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ISOENZYME VARIATION IN THE GENUS *PISUM* II. ELECTROPHORETIC PATTERNS OF ALCOHOL DEHYDROGENASE AND ISOCITRATE DEHYDROGENASE FROM COTYLEDONS OF UNGERMINATED SEEDS[1]

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Summary. Electrophoretic analysis of alcohol dehydrogenase (ADH) and isocitrate dehydrogenase (IDH) of cotyledons of ungerminated seeds was performed in 108 *Pisum* accessions, representing various taxonomic units. The work was a continuation of on-going electrophoretic investigations on isoenzyme variation in *Pisum*. Seven electrophoretic phenotypes of ADH and two phenotypes of IDH have been distinguished in the investigated material. The obtained results are discussed with reference to classification and identification of *Pisum* genetic resources.

Investigations of isoenzyme variation in *Pisum* have been recently undertaken in this laboratory. One hundred eight *Pisum* forms, covering most of the annual *Pisum* taxa recorded by Lehmann (1954), were analysed for electrophoretic patterns of the following enzyme systems: leucine aminopeptidase (LAP) and amylases (Amy) of cotyledons of ungerminated seeds, glutamate oxaloacetate transaminase (GOT) of leaves, and peroxidases (PX) of roots (Przybylska et al. 1982).

This paper presents further data on isoenzyme variation in the genus *Pisum*. The *Pisum* accessions used in the above mentioned investigations were analysed for electrophoretic patterns of alcohol dehydrogenase (ADH) and isocitrate dehydrogenase (IDH) in ungerminated seeds.

MATERIAL AND METHODS

PLANT MATERIAL

The 108 investigated *Pisum* accessions represent 5 annual species (Lehmann 1954): *P. elatius* (4 accessions), *P. humile* (3 accessions), *P. sativum* (85 accessions), *P. abyssinicum* (11 accessions), *P. fulvum* (5 accessions). The list of the investigated

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materials, with the data indicating their origin and source, was presented in an earlier paper (Przybylska et al. 1982). The seeds used for analyses were harvested during the years 1979 - 1982 from plants grown in a greenhouse at the Institute of Plant Genetics of the Polish Academy of Sciences in Poznań. As a rule, seed samples from one-year-harvest were examined. In the case of several accessions obtained from the Weibullsholm Plant Breeding Institute in Landskrona, Sweden, 3 - 5 samples harvested in different years were analysed. The analysis covered both original and reproduced samples. All the seed samples examined were stored at room temperature.

Cotyledons of ungerminated seeds of at least 6 individuals of each accession were analysed for electrophoretic patterns of ADH and IDH. In addition, mixed samples of embryonic axes from 5 - 10 seeds of seven selected *Pisum* accessions were examined for electrophoretic patterns of ADH.

ANALYTICAL TECHNIQUES

Cotyledons and embryonic axes of dry ungerminated seeds were crushed to the meal and the proteins were extracted with 0.09 M tris-borate buffer, pH 7.5, containing 0.004 M EDTA, for 1 h at 4°C. The ratio of the meal to the extracting solution (w/v) was 1: 3 (cotyledons) or 1 : 10 (seed axes).

Electrophoretic separation of ADH was performed in a 13% starch gel, in the buffer system described by Rasmuson and Rudin (1971); the electrode buffer was 0.09 M tris-borate, pH 7.5, containing 0.004 M EDTA. For gel preparation the electrode buffer was diluted 1 : 19 with distilled water. Electrophoretic separation of IDH was performed in 14% starch gel, in the tris-citrate buffer system I, pH 7.0, described by Shaw and Prasad (1970). Electrophoresis was carried out at 2 mA/cm², for 4 - 5 hrs.

Enzyme activity of ADH was detected by incubating gels, for 40 min. at 37°C, in darkness, in the reaction mixture containing: 10 mg nitro blue tetrazolium (NBT), 1 mg phenazine methosulphate (PMS), 4 mg nicotinamide adenine dinucleotide (NAD), 2 ml 95% C_2H_5OH, 30 ml 0.1 M glycine-NaOH buffer, pH 8.7. For detection of IDH activity gels were incubated for 2 hrs at 37°C, in darkness, in the mixture containing: 10 mg NBT, 1 mg PMS, 3 mg nicotinamide adenine dinucleotide phosphate (NADP), 60 mg sodium isocitrate, 1 ml 1% $MgCl_2$, 30 ml 0.2 M tris-HCl buffer, pH 8.0.

Gels were fixed in 50% methanol, wrapped in foil and stored in a refrigerator for later reference.

RESULTS AND DISCUSSION

As indicated in Materials and Methods, for several *Pisum* accessions both original seed samples and samples reproduced in different years were analysed. No changes attributable to seed storage were noticed in the IDH patterns. In the case of

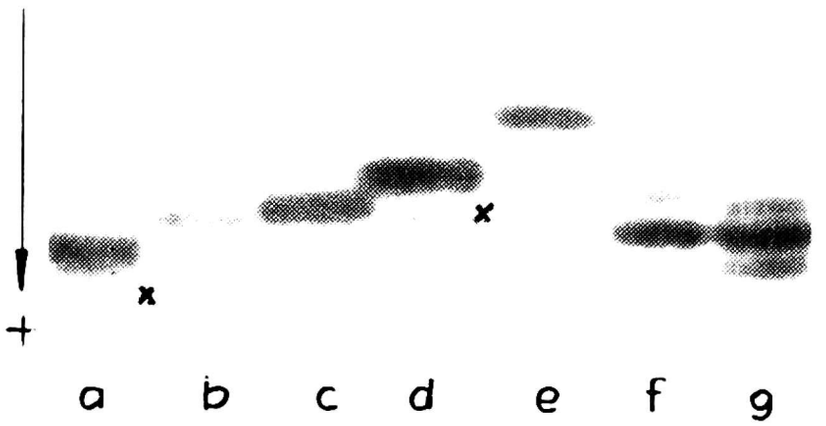

Fig. 1. ADH phenotypes revealed in cotyledons of the examined *Pisum* accessions. Bands occassionally observed and not considered in distinguishing the phenotypes are marked with an asterisk. The presented phenotypes, "a"-"g", are produced by the following accessions: "a" — W 808, *P. abyssinicum*; "b" — JI 691, *P. abyssinicum*; "c" — W 110, *P. sativum*; "d" — W 1935, *P. sativum*; "e" — JI 224a, *P. fulvum*; "f" — W 1896, *P. sativum*; "g" — W 1884, *P. sativum*

Seed sources: lines designated with the letter "W" were obtained from the Pisum Gene Bank at Weibullsholm Plant Breeding Institute, Landskrona, Sweden; those with the letters "JI" — from the John Innes Institute, Norwich, England (*P. fulvum* JI 224a was isolated from the accession labelled JI 224)

Fig. 2. ADH phenotypes revealed in embryonic axes of *Pisum* accessions representing the variation shown in Fig. 1. From left to right the accessions indicated in Fig. 1

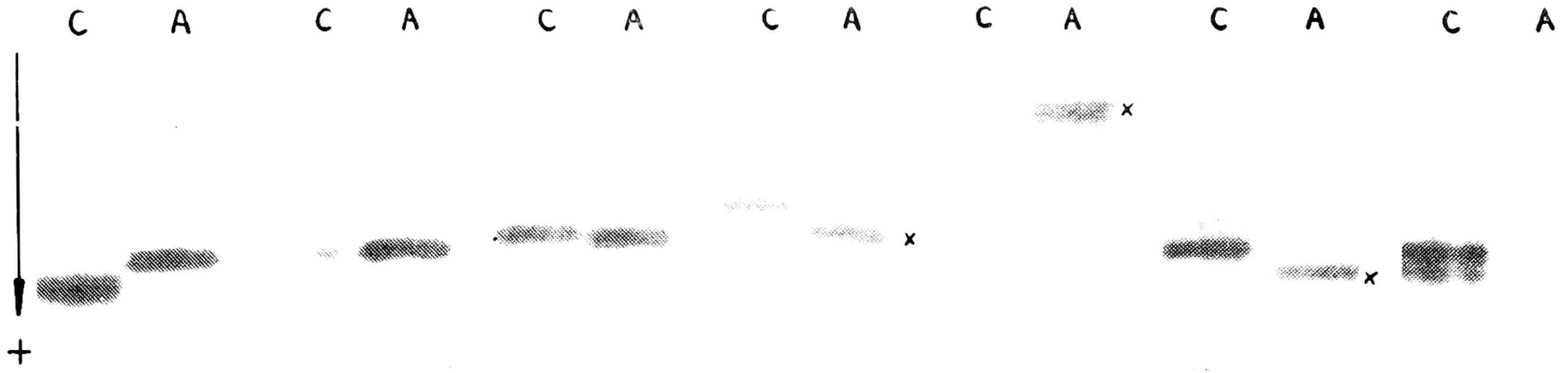

Fig. 3. Gelsshowing compared side-by-side ADH phenotypes of cotyledons (C) and embryonic axes (A) of *Pisum* accessions representing the variation shown in Fig. 1. From left to right the accessions indicated in Fig. 1. Bands characteristic of embryonic axes and sporadically observed on ADH zymograms on cotyledons are marked with an asterisk

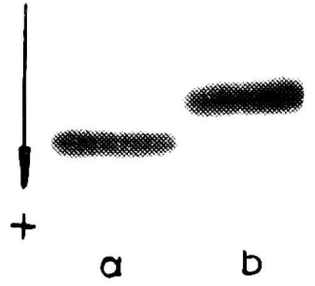

Fig. 4. IDH phenotypes revealed in cotyledons of the examined *Pisum* accessions. The presented phenotypes are produced by the following accessions: "a" — W 936, *P. humile*; "b" — W 110, *P. sativum*

ADH, seeds stored for more than five years tended to show a decreased enzyme activity. Seeds of some accessions stored for more than ten years showed no bands on ADH zymograms.

ALCOHOL DEHYDROGENASE (ADH)

Seven electrophoretic phenotypes of ADH, designated "a" — "g", were distinguished in the examined material (Fig. 1). Phenotypes "a" — "e" show one major band, while phenotypes "f" and "g" show two and four bands, respectively. On zymograms of one of the *Pisum* forms investigated (*P. fulvum* VIR 3397) no band of ADH activity was observed. At this stage of investigation, genetic interpretation of the distinguished ADH phenotypes cannot be offered.

In many cases, some individuals of particular accessions showed "additional" weak bands. These bands were not taken into consideration when distinguishing the electrophoretic phenotypes. The "additional" bands were suspected to be attributable to contamination by material from embryonic axes. It also seems that there is no demarcation line between cotyledons and an embryonic axis; thus, protein bodies which are organelles characteristic of mature cotyledons, were observed in embryonic axes of various plant species (Ashton 1976).

Electrophoretic analysis of embryonic axes was performed for several *Pisum* accessions. Fig. 2 presents the ADH patterns of embryonic axes of 7 *Pisum* accessions having different ADH phenotypes of cotyledons. In Fig. 3, the ADH phenotypes of cotyledons and of embryonic axes of the 7 accessions are compared side-by-side.

Comparing Fig. 2 with Fig. 1 one may notice that the variation of ADH phenotypes of embryonic axes is not parallel to that of cotyledons. Four accessions having different cotyledon phenotypes (phenotypes "a" — "d") show a uniform embryonic axis phenotype, with a single intense band. One of the remaining accession shows one band migrating more slowly, and two accessions reveal a faster moving band. It is worth of noticing that embryonic axes provide additional ADH markers.

A side-by-side comparison of ADH phenotypes of cotyledons and embryonic axes showed that only some of the "additional" bands observed on zymograms of cotyledons may be attributed to embryonic axes. Such bands are indicated in Fig. 3. The remaining "additional" bands may be characteristic of certain individuals or they may represent artefacts, as was observed by other investigators (Tanksley 1979; Oakeshott et al. 1982).

Regarding the distribution of ADH phenotypes distinguished in the cotyledons of the 108 examined *Pisum* accessions, as many as four phenotypes, "b", "d", "e", "g", were found in single accessions. The rare phenotypes were detected in the following accessions: "b" — in *P. abyssinicum* JI 691, "d" — in *P. sativum* W 1935, "e" — in *P. fulvum* JI 224a, and "g" — in *P. sativum* W 1884. As pointed out in the previous paper, *Pisum* accessions showing rare electrophoretic phenotypes may significantly contribute to the total variation range within collections. They also serve as useful identifiers. In this connection it should be mentioned that of

the *Pisum* accessions found to have rare ADH phenotypes, *P. fulvum* JI 224a was reported to show rare electrophoretic phenotypes of also three other enzyme systems: amylases and esterases from cotyledons of ungerminated seeds and root peroxidases (Przybylska et al. 1982; unpublished data).

The phenotype "a" was observed only in *Pisum abyssinicum*. Except *P. abyssinicum* JI 691, having the rare phenotype "b", all the investigated *P. abyssinicum* accessions show the phenotype "a". As *P. abyssinicum* accessions were reported to be quite uniform with respect to electrophoretic patterns of both storage (Przybylska et al. 1983) and enzyme proteins (Przybylska et al. 1982), the distinction of one form with respect to the ADH pattern should be stressed.

The phenotype "c" was the most commonly occurring; it was found in *P. elatius* (4 accessions), *P. humile* (3 accessions), *P. sativum* (75 accessions) and *P. fulvum* (3 accessions). It should be mentioned that in the group of accessions having the phenotype "c" a marked variation was observed in the intensity of the characteristic band; the band was especially intensive in *P. sativum* ssp. *sativum* convar. *medullare* and *medullo-saccharatum* (Lehmann 1954).

The phenotype "f" was found in 8 accessions of *P. sativum*.

ISOCITRATE DEHYDROGENASE (IDH)

Two single-banded phenotypes of IDH, designated "a" and "b", were found in the cotyledons of the investigated *Pisum* accessions (Fig. 4); the fast moving band characteristic of the phenotype "a" seemed to be less intense. As the distinguished phenotypes show alternatively occurring bands they are probably controlled by alleles at one locus.

Of the two IDH phenotypes, the phenotype "b" was found to be more commonly occurring. The phenotype "a" was found only in *P. fulvum*, in two *P. humile* accessions, in *P. elatius* Gat. 255, and in some individuals of *P. sativum* W 1917.

One of the main objectives of the investigation of isoenzyme variation in *Pisum*, carried out in this laboratory, is to identify biochemical markers to be used for identification and classification of genetic resources. As indicated in the introduction, the 108 *Pisum* forms used in this work have already been analysed for four other enzyme systems. Over 30 accessions were found to have unique multienzyme phenotypes, while the others could be separated into 22 groups, each group comprising from 2 up to 11 electrophoretically undistinguishable forms (Przybylska et al. 1982). Due to the presently reported variation in the ADH electrophoretic pattern of cotyledons of ungerminated seeds, 8 of the above mentioned 22 groups could be further subdivided. This resulted in some additional accessions with unique multienzyme phenotypes. Distinction of two IDH phenotypes did not alter the grouping of the *Pisum* forms reported in the previous paper (Przybylska et al. 1982).

As shown above, the ADH phenotypes distinguished in the cotyledons of the examined *Pisum* accessions significantly contribute to an overall isoenzyme varia-

tion detected so far in the genus. It is also to be reminded that embryonic axes were found to provide additional ADH markers. On the other side, some critical remarks concerning usefulness of the ADH system in seeds for the routine electrophoretic analysis of *Pisum* genetic resources should be made.

For many reasons cotyledons of mature seeds constitute a particularly suitable material for routine screening of isoenzyme variation. However, in the case of the ADH system different problems arise. Occurrence of artefacts on zymograms makes identification of ADH phenotypes difficult in the routine analysis. Also variation in band intensity creates problems in screening. The variation, reflecting most probably the variation in the total enzyme activity, may be due to both genetic and environmental factors including seed storage.

The use of embryonic axes for routine analyses should not be generally recommended as it involves seed destruction. Moreover, because of a low ADH activity of a single embryonic axis, screening for individual variation is not possible under the applied conditions of electrophoretic analysis.

In view of the above limitations, the ADH system in *Pisum* seeds does not seem advisable for the routine electrophoretic analysis.

Detailed data concerning distribution of ADH and IDH phenotypes will be published later, together with other data on isoenzyme variation.

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ZMIENNOŚĆ IZOENZYMÓW U RODZAJU *PISUM*
II. ELEKTROFORETYCZNE FENOTYPY DEHYDROGENAZY ALKOHOLOWEJ I DEHYDROGENAZY IZOCYTRYNIANOWEJ W LIŚCIENIACH DOJRZAŁYCH NASION

Streszczenie

Kontynuując badania polimorfizmu izoenzymów u *Pisum* przeprowadzono elektroforetyczną analizę dehydrogenazy alkoholowej (ADH) i dehydrogenazy izocytrynianowej (IDH) w liścieniach dojrzałych nasion. W obrębie badanego materiału, obejmującego 108 form *Pisum* reprezentujących różne jednostki taksonomiczne tego rodzaju, wyróżniono 7 elektroforetycznych fenotypów ADH i 2 fenotypy IDH. Uzyskane wyniki omawia się w nawiązaniu do klasyfikacji i identyfikacji zasobów genowych rodzaju *Pisum*.

ИЗМЕНЧИВОСТЬ ИЗОЭНЗИМОВ У РОДА *PISUM*
II. ЭЛЕКТРОФОРЕТИЧЕСКИЕ ФЕНОТИПЫ СПИРТОВОЙ И ЛИМОННОКИСЛОЙ ДЕГИДРОГЕНАЗ В СЕМЯДОЛЯХ ЗРЕЛЫХ СЕМЯН

Резюме

Продолжая исследования полиморфизма изоэнзимов у *Pisum* был проведён электрофоретический анализ спиртовой (ADH) и лимоннокислой (IDH) дегидрогеназ в семядолях зрелых семян. В пределах исследуемого материала, охватывающего 108 форм, представляющих собой различные таксономические единицы рода, было выделено 7 электрофоретических фенотипов ADH и 2 фенотипа IDH. Полученные результаты рассматриваются в их связи с классификацией и идентификацией генных ресурсов рода *Pisum*.