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Cultivar distinction in sugar beet *(Beta vulgaris L.)* by electrophoretic genotype determination

Sortsadskillelse i sukkerroer ved elektroforetisk genotypebestemmelse

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Summary
Eighteen cultivars of monogerm, triploid sugar beet have been subjected to genotype determination using three allozyme systems. Glucosephosphateisomerase (Gpi-2), malate dehydrogenase (Mdh-1) and phosphoglucomutase (Pgm-1) were assayed by specific staining after horizontal starch gel electrophoresis. The same systems were used as the ploidy test in triploid cultivars where deviating types (di- and tetraploids) were controlled by chromosome counting using phase contrast microscopy. The method is proposed as a routine analysis performed on freeze dried samples of leaf materials, independent of season.

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Key words: Sugar beet, *Beta vulgaris,* cultivar distinction, electrophoresis, isoenzymes, ploidy test.

Resumé
Et voksende problem inden for den lovpligtige sortsafprøvning af sukkerroer er at adskille et stigende antal nyanmeldte sorter fra sortimentet på EF-sortslisten. En mulig løsning på problemet er anvendelsen af genetisk analyse ved hjælp af elektroforetisk isoenzymadskillelse.

Genotypebestemmelse af 18 sorter triploide, monogerme sukkerroer er blevet foretaget med tre allozymsystemer, glucosephosphatisomerase (Gpi-2), malatdehydrogenase (Mdh-1) og phosforglucomutase (Pgm-1), der blev specifikt farvet efter horisontal stivelsesgel elektroforese. De samme systemer blev benyttet som ploidi test i triploide sorter, hvor afvigende typer (di- og tetraploider) blev kontrolleret ved kromosomtælling i fasekontrast mikroskopi. Metoden foreslås som sæsonuafhængig rutineanalyse udført på frysetørret bladmateriale.

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Nøgleord: Sukkerroe, *Beta vulgaris,* sortsadskillelse, elektroforese, isoenzymer, ploidi test.

Introduction

A growing problem within the compulsory cultivar testing of sugar beet is to distinguish an increasing number of cultivars newly applied from cultivars included in the European Common Catalogue. Particularly within the monogerm triploid cultivars the morphological criteria for distinctness has proved to be unsatisfactory for securing a certain distinctness over a two year period. In several cases this has caused high yielding cultivars to be rejected. As the morphological characters are influenced by extreme environmental conditions (e.g. climatic extremes in Denmark 1982 and 1983) this situation is unsatisfactory for both plant breeders and testing authorities.

This paper shows the results from an experimental project the purpose of which is to illustrate the possibility of using isoenzymes, supposed to be neutral selection and independent of environment as characteristics of *Beta vulgaris* cultivars.

Monogerm, triploid sugar beet is produced by harvesting a diploid, malesterile line, containing the genes for monogermity after pollination with a tetraploid line as pollen donor. Triploid sugar beet may contain a small quantity of di- and tetraploid plants (below 3%) which is controlled by microscopy.

Electrophoretic methods using allozyme systems for genetic analysis of outbreeders have been published earlier for ryegrass (3, 9, 13 and others) and for sugar beet (5).

Three allozyme systems were selected for horizontal starch gel electrophoresis: glucosephosphateisomerase (E.C. 5.3.1.9), Gpi-2, malate dehydrogenase (E.C. 1.1.1.37), Mdh-1 and phosphoglucomutase (E.C. 2.7.5.1), Pgm-1. Only two different alleles are common in these systems in sugar beet which simplifies the zymogram patterns suited for routine analysis.

Material and methods

Plant matrial

Analyses were carried out on 18 cultivars from DUS (Distinctness, Uniformity and Stability) test plots. The cultivars are named alphabetically from A to R and they all belong to the same collection of monogerm triploid sugar beets with medium sugar content. Cultivar A is a newly applied cultivar and the remaining 17 cultivars are all from the European Common Catalogue. Cultivar A was represented by six different seed lots from three years and two geographically different seed origins (Italy and USA). Furthermore, three different seed lots from cultivar B closest to the new cultivar were used. The remaining cultivars were represented by only one seed lot each.

The analysed plant material was either fresh or freeze-dried leaf material. In both cases the material was collected row by row from fully developed photosynthetically active leaves. 0.5 cm^2 of fresh leaves (sampled with cylinder drill) or 10 mg of freeze-dried material was used. For the analysis of different seed lots of cultivar A and B which is not represented in the DUS test plot, seed leaves from plants germinated in climate chamber were used.

The zymograms proved to be constant in all stages of the plants (seed leaves and leaves of 1st and 2nd year beets) but in the seed leaves the Pgm-1 system appears with weak band intensities and this system is partly omitted from the analysis of seed lots.

Freeze drying

Samples from the DUS test plots taken with a cylinder drill, diameter 3 cm, were placed in fresh state in small freeze drying equipment on needles, 50 samples on each. Up to 2,000 samples corresponding to about 500 g fresh weight were handled in one operation (6).

In the initial phase of the freeze drying (actual vacuum drying) the sample temperature decreased within two minutes to a minimum temperature between 0 and –21° C, depending on the sample weight and the humidity state of the sample.

The process is terminated within a week after which the material can be stored for a few weeks

in self-sealing plastic bags in a refrigator at 5° C without any change in the zymograms.

Storing under nitrogen at –21° C in self-sealing plastic boxes has shown unchanged zymograms after more than 3 years and is recommended for long term storage.

Sample application

The plant material was crushed in perspex frames with 100 µl 0.5% 2-mercaptoethanol and the crude extract absorbed in ordinary ashfree filter paper wicks (Frisenette No. 616) 3 × 8 mm. The paper wicks were removed after 20–30 minutes of electrophoresis.

Electrophoresis

The electrophoretic separation as presented earlier (*Itenov*, 1983) was performed in 10 mM histidine pH 7.0 as gel buffer and 0.135 M TRIS / 0.043 M citric acid pH 7.0 (11).

The electrophoresis was carried out over four hours with 13% starch gels founded in glass frames 250 × 130 × 8 mm at 100 mA constant current or over 17 hours with 15% starch gels in 250 × 170 × 8 mm frames at 140 V constant. The cooling was performed in cold storage at 5° C or with water cooling below 9° C.

Staining

After electrophoresis the gel was sliced in four layers using the lower three for enzyme staining. Assaying in dark at room temperature as follows:

Gpi, 1 hour in 100 ml 0.1 M Tris/HCl pH 8.0 containing 5 mg NADP, 15 mg fructose-6-phosphate, 6 units glucose-6-phosphate dehydrogenase, 100 mg $MgCl_2$, 18 mg MTT, 0.6 mg PMS.

Mdh, 2–3 hours in 90 ml 0.1 M Tris/HCl pH 7.0, 10 ml lM Na-malate pH 7.0, 5 mg NAD, 18 mg MTT, 1.2 mg PMS.

Pgm, 3–4 hours in 100 ml 0.1 M Tris/HCl pH 7.0, 5 mg NADP, 12.5 mg alpha-D-glucose-1-phosphate, 6 units glucose-6-phosphate dehydrogenase, 50 mg $MgCl_2$, 18 mg MTT, 0.6 mg PMS.

Results and Discussion

Genotype determination

The normal zymograms for Gpi-2, Mdh-1 and Pgm-1 have shown to contain two alleles in each system confirmed by analyzing more than 10,000 plants of sugar beet (included diploid cultivars).

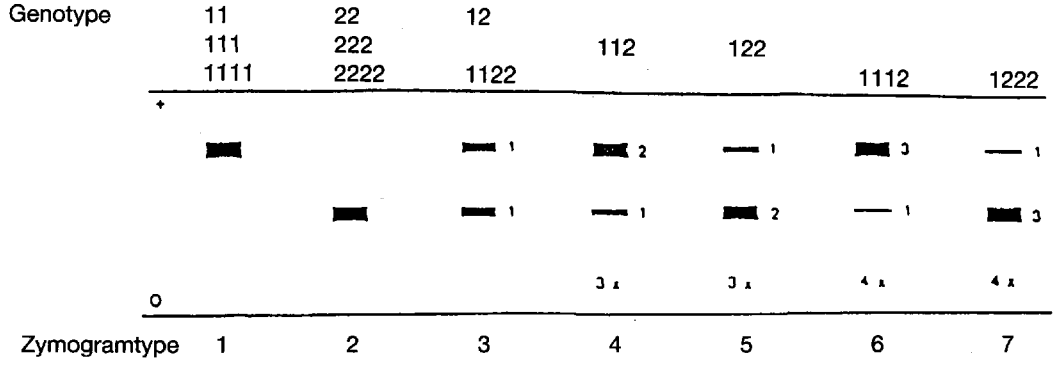
The zymograms for Gpi-2 and Mdh-1 equalize those expected from a dimeric allozyme system with two alleles noted as 1 or 2 coding for fast or slow migrating enzyme (fig. 1). Homozygote plants appear with one band of very strong intensity, heterozygote plants with three bands two of which equalize the homodimeric bands concerning the migrating distance and the third band representing the hybrid band with intermediate migrating distance. The band intensities, which is caused by additive gene dosage and free pairing of subunits by chance, show the ploidity level according to one, two, three or four gene loci. Relative strength of the theoretically calculated band intensities within each heterozygote zymogram pattern is noted with small figures to the right of each band.

Pgm-1 is a monomeric allozyme system in *Beta vulgaris* with two alleles 1 and 2 coding for a fast and a slow migrating enzyme (fig. 2). Homozygote plants appear with one band of strong intensity. Heterozygote plants show two bands the relative intensities of which reflects the ploidy. The Pgm-1 system is in relation to separation distance situated immediately in front of the bands of a Pgm-2 system always appearing with two bands of equal intensity (not shown in fig. 2).

For all three systems the same zymogram patterns have been found in di-, tri- and tetraploid cultivars of equal ploidy (*Itenov et al.* in preparation). Diploids only contain three types, 1, 2 and 3 (genotype 11, 22 and 12), triploids contain four types, 1, 2, 4 and 5 (genotype 111, 222, 112 and 122) and tetraploids contain five types, 1, 2, 3, 6 and 7 (genotype 1111, 2222, 1122, 1112 and 1222), cfr. fig. 1 and fig. 2. The efficiency of the three systems used for genotype determination in monogerm triploids is equal.

Zymogramtypes of Gpi-2 and Mdh-1

Fig. 1 and 2.
Zymogram patterns for di-, tri- and tetraploid plants in dimeric allozyme systems of Gpi-2 and Mdh-1, Fig. 1, and monomer allozyme Pgm-1, Fig. 2.
Zymogram 1 and 2 show homozygote plants. The polidy is masked and can be 2x, 3x or 4x (or others) noted as genotype 11, 111 or 1111 for type 1 and genotype 22, 222 or 2222 for type 2.
Zymogram 3 shows heterozygote plants representing either 2x (genotype 12) or 4x (genotype 1122) giving a symmetric pattern.
Zymogram 4 and 5 show asymmetric heterozygote pattern representing 3x plants (genotype 112 and 122).
Zymogram 6 and 7 show asymmetric heterozygote pattern representing 4x plants (genotype 1112 and 1222).



Zymogramtypes of Pgm-1

By using weighted freeze-dried material difficulties due to variation in the applied amount of material seldom arise. Otherwise variation in the intensities of zymograms from different plants may give problems in the determination of zymogramtype in the case of too strongly or too weakly coloured bands. This is especially a problem in the Pgm-1 system in the distinction between genotype 112 and 1112 or between type 122 and 1222.

Besides the two common ones one rare allele of sugar beet in the Mdh-1 system has been observed in three cultivars with a frequency below 3%. This allele codes for a very fast moving subunit which hybridize with the subunits of the two common alleles. In one type of triploid fodder sugar beet about 25% of the plants show the presence of this allele, which possibly arise by hybridization of sugar beets with wild species of *Beta vulgaris* (2) or from other types of cultivated beets (fodder beet or leaf beet) where this allele is shown to be present (*Itenov et al.* in preparation).

Ploidy control

In two triploid cultivars the plants of deviating zymograms (di- and tetraploids) were rediscovered in the test field and the ploidy level was ascertained by chromosome counting by phase contrast microscopy.

Homozygosity in all three systems in the same plant which masks the ploidy is found with a frequence between 0 and 5% in the tested cultivars. In diploid and anisoploid cultivars this frequence lies between 5 and 20%. Improvement of the method for ploidy control could include further one or more dimeric allozyme systems by means of which polyploid (anisoploid) cultivars could be tested for ploidy. The method is supposed to supplement or substitute the traditional ploidy control (4).

Allele frequencies

The frequencies of the four different genotypes in each of the tested triploid cultivars seem to be close to those expected in a population according to the Hardy-Weinberg »law«, which states that the gametic constitution of a population remains constant in the abscence of mutation, random changes or selection (7).

The genotype distribution in a triploid cultivar obtained by hybrid breeding of a paternal and a maternal line with the same allele frequencies and both in genetic equilibrium can be expressed as the binomial $(p + q)^3$, where p and q represent the relative allele frequencies of allele 1 and 2 respectively. In this case equilibrium is achieved after random mating and the contribution to the zygote by the tetraploid males are expressed by $(p + q)^2$ and the contribution by the diploid females are expressed by $(p + q)$. Under random chromosomal segregation (7) the number of plants belonging to the different genotypes can be stated as:

$$\mu_{111} = np^3$$

$$\mu_{112} = 3np^2(1\text{-}p)$$

$$\mu_{122} = 3np(1\text{-}p)^2$$

$$\mu_{222} = n(1\text{-}p)^3$$

where μ_{111}, μ_{112}, μ_{122} and μ_{222} are the expected frequencies of the triploid genotypes 111, 112, 122 and 222 and p is the relative frequency of allele 1, n is the total number of plants:
$n = n_{111} + n_{112} + n_{122} + n_{222}$.

To test whether the frequencies of the genotypes could be in accordance with this law the χ^2-goodness of fit test is used.

The test statistic is

$$\chi^2 = \frac{(n_{111}-\mu_{111})^2}{\mu_{111}} + \frac{(n_{112}-\mu_{112})^2}{\mu_{112}} + \frac{(n_{122}-\mu_{122})^2}{\mu_{122}} + \frac{(n_{222}-\mu_{222})^2}{\mu_{222}}$$

with 2 degrees of freedom. n_{111}, n_{112}, n_{122} and n_{222} is the number of plants with each of the 4 genotypes.

To take into account the large number of independent tests for each system the probability

$$P = 1-\sqrt[k]{0.95}$$

is used as the 5% level of significance. Here k is the number of seed lots for the system in question. The χ^2-goodness of fit test showed that the frequencies of the four genotypes differed significantly at the 5% level of significance only in a few cases (cultivar J and P in the Mdh-1 system and cultivar A_3 and R in the Pgm-1 system). It should be noted that the data should only follow the Hardy-Weinberg »law« as an approximation because in practice the paternal and maternal line will not have exactly the same allele frequency. However, the χ^2-goodness of fit test shows that this approximation seems satisfactory. The majority of the data genotype frequencies can thus be summarized in one of the allele frequencies, e.g. the allele carrying the fastest allozyme.

For the 18 cultivars the frequency of the allele coding for the fastest moving allozyme ranged between 0.50 and 0.79 for the Gpi-2 system, between 0.44 and 0.91 for the Mdh-1 and between 0.39 and 0.79 for the Pgm-1 system (Table 1).

Genetic distance

The difference between arcsin transformed values of the relative frequencies of the allele carrying the fastest allozyme is used as a measure of distance between varieties. Thus

$$\delta = | \theta_i - \theta_{i'} | \quad \text{for each system.}$$

Table 1. Data summary.

	Gpi-2			Mdh-1			Pgm-1		
Variety	p	number of plates	number of plants	p	number of plates	number of plants	p	number of plates	number of plants
A_1	0.629	7	184	0.763	6	173	0.500	1	30
A_2	0.618	5	144	0.761	5	145	.	0	.
A_3	0.618	12	349	0.806	12	350	0.500	5	130
A_4	0.599	5	143	0.791	5	145	.	0	.
A_5	0.586	6	141	0.773	4	103	0.536	1	28
A_6	0.594	4	114	0.778	4	123	0.543	4	113
B_1	0.551	6	159	0.739	5	134	.	0	.
B_2	0.590	10	175	0.728	10	163	.	0	.
B_3	0.619	10	244	0.718	10	232	.	0	.
C	0.522	5	143	0.678	5	144	0.586	1	29
D	0.746	8	189	0.737	8	193	0.643	8	187
E	0.784	10	182	0.506	10	170	.	0	.
F	0.738	7	183	0.553	7	173	0.682	7	177
G	0.655	5	144	0.706	5	137	.	0	.
H	0.558	7	197	0.756	7	198	.	0	.
I	0.503	9	222	0.795	9	213	0.675	8	197
J	0.581	9	217	0.531	9	219	0.615	8	186
K	0.785	4	87	0.686	4	88	0.655	4	88
L	0.709	8	229	0.908	8	228	0.390	6	177
M	0.644	8	195	0.653	8	195	0.635	6	146
N	0.762	5	101	0.729	5	101	0.663	5	101
O	0.618	11	254	0.545	11	254	0.656	11	260
P	0.580	9	255	0.786	8	223	0.506	9	249
Q	0.636	11	251	0.444	12	269	0.499	11	240
R	0.577	9	237	0.492	10	257	0.786	7	187

θ_i and $\theta_{i'}$ are the arcsin transformed allele frequencies of the two cultivars compared.

Figs 3a and 3b show the distribution of cultivars and seed lots. It is seen that the newly applied cultivar A is very close to P, H, B and G.

To test whether the obtained distances were significantly greater than zero a weighted analysis of variance was performed using the arcsin transformed values of the relative frequencies of allele »1« on each plate. A weighted analyses of variance was used for two reasons:

First as the plants on each plate might arise from different plots in the field, it could be expected that the variability among plates would be larger than expected from the binomial distribution. The analysis of variance was used in order to take this possible enlargement of the variance into account.

Secondly because the number of plants ranged from 4 to 32, each plate had to be given different weights.

In the analysis each plate was given a weight proportional to the number of plants on it. The weighted analysis of variance is described in more details by *Scheffé* (10) page 19 to 21 with an example on page 85 to 86.

This analysis showed that the pooled variance was somewhat larger than would be expected from the binomial distribution for the Mdh-1 and Pgm-1 systems. For the Mdh-1 system the pooled variance was 1.22 times larger than would be expected. For the Pgm-1 system it was 1.25 times larger than would be excepted. For the Gpi-2 systems the quotient between the pooled variance and the expected variance was 0.99, which is very close to unity.

Based on the pooled error variance all pairwise t-tests were calculated (Table 2a and 2b).

If the methods are to be useful it should be a requirement that none of the seed lots from the same variety show a distance significantly different from zero. From Table 2a and 2b it is seen that

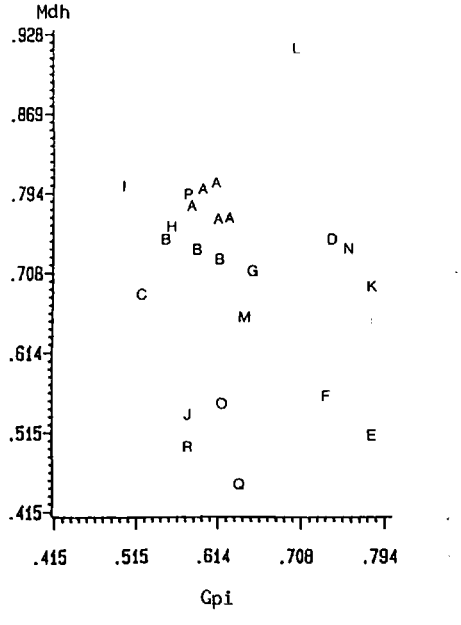


Fig. 3a. The figure shows the distribution of 18 cultivars when the Gpi-2 and Mdh-1 systems are at use. The axes are scaled according to arcsin ($\sqrt{p}$), where p is the relative frequence of the fastest allele. Cultivars are identified by letters.

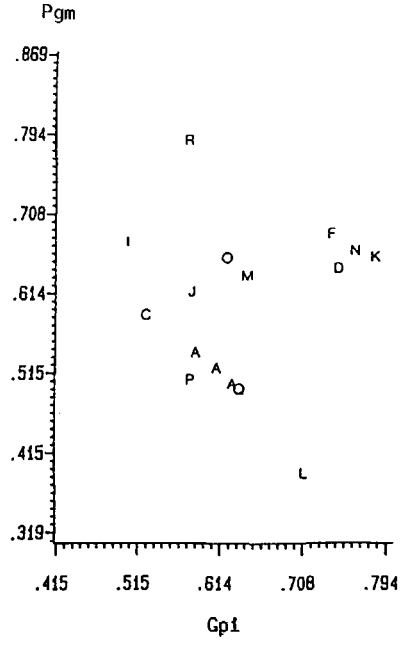


Fig. 3b. The figure shows the distribution of 18 cultivars when the Gpi-2 and Pgm-1 systems are at use. The axes are scaled according to arcsin ($\sqrt{p}$), where p is the relative frequency of the fastest allele. Cultivars are identified by letters.

	A_1	A_2	A_3	A_4	A_5	A_6	B_1	B_2	B_3	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
A_1	-									xx		xx	xx				xx		xx	xx		xx		xx	xx
A_2		-								x		xx	xx				xx		xx	xx		xx		xx	xx
A_3			-				x	xx	xx	xx	xx	xx	xx	xx	x		xx	xx	xx	xx	x	xx		xx	xx
A_4				-				x	x	xx		xx	xx	xx			xx	xx	xx	xx		xx		xx	xx
A_5					-					xx		xx	xx				xx		xx	xx		xx		xx	xx
A_6						-				xx		xx	xx	x			xx		xx	xx		xx		xx	xx
B_1	x		x				-					xx	xx				xx		xx	xx		xx		xx	xx
B_2								-				xx	xx			x	xx		xx	x		xx		xx	xx
B_3							x		-			xx	xx			xx	xx		xx	x		xx	xx	xx	xx
C	xx	xx	xx	x		x		x	xx	-		xx	xx		x	xx	xx		xx			xx	xx	xx	xx
D	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	-	xx	xx			x	xx		xx	xx		xx		xx	xx
E	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx		-		xx	xx	xx		xx	xx	xx	xx		xx		
F	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx			-	xx	xx	xx		xx	xx	xx	xx		xx	xx	
G					x		xx	x		xx	xx	xx	xx	-		xx	xx		xx			xx	xx	xx	xx
H	x		x						x		xx	xx	xx	xx	-		xx		xx	xx		xx		xx	xx
I	xx	xx	xx	xx	xx	xx		xx	xx		xx	xx	xx	xx	x	-	xx	xx	xx	xx	x	xx		xx	xx
J											xx	xx	xx	x		xx	-	xx	xx	xx	xx		xx	xx	
K	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx				xx	xx	xx	xx	-	xx			xx	xx	xx	xx
L	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx		xx			xx	xx	xx	x	-	xx	xx	xx	xx	xx	xx
M							xx			xx	xx	xx	xx		xx	xx	x	xx	x	-	x	xx	xx	xx	xx
N	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx				xx	xx	xx	xx			xx	-	xx		xx	xx
O							x			xx	xx	xx	xx		x	xx		xx	xx		xx	-	xx	xx	
P											xx	xx	xx	xx		xx		xx	xx	x	xx		-	xx	xx
Q							xx			xx	xx	xx	xx		xx	xx	x	xx	xx		xx		x	-	
R											xx	xx	xx	xx		xx		xx	xx	x	xx			x	-

Table 2a. Pairwise comparisons of varieties and origin of seeds. Comparisons below the diagonal are for the Gpi-2 system and comparisons above the diagonal are for the Mdh-1 system.

x = difference significant at the 5% level
xx = difference significant at the 1% level

B_1 is significantly different from B_3 for the Gpi-2 system. However, by using Bonferroni's inequality (8) this pair is not significant. Bonferroni's inequality takes into account that there are six ways (B_1-B_2, B_1-B_3 and B_2-B_3 for each of two systems) in which cultivar B may show significance.

Cultivar distinction

A cultivar newly applied (named A) was analysed together with 17 cultivars from the EEC Common Catalogue (B to R). Of these five cultivars (B, C, D, E and F) could not be distinguished over a two year period by morphological characters when following the guidelines UPOV (1979) and A.B.1. (1972). From Table 2a and 2b it appears that all seed lots of cultivar A can be separated from cultivar C, D, E and F, which is four out of the five cultivars that could not be distinguished over a two year period by morphological measures. However, it is not quite clear whether cultivar A can be separated from cultivar B, the cultivar which is closest to cultivar A. Only seed

	A_1	A_2	A_3	A_4	A_5	A_6	B_1	B_2	B_3	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
A_1	-						x			xx	xx	xx	xx		x	xx	xx	xx	xx	xx	xx	xx		xx	xx
A_2	-	-					x			x	xx	xx	xx			xx	xx	xx	xx	xx	xx	xx		xx	xx
A_3		-	-				x	xx	xx	xx	xx	xx	xx	xx	x	xx	xx	xx	xx	xx	xx	xx		xx	xx
A_4	-	-	-	-				x	x	xx	xx	xx	xx	xx		xx	xx	xx	xx	xx	xx	xx		xx	xx
A_5		-		-	-					xx	xx	xx	xx	x		xx	xx	xx	xx	xx	xx	xx		xx	xx
A_6		-		-		-				xx	xx	xx	xx	x		xx	xx	xx	xx	xx	xx	xx		xx	xx
B_1	-	-	-	-	-	-	-		x		xx	xx	xx	xx			xx	xx	xx	xx	xx	xx		xx	xx
B_2	-	-	-	-	-	-	-	-		x	xx	xx	xx	x	x	xx	xx	xx	xx	x	xx	xx		xx	xx
B_3	-	-	-	-	-	-	-	-	-	xx	xx	xx	xx		x	xx	xx	xx	xx	x	xx	xx	xx	xx	xx
C		-		-			-	-	-	-	xx	xx	xx	xx	x	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
D	x	-	xx	-		xx	-	-	-		-	xx	xx			x	xx		xx	xx		xx	xx	xx	xx
E	-	-	-	-	-	-	-	-	-	-	-	-		xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
F	xx	-	xx	-	x	xx	-	-	-			-	-	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	xx	xx	xx	xx	xx		xx	xx	xx	xx	xx
H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	xx	xx	xx	xx	xx	xx		xx	xx
I	xx	-	xx	-	x	xx	-	-	-			-		-	-	-	xx	xx	xx	xx	xx	xx	xx	xx	xx
J		-	xx	-			-	-	-			-	x	-	-		-	xx	xx	xx	xx		xx	xx	
K	x	-	xx	-		x	-	-	-			-		-	-			-	xx	xx		xx	xx	xx	xx
L		-	xx	-	x	xx	-	-	-	xx	xx	-	xx	-	-	xx	xx	xx	-	xx	xx	xx	xx	xx	xx
M	x	-	xx	-		x	-	-	-			-		-	-				xx	-	xx	xx	xx	xx	xx
N	x	-	xx	-		xx	-	-	-			-		-	-				xx		-	xx	xx	xx	xx
O	x	-	xx	-		xx	-	-	-			-		-	-				xx			-	xx	xx	xx
P		-		-			-	-	-		xx	-	xx	-	-	xx	xx	xx	xx	xx	xx	xx	xx	-	xx	xx
Q		-		-			-	-	-		xx	-	xx	-	-	xx	xx	xx	xx	xx	xx	xx	xx		-	xx
R	xx	-	xx	-	xx	xx	-	-	-	xx	xx	-	xx	-	-	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	-

Table 2b. Pairwise comparisons of cultivars and origin of seeds. Comparisons below the diagonal are for the Pgm-1 system and comparisons above the diagonal are a summary of both two or all three systems.

– = no comparison possible
x = difference significant at the 5% level
xx = difference significant at the 1% level

lots A_3 can be separated from all the seed lots of cultivar B. This is the seed lot where the largest number of plants (349) have been analyzed, which indicates that the two cultivars can be separated using the Mdh-1 system, but only if a very large number of plants are analyzed.

As shown by Table 2 and Fig. 3 cultivar A cannot be separated from cultivar P and neither could all seed lots of cultivar A be separated from cultivar G and H. However, those three cultivars can be separated from cultivar A by means of morphological characters. All seed lots of cultivar B cannot be separated from cultivar A, C, G, H and P, but cultivar B can be separated from these by morphological characters, except A.

Of the remaining 120 comparisons not involving cultivar A and B only ten are not distinct at the 5% level of significance in at least one of the systems. This seems to be a high frequency of separation when only three measures are used.

Conclusion

The proposed method consisting of three allozyme systems seems to be able to separate a great part of the examined cultivars. It is not able to separate two cultivars which could neither be separated by means of morphological characters. Since these two cultivars produced by the same plant breeding company could be of close genetical relationship this would be expected.

Since the systems seem to be as efficient as morphological characters and cheaper to use than those it is supposed that the method could advantageously be used as a supplement or a substitute of some of the morphological characters.

The application of freeze-dried leaf material has shown to be advantageous, in making the method independent of season.

The season of independence of the method and the information of ploidy which is achieved beside the results of allele frequencies used in cultivar distinction make the method suitable for a routine analysis.

Since the method of breeding (maintaining of the parental populations) can affect the stability of the cultivars in respect to allele frequencies this should be taken into consideration when the method is used in test for Distinctness, Uniformity and Stability.

Before the method is used as a routine analysis further investigations on the stability of the allele frequencies (genotypes) in seed lots from different years and from different geographical breeding places must be carried out.

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