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Enzyme and storage protein electrophoresis in varietal identification of sugar beet

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Summary. Different methods of classification, based on total protein patterns as well as on specific isoenzyme patterns, were compared in order to establish an identification system for sugar beet varieties and lines. Single seed patterns and bulk extractions of total and fractionated proteins were compared on SDS-PAGE. Due to important intra-populational variation contrasting with similarity at the varietal level no method proved to be sufficiently discriminatory. Twelve sugar beet lines have been genotypically fingerprinted on the basis of five allozyme systems. The allele frequencies of each variety have been measured by using 60–100 individuals. From the data, genetic distance coefficients have been calculated in order to group the different entries by cluster analysis. In addition, a comparison has been made between two seed lots independently obtained from the same parental lines, to test the stability over years. Seeds from the same parental lines, but produced in different localities (Denmark, Italia and USA), were compared to test the influence of the environment on the classification. It has been concluded that isozymes could provide a useful tool for cultivar distinction. The variability at the level of allele frequencies within localities was small. The stability of different generations of the strains was relatively constant. Different strains originating from the same seed firm were less distinct than strains originating from different seed firms.

Key words: Varietal identification – Electrophoresis – Isozymes – Fractionated proteins

Introduction

Classifying sugar beet cultivars has proven to be very difficult. The outbreeding nature of this crop, and the breeding system itself, facilitate an accumulation of variability within the basic populations. Multiway crosses further increase the chance of heterozygosity. Very few morphological or physiological traits can be used as discriminatory factors. Characteristics of economical interest are yield and sucrose content, resistance to diseases, juice purity and mono- versus polygermity. However, most of these characteristics are highly dependent on environmental conditions.

Therefore, a critical factor that determines the usefulness of an additional technique is not only its discriminatory power but also how the markers are influenced by both environmental factors and the developmental phase of the plant (Smith and Smith 1986; Pedersen and Simonsen 1987).

In several crops, the use of molecular markers offers a possible alternative to this need. The possibilities, however, depend greatly on the crop under study. In the electrophoresis of cereals, where patterns of storage proteins have been proposed to distinguish varieties, the need for standard reference methods is necessary (Cooke and Morgan 1986; Draper 1987). In maize, a distinction by means of enzyme polymorphism has been accepted by the CTPS (Comité Technique Permanent de la Selection des plantes culivées) in France (1990), but isozymes are still not regarded as suitable for testing the uniformity and stability of the varieties. In sugar beet Itenov and Kristensen (1985) demonstrated that allozyme screening combined with relative band intensities could represent a promising tool for distinguishing monogerm triploid varieties.

DNA restriction fragment length polymorphisms offer an advanced technique, probably applicable to any crop, with an extensive range of possibilites (Bernatsky and Tanksley 1989; Aicher and Saunders 1990). The most important drawbacks, however, are the high cost, the

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sophisticated technology involved and the requirement for standardized probe collections. Therefore, the evaluation of the possibilities of relative simple techniques (e.g., isozyme and protein patterns) remains important.

This study has four objectives:

(1) To assess the discriminatory power of proteins and isozymes for the characterization of sugar beet cultivars,
(2) to investigate the usefulness of distance coefficients to distinguish varieties,
(3) to measure the stability of sugar beet cultivars with isozymes,
(4) to investigate the influence of the environment upon the data obtained.

Materials and methods

Total protein analysis

The protein patterns of the cultivars Massabel, Viva, Kawevera, Kawegigamono, Zwaanpoly, Zeppo, Zaricco, Magnamono, Vermon and Donor, as well as the experimental lines Z97 (high sugar content) and E1 (high yield), were determined by SDS-PAGE electrophoresis. Single seeds without an operculum were crushed in extraction buffer and kept in a boiling water bath for 2 min. The extraction buffer was composed of 0.05 *M* Tris-HCl pH 6.8, 18% (v/v) glycerol, 2.5% (v/v) β-mercaptoethanol and 2% (w/v) sodium dodecyl sulphate (SDS).

Protein fractionation, based on solubility characteristics, was carried out by adapting the method of Rahman et al. (1982). Seeds were extracted in water-saturated butanol and shaken on a vortex for 30 min. For single seeds, 50 µl was added per seed while for bulk extractions 20 seeds were extracted in 700 µl of solvent. This procedure was repeated three times. After each extraction, the supernatant was collected by centrifugation (5 min at 12,000 *g*) and pooled (fraction A). The pellet was subsequently extracted three times with 1 *M* NaCl in order to collect the salt-soluble fraction (fraction B); three times using 50% (v/v) propane-1-ol + 2% (v/v) β-mercaptoethanol for the extraction of the prolamins (fraction C) and finally three times with 0.05 *M* borate buffer pH 10, 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol for the solubilization of the glutelins (fraction D). All fractions were dialysed overnight against water and concentrated by lyophilization. Thereafter the samples were resuspended in extraction buffer and incubated in boiling water for 2 min. SDS-page and staining was performed as previously described (Van Geyt and Smed 1984) using polyacrylamide concentrations of respectively 7.5% and 15%.

Isozyme analysis

Seven different sugar beet cultivars were investigated for their enzyme polymorphism: Viva, Massabel, Monyx and Diana from SES-ICI seeds, Kws 2 and Tosca from KWS, Regina from Ringot.

Five different strains from Maribo, kindly provided by Dr. Smed, were investigated to test stability and environmental influence. In experimental line M8301, with subpopulations P103 (harvest Denmark 1981), P203 (harvest Denmark 1982 and P10 (harvest Italy 1982), and in the variety Magnamono, with subpopulations P19 (harvest Italy) and P34 (harvest USA), the years and the location of basic seed production differ.

Per line 60–100 randomly chosen individuals (single plants and/or seeds) were investigated. Alcohol dehydrogenase (ADH), malate dehydrogenase (Nad-MDH) and aconitase (ACO) were tested at the seed level. Young leaf material was used for testing leucine aminopeptidase (LAP) and isocitrate dehydrogenase (ICD).

The enzyme systems ADH, MDH, ACO and ICD were visualised by starchgel electrophoresis as described previously (Van Geyt and Smed 1984; Van Geyt and Jacobs 1986; Smed et al. 1989; Van Geyt et al. 1990). LAP isozymes were separated on a polyacrylamide gel using a PAGE 1 system (Van Geyt and Smed 1984) and stained in 100 ml 0.05 *M* Phosphate buffer pH 6.0, 30 mg L-leucil β naphthylamide, 100 mg Fast Garnet. The nomenclature as described by Smed et al. (1989), and Van Geyt et al. (1990) was followed.

Statistical analysis

Associations among cultivars were based on the Rogers Genetic Distance Coefficient. Clusters were determined by the Unweighted Pair Group Method (UPGMA). Calculations were made using the computer program of Swofford and Selander (1981).

Results and discussion

Fractionated proteins

The technical reproducibility of the different extraction and electrophoretical methods was very high. The resolution of the bands decreased following overnight runs at low electrical conditions (constant voltage of 70 V). The thickness (0.75 or 1.5 mm) of the gels, or small differences in protein concentrations between the different samples (up to two-fold dilutions), did not influence the pattern. To increase the resolution at the level of the higher molecular weight proteins, a decreased gel concentration of 12 or even 7.5 instead of 15% was preferred.

In a first set of experiments, the total, as well as the fractionated, proteins extracted from single seeds have been compared. Figure 1 shows a typical example of the comparison of the total protein patterns separated respectively on a 15% and 7.5% PAGE gel. Polymorphism could be detected between different individuals from the same line as well as between the lines. Differences have been observed in the presence or absence of bands as well as in their relative intensities. The intravarietal difference was so marked that classification of the cultivars became extremely difficult. Indeed, if enough individuals were considered, most types of band migrations were found within the lines.

In order to compare the overall variation between lines, the seeds were extracted in bulk. By mixing 20 seeds the patterns became completely comparable between lines. If minor differences were detected, these could not be seen as specifically present in a line. The buffering effect of the bulk extraction almost completely masked the individual genotypic differences. Separating the total proteins in fractions according to their different solubility characteristics did not provide any extra information.

In order to evaluate this technique for classification purposes, a preliminary screening from seed patterns of

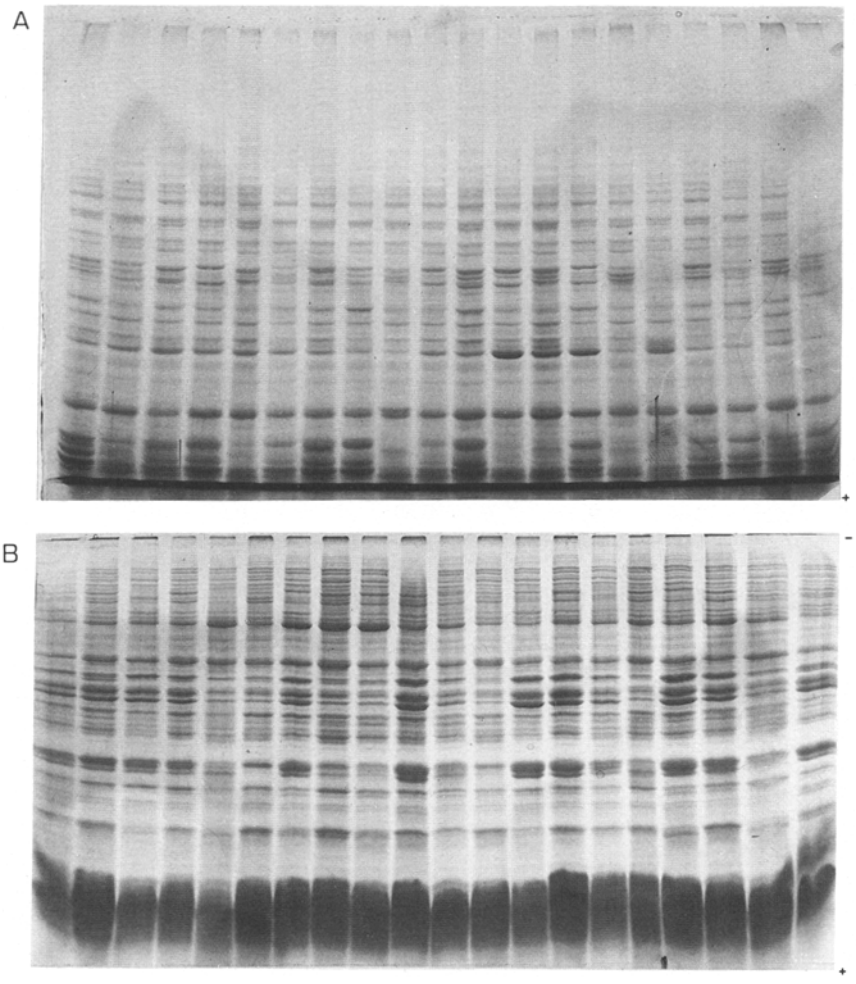

Fig. 1 A, B. Total protein patterns obtained by SDS-PAGE electrophoresis on respectively a 7.5% (**A**) and a 15% (**B**) acrylamide concentration. Each sample represents a single seed extraction. *Lanes 1, 3, 5* etc. show samples of the experimental line E1, whereas *lanes 2, 4, 6* etc. show samples from the experimental line Z97

the various wild species was carried out. The patterns within species of the *Vulgares* section were similar. Only minor differences could be found between *B. maritima* L. and the cultivated beets. The patterns of the two other *Beta* sections, however, are clearly different. It is interesting to note that *B. procumbens* Chr. Sm. and *B. webbiana* Moq. showed similar patterns while *B. patellaris* Moq. was clearly different. This observation favours the theory that the first two could be considered as ecotypes rather than distinct species. The clear differences observed between these patterns and that of *B. patellaris* indicate that *B. patellaris* is a distinct species and not a polyploid form of *B. procumbens* or *B. webbiana*. Similar observations were made by Curtis (1968) when studying fruit shape, by Mikami et al. (1984), Kishima et al. (1987) and Fritzsche et al. (1987) when studying organelle DNA, by Wagner et al. (1989) when observing isozymes, and by Mita et al. (1991) after RFLP analysis. A different point of view was proposed by Santoni and Bervillé (1992). They suggested that *B. webbiana* had been introgressed by the diploid ancestor of *B. patellaris*, resulting in a hybrid form recognized as *B. procumbens*.

Enzyme polymorphisms

Five enzyme systems have been investigated and eleven alleles have been detected in the cultivars studied. All tested cultivars were highly polymorphic for *Nad-Mdh1, Icd1* and *Lap1* and much less polymorphic for *Adh1* and *Aco1*. The frequencies of the allele F (controlling the fast moving band), S (controlling the slow moving band) and M (controlling an intermediary migrating band) and the frequency of heterozygotes (H), estimated for each population, were counted (Table 1).

(1) Alcohol dehydrogenase (ADH). ADH1 is a dimeric enzyme (Van Geyt and Jacobs 1986). In the populations studied there were two allelic forms controlling, respectively, a fast migrating band, designated as F, and a slower migrating band (S) with very low activity. The heterozygous type is characterized by a three-band pattern corresponding to the fast and slow bands plus an intermediary migrating band. This system is only active in seeds and young seedlings. In all the tested cultivars the F allele has a much higher frequency than the S allele.

Table 1. Estimations of the frequency of the F (fast moving), S (slow moving) and M (moving between F and S) bands, the frequency of the heterozygotes (H) and the number of tested plants (N) for each population. The mean frequencies of the alleles are shown in the last column

Enzyme system	Population												Mean allele freq.
	Viva	Monyx	Regina	Two	Massa-bel	Diana	Tosca	Magnamono			M8301		
								P 19	P 34	P 103	P 203	P 10	
ADH													
N	60	59	58	60	78	60	64	73	68	62	68	67	
F	0.87	0.92	0.91	1.00	0.96	0.98	0.95	1.00	0.99	1.00	0.99	0.99	0.96
S	0.13	0.09	0.09	0.00	0.04	0.03	0.05	0.00	0.02	0.00	0.02	0.02	0.04
H	0.17	0.17	0.17	0.00	0.08	0.05	0.09	0.00	0.03	0.00	0.03	0.02	
MDH													
N	70	60	86	64	88	68	74	80	68	99	109	85	
F	0.53	0.53	0.57	0.47	0.65	0.52	0.77	0.71	0.74	0.63	0.67	0.67	0.62
S	0.47	0.47	0.43	0.53	0.35	0.49	0.23	0.29	0.27	0.37	0.33	0.34	0.38
H	0.86	0.83	0.78	0.90	0.65	0.45	0.46	0.59	0.50	0.75	0.65	0.67	
ICD													
N	58	58	55	55	60	58	59	62	76	62	65	60	
F	0.91	0.72	0.54	0.91	0.76	0.70	0.84	0.69	0.64	0.59	0.59	0.60	0.71
S	0.09	0.28	0.46	0.09	0.24	0.30	0.16	0.32	0.36	0.41	0.41	0.40	0.29
H	0.17	0.45	0.85	0.18	0.45	0.60	0.32	0.53	0.65	0.82	0.75	0.63	
ACO													
N	87	108	73	91	69	78	83	77	95	103	76	83	
F	0.01	0.06	0.00	0.50	0.07	0.01	0.13	0.03	0.04	0.06	0.04	0.05	0.08
S	9.99	0.94	1.00	0.50	0.94	0.99	0.87	0.97	0.96	0.94	0.96	0.95	0.92
H	0.01	0.11	0.00	1.00	0.13	0.01	0.25	0.07	0.08	0.12	0.08	0.10	
LAP													
N	62	53	75	60	59	78	60	70	77	66	84	66	
F	0.72	0.52	0.57	0.17	0.73	0.76	0.78	0.51	0.51	0.59	0.49	0.62	0.58
S	0.15	0.26	0.38	0.43	0.20	0.17	0.13	0.16	0.22	0.17	0.30	0.24	0.23
M	0.15	0.22	0.03	0.41	0.08	0.06	0.08	0.34	0.27	0.24	0.21	0.14	0.19
FM	0.29	0.41	0.04	0.15	0.12	0.12	0.17	0.54	0.38	0.44	0.30	0.26	
MS	0.00	0.02	0.03	0.00	0.03	0.01	0.00	0.43	0.08	0.03	0.11	0.03	
FS	0.27	0.40	0.65	0.18	0.36	0.33	0.27	0.10	0.18	0.23	0.30	0.38	

Kws 2, Massabel, Diana, Tosca, Magnamono (P19, P34) and M8301 (P103, P203, P10) show an allele frequency of less than 0.05 for the *Adh1*-S allele type. Except in Kws 2, P19 and P103, where no heterozygous plants could be detected, low numbers of heterozygous plants were present in all the other populations investigated.

(2) Malate dehydrogenase (Nad-MDH). The Nad-Mdh1 gene (Van Geyt et al. 1990), expressed in seeds and leave of all developmental stages, proved to be an excellent marker. This gene codes for a dimeric enzyme with two allelic forms, a fast form (F), and a slow form (S). The enzyme Nad-MDH1 was highly polymorphic in the populations investigated. Except in the Maribo populations, were the S allele is less represented than the F allele, both F and S alleles were represented in equal amounts.

(3) Isocitrate dehydrogenase (ICD). This enzyme was expressed at all stages of development, but the level of activity is much lower in seeds (Smed et al. 1989). Three different types of zymograms were detected. Two homozygous types expressed three bands, one fast moving (F) and the other one slow moving (S). The heterozygous plants are characterized by five bands at the corresponding positions.

Icd1 is highly polymorphic and both allelic forms are represented in variable amounts in the populations tested. In general the S allele has a lower frequency than the F allele.

(4) Aconitase (ACO). To-date three different allelic forms have been identified within *B. vulgaris* and designated as *Aco1* A, *Aco1* B and *Aco1* C, in order of increasing mobility (Van Geyt et al. 1990). The gene codes for enzymes which are active as monomers.

In the populations analysed only *Aco1* A (S) and *Aco1* B (F) were detected and a low degree of polymorphism was present. Aconitase proves to be poorly polymorphic in Viva, Regina, Diana, Magnamono and M8301; a frequency of 0.05 or less was determined for the

F allele (Table 1). On the other hand, line Kws 2 is highly polymorphic and both allelic forms have a frequency of 50%.

(5) Leucine aminopeptidase (LAP). LAP1 is active in all stages of development but the activity is very poor in seeds and seedlings. So far three allelic forms have been detected in *B. vulgaris* controlling a fast (F), a slow (S) and an intermediary (M) moving band. LAP1 is a monomeric enzyme. The band pattern consists of one band in homozygotes or two bands in heterozygotes. The migration distance between M and S is very small, a high resolution of the gel being required to discriminate between both allelic forms.

The three allelic forms of *Lap1* were present in all investigated populations except for the cultivar Regina were the M allele has a frequency of only 0.03. In general the M allele has a low frequency except in Kws 2 where 40% of the allelic forms is M. Magnamono and M8301 also contain a high level of M alleles. In most of the tested populations it is the F allele which is the most frequent.

In general, sugar beet cultivars are highly polymorphic although very few different allelic forms occur. This implies that no single population can be characterized by one typical allelic form. Therefore, it is necessary to evaluate the difference between cultivars by taking into account the allele frequencies. An important factor to consider here is the difference in ploidy level and the sometimes amphiploid character of sugar beet cultivars. In order to use allele frequencies in the calculations the number of chromosomes have to be known and the relative allele dosages in each plant need to be estimated.

Only heterozygous plants can be considered in order to use the relative intensities of the bands formed by homo- and heteromers as an internal control. However the relative intensities of the bands were influenced by the developmental stage, the type of tissue used, and also the duration of the period of gel staining. Even if these parameters are taken into account, the interpretation of the electrophoresis pattern remains rather difficult. The use of a densitometer, to estimate quantitative differences between bands, offers a very expensive and time consuming alternative, especially if a large number of samples have to be screened. Moreover 3x sugar beet cultivars often contain small amounts of 2x and 4x plants. Thus, an additional control of the ploidy level of the varieties and the patterns of the parents needs to be employed.

As the main aim of cultivar identification requires a rapid, and easily reproducible method, chromosome counting and dosage estimates are not ideal for this purpose. Therefore, the determination of band frequencies are proposed here; the absence or presence of an allelic form for each plant being visualised by electrophoresis. Table 2 shows the estimates of distance (Rogers Genetic Distance) calculated between the 12 tested populations. A better survey of the interactions between the populations is given by a cluster analysis (UPGMA) calculated from the distance coefficients (Fig. 2).

Discrimination between cultivars

The data show that the measured distances between the populations are relatively small. The largest distance was measured between population Kws 2 and the other cultivars and was only 26%. Although the measured distances were small, the interactions between the populations were still in accordance with the expected data (Fig. 2).

In the UPGMA analysis five clusters can be distinguished. One contains two subclusters: the Maribo cultivars M8301 (P_{10}, P_{203}, P_{n03}), and Magnamono (P_{ng}, P_{34}). A second contains the cultivar Regina from Ringot. The third cluster consists of the related SES cultivars Viva,

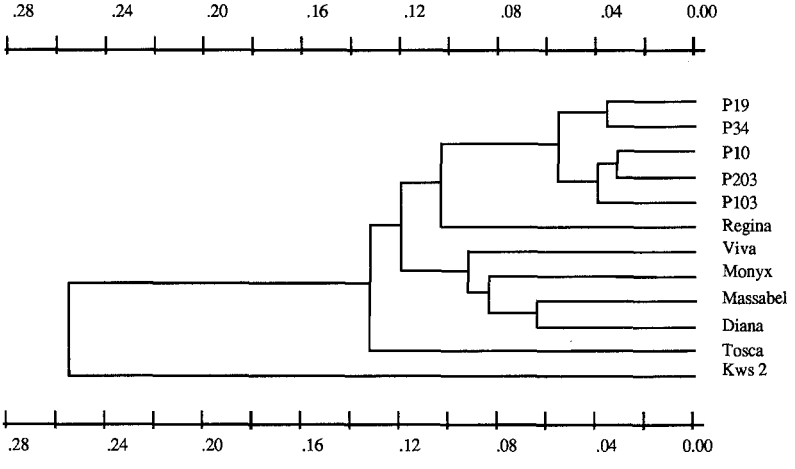

Fig. 2. Interactions between the population are represented by the use of the UPGMA cluster

Table 2. The Rogers genetic distance as calculated between the 12 tested populations

Population	Viva	Monyx	Regina	Kws 2	Massa-bel	Diana	Tosca	P 19	P 34	P 103	P 203	P 10
Viva	×											
Monyx	0.089	×										
Regina	0.135	0.088	×									
Two	0.23	0.217	0.292	×								
Massabel	0.108	0.091	0.144	0.246	×							
Diana	0.083	0.073	0.096	0.259	0.065	×						
Tosca	0.118	0.138	0.179	0.265	0.125	0.115	×					
P 19	0.151	0.087	0.135	0.248	0.148	0.104	0.125	×				
P 34	0.159	0.084	0.117	0.262	0.151	0.111	0.118	0.033	×			
P 103	0.141	0.08	0.091	0.258	0.128	0.094	0.137	0.059	0.051	×		
P 203	0.161	0.078	0.086	0.256	0.149	0.109	0.144	0.056	0.035	0.04	×	
P 10	0.137	0.085	0.082	0.273	0.121	0.085	0.119	0.065	0.046	0.031	0.028	×

Monyx, Massabel and Diana. The two KWS cultivars, Tosca and Kws 2, are relatively distinct and belong to two different clusters. The cultivars Magnamono and M8301 are so close (only 6% distance) that there is clearly no marked distinction between them.

Stability

The usefulness of allele frequencies in allozyme systems as a means of determining genetic distances, requires stability of these frequencies (independent production lots) in both different environments (production sites) and generations (years of production).

To estimate this variability two different approaches have been employed. First, seed lots of one variety produced in different years have been tested. Second, seed production on the same base carried out in the same year but in different sites gives an idea of the influences of external factors upon the measurements made. Table 2 and Fig. 2 show that the differences between P19 and P34 are very small. This implies that the environment had a very small impact upon the data. The same conclusion was drawn when the populations P10, P103, P203 were considered. In addition this line remained stable over the years studied.

Conclusion

Total as well as fractionated proteins can be clearly and reproducibly separated by means of SDS-Page. However, the intravarietal variation was so important in single seed patterns that classification of the cultivars became extremely difficult. Referring to the enormous variation found within the lines the possibility exists that, if enough individuals are screened, most of all migration positions exist within each line. Therefore, several questions arise concerning the usefulness of these patterns for specifying lines and cultivars. Using bulk extraction methods, the buffering effect of the bulk extraction almost completely masked the individual genotypic differences.

A possible alternative is to identify cultivars with isozymes. This has the advantage that the results are genetically interpretable, that the populations are classified according to the naturally occurring allelic variants and to the structure of the populations (allele frequencies). It was also demonstrated in the present paper that the environment has no effect upon the results. The measured distances between both samples of the Magnamono cultivar and both samples of the experimental line M8301 were less than 4%.

The choice of enzymes is dependent on their natural variation and their constancy in expression during the plant development and can be chosen in such a way that each chromosome is represented by at least one isozyme. A great deal of polymorphism is assumed to exist in the investigated populations due to the outbreeding nature of beet. In fact, while the investigated varieties were heterozygous the number of allelic forms for each isozyme system was relatively low in comparison with other plants. This, in combination with the rather small gene pool of the cultivated beet (Van Geyt et al. 1990), is certainly one of the reasons for the unsuitability of protein patterns as a means of identifying cultivars. Enzyme polymorphism is proposed here as an alternative. With enzyme studies it has been demonstrated that the tested cultivars are clearly distinct. Even relatively small genetic distances could be detected (Fig. 2).

In France, two maize cultivars can be defined as distinct by their enzyme polymorphism alone, if they satisfy the conditions proposed by the CPTS. A clearcut definition of the distinctness of sugar beet cultivars could also be established in the sense of acquired "minimal distance". A standardized proposal of distance calculations could then be adopted.

The tested populations remained stable over the years. A comparable set up could, therefore, be established to test the stability and uniformity of new applied varieties.

In general, it can be concluded that isozymes represent a useful tool for cultivar distinction, but certain drawbacks inherent to the crop studied have to be considered. An isozyme-based classification could also be useful in complementing agronomical or semi-agronomical characteristics.

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