ISOZYMES OF ACID PHOSPHATASE AND LEUCINE AMINOPEPTIDASE AS BIOCHEMICAL MARKERS FOR PURITY TESTING IN RAPESEED ANDROGENETIC LINES

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Extracts of cotyledons of *Brassica napus* plants (seed progenies of doubled haploid plants) were separated by electrophoresis on polyacrylamide gels and stained for acid phosphatase (ACP – E.C. 3.1.3.2.) and leucine aminopeptidase (LAP – E.C. 3.4.11.1) enzymes to investigate the possibility of utilising isoenzymes as markers of homogeneity (purity) of plant populations. One zone of activity for acid phosphatase and two zones of activity for leucine aminopeptidase were identified on gels, some variation in isozyme patterns occurred in several samples. This method is appropriate and consistent for testing the homogeneity of populations of progenies of doubled haploid (D.H.) plants.

Brassica; doubled haploid lines; enzyme electrophoresis; genetic markers; isozymes; PAGE

INTRODUCTION

Isozyme analysis has become a useful tool for various applications in plant genetics and breeding. Isozyme markers have been specifically applied in genetic and taxonomic studies, in breeding programmes of the family *Brassicaceae*.

In *Brassica napus* – rapeseed – isozymes have been used in taxonomic studies (Vaughan, Waite, 1967; Truco, Arús, 1987) and to identify and confirm interspecific hybrids (Sundberg, Glimelius, 1986, 1991). Enzyme polymorphism detected by gel electrophoresis has been proposed as a powerful tool for the testing of purity of F_1 hybrids (Nijenhuis, 1971; Woods, Thurman, 1976; Wills et al., 1979; Wills, Wiseman, 1980). In recent years it has been reported that isozymes as biochemical genetic markers have been used for characterization and cultivar identification in *Brassicas* (Arús et al., 1985; Mündges et al., 1990).

Isozymes as biochemical markers in plant genetic studies and breeding programmes have some advantages: the alleles at most isozyme loci are

co-dominant, and cause no deleterious changes in plant phenotype. This co--dominance also allows homozygotes to be distinguished from heterozygotes. The equipment and materials needed for screening the isozyme banding patterns (zymograms) of plants are relatively inexpensive and it is possible to screen large numbers of plants rapidly. The integrity of the whole plant can be maintained in that only small sample amounts of plant tissue are needed (Moore, Collins, 1983).

The objectives of this paper were to analyze the purity (homogeneity) of breeding lines originated from doubled haploid plants (D.H. plants) obtained after colchicine treatment from haploids regenerated from anther culture of *Brassica napus*. Androgenetic breeding lines were obtained from winter rapeseed varieties Jet Neuf, Darmor, Ceres, Solida, and F_1 hybrids of Ceres x Solida. Methods of cultivation and regeneration of plants describes Bohá-čová (1990), D.H. plants are used in a selection programme at the Slapy breeding station (near to Tábor, Czech Republic).

MATERIALS AND METHODS

Growth of plant material: Plants were cultivated in a growth chamber, in sterile Vermiperl (perlite), under a 16 hour photoperiod with a 25 °C/ /15 °C day-night temperature regime. Plants were harvested and analyzed on the 6th day after germination, when cotyledons were fully expanded. The origins of the D.H. lines are shown in Tab. I.

I. The origins of the doubled-haploid lines

Line	Origin
11	Jet Neuf 24
12	Darmor 25
13	Ceres 31
14	Ceres 32
15	Ceres 33
16	Solida 27
17	Solida 54
18	F_1 Ceres x Solida 350
19	F_1 Ceres x Solida 404

Extraction of enzymes: From each analyzed breeding line – progeny of D.H. plant, 20–30 plants from germinated seed were analyzed. One cotyledon from each plant was analyzed. Tissue was ground in an Eppendorf tube with a glass rod in extraction buffer on ice. Three different extraction systems were used:

a) 10 mM Tris (Trizma) – HCl, pH = 7.2, 1 mM 2-mercaptoethanol
b) 2% reduced glutathione, titrated to pH = 7.6 with 2M Tris (Trizma)
c) 10 ml 0.5 M Tris-HCl, pH = 6.8, 1 ml 2-mercaptoethanol, 10 ml glycerol, 5 mg bromophenol blue, 79 ml water, to 100 µl of this extraction buffer add 20 µl PMSF (10 mg/1 ml isopropanol).

50 µl of extraction buffer was added per cotyledon. The crude extract was centrifuged at 4 °C at 12 000 g for 8 minutes. The supernatant was transferred to a new tube and 10 µl of loading buffer (60 ml water, 40 ml glycerol, 10 mg bromophenol blue) was added. Samples were immediately frozen and stored at –70 °C.

Polyacrylamide gel electrophoresis: Isozymes were separated into discrete bands by vertical slab polyacrylamide gel electrophoresis (PAGE) with the resolving gel containing 7.5% (w/v) acrylamide and 0.2% (w/v) N,N'--methylenebisacrylamide (BIS). The stacking gel contained 3.75% (w/v) acrylamide and 0.1% (w/v) BIS. Compositions of resolving, stacking gels and buffers and electrode buffer are shown in Tab. II.

45 µl of enzyme extract were applied per well. Electrophoresis was performed at 6 °C for 4–5 hours under constant current (30 mA), using a voltage range 70–150 V.

II. Composition of solutions for PAGE

		Resolvig gel	Stacking gel
AC/BIS	ml	15	2.5
Buffer A	ml	7.5	–
Buffer B	ml	–	5
Sodium sulfite	µl	180	60
Ammonium persulfate	µl	300	150
TEMED	µl	30	30
Water	ml	37.5	12.5

AC/BIS:	30 g acrylamide, 0.8 g BIS / 100 ml
Buffer A:	7.27 g Tris, 48 ml 1M HCl, pH = 7.5 / 100 ml
Buffer B:	6 g Tris, 48 ml 1M HCl, pH = 6.8 / 100 ml
Na_2SO_3:	saturated aqueous solution
$(NH_4)_2S_2O_8$:	1.5 g in 10 ml water
Electrode buffer:	9.27 g boric acid, NaOH to pH = 7.2, final volume 1 000 ml

Enzyme activity staining: Immediately after electrophoresis, gels were rinsed with distilled water, equilibrated for 15 minutes with buffer used for staining at 4 °C in the dark, and than stained to detect enzyme activity. Investigations were made on acid phosphatase E.C. 3.1.3.2. and leucine aminopeptidase E.C. 3.4.11.1. isozyme systems (W e b b , 1984). Staining solutions for the isozymes were:

LAP – leucine aminopeptidase: 100 ml 0.08M Tris-maleate buffer, pH = 6.0, 40 mg L-leucine-β-naphthylamide.HCl, 50 mg Fast Black K salt, 1 ml 1M magnesium chloride, 28 °C, 1–2 hours in the dark.

ACP – acid phosphatase: 100 ml buffer (9.3 ml glacial acetic acid, 5.1 g NaOH, water to 900 ml, pH = 5.0), 50 mg β-naphthylphosphate (1% solution in 50% acetone), 100 mg Fast Black K salt, 1 ml 1M magnesium chloride, 28 °C 2–3 hours in the dark.

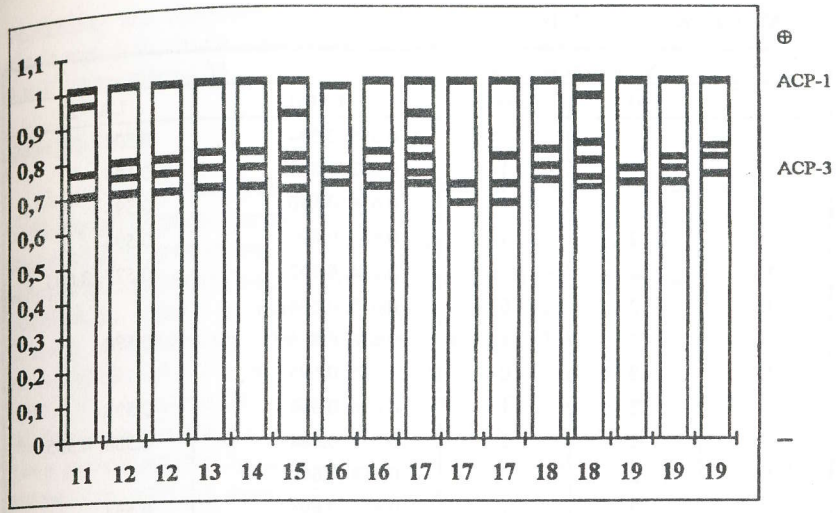
RESULTS

Results for acid phosphatase and leucine aminopeptidase evaluation are shown in Tabs. III and IV and in Figs. 1 and 2.

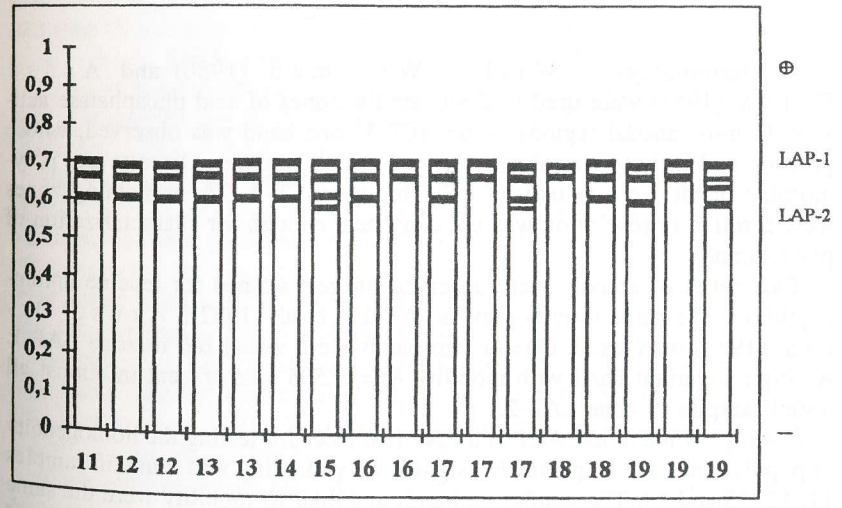
III. ACP isozymes in tested D.H. lines

Sample	Number of isozymes		*Rf*	
	ACP-1	ACP-3	ACP-1	ACP-3
11	1	3	1.0	0.697, 0.758, 0.955
12	1	3	1.0	0.697, 0.742, 0.787
13	1	3	1.0	0.704, 0.758, 0.803
14	1	3	1.0	0.704, 0.758, 0.803
15	1	4	1.0	0.697, 0.750, 0.789, 0.908
16	1	2	0.985	0.712, 0.750
	1	3	1.0	0.705, 0.758, 0.803
17	1	2	0.985	0.712, 0.750
	1	3	1.0	0.705, 0.758, 0.803
	1	5	1.0	0.712, 0.742, 0.788, 0.833, 0.909
18	1	3	1.0	0.723, 0.762, 0.808
	1	5	1.0	0.705, 0.721, 0.770, 0.819, 0.951
19	1	2	1.0	0.715, 0.754
	1	3	1.0	0.715, 0.754, 0.785

ACP-1, ACP-3 = zones of activity, according to A r ú s and O r t o n (1983)



1. Acid phosphatase isoenzymes; 11 to 19 – origin of samples in Tab. I



2. Leucine amonopeptidase isoenzymes; 11 to 19 – origin of samples in Tab. I

IV. LAP isozymes in tested D.H. lines

Sample	Number of isozymes		*Rf*	
	LAP-1	LAP-2	LAP-1	LAP-2
11	2	1	0.656, 0.694	0.598
12	2	1	0.652, 0.684	0.595
13	2	1	0.658, 0.690	0.595
14	2	1	0.656, 0.694	0.598
15	2	(2)	0.654, 0.692	0.577, 0.603
16	2	0	0.656, 0.694	
	2	1	0.656, 0.694	0.598
17	2	0	0.656, 0.695	
	2	1	0.656, 0.694	0.598
	2	2	0.652, 0.684	0.588, 0.595
18	2	0	0.658, 0.690	
	2	1	0.656, 0.694	0.598
19	2	0	0.656, 0.694	
	2	1	0.652, 0.684	0.589
	3	1	0.636, 0.648, 0.688	0.584

LAP-1, LAP-2 = zones of activity, according to Arús and Orton (1983)

The terminology of Wills, Wiseman (1980) and Arús, Orton (1983) were used to designate the zones of acid phosphatase activity. In most anodal regions (zone ACP-1) one band was observed, whose position and activity was almost the same in all samples. Discrete and polymorphic bands were visualized only in zone ACP-3. The remaining zones were inactive or resolution was not consistent enough for characterization of plant sample.

Two zones of activity were observed on gels stained for leucine aminopeptidase. The same terminology as Arús et al. (1982), Arús, Orton (1983) were used. One or two bands were visualized in zone LAP-1. A single invariant band with mobility *Rf* = 0.595 was present in almost all tested samples in zone LAP-2.

It was possible to use ACP and LAP isozymes for testing the homogeneity of populations of D.H. plants. No significant variability was found in samples 11, 12, 13 and 14. The number of bands and their *Rf* mobility were the same in all analyzed seedlings. In contrast samples 15, 16, 17, 18 and 19 showed differences in number and mobility of visualized isozymes. High degree of

variability was observed in samples 16, 17, 18 and 19 only. In the remaining plant material, only a small number of plants showed differences from „standard" zymograms.

DISCUSSION

Conditions for isozyme analysis in purity testing and the identification of D.H. rapeseed plant populations were optimized. The age and developmental stage of analyzed plants is very important for the interpretation of results, since electrophoretic segregation patterns are quite dissimilar in seedlings' cotyledons and/or in leaves of older plants (Arús, Orton, 1983; Čurn, unpublished). The best extraction method was with modified glutathione buffer (Arús et al., 1991; Delourme, Foisset, 1991) – extraction system */b*. Extraction with 2-mercaptoethanol and PMSF (Lönnendonker, Schieder, 1980; Mündges et al., 1990) gave poorer results. In several cases enzyme activity was influenced by the extraction method employed, some extractions exhibited a strong background, nonspecific staining and indistinguishable bands. Enzyme extracts (with added glycerol and bromophenol blue) were stored at –70 °C for several months without any apparent changes of activity.

In this study for separating isozyme variants to discrete bands nondenaturing discontinuous polyacrylamide gel electrophoresis was used with modified resolving (running) gel and electrode buffers. With standard PAGE methods (Laemmli, 1970; Hames, Rickwood, 1990; Shields et al., 1983) consistent and reproducible results were not obtained. Staining of enzyme activity was poor. In particular staining for acid phosphatase was insufficient in PAGE systems with a more alkaline pH (Arús, Orton, 1983; Shields et al., 1983).

Staining recipes were modified according to Wendel, Weeden (1990) and Vallejos (1983). Staining with α-naphthylphosphate as substrate for acid phosphatase and Fast Blue BB salt or Fast Blue RR salt (Wendel, Weeden, 1990) did not give clear results. Zymograms were quite indistinct; in some ACP-1 there was 1 band, in zone ACP-3 several very indistinctly visible bands were apparent.

In *Brassicas*, ACP isozymes and LAP isozymes have been used in studies examining electrophoretic variability as a tool in testing hybrid seed purity (Nijenhuis, 1971; Woods, Thurman, 1976; Wills et al., 1979; Arús et al., 1982).

These investigations were directed to analyze purity (or homogeneity) of plant populations obtained from anther cultures of rapeseed. Isozyme analysis is an appropriate tool for these purposes. With this method, it is possible to

simply investigate variability between regenerated plants, in early developmental phases, without affecting the integrity of the whole plant.

Variability observed in samples 16, 17, 18 and 19 may have originated from several sources. These include: 1) regenerated plants may not have been haploid, but may have regenerated from diploid cells, 2) variability may have occurred during propagation *in vitro* or 3) variability may have occurred during sexual propagation of the plants.

For plant breeders should be quite helpful to have simply screening method which can in early developmental stages distinguish heterozygous plants. We can recommend the lines with observed variation in isozyme patterns to be eliminated from breeding-selection programmes. This approach can be extended to the identification of homogeneous populations derived from doubled haploid plants of other important crop plants.

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Izoenzymy kyselé fosfatázy a leucin aminopeptidázy jako biochemické markery pro testování čistoty androgenetických linií řepky.

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Analýza izoenzymového spektra pomocí elektroforézy na gelovém nosiči má již poměrně široké uplatnění i v genetice a šlechtění brukvovitých rostlin. Izoenzymové

markery se používají pro taxonomické studie a pro identifikaci a potvrzení hybridů po somatické hybridizaci. Pravděpodobně nejvýznamnější aplikací je využití enzymového polymorfismu detekovaného po elektroforéze k testování čistoty F_1 hybridů, odrůd a novošlechtění. V posledních letech se izoenzymy začínají používat i jako biochemické markery pro identifikaci odrůd.

Cílem této studie byla analýza čistoty (homogenity) šlechtitelských linií ozimé řepky. Tyto androgenetické linie byly získány jako potomstva D.H. rostlin, regenerovaných z prašníkových kultur a ošetřených kolchicinem. Linie byly odvozeny u odrůd ozimé řepky Jet Neuf, Darmor, Ceres a Solida a u F_1 hybridů Ceres x Solida. Osivo testovaných linií jsme získali za šlechtitelské stanice Slapy u Tábora.

Rostliny byly kultivovány v klimaboxu ve sterilním perlitu při 16hodinové fotoperiodě a teplotě 25 °C/15 °C den/noc. Analýzy byly prováděny šestý den po vzejití, při plně rozvinutých děložních lístcích. Extrakce enzymů byla prováděna z jednoho děložního lístku v 50 µl extrakčního pufru; jako nejvhodnější pro extrakci se osvědčil 2% glutationový pufr. Elektroforéza probíhala v polyakrylamidovém 7,5% gelu v diskontinuálním nedenaturačním systému.

Výsledky shrnují tab. III a IV a obr. 1 a 2. U androgenetických linií 11 (Jet Neuf 24), 12 (Darmor 25), 13 (Ceres 31) a 14 (Ceres 32) nebyla zjištěna žádná variabilita ve spektru sledovaných izoenzymů. Naopak zejména u linií 16 (Solida 27), 17 (Solida 54), 18 (F_1 Ceres x Solida 350) a 19 (F_1 Ceres x Solida 404) byly zjištěny poměrně četné rozdíly v počtu a mobilitě izoenzymů.

Tato variabilita může pocházet z několika zdrojů:

- regenerované rostliny nebyly primárně haploidní, ale diploidní – regenerované z diploidních buněk
- variabilita se objevila během propagace *in vitro*
- variabilita se mohla objevit během množení linií semeny (cizí sprášení).

Metoda izoenzymové analýzy může sloužit i jako rychlá screeningová metoda a napomoci šlechtiteli při hodnocení homogenity materiálu.

Brassica; řepka olejka; androgenetické linie; enzymová elektroforéza; genetické markery; izoenzymy; PAGE

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