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PROTEINS AND ENZYMATIC SYSTEMS IN THREE VARIETIES OF *HELIANTHUS ANNUUS* L.

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ABSTRACT. The paper reports results of analysis of protein and eight enzymatic systems of three varieties of *Helianthus annuus* L. (Wielkopolski, Coril, and Frankasol) performed by three electrophoretic methods. Electrophoretic analyses allowed finding protein and isoenzymes markers for each of variety. The two interline hybrids Coril and Frankasol shared more features in common than the population variety Wielkopolski with each of the former. Mean values of the polymorphism index for the protein and the eight enzymes indicate that the variety Wielkopolski is more homogeneous than Coril and Frankasol.

Key words: electrophoresis, enzymes, isoelectrofocusing, proteins, sunflower, variability

Introduction

Along with rape, soya-bean and different varieties of oil palm, sunflower (*Helianthus annuus* L.) belongs to most often cultivated oil plants in the world. The species originates from southwestern USA. In Europe, mainly in Central Europe, it is a commonly grown oil plant. Till recently, in Poland only the population variety of sunflower Wielkopolski was cultivated. This variety gives relatively high yield in various environmental conditions (**Toboła** and **Muśnicki** 1997), however, it has been replaced by interline hybrids which bring even higher yield. The interline hybrids showed lower variation of the morphological features and chemical composition of achene than population variety (**Kluza** and **Muśnicki** 1998).

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Koranyi (1988) demonstrated that SDS-PAGE is a proper method for differentiation of lines and hybrids, although it is difficult to use on a large scale because of a large number of bands in the patterns e.g. fifty band. Analysis of 11S globulin (heliantin) permits differentiation the inbred lines, varieties and hybrids. In particular, polymorphism of heliantin brings the information on homogeneity and differences between sunflower varieties (**Anisimowa** et **al.** 1991). Analysis of fifty two lines of sunflower performed by electrophoretic separation of eight enzymatic systems in starch gels has proved that only forty five of these lines can be precisely identified (**Quillet** et **al.** 1992).

Genome structure investigation, including DNA polymorphism determination, has made it possible to assess genetic differentiation and characterise the plant studied. For example, for the sunflower, RAPD analysis of the Australian cross-sections based on a comparison of sixteen genotypes, has proved that from the total number of 158 markers 133 were polymorphous in pair-wise (**Lawson** et **al.** 1994).

The main aim of the study was to find proteins or isoenzymes characteristic of the three *Helianthus annuus* varieties studied, determination of their polymorphism and finally comparison these varieties. The study also tested the suitability of the methods for analysis of proteins including isoenzymes from seedling and young leaves.

Materials and methods

The study was performed on twenty individuals representing each of the three varieties of *Helianthus annuus.* One of them is the Polish population variety Wielkopolski bred by IHAR. The other two are interline hybrids: Coril, breed by Pioneer, the USA, and Frankasol, bred by a French firm Cargill. The seeds of Wielkopolski come from Borowiec IHAR station and other two from Pionier and Cargill Company.

The seeds were germinated on Petri dishes in dark and single 5-day old roots were collected and kept in –70°C. From each of variety twenty individuals were analysed. From each young plants two (2 cm long) leaves were collected and kept in –70°C.

Starch gel electrophoresis

The proteins were extracted from two young leaves, homogenised for 30 min at 4ºC with 120 µl 0.1 M TRIS-HCl buffer of pH 7.2 containing 1% dithiotreitol and 0.1% Triton X-100. After centrifugation, crude extracts were absorbed onto Whatman 3 MM (4 × 12 mm, 9 mg protein) and applied into starch gels. Starch gel electrophoresis was conducted in 12.5% gel, (Conaught) with 0.2 M lithium-borate of pH 8.3 as electrode buffer. The gel buffer was composed of nine parts of 0.05 M TRIS-citric acid of pH 8.3 and one part of the electrode buffer (**Greneche** et **al.** 1991). The slice of the gel was incubated in the stain solution for phosphoglucose isomerase – PGI (**Hayward** and **McAdam** 1977, **Lewis** et **al.** 1980).

Polyacrylamide gel electrophoresis (PAGE)

The same crude extracts as for starch gel electrophoresis were used for polyacrylamide gel electrophoresis (30 µ1 extract, containing 18 mg of proteins, was applied into the pocket of stacking gel). Electrophoresis was conducted in 10% polyacrylamide slabs (1 mm in thickness, 8 cm path of separation) under stable power of 10 mA/cm^2. The proteins in gels were stained with Comassie Brillant Blue G-250 and for diaphorase (DIA), shikimic dehydrogenase (SkDH) and malate dehydrogenase (MDH) activity.

Isoelectrofocusing (IEF)

The proteins from 5-day seedlings grown in sterile sand on Petri dishes were extracted for 30 min at 4ºC by 1% dithiotreitol in water (50 µl). The crude extract was absorbed onto Whatman 3 MM paper 4 × 4 mm (3 mg of the proteins) placed on polyacrylamide slab near the cathode. The isoelectric focusing (IEF) separations were performed in polyacrylamide gel slabs containing Servalyt pH 3-10 range (for esterases – EST) and of pH 3-7 (for peroxidase – PX, acid phosphatase – ACP, glucose-6-phosphate dehydrogenase – 6-PGD). The gel slabs of 0.4 mm thickness were photochemically polymerised under UV light in the presence of riboflavin as a catalyst. The separation was run over of 5-cm distance. The Whatman 3 MM electrode bridges were 15 mm wide stripes saturated with 0.5 M NaOH (cathode) and 0.5 M H_3PO_4 (anode) touching the cross-section of the gel slabs. The separation was conducted over 100 min with voltage increasing from 50 V to 300 V.

Protein concentration in crude extracts was determined according to **Bradford** (1976).

The bands on the gels were detected by laser densitometer UltroScan LKB and scanned using SHARP JX 330 scanner. Image master 2-D Elite program (Pharmacia Biotech) was using for gels analysis.

The polymorphism of the varieties was described by the frequency of bands corresponding to particular isoenzymes. On the basis of the frequencies of occurrence of all bands in the isoenzymes patterns corresponding to a given variety the polymorphism index (PI) was calculated (**Marshall** and **Jain** 1969). To compare the varieties of *H. annuus*, for general zymograms of each enzymatic system (taking into regard all bands, even those appearing in the pattern of single representatives of a given variety) the vales of similarity coefficients were calculated (**Sneath** and **Socal** 1973).

Results

Proteins. Thirty-nine bands patterns of proteins were obtained for the three varieties of *H. annuus* studied (Fig. 1). As follows from Table 1, the majority of them provided quantitative differentiation of the varieties. However, a more detailed analysis showed that bands no. 8 and no. 38 were found only in 50% and 6.3% individuals representing

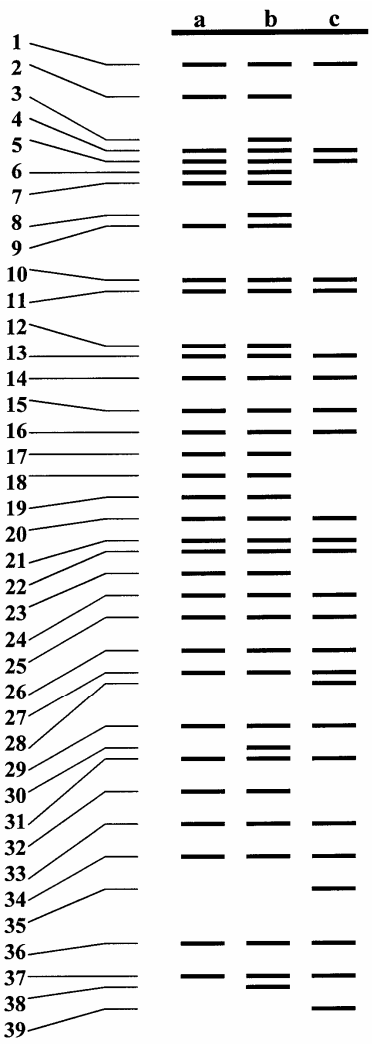

Fig. 1. Proteins from seeds of Wielkopolski (a), Coril (b) and Frankasol (c) varieties after poliacrylamide gel ectrophoresis

Ryc. 1. Białka nasion odmian Wielkopolski (a), Coril (b) i Frankasol (c) rozdzielonych metodą elektroforezy w żelu poliakryloamidowym

Coril, respectively, no. 28 was present in 31.3% and no. 35 in all individuals representing Frankasol. The three varieties were also differentiated by the lack of certain peptides, e.g. band 2 was absent in the patterns of Frankasol, while in those of Wielkopolski and Coril it was present in the patterns of all samples (Tab. 1). The varieties Wielkopolski and Coril shared the greatest number of common features (87% similarity), while the former and Frankasol – the lowest (56% similarity).

Taking into regard the total proteins, the most homogeneous proved Frankasol (PI = 0.029), less homogeneous was Wielkopolski (PI = 0.057), and the least Coril (PI = 0.075).

Phosphoglucose isomerase (PGI), separated in starch gel, showed the one- and three-band patterns corresponding to the genotypes "aa", "bb" and "ab" (locus 2). The most frequent genotype in Wielkopolski variety was "aa" found in 70% individuals, and heterozygotes "ab" were found in 30% individuals. From among the individuals of interline hybrid Coril, 15% were heterozygotes and the remaining ones were "aa" homozygotes. The majority (60%) of the Frankasol samples were "ab" heterozygotes (60%), 30% were "aa" homozygotes and the remain were "bb" homozygotes, which was specific of this variety.

Diaphorase (Dia). Ten isoenzymes of diaphorase were obtained (Fig. 2 a). Isoform no. 9 was detected only in Wielkopolski variety (65% individuals), and no. 2 only in Coril (80% individuals). The isoform no. 3 was not detected for Wielkopolski while it was present in the patterns obtained for other two varieties. Three isoenzymes labelled as 6, 8 and 10 were found in all individuals studied except one of the Wielkopolski variety. The qualitative comparison has shown that the diaphorase of Coril and Frankasol individuals differ by only one isoform (90% similarity), Wielkopolski and Coril by two (80%) and Wielkopolski and Frankasol by three (70%). The other differences were quantitative (Tab. 2). The least variable was the population variety of Wielkopolski (PI = 0.078), while the two interline hybrids Coril and Frankasol were polimorphic in the same degree (PI = 0.116).

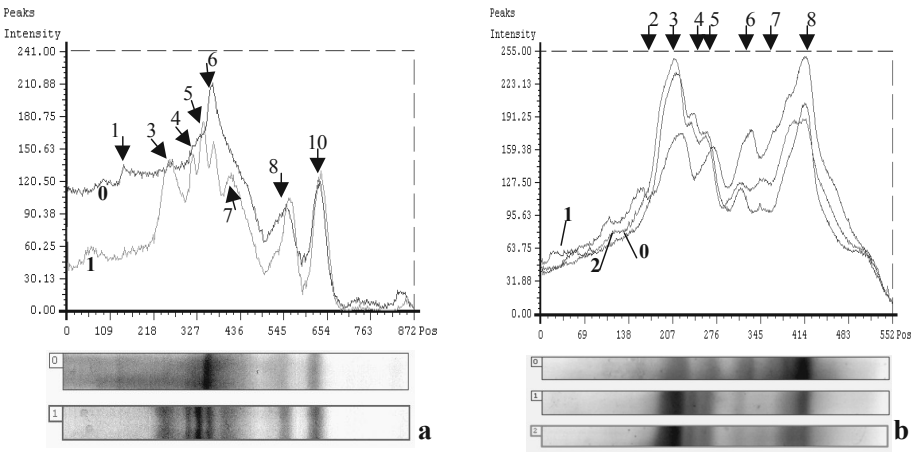

Fig. 2. Diaphorase from leaves of two individuals of Coril variety after separation in poliacrylamide gel (a) and peroxydases from leaves of two individuals of Wielkopolski variety after separation by isoelectrofocusing (b) as an examples of densitometry analysis of isoenzymes

Ryc. 2. Przykłady densytometrycznej analizy rozdziałów diaforazy u dwóch osobników odmiany Coril po rozdziale w żelu poliakryloamidowym (a) oraz peroksydaz rozdzielonych metodą izoelektroogniskowania dla dwóch osobników odmiany Wielkopolski (b)

Malate dehydrogenase (MDH) gave an **e**ight-band pattern (Tab. 2). Two of them labelled as no. 1 and 2, were observed in all individuals of Coril, and in 80% and 70% respectively of the Frankasol. In Wielkopolski variety these isoform and that no. 7 were not detected. No. 7 occurred in all samples of Frankasol and in 35% of Coril individuals. The fastest electrophoretic mobility isoform no. 8 was found in 55% of Frankasol individuals only. The qualitative comparison revealed 88% similarity between Coril and Frankasol (one differ isoform), 63% similarity between Wielkopolski and Coril (three differ isoforms) and 50% similarity between Wielkopolski and Frankasol (four differ isoforms).

As far as diaphorase is concerned, the least variable proved Wielkopolski with PI = 0.037 and the most Frankasol with PI = 0.157, while for Coril PI = 0.060.

Shikimic dehydrogenase (SkDH). Coril proved monomorphous with respect to the three SkDH isoenzymes. The population variety Wielkopolski was more polymorphic (PI = 0.085), since isoenzyme no. 1 was found in all its samples and no. 2 and no. 3 in seventeen of them. Frankasol was the most polymorphic (PI = 0.159). All individuals showed no. 2 which always appeared with no. 3.

Esterases (Est). In the three varieties studied, thirteen different isoenzymes of esterases were detected, of which eleven were separated at the pH range 4-5 and two at pH 9.1 and 9.5 (no. 1 and 2). In most cases the isoenzymes detected occurred at different frequencies in the three varieties or a given isoenzyme was observed in two of the three varieties. No. 6 was observed in all individuals of the three varieties. No. 7 was detected in the patterns of all individuals of Wielkopolski only. No. 12 was found in

five samples for Frankasol, whereas for Wielkopolski and Coril it was present in the patterns of all

Table 1

Frequencies of proteins from twenty individuals of three sunflower varieties (%). Bands were numbered from cathode to anode
Częstości białek dla dwudziestu osobników trzech odmian słonecznika (%). Prążki numerowano od katody do anody

Number of bands Numer prążka	Wielkopolski	Coril	Frankasol
1	81.3	100	100
2	100	100	0
3	0	6.3	0
4	50	100	18.8
5	50	100	18.8
6	12.5	37.5	0
7	25	43.8	0
8	0	50	0
9	31.2	31.2	0
10	100	75	100
11	100	93.4	100
12	87.5	50	0
13	87.5	100	100
14	50	100	0
15	100	100	100
16	31.2	68.8	0
17	100	100	100
18	43.8	43.8	0
19	100	100	0
20	100	100	100
21	100	100	100
22	25	68.8	50
23	100	18.8	0
24	100	100	100
25	100	100	100
26	100	100	87.5
27	100	100	31.3
28	0	0	31.3
29	100	100	100
30	0	56.3	0
31	100	100	100
32	81.3	100	0
33	100	100	100
34	100	100	100
35	0	0	100
36	100	50	100
37	100	100	100

38	0	6.3	0
39	0	100	100

individuals (Tab. 2). The isoenzyme assigned as no. 9 was present in the patterns the Wielkopolski and no. 13 in those of Coril variety only. A distinguishing feature of Frankasol representatives was the absence of isoforms 1, 4, 9 and 13 in their patterns.

Taking into account all esterase isoenzymes, disregarding their frequencies, the similarity between Frankasol and Coril can be assessed as 77%, while the similarity of Wielkopolski to each of the two former – as 69%. The greatest polymorphism was found for Wielkopolski (PI = 0.126), while the least for Frankasol (PI = 0.053) and the intermediate for Coril (PI = 0.092).

Peroxydases (PX). The patterns revealed the presence of eight isoenzymes whose isoelectric points were in the pH range from 3.9 to 4.7 (Fig. 2 b). No. 1 was present only in 60% individuals of Coril, while no. 3 only in 55% individuals of Wielkopolski. Isoform 2, 4 and 7 were present in all sixty individuals of the three varieties and the other isoforms were detected at different frequencies (Tab. 2). The polymorphism of the three varieties studied was similar, and the PI values determined for them were Wielkopolski – 0.093, Coril – 0.099 and Frankasol – 0,083.

As follows from a qualitative comparison the value of the similarity coefficient obtained from synthetic electrophoregram the varieties Wielkopolski and Coril was 75%, while for Wielkopolski and Frankasol as well as Coril and Frankasol it was 88%.

Acid phosphatase (ACP). After separation by isoelectrofocusing method**,** ten ACP isoenzymes with isoelectric points in the pH range from 3.6 to 4.8 were stained. As follows from Table 2, two of them, corresponding to bands no 1, 2 were observed in single individuals representing Wielkopolski and Coril. Three others corresponding to no 5, 7 and 8 were detected in all individuals of Coril and Frankasol, whereas only no. 5 was observed in all samples of Wielkopolski and the other two in the majority of Wielkopolski. Isoform no. 3 can be assumed as characteristic of Frankasol, although it occurred only in 30% of its individuals (no. 9 only in Coril – 5% of individuals). According to the values of the similarity coefficient, the variety Wielkopolski was similar to each of the other two in 90%, while the other two were similar in 80%. The polymorphic index was similar or somewhat lower than in the case of the other enzymatic systems: Wielkopolski – PI = 0.065, Coril – PI = 0.085, Frankasol – PI = 0.074.

Glucose-6-phosphate dehydrogenase (6-PGD). The three 6-PGD isoforms were detected in the narrow pH range 4.5, 4.7 and 4.9 (Tab. 2). The differences among the varieties studied were exclusively quantitative. Band no. 3 occurred in the patterns of all samples, except one individual of the variety Wielkopolski. No. 2 was absent in six samples of Coril individuals, and no. 1 was the least frequent in all three varieties. The greatest polymorphism was observed in Coril – PI = 0.150, then in Wielkopolski – PI = 0.104, and Frankasol – PI = 0.075.

Taking into account the results obtained for all the enzymatic systems, the greatest number of common isoenzymes shared the interline hybrids, which were found similar in 87%, and the population variation of Wielkopolski was similar to each of them in 83%. The most polymorphic was Frankasol (mean PI = 0.104), while for the two other varieties the mean PI value was the same: PI = 0.085.

Table 2

Frequencies of isoforms of six enzymes from twenty individuals of three sunflower varieties (%). Isoenzymes were numbered from cathode to anode
Częstości izoform sześciu układów enzymatycznych dla dwudziestu osobników trzech odmian słonecznika (%). Izoenzymy numerowano od katody do anody

Enzyme systems System enzymatyczny	Number of band Numer prążka	Wielkopolski	Coril	Frankasol
1	2	3	4	5
Est	1	25	25	0
	2	35	45	15
	3	35	60	100
	4	25	50	0
	5	15	65	15
	6	100	100	100
	7	0	100	100
	8	75	100	100
	9	30	0	0
	10	20	0	70
	11	15	100	100
	12	100	100	65
	13	0	5	0
PX	1	0	60	0
	2	100	100	100
	3	55	0	0
	4	100	100	100
	5	100	20	50
	6	55	30	25
	7	100	100	100
	8	45	25	35
ACP	1	5	5	0
	2	5	5	0
	3	0	0	30
	4	15	45	45
	5	100	100	100
	6	15	45	90
	7	95	100	100
	8	90	100	100
	9	0	5	0
	10	20	30	25
6-PGD	1	35	40	65
	2	100	70	100
	3	95	100	100

Table 2 – cd.

1	2	3	4	5
Dia	1	95	90	55
	2	0	80	0
	3	0	25	40
	4	90	50	35
	5	85	55	30
	6	95	100	100
	7	60	35	60
	8	100	100	100
	9	65	0	0
	10	100	100	100
MDH	1	0	100	80
	2	0	100	70
	3	100	65	70
	4	100	100	25
	5	45	15	60
	6	100	100	100
	7	0	35	100
	8	0	0	55

Discussion

The three varieties studied are stabilised cultivated forms, they were characterised by relatively great polymorphism. The population varieties are considered more polymorphous than the interline hybrids (**Kluza** and **Muśnicki** 1998). After protein analysis the variety of Wielkopolski was characterised by lower polymorphism, but the differences between the mean values of PI seem insignificant (the significance of these differences was not tested). For the natural populations, such as e.g. *Anthyllis vulneraria* L. the values of PI were high (**Kalinowski** and **Bartkowiak** 1979, **Kalinowski** et **al.** 1979). The synthetic cultivated forms, such as the interline hybrids studied, are subjected to artificial selection which reducing variability. Increased polymorphism may be a result of the fact that *H. annus* is both open pollinated and self-pollinated species.

Starch gel electrophoresis is the most popular method allowing genetic interpretation of data but it has insufficient discriminative value (**Quillet** et **al.** 1992). An important finding this method provided in our study was that the "bb" homozygotes **PGI-**2 occurred only in Frankasol. Electrophoresis in polyacrylamide slabs (SDS-PAGE), proved successful in differentiating the varieties (**Koranyi** 1988). The separations in the polyacrylamide slabs in non denaturing conditions (PAGE), revealing both qualitative and quantitative differences between three sunflower varieties. Analysis of general proteins for Coril and Frankasol revealed two bands characteristic of each of them. One isophorm of **Dia** was characteristic of Coril and another one of Frankasol. The PAGE

method applied for analysis of MDH led to identification of only one band characteristic of the Frankasol variety and no specific isoforms of SkDH. The third method used was isoelectrofocusing, considered as providing the best resolution, but on condition that apart from proteins the enzymes, which remain active having reached the isoelectric point, are also analysed. (i.e. **Höfelman** et **al.** 1983, **Kalinowski** et **al.** 1982). As shown above, analysis of esterases, peroxidase and acid phosphatase by this method very well differentiates the three varieties of *H. annuus*. Analysis of Est, PX and ACP allowed finding of two isoforms for each of them and no one for 6-PGD. As evidenced by the above results, the three electrophoretic methods allowed easy characterisation of the sunflower varieties studied.

One-dimensional electrophoretic methods are useful for determination of variability in a variety but very often do not allow finding markers differentiating varieties or lines of plants. Electrophoretic patterns of albumins and globulins separated in poliacrylamide gels (SDS-PAGE), proved insufficient for differentiation of cultivated varieties of sunflower (**Canella** et **al.** 1982). A reliable method of precise differentiation of forms is the two-dimensional electrophoresis in polyacrylamide gels (**Thiellement** et **al.** 1999, **Grög** et **al.** 1992, **Picard** et **al.** 1997). As presented above, the results obtained by the electrophoretic methods allowed identification of proteins and enzymatic markers of the three sunflower varieties. The methods proved reliable in differentiation of the three taxa. The values of similarity coefficients for particular enzymes and total proteins obtained for the three varieties were much different. It seems that only analysis of their mean values permits better interpretation of affinity between analysing varieties.

The material subjected to analysis were the roots of seedlings and young leaves. Attempts at performing protein and enzyme separation from seeds failed, despite a great content of fats in them. The best patterns after electrofocusing were obtained when proteins were extracted from the roots, however, the volume of extracts from individual plants was too small to perform separations both in polyacrylamide slabs and starch gels. Young leaves were proper materials for analysing several enzymes from single plants.

Conclusions

The three sunflower varieties, being stabilised cultivated forms are relatively highly polymorphic because the sunflower is both open- and self-pollinated. Most of differences between varieties were quantitative but six enzymes allow a detection of specific isoenzymatic markers. One-dimensional electrophoretic methods can be successfully used for identification and genetic purity testing of sunflower varieties. We suppose that in case a greater number of varieties is analysed, identification of the protein markers would not be so easy. The results concerning the polymorphism of the *H. annus* varieties studied allow us to conclude that 20 individuals are the minimum number needed to analyse variability of a variety. A greater number of samples representing each variety simply increases the probability of detecting rarely occurring isoenzymes.

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BIAŁKA I UKŁADY ENZYMATYCZNE U TRZECH ODMIAN *HELIANTHUS ANNUUS* L.

S t r e s z c z e n i e

W pracy przedstawiono wyniki analiz białek i ośmiu układów enzymatycznych, rozdzielanych trzema metodami elektroforezy, u trzech odmian *Helianthus annuus* L. (Wielkopolski, Coril i Frankasol). Elektroforetyczne analizy umożliwiły znalezienie białkowych i izoenzymatycznych markerów dla trzech odmian. U dwóch odmian międzyliniowych mieszańców Coril i Frankasol wykryto więcej cech wspólnych niż u wymienionych odmian i odmiany Wielkopolski. Średnie wartości indeksu polimorfizmu, obliczone dla białek i ośmiu układów enzymatycznych, pokazały, że odmiana Wielkopolski była bardziej wyrównana niż odmiany Coril i Frankasol.