolution of bands which were found to be located in a characteristic number of distinct activity zones per enzyme system on the gels. To further improve the separation of isozyme variants, the buffer systems given in *table 1* were tested. *Table 2* summarizes the results obtained for ten different enzyme systems comprising AAT, ACO, ADH, IDH, LAP, MDH, 6PGDH, PGI, PGM, and SKDH. The buffer system which we found to be most resolving for each enzyme system, is given separately. In accordance with the optimal separation systems given, it is possible to simultaneously perform electrophoretical separation of several different enzymes, *e.g.* ACO, PGM and SKDH on one single gel of 1 cm thickness which was

cast previously. By using *e.g.* a gel slicer as described in ROTHE (1994), 5 2 mm thick layers are obtained by slicing the gel horizontally following electrophoresis. It should be noted that the other enzymes which are mentioned in MAURER and TABEL (1995b) [*i.e.* acid phosphatase (E.C. 3.1.3.2), diaphorase (E.C. 1.6.4.3), esterase (E.C. 3.1.1.1), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), glutamate dehydrogenase (E.C. 1.4.1.2), malic enzyme (E.C. 1.1.1.40), and sorbitol dehydrogenase (E.C. 1.1.1.14)] were also tested by using all the buffer systems given in *table 1*. Since no significant improvement in the separation of these enzymes could be obtained so far, they are not included in *table 2*.

The variation of banding patterns observed per enzyme system is also included in *table 2* and may be assumed to reflect the genetic diversity of the *Tilia cordata* samples studied so far. The extent of variability is classified in the (arbitrarily) chosen groups "low" which means up to 5 different patterns; "high", 6 to 10 different patterns; and "very high", more than 10 different patterns. As an example, a typical zymogram obtained for SKDH as given in *figure 1* may illustrate the very high variability found for this enzyme system.

Table 2 also gives the number of activity zones which were observed on the gels for the different linden enzyme systems due to being clearly separated from each other. This figure can be judged to give, as a first indication, the number of gene loci which code for the enzyme system studied. However, by comparing the respective numbers of subcellular isozymes found in plants per enzyme system and by considering their basic quarternary (subunit) structures (quoted by WEEDEN and WENDEL, 1989), the corresponding numbers of activity zones obtained for the different linden enzymes were found to be higher for IDH, MDH, PGM, 6PGDH, and PGI (*cf. Table 2*). This observation supports the hypothesis that the indigenous diploid *Tilia* species descend from a hexaploid ancestor (SEITZ, 1951; HATTEMER *et al.*, 1993).

Table 2. – A compilation of the data obtained for a variety of Tilia cordata enzyme systems by performing starch gel electrophoresis as well as literature data referring to the number of isozymes and to the quarternary (subunit) structure reported for these enzymes.

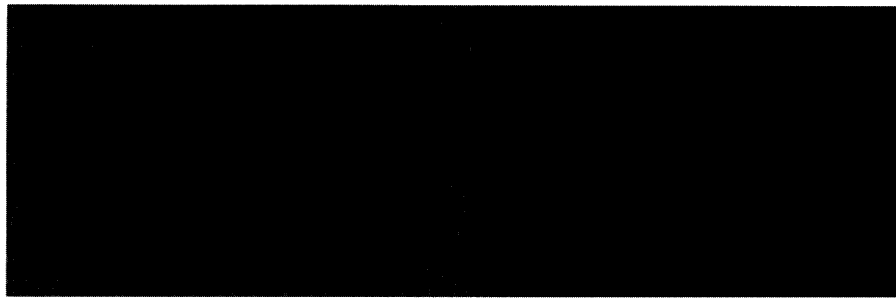| enzyme system (abbr.; E.C. ref.)	separation buffer systems (cf. table 1)		number of activity zones observed on gels	variation of banding patterns observed on gels	number of isozymes	quartenary structure [WEEDEN and WENDEL (1989)]	remarks
	systems tested	most resolving system					
Aconitase (ACO; E.C. 4.2.1.3)	5	5	3	low	1-3	monomer	additional faint bands in the anodal as well as in the catodal zones which stain very weak as compared to the others
Alcohol Dehydrogenase (ADH; E.C. 1.1.1.1)	3, 5, 6, 7, 8	8	3	low	1-3	dimer	generally weak staining
Aspartate Aminotransferase (AAT; E.C. 2.6.1.1)	2, 3, 4	2	4	very high	4	dimer	slightly blurred bands in the anodal zone
Isocitrate Dehydrogenase (IDH; E.C. 1.1.1.41)	5, 8	5	3	low	1	dimer	the most anodal zone stains very intensive as compared to the others
Leucine Aminopeptidase (LAP; E.C. 3.4.11.1)	3	3	2	high	2	monomer	the anodal zone stains more intensive as compared to the others
Malate Dehydrogenase (MDH; E.C. 1.1.1.37)	5, 7, 8	5, 8	4	high	3	dimer	in the slightly blurred anodal zone bands become visible in particular with system 8
Phosphoglucomutase (PGM; E.C. 2.7.5.1)	2, 3, 5	5	3	high	2	monomer	most anodal zone stains more intensive as compared to the others
6-Phosphogluconate Dehydrogenase (6PGDH; E.C. 1.1.1.44)	5, 7	5	3	low	2	dimer	
Phosphoglucose Isomerase (PGI; E.C. 5.3.1.9)	2, 5	2	4	high	2	dimer	
Shikimate Dehydrogenase (SKDH; E.C. 1.1.1.25)	5	5	2	very high	1-2	monomer	

Figure 1. – Photo of a gel showing the patterns for the enzyme shikimate dehydrogenase (SKDH) as obtained from different seed orchard clones studied. Top of the gel: origin; migration of bands: from the top (–) to the bottom (+).

In this case copies of identical gene loci located on different chromosomes might have evolved independently from each other at least partly bringing about nonidentical isozymes which become visible in individual activity zones on the gel. Genetic analysis to be performed with controlled crosses will further elucidate this question.

Application in practice of the tested linden enzyme systems

Following the successful resolution of isozymic forms of the above given enzyme systems, the method was used in practice for clone identification. This special kind of applying isozyme analysis is performed by taking the characteristic isozyme banding patterns of an enzyme system obtained on the phenotypic level, *i.e.* the isozyme banding patterns which are observed in the zymograms of any enzyme system can be used directly for the distinct characterization of an individual without any knowledge of the relevant genotype (CHELIAK, 1993).

In the first application, *Tilia cordata* clones constituting a seed orchard were identified. For this purpose, the banding patterns obtained per enzyme system per clone were combined with each other. This brought about the highly specific characterization of each individual clone and makes possible the unequivocal differentiation of one clone from any other. Moreover, these characteristics present the basic data for clearing up the genetic control of the studied enzymes by performing controlled crosses between these clones.

By using these enzyme systems it could be demonstrated in another study that linden trees growing in a country road avenue should be of clonal origin. Due to their obviously uniform outward appearance, it was assumed that the trees must be closely related with each other. Isozyme analysis performed on 40 out of 300 individual trees revealed invariable banding patterns for the enzyme systems isolated from crown leaves. In addition the enzyme systems isolated from leaves of root shoots which could be found at some (but unfortunately not at all individual trees) were also subjected to isozyme analysis. The banding patterns obtained per enzyme system differed not only from each other when the root shoots of the different individual trees were compared. Within an individual tree, significant differences became also evident by comparing the banding patterns obtained from leaves of root shoots and those obtained from crown leaves. From these findings it was concluded that the trees studied should be graftings since different patterns were obtained for the same enzyme systems in leaves of root sprouts (*i.e.* understock) and those in crown (*i.e.* scion) leaves per individual tree. Moreover, the uniformity of the banding patterns obtained per enzyme system of the crown leaves from all the individual trees studied makes it appear to be obvious that the avenue trees – at least those

under study – must originate from a single clone. In general these findings may confirm that for the purpose of making avenues look uniform in their outward appearance, clonal tree material was and still is frequently used when they are founded (J. KLEINSCHMIT, NFV Escherode, personal communication). By applying the method of isozyme analysis, it is possible to prove this fact, in particular if any other relevant information is lacking.

Conclusions

When this research was started in order to apply isozyme analysis to the indigenous linden species, no relevant data could be found in literature. With the methodical details described here, it is possible now to separate, by means of starch gel electrophoresis, isozymic forms of a variety of enzyme systems originating from primary or secondary metabolism, respectively. Thus on this basis investigations can be extended now to clear up, by genetic analysis using controlled crosses, the genetic control of the different enzyme systems studied here.

Acknowledgement

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Genetic Variation in High Elevated Populations of Norway Spruce (*Picea abies* (L.) KARST.) in Switzerland[1])

By G. MÜLLER-STARCK[2])

Abstract

20 autochthonous populations of Norway spruce (*Picea abies* (L.) KARST.) were studied which are located in mountainous and subalpine vegetation zones in Switzerland. For each of 2000 trees, multilocus genotypes were identified at 18 enzyme coding gene loci.

Genetic inventories revealed large genetic variation within populations and relatively small interpopulational variation. Compared to results from inventories in lower elevated regions northward and southward of the Alps, intrapopulational variation is not smaller in high elevated populations. Generally, frequency distributions of genetic types tend to deviate substantially from evenness.

The geographic variation of allele frequencies supports the hypothesis of postglacial re-immigration extra to the commonly accepted east-west routes. Results serve as criteria for gene conservation *in situ*. Preservation of genetic variability is required particularly under changing environmental conditions which challenge Alpine forest ecosystems.

Key words: Enzyme gene loci, genetic variation, heterozygosity, diversity, differentiation, gene conservation, Norway spruce (*Picea abies* (L.) KARST.).

FDC: 165.3; 165.5; 174.7 *Picea abies;* (494); (234.31).

Introduction

Norway spruce (*Picea abies* (L.) KARST.) is a predominant tree species which ranges from low elevations up to the subalpine vegetation zones. This species is subjected to a very large variety of site conditions. In particular, Norway spruce is an essential element of high elevated forest ecosystems which provide substantial benefits for the human society with respect to various protection and social functions. Generally, Norway spruce belongs to those tree species which are economically significant. Norway spruce is the most common tree species in Switzerland. It covers approximately 40 % of the total forest area and makes up 49 % of the total wood stock.

The postglacial re-immigration of spruce predominantly occurred from the eastern to the western part of the Alpes (e.g. KRAL, 1979; BURGA, 1988). Generally, re-immigration resulted in a competition between Norway spruce and pioneer species such as birches or pines which immigrated earlier. Silver fir (*Abies alba* MILL.) re-immigrated nearly at the same time as Norway spruce but predominantly the opposite way, i.e. from the western to the eastern parts of the Alps.

The development of the spruce habitat was severely affected by men. Inferences such as grazing or clearing by fire started already during the Neolithic Age around 3500 B.C. and became intensified during the Roman era and succeeding periods (e.g. exploitation following mining, salt works, charcoal-burning). These and other forms of ancient forest utilization modified substantial parts of high elevated forest ecosystems or replaced them. At least during the last two centuries, forestry interfered in the geographic distribution and the composition of tree species. Norway spruce was favoured by forest management because of its economic significance.

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