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METHODOLOGICAL APPROACHES TO SIMPLE ENZYME POLYMORPHISM ANALYSES OF AMARANTH SPECIES (*AMARANTHUS* SP.)

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Amaranths have drawn a great interest in last years as an agronomic crop, alternative energy source and ornamental plants around the world. Several of them are cultivated as the leaf vegetables, cereals, or as the colourful, quick growing garden plants. Increasing demand for the breeding and proteomic study in *Amaranthus* species led us to test some methodological approaches to enzyme polymorphism analysis. For these experiments genotypes of *Amaranthus cruentus* L. (genotype Ficha) and mutant line of hybrid K-433 were selected, characterized by a good seed quality and quantity, suitable for food production. The attention was devoted to test of published methodology, which were slightly modified for enzyme (ACP, ADH, CAT, DIA, GOT, IDH, MDH, PGD, PGI and PGM) multiplicity analysis for amaranths and testing of feasibility of chosen analysed organ weights, dimensions of Whatman No. 2 wicks and different volumes of extract buffer. Results from enzyme multiplicity analysis are presented by means of photographed fingerprints and phenotypes are expressed in diagrams where positions of zymograme bands are marked by using factors of relative mobility (Rm).

Key words: *Amaranthus* sp., isoenzymes, horizontal starch gel electrophoresis, methodology, molecular markers, isozymograms

INTRODUCTION

In 2009 lot of scientists and researchers remember fifty years anniversary of the first coining the term "isozymes" to describe, by Markert and Moller (1959), the different molecular forms of an enzyme in a species that share a common catalytic activity. During the last five decades much work was done concerning enzyme polymorphism or plant enzyme multiplicity. Most of the scientific works was focused on identification of plant enzyme polymorphism diversity for purposes of population genetics, selection and breeding. In this manner the plant species of high economical importance, such as maize (*Zea mays* L.), soybeen (*Glycine max*. [L.] Merr.), sunflower (*Helianthus annuus* L.), pea (*Pisum sativum* L.) etc., were intensively studied. This effort led to the creation of standardized analytical methodologies (Cardy et al. 1980; Stuber et al. 1988; Greneche & Giraud 1989; Bourgoin-Greneche & Lallemand 1993), which enabled unification and cooperation in analyzing and genetic interpretation of enzyme polymorphism by research and testing laboratories in many countries. In spite of the considerable progress in enzyme polymorphism analysis in above mentioned species, amaranths belong to plant species which have not been so extensively studied. It is perhaps due to lover economic importance of this minor and underutilised crop or low allozymic variation among amaranth populations documented by research. Jain et al. (1980) presented a summary of var-

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iation studies based on morphological and allozyme markers and reported the marked contrast between the high level of morphological polymorphism and almost monomorphic allozyme loci. The low allozyme variation in total 52 amaranth populations and two cultivars were described by Yudina et al. (2005). The more accurate estimates of genetic diversity and of relationships within and among crop species and wild relatives of amaranth were generated by means of complementary approach using information from both isozymes and RAPD analyses (Chan & Sun 1997). On the contrary, a high degree of polymorphism was found within and between populations of amaranth even in neighbouring populations, as characterized by different alleles or isoenzymes (Jacobsen & Mujica 2003).

Amaranths have drawn a great interest in the last years as an agronomic crop, alternative energy source and ornamental plants around the world. Several of them are cultivated as the leaf vegetables, pseudocereals, or as the colourful, quick growing garden plants. Importance of the breeding work on grain amaranth by inducing genetic variability through mutagenesis has been suggested by Gajdošová et al. (2008). Since 1985, the work on grain amaranth cultivars has been conducted in Ukraine. The study of genetic diversity of amaranth cultivars of the Ukrainian selections started since 2008 using the isoenzyme systems (Goptsiy et al. 2008).

Increased need for growing and breeding of the amaranth cultivars and tendency to utilize induced mutagenesis for increasing genetic variability of breeding material led us to test methodological approaches for simple enzyme polymorphism analyses of amaranth species (*Amaranthus* sp.). The main objective of the present study was to devote attention to testing the methodology published by Stuber et al. (1988), slightly modified for enzyme multiplicity analysis for amaranths and to testing feasibility of chosen analysed organ weights, dimensions of Whatman No. 2 wicks and different volumes of extract buffer.

MATERIAL AND METHODS

Seed sources and sample population. Genotypes of *Amaranthus cruentus* L. (genotype Ficha) and mutant line of hybrid K-433 (D 279/1) were selected for these experiments, characterized by a good seed quality and quantity, suitable for food production. Both seed samples were obtained from the collection of Gene Bank of the Research Institute of Plant Production Praha-Ruzyne, Czech Republic. *Amaranthus cruentus* L. genotype Ficha is a medium early cultivar with big seeds (WTS 0.85 g), light in colour. *Amaranthus* hybrid K-433 is a plant breeding material, product of interspecific hybridization (*A. hypochondriacus* L. × *A. hybridus* L.) originated from Rodalo Research Center in Pennsylvania, USA. It is medium early genotype, with light-coloured seeds (WTS 0.73 g).

In the first experiment, the dry seed samples of *Amaranthus cruentus* L. and hybrid K-433 in different weight 10, 50, 100, 150, 200 mg (that is 13, 65, 130, 195, 235 and 14, 70, 135, 210, 270 seeds, respectively), as well as three dimensions of Whatman No. 2 wicks (11 × 1.5, 11 × 2.0 and 11 × 3.0 mm) were tested. In the second experiment, the samples of seedlings after one, three and six days of germination in the same weights as seed samples have been used. The dimension of Whatman No. 2 wicks was uniform in the second experiment – 11 × 1.5 mm. Seed germination and growth of seedlings took place in thermostat on wet filter paper enriched by redistilled water in Petri dishes under dark at 25°C and 98% RH. For comparing mobility of zones of enzyme activities of amaranth with mobility of zones of maize coleoptile section enzyme activities, extract from coleoptile section after five days cultivation of maize single-cross hybrid grain (Sc 3098 × 3150, Sempol Holding Inc., Trnava, Slovak Republic) under the same cultivation conditions have been used.

Electrophoresis. We employed one type of electrophoresis, the standard technique (for coleoptile section of maize) of horizontal starch gel electrophoresis according to Stuber et al. (1988) and recently published by Múdry and Juráček (2001). This method was used for analysis of amaranth enzyme polymorphism in acid phosphatase (ACP, E.C. 3.1.3.2), alcohol dehydrogenase (ADH, E.C. 1.1.1.1), catalase (CAT, E.C. 1.11.1.6), diaphorase (DIA, E.C. 1.6.99.), ß-glucosidase (GLU, E.C. 3.2.1.21), glutamateoxaloacetate transaminase (GOT, E.C. 2.6.1.1), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), malate dehydrogenase (MDH, E.C. 1.1.1.37), 6-phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), phosphoglucoisomerase (PGI, E.C. 5.3.1.9), and phosphoglucomutase (PGM, E.C. 2.7.5.1). Extracts were done from maize coleoptile

sections, dry seeds and seedlings of amaranth by hand crashing of samples using glass stick in mortar on ice with addition of several grains of pure sand (only for dry seeds of amaranth). Extraction solution contained 5 ml of water, 0.84 g of sucrose and 0.42 g of sodium ascorbate. Doses of the extraction solution were applied as follows: 20 µl/coleoptile section eleven mm long of maize; 10/10, 50/50, 100/100, 100/150 and 100/200 (µl mg^{-1}) at dry seeds or seedlings of amaranth. Starch gels contained 77.31 g of hydrolyzed potato starch for electrophoresis (Starch Art Corporation, Smithville, USA), 15 g of sucrose and 600 ml of gel buffer. The compositions of buffers are shown in Table 1. The extracts were inserted into gels by means of paper wicks (Whatman No. 2) approximately 3 cm from the cathodic end. An electrophoretic separation was running in refrigerator at 4°C.

After finishing of separation the gels were cut horizontally into several thin slices (appr. 1.2 mm thick) and placed into boxes for staining of zones of enzymatic activity in buffer systems. The compositions of buffer solutions for preparation of staining solutions are shown in Table 2.

Composition of staining media for individual enzymes:

ACP: 50 ml 0.1 mol dm^{-3} sodium acetate-acetic acid buffer (pH 5.0), 50 mg Fast Garnet GBC Salt, 50 mg $MgCl_2$, 50 mg α-naphtyl acid phosphate (sodium salt).

ADH: 50 ml 0.05 mol dm^{-3} Tris-HCl buffer (pH 8.0), 1 ml 95% ethanol, 20 mg ß-nicotinamide adenine dinucleotide, 20 mg MTT, tetrazolium thiazolyl blue, 5 mg phenazine methosulphate.

CAT: 500 mg pottasium ferricyanide, 500 mg ferric chloride, 50 ml H_2O, 0.01 % H_2O_2.

DIA: 50 ml 0.01 mol dm^{-3} Tris-HCl buffer (pH 9.1), 500 mg polyvinylpyrrolidone 40, 4 mg 2,6-dichlorophenol indophenol, 5 mg ß-nicotinamide adenine dinucleoitide reduced, 40 mg tetrazolium thiazolyl blue.

GLU: Solution 1: 50 ml 0.05 mol dm^{-3} potassium phosphate buffer (pH 6.5), 1 g polyvinylpyrrolidone 40, 100 mg Fast blue BB salt. Solution 2: 50 mg 6-bromo-2-naphthyl-ß-D-glucoside in 5 ml N,N-dimethyl formamide.

GOT: 50 ml deionized water, 50 mg Fast blue BB salt, 18.25 mg α-ketoglutaric acid, 66.5 mg L-as-

T a b l e 1

Electrode and gel buffers formulae (Stuber et al. 1988)

System	Electrode buffer	Gel buffer
B pH 5.7	0.065 mol dm^{-3} L-histidine, 0.02 mol dm^{-3} citric acid.H_2O, pH adjusted with citric acid	0.009 mol dm^{-3} L-histidine 0.003 mol dm^{-3} citric acid.H_2O (1:6 dilution of electrode buffer)
C pH 8.3	0.19 mol dm^{-3} boric acid, 0.04 mol dm^{-3} lithium hydroxide, pH adjusted with LiOH	9 parts Tris-citric acid buffer (0.05 mol dm^{-3} Trizma base, 0.007 mol dm^{-3} citric acid.H_2O, pH 8.3) + 1 part Electrode C buffer
D pH 6.5	0.065 mol dm^{-3} L-histidine, 0.007 mol dm^{-3} citric acid.H_2O, pH adjusted with citric acid	0.16 mol dm^{-3} L-histidine 0.002 mol dm^{-3} citric acid.H_2O (1:3 dilution of electrode buffer)
F pH 7.0	0.135 mol dm^{-3} Trizma Base, 0.04 mol dm^{-3} citric acid.H_2O, pH adjusted with citric acid	0.009 mol dm^{-3} Trizma Base 0.003 mol dm^{-3} citric acid.H_2O (1:14 dilution of electrode buffer)

T a b l e 2

Staining buffers, gel system, power and time (Stuber et al. 1988)

Enzyme	Buffer pH	Components	
ADH, DIA, IDH, PGI/6-PGD	8.0	0.05 mol l^{-1} Trizma base, titrate to pH 8.0 with HCl	
ACP	5.0	0.1 mol l^{-1} Sodium acetate.3 H_2O, titrate to pH 5.0 with 1 N HCl	
CAT	–	–	
GLU	6.5	0.05 mol l^{-1} potassium phosphate (mono), titrate to pH 6.5 with 5 N NaOH	
PGM	8.5	0.1 mol l^{-1} Trizma base, titrate to pH 8.5 with HCl	
MDH	9.1	0.1 mol l^{-1} Trizma base, titrate to pH 9.1 with HCl	
System	Power (Watts)	Time (hours)	System is optimal for:
B	17.0	7.25	ACP, GLU, MDH
C	12.0	6.0	ADH, GOT
D	16.0	6.5	CAT, IDH, PGM, 6-PGD, PHI
F	15.0	6.5	DIA

partic acid, 250 mg polyvinylpyrrolidone 40, 25 mg ethylenediamine tetraacetic acid, disodium salt, 0.71 g Na_2HPO_4.

IDH: 50 ml 0.05 mol dm^{-3} Tris-HCl buffer (pH 8.0), 50 mg $MgCl_2$, 150 mg DL-isocitric acid, trisodium salt, 5 mg ß-nicotinamide adenine dinucleotide phosphate, sodium salt, 5 mg tetrazolium thiazolyl blue, 1 mg phenazine methosulphate.

MDH: 50 ml 0.1 mol dm^{-3} Tris-HCl buffer (pH 9.1), 100 mg DL-malic acid, neutralized, 20 mg ß-nicotinamide adenine dinucleotide reduced, 10 mg nitro blue tetrazolium, 1.25 mg phenazine methosulphate.

PGD: 50 ml 0.05 mol dm^{-3} Tris-HCl buffer (pH 8.0), 20 mg 6-phosphogluconic acid, trisodium salt, 50 mg $MgCl_2$, 5 mg ß-nicotinamide adenine dinucleotide phosphate, sodium salt, 5 mg tetrazolium thiazolyl blue, 1.5 mg phenazine methosulphate.

PGI: 50 ml 0.05 mol dm^{-3} Tris-HCl buffer (pH 8.0), 50 mg D-fructose-6-phosphate, trisodium salt, 50 mg $MgCl_2$, 5 mg ß-nicotinamide adenine dinucleotide phosphate, sodium salt, 5 mg tetrazolium thiazolyl blue, 1.5 mg phenazine methosulphate, 10 units of NADP-dependent glucose-6-phosphate dehydrogenase.

PGM: 50 ml 0.1 mol dm^{-3} Tris-HCl buffer (pH 8.5), 100 mg $MgCl_2$, 250 mg α-D-glucose-1-phosphate, disodium salt, 10 mg ß-nicotinamide adenine dinucleotide phosphate, sodium salt, 7.5 mg tetrazolium thiazolyl blue, 1 mg phenazine methosulphate, 37.5 units of NADP-dependent glucose-6-phosphate dehydrogenase.

Alleles at analysed maize loci are classified according to their migration distances. For MDH loci, larger numbers correspond to greater anodal migration. For other enzymes larger numbers correspond to slower migration. Mmm is a modifier locus, the alleles of which affect the migration of certain MDH bands (Goodman et al. 1980; Newton & Schwartz 1980). *Glu1: 6–7* means that enzymes encoded by the alleles 6 and 7 co-migrated (Bourgoin-Greneche et al. 1998). The zones of isoenzymes activity in isozymograms (fingerprints) of amaranths have been photographed, expressed by using factors of relative mobility (Rm) and diagram constructions.

RESULTS AND DISCUSSION

The main role for successful analysis and correct interpretation of enzyme polymorphism plays complete fingerprints clearly distinguishing each of their bands or spots. There exist many factors affecting the readability of isozymograms, such as cultivation through homogenization and extraction of biological material (chosen individual seed or seedling as sample, seed or seedling mixture, part of plant, organ or tissue, ontogenetic stage of biological material, type of extraction buffer), starch gel preparing (percentage of hydrolyzed starch and cooking temperature of gel, gel buffer system), immersion of wicks soaked by extract (Whatman No. 2 or 3 and wick dimensions), electrophoresis (constant voltage, power or current, time in hours), slicing of gel (thickness of gel plate) and gel

staining (staining buffers and composition of staining solutions). From this point of view, the most important factor seems to be enzyme activity in analysed extract volume.

Because of absence of the universal methodology for enzyme polymorphism analysis for the majority of plant species, there can be some problems when comparing results obtained in different laboratories. According to our and other laboratories experimental experiences, slightly modified methodology of horizontal starch gel electrophoresis published by Stuber et al. (1988) and Bourgoin-Greneche et al. (1998) for coleoptile section of maize seedling can be successfully utilised for study of enzyme multiplicity in pea, soybeen, grass pea, chickpea seeds and cotyledons of sunflower (Bourgoin-Greneche & Lallemand 1993; Múdry & Juráček 1998, 1999a,b; Múdry et al. 1998), coleoptiles, leaves and roots of maize seedlings (Múdry & Dragúň 2007), leaf and root tissues regenerated from anther culture of maize (Uváčková et al. 2008) or leaf tissues of sugar beet (Engelhardt & Múdry 1999; Engelhardt & Bežo 2000). The results on influence of some modifications of experimental conditions on amaranth isozymograms and convenience of methodology published by Stuber et al. (1988) for enzyme polymorphism analysis of amaranths are presented by the electrophoretic phenotypes (Fig. 1–7), a scheme of isoenzyme patterns observed for all group of eleven analyzed enzymes (Fig. 8) and by Table 3 and 4 summarizing effects of studied factors on the electrophoretic phenotypes.

The eleven enzymes which were chosen for experimental work belong to the most frequently studied enzymes with plant genotyping in genetics, breeding and seed improvement.

Acid phosphatase fingerprints of amaranths are shown in Figure 1. No differences in enzyme multiplicity between these two species were detected. Dimeric structure of ACP (one or three banded fingerprints) was confirmed for seedlings of amaranths with two true leaves fully expanded Jain et al. (1980) and Hauptli and Jain (1984). However, our results supported seven to eight electrophoretic patterns of isoenzymes, similarly as it was found in shoot apices of *Amaranthus viridis* L. published by Sawhney et al. (1981). A scheme of ACP isoenzyme pattern observed for amaranth species is expressed in Figure 8.

Alcohol dehydrogenase – our fingerprints and previously published works (Jain et al. 1980; Hauptli & Jain 1984) confirmed the low isoenzyme variability for amarants. ADH synthesis in amaranth is controlled by a single *Adh* locus with two alleles *Adh* F (fast) and *Adh* S (slow). Fast variant occurred frequently in population of various amaranth species, whereas slow variant was rare (Yudina et al. 2005, 2008). Alcohol dehydrogenase isoenzymes of amaranths have dimeric structure. A monomorphic fingerprint and a scheme of ADH isoenzyme pattern observed for our amaranth species are expressed in Figure 2 and 8.

Catalase and **ß-glucosidase** – while CAT and GLU isozymograms of control sample were complete, isozymograms of amaranth CAT were weak, smudged and isozymograms of amaranth GLU absented for majority of the samples (Table 3, 4; Fig. 8). We have no evidence of amaranth CAT and GLU isozymograms from the literature but in most plants there are one, two or three banded fingerprints for CAT (Jebara et al. 2010; Magbanua et al. 2007; Mangal & Sharma 2002).

Diaphorase – polymorphism of amaranthus diaforase belongs to poorer among studied enzymes. Two loci *DIA – Dia1* (three alleles) and *Dia2* (five alleles) have mentioned Pratt and Clark (2001) for *Amaranthus rudis* J. D. Sauer and *Amaranthus tuberculatus* (Moq.) J. D. Sauer. Our methodological analysis showed unclear two and five banded fingerprints. Because of low isoenzyme activity of *DIA* and dark background of gel plate further methodological study is needed (Fig. 8).

Glutamateoxaloacetate transaminase (Aspartate aminotransferase AAT) – the phenotype of enzyme multiplicity of GOT for all samples of amaranths we have analysed was monomorphic, one of two banded (Fig. 8). The bands (spots) in GOT isozymograms were weak and hence are not presented as phenotypes in this work. According to published phenotypes (one-, two- and five-banded phenotypes) for GOT polymorphism of amaranths, there are present one or two loci responsible for their expression (Jain et al. 1980; Hauptli & Jain 1984; Warwick & Black 1986; Kirkpatrick 1995; Pratt & Clark 2001).

Isocitrate dehydrogenase polymorphism in all our analysed samples of amaranths was monomorphic with one of the two-banded phenotypes (Fig. 3 and 8). Null-, one-, two- and three-banded phenotypes for amaranth were published by Warwick and Black (1986), Kirkpatrick (1995) and Yudina et al. (2005, 2008).

Malate dehydrogenase – Hauptli and Jain (1984)

were the first who studied MDH in amaranth. They have reported that quaternary structure of MDH is a monomer controlled by a single locus with three alleles. Yudina et al. (2005, 2008) had suggested that

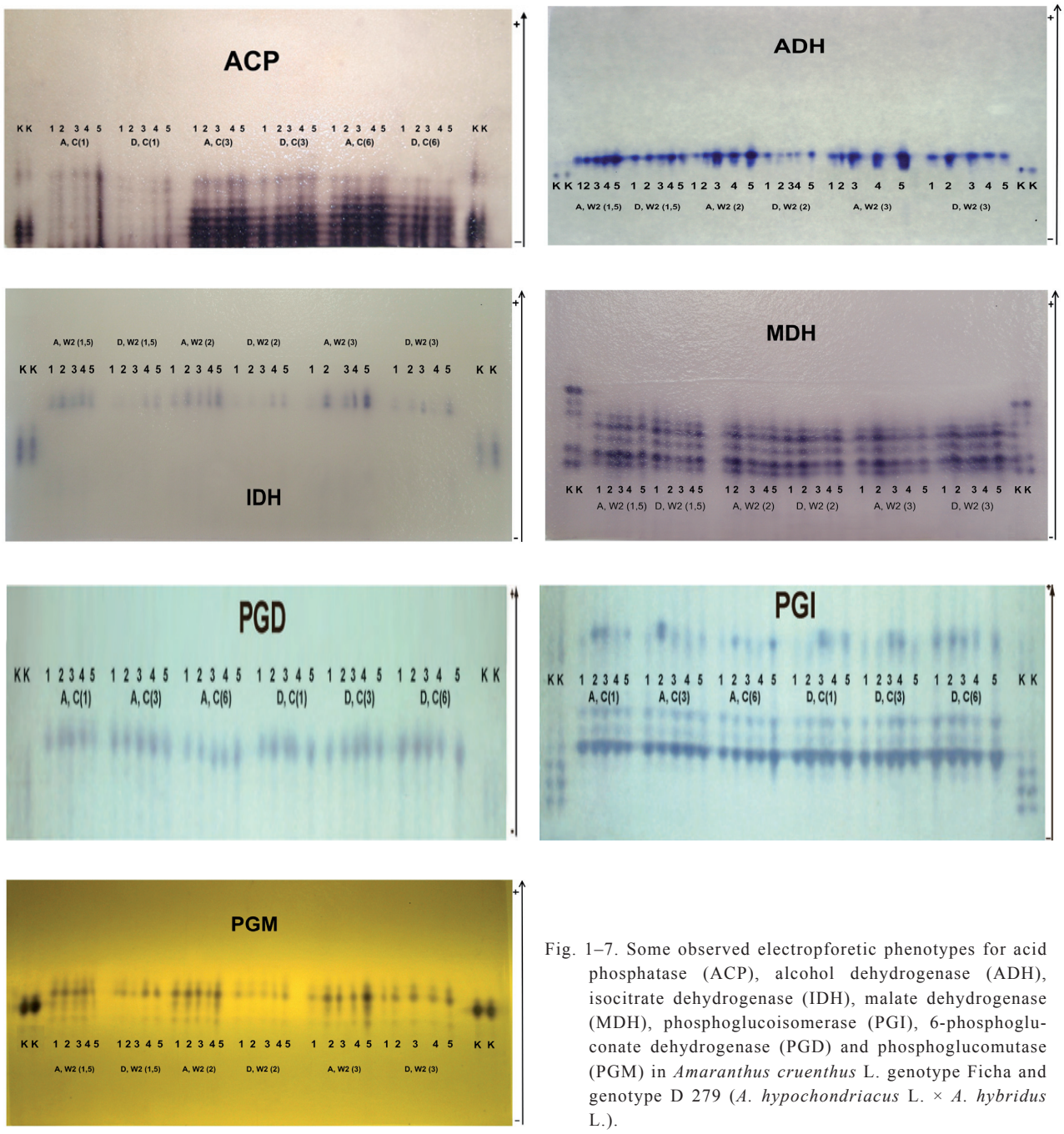

Fig. 1–7. Some observed electropforetic phenotypes for acid phosphatase (ACP), alcohol dehydrogenase (ADH), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), 6-phosphogluconate dehydrogenase (PGD) and phosphoglucomutase (PGM) in *Amaranthus cruenthus* L. genotype Ficha and genotype D 279 (*A. hypochondriacus* L. × *A. hybridus* L.).

Abbreviations in Fig. 1−7: K (control) – maize coleoptile section (single-cross hybrid Sc 3098 × 3150), genetic interpretations of maize isozymograms (loci and alleles) − *Acp1: 2/4, Adh1: 4/4, Idh1: 4/4, Idh2: 4/6, Mdh1: 6/6, Mdh2: 3/3, Mdh3: 16/16, Mdh4: 12/12, Mdh5: 12/12, Mmm: M/M, Pgd1: 2/3.8, Pgd2: 5/5, Pgi1: 4/4, Pgm1: 9/9 and Pgm2: 4/4*; **A1** (seeds), **A2** (seedlings) – samples of *Amaranthus cruentus* L. genotype Ficha; **D1** (seeds), **D2** (seedlings) – samples of *Amaranthus* genotype D 279 (*A. hypochondriacus* L. × *A. hybridus* L.); **W2 (1.5), W2 (2.0), W2 (3.0)** − Whatman No. 2 wicks dimensions (11 × 1.5, 11 × 2.0 and 11 × 3.0 mm, respectively); **C(1), C(3)** and **C(6)** − samples of amaranth seedlings (one, three and six days of germination, respectively); **1, 2, 3, 4** and **5** – number of sample weights in mg versus extract solution volumes in µl (10/10, 50/50, 100/100, 150/100 and 200/100 mg μl^{-1}, respectively).

MDH is controlled by two non-allelic genes – monomorphic *Mdh1* and polymorphic *Mdh2* that has three alleles. The MDH multiplicity from one- to five-banded was published. In our analysis, fingerprints of MDH polymorphism were monomorphic for all amaranth samples with five-banded phenotypes (Fig. 4 and 8).

6-phosphogluconate dehydrogenase – one-banded and monomorphic phenotype occurred in our analyses (Fig. 5 and 8). Isozyme patterns of amaranth PGD were mentioned only in one work published by Warwick and Black (1981). They presented three isozyme phenotypes one four- and two two-banded.

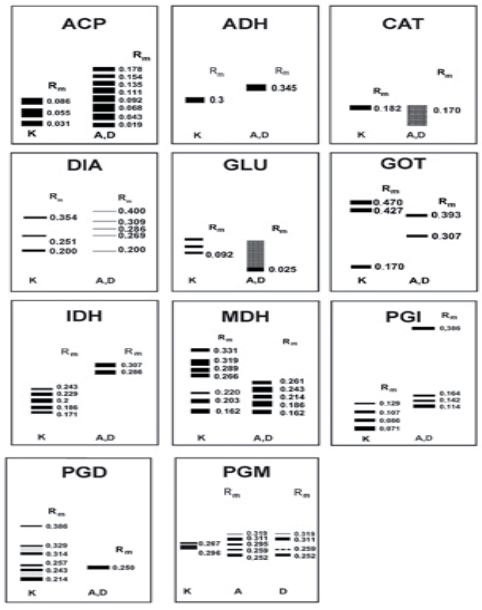

Fig. 8. A scheme of isoenzyme patterns observed for acid phosphatase (ACP), alcohol dehydrogenase (ADH), catalase (CAT), diaphorase (DIA), ß-glucosidase (GLU), glutamateoxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), 6-phosphogluconate dehydrogenase (PGD) and phosphoglucomutase (PGM) in *Amaranthus cruenthus* L. genotype Ficha and genotype D 279 (*A. hypochondriacus* L. x *A. hybridus* L.). Rm – the factor of relative mobility.

Phosphoglucoisomerase – all allozyme phenotypes for all amaranth samples were four-banded and monomorphic (Fig. 6 and 8). The four-banded phenotypes of PGI isozymograms are documented also by Hauptli and Jain (1984), Warwick and Black (1986) and Kirkpatrick (1995). Pratt and Clark (2001) published that two loci (*Pgi1* and *Pgi2*) are responsible for PGI polymorphism.

Phosphoglucomutase – polymorphism of amaranth phosphoglucomutase belongs to poorer among studied enzymes. In our fingerprints four- and three-banded phenotypes with one very weak uppermost band for *Amaranthus cruenthus* L. genotype Ficha and genotype D 279 (*A. hypochondriacus* L. × *A. hybridus* L.), respectively, were detected. These two genotypes are distinguishable each other by enzyme polymorphism of PGM. Only one work was published (Pratt & Clark 2001) mentioning that one locus of PGM with six possible alleles for *Amaranthus rudis* and *Amaranthus tuberculatus* was detected.

Effects of Whatman No. 2 wick dimensions and amaranth seed extract concentrations on quality of isozymograms are expressed in Table 3. According to visual evaluation of isozymograms their quality in relation to genotype Ficha decreased in direction of MDH > ADH > PGI > PGD > IDH > PGM > ACP > GOT > CAT > DIA, GLU; while for genotype D 279 decreased in direction of MDH > ACP > PGI, PGD > ADH > IDH > PGM > GOT > CAT > DIA, GLU; and for both genotypes in direction MDH > ADH > PGI > PGD > ACP > IDH > PGM > GOT > CAT > DIA and GLU. Effect of Whatman No. 2 wick dimensions on quality of isozymograms was low but slightly increased to dimension of 11 × 3.0 mm. Extract concentration from samples number 1, 2 and 3 had little effect on isozymograms quality, however quality increased with extract concentration from samples number 4 to 5. The quality of genotype Ficha isozymograms was little higher than those of genotype D 279. Great disadvantage for quick manual preparing of extracts from amaranth seed samples is too high stiffness of seeds and low extract volume in the case of sample no. 1 (volume not sufficient for more than two Whatman No. 2 wicks).

Effects of cultivation and amaranth seed extract concentrations on quality of isozymograms are expressed in Table 4. The quality in relation to genotype decreased for Ficha in direction of MDH > ACP > ADH, PGI, PGD > GOT, IDH > PGM > CAT, DIA, GLU; while for D 279 of MDH > ACP, ADH > GLU > PGI, PGD, PGM > IDH > GOT > CAT, DIA and for both genotypes of MDH > ACP > ADH > PGI > PGD > PGM > IDH > GOT > GLU > CAT and DIA. Effect of length of cultivation on quality of isozymograms was evident and decreased 1 – day > 3 – days > 6 –

days of cultivation with minimal differences between 3 and 6 days of cultivation. Higher isoenzyme activity in extracts from one day cultivation is connected with hard and time consuming manual preparing of extracts (stiff germinating seeds). Extract concentration from samples number 1, 2 and 3 had no or very little effect on isozymogram quality which increased with extract concentration from samples number 4 to 5. In this case the quality of genotype Ficha isozymograms was lower than those of genotype D 279.

T a b l e 3

Effect of Whatman No. 2 wick dimensions and amaranth seed extract concentrations on quality of isozymograms

Genotype	*Amarantus cruentus* L. „Ficha“															*A. hypochondriacus* L. × *A. hybridus* L. „D 279“													
Whatman No. 2 wick dimensions	11 × 1.5 mm					11 × 2.0 mm					11 × 3.0 mm					11 × 1.5 mm					11 × 2.0 mm					11 × 3.0 mm				
Number of sample	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Enzyme																														
ACP	+	+	+	+	+	++	++	++	++	++	–	+	–	–	++	+++	+++	+	+++	+++	++	+++	+	+	+	++	++	+++	+++	+++
ADH	++	++	++	++	+++	++	++	+++	++	+++	++	++	++	+++	+++	+++	++	++	++	++	++	+	+	+	++	++	++	+++	++	++
CAT	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
DIA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GLU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GOT	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+
MDH		++	++	++	++	++	++	++	++	++	+	++	++	++	++	+	+	+	++	++	+	+	+	++	++	+	+	++	+	++
MDH	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
PGI	+++	++	++	++	+++	++	++	++	++	++	++	++	++	++	+++	++	++	++	++	++	++	++	++	++	+++	++	++	++	++	++
PGD	++	++	++	++	+++	++	++	++	++	++	++	++	++	++	+++	++	++	++	++	++	++	++	++	++	+++	++	++	++	++	++
PGM	++	++	++	++	++	++	++	++	++	+++	–	+	–	++	++	–	–	–	++	++	–	–	–	–	++	++	++	+	+	++

Abrevations:
+++ very strong band (spot) intensity;
++ good band (spot) intensity;
+ weak band (spot) intensity;
– very weak nearly no band (spot) intensity;
1, 2, 3, 4 and 5 number of sample weights in mg versus extract solution volumes in µl
(10/10; 50/50; 100/100; 150/100 and 200/100 mg μl^{-1}, respectively)

CONCLUSION

Our experimental results and experiences with testing of slightly modified methodology for quick testing amaranth enzyme multiplicity published by Stuber et al. (1988) for coleoptile section of maize led us to the following conclusions: a) differences between amaranth seed and seedling fingerprint qualities are low

T a b l e 4

Effect of cultivation and amaranth seedling extract concentrations on quality of isozymograms

Genotype	*Amarantus cruentus* L. „Ficha“															*A. hypochondriacus* L. *x A. hybridus* L. „D 279“													
Cultivation (days)	1 day					3 days					6 days					1 day					3 days					6 days				
Number of sample	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Enzyme																														
ACP	++	++	++	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
ADH	+++	+++	+++	+++	+++	++	++	++	++	++	+	+	+	+	+	+++	+++	+++	+++	+++	++	++	++	++	++	++	++	++	++	++
CAT	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DIA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GLU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	++	++	++	++	++	+++	+++
GOT	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	++	++	++	++	++	–	–	–	–	–	+	+	++	++	++
MDH	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+
MDH	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
PGI	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
PGD	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
PGM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

Abrevations:
+++ very strong band (spot) intensity;
++ good band (spot) intensity;
+ weak band (spot) intensity;
– very weak nearly no band (spot) intensity;
1, 2, 3, 4 and 5 number of sample weights in mg versus extract solution volumes in µl (10/10; 50/50; 100/100; 150/100 and 200/100 mg μl^{-1}, respectively)

but more practical for quick testing were 3 – days cultivated seedlings; b) effect of Whatman No. 2 wick dimensions on quality of isozymograms was low – it is known that increasing width of wicks decreases one gel sample capacity; c) extract concentration number 5 (100/200 µl mg^{-1}) was the best for dry seed and seedling samples. According to isozymograms which are documented in Figure 1–7 is clear, that tested methodology is suitable for analyses of MDH, ADH, PGI, PGD, IDH, PGM and ACP multiplicity in amaranth species.

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