Additional Useful Explanations

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Part I

Introduction

The following Annex contains a list of characteristics based on isozyme markers revealed by electrophoresis and a description of the method to be used. UPOV decided to place these characteristics in an Annex to the Test Guidelines, thereby creating a special category of characteristic, because the majority of the UPOV members is of the view that it is not possible to establish distinctness solely on the basis of a difference found in a characteristic based on isozyme markers revealed by electrophoresis. Such characteristics should therefore only be used as a complement to other differences in morphological or physiological characteristics. UPOV reconfirms that these characteristics are considered useful but that they might not be sufficient on their own to establish distinctness. They should not be used as a routine characteristic but at the request or with the agreement of the applicant of the candidate variety.

For the analysis of hordeins; polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS PAGE) is recommended. Hordeins are encoded by three compound loci known as Hor-1, Hor-2 and Hor-3 located on chromosome 5 (Hor-1 and Hor-2 on the short arm, Hor-3 on the long arm). There are a number of alleles at each locus and the analysis of hordeins is based on the recognition of these alleles from proteins, which appear on gels as a series of well-defined bands or patterns of bands. The loci encode different groups of electrophoretically separable proteins, known as B-; C- and D-hordeins in decreasing order of mobility. The alleles at each locus can be designated by letters or numbers, or a combination of both. The relative electrophoretic mobilities (REMs) of each of the bands can also be determined.

If only C-(Hor-1) and B-(Hor-2) hordeins are of interest, then the standard reference acid PAGE method of the International Seed Testing Association (ISTA) could be used.

Part II

Characteristics derived by Protein Polymorphism

The following table indicates the REM values of the main bands present in the B-, C-.and D-hordein alleles analyzed with the SDS PAGE method and the Acid PAGE method. In comparing both methods, it should be noted that the example varieties and notes given for the individual states of expression are identical in both methods.

	Characteristics Band position in SDS PAGE method	Band position in Acid PAGE method	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
29.	**QL VG**			
	D-Hordein composition: **allele expression at locus Hor-3**			
	band 34		(W) California	1
	band 33		(W) Medina	2
	band 35		(W) Saturn	3
	band 32.5		(W) Iris	4
	band 32		(W) Princesse	5
30.	**QL VG**			
	C-Hordein composition: **allele expression at locus Hor-1**			
	bands 62+65+68	bands 27+30+32+37+39	(W) California	1
	bands 62+65+66+68	bands 27+30+32+34+37+39	(W) Lomerit	2
	bands 65+68	bands 27+30+32+37	(W) Medina	3
	bands 66.5+71	bands 32+37+41	(W) Sandra	4
	bands 61.5+66.5+71	bands 27+30+32+37+39+41	(S) Meltan	5
	bands 65	bands 32+37+38	(S) Armada	6
	bands 60 +67.5+68.5	bands 35+38	(W) Roseval	7
	bands 61+65+68+73	bands 32+37+39+41	(W) Semper	8
	bands 60+69+72	bands 38+41+42	(S) Sydney	9
	bands 64+66.5	bands 30+32+37	(W) Saturn	10
	bands 67+71	bands 34+37	(S) Pastello	11
	bands 65+68+69+70	bands 34+39+41+42	(W) Albacete	12
	bands 61.5+68+71	bands 31+34+37+38+41	(W) Borwina	13
	bands 65+67.5	bands 32+37+41+43	(W) Kendo	14
	bands 65.5+70.5		(W) Delita	15
	bands 66+70.5		(W) Maybrit	16
31.	**QL VG**			
	B-Hordein composition: **allele expression at locus Hor-2**			
	bands 79+86+88+100	bands 71+79+83+86+94+100	(S) Quench	1
	bands 79+88+91+95+97+101	bands 71+82+89+100	(S) Overture	2
	bands 79+91+92+95+97+101	bands 76+82+83+86+100	(S) Hellana	3
	bands 75+82+87+91+97	bands 66+71+76+86+93+100	(W) Caribic	4
	bands 79+86+88+97+101	bands 71+78+79+90+94	(W) Piroline	5
	bands 78+84+95+101	bands 76+81+94	(W) Ingmar	6
	bands 79+90+91+94+100	bands 71+72+75+82+85+86+100	(S) Sebastian	7
	bands 78+86+91+95+100	bands 72+76+79+90+94	(W) Sandra	8
	bands 79+82+88+91+92+100	bands 71+76+79+86	(S) Ebson	9
	bands 76+79+86+88+100	bands 71+78+83+86+94+100	(S) Trebon	10

Characteristics Band position in SDS PAGE method	Band position in Acid PAGE method	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
bands 79+86+89+92+95+101	bands 71+79+83+86+90	(W) Sigma	11
bands 79+95+101	bands 71+76+79	(W) Midas	12
bands 78+89+92+101	bands 71+89	(W) Lomerit	13
bands 75+78+79+81+89+101	bands 79+83+86+90	(W) Findora	14
bands 75+78+79+81+83+86+88+94+95+100	bands 67+69+71+72+78+79+85+89+94	(W) Caresse	15
bands 81+84+88+90+101	bands 71+79+83+88+94	(W) Reseda	16
bands 75+78+79+81+83+86	bands 69+76+79+83+93	(W) Baronesse	17
bands 82+88+100	bands 71+72+79+85+86+91+100	(W) Albacete	18
bands 81+100	bands 72+76+100	(S) Basic	19
bands 75+79+83+89+91	bands 61+71+76+79+83	(W) Camargue	20
bands 79+84+92	bands 76+81+94+100	---	21
bands 79+91+92		(W) Libelle	22
bands 75+79+91+92+95+97+101		(W) Anja	23
bands 75+79+90+94+99		(W) Hiberna	24
bands 79+(83-85)+(89-91)+(94-96) +102		(W) Jerka	25

Part III

Description of the Method to be used

1. SDS PAGE Method for Analysis of Hordeins from *Hordeum vulgare*

1.1 Apparatus and equipment

Any suitable vertical electrophoresis system can be used; provided that the gels can be kept at a constant temperature. A gel thickness of no more than 1.5 mm is recommended. The power supply used should be capable of delivering both constant current and constant voltage output.

1.2. Chemicals

All chemicals should be of 'Analytical Reagent' grade or better.

Acrylamide (specially purified for electrophoresis)
Bisacrylamide (specially purified for electrophoresis)
Tris (hydroxymethyl) methylamine (TRIS)
Sodium dodecyl sulphate (SDS)
Ammonium persulphate (APS)
2-mercaptoethanol
TEMED (NNN'N'-tetramethylethylenediamine)
Trichloroacetic acid (TCA)
Hydrochloric acid
Glacial acetic acid
Glycine
n-Butanol
Pyronin
Glycerol (d = 1.256)
Methanol
Coomassie Brilliant Blue R-250 (or equivalent)
Coomassie Brilliant Blue G-250 (or equivalent)

1.3 Solutions

1.3.1 Extraction solution

Stock solution:
6.25 ml 1M TRIS HCl buffer; PH 6.8 (see 1.3.3.2)
12.05 ml distilled water
2g SDS
10 mg Pyronin
10 ml glycerol
This solution can be stored for 2 months at 4°C.

Immediately before use; extraction solution is prepared as follows:

28.33 ml stock buffer solution plus 7.91 ml 2-mercaptoethanol made up to 100 ml with distilled water. This solution must be prepared immediately prior to use and cannot be stored.

1.3.2 Electrophoresis (running) buffer

Stock solution:
141.1 g glycine
30.0 g TRIS
10.0 g SDS
made up to 1 liter with distilled water.

Immediately before use; the stock solution is diluted 1:10 with distilled water.

The stock buffer solution can be stored for 2 months at room temperature. Do not store the diluted buffer more than one week. The pH of the buffer must be close to 8.3.

1.3.3 Gel preparation solutions

1.3.3.1 Stock resolving gel buffer (1M TRIS HCl pH 8.8)

121.14 g TRIS plus approximately 20 ml HCl (d = 1.19) made up to 1 liter with distilled water. This buffer can be stored at 4°C for 2 months.

1.3.3.2 Stock stacking gel buffer (1M TRIS HCl; pH 6.8)

121.14 g TRIS plus approximately 78 ml HC1 (d = 1.19) made up to 1 liter with distilled water. This buffer can be stored at 4°C for 2 months.

1.3.3.3 10% (w/v) SDS solution

10g of SDS dissolved in distilled water and made up to 100 ml. This solution can be stored at 4°C for 2 months. Prior to use; stir and heat gently to re-dissolve the SDS; if it comes out of solution.

1.3.3.4 1% (w/v) ammonium persulphate solution

1 g of APS dissolved in distilled water and made up to 10 ml. This solution must be prepared immediately prior to use.

1.3.3.5 Stock acrylamide solution

51.98 g acrylamide made up to 100 ml with distilled water.

1.3.3.6 Stock bisacrylamide solution

0.3185g bisacrylamide made up to 130 ml with distilled water.

1.3.4 Staining solutions

1.3.4.1 0.25g Coomassie Brilliant Blue G-250 plus 0.75g Coomassie Brilliant Blue R-250; made up to 100 ml with water.

1.3.4.2 55 g TCA; 65 ml glacial acetic acid; 180 ml methanol plus 25 ml solution 1.3.4.1; made up to 1 liter with distilled water.

1.4. Procedure

1.4.1 Protein extraction

Individual seeds are ground using a hammer (or other device). Ground seed meal is mixed with diluted sample extraction buffer (1.3.1) in a 3 ml polypropylene hemolyse or similar tube with a screw-on cap. The ratio of meal/extraction buffer is 50 mg/0.75 ml. The samples are extracted for 2 hours at room temperature; mixed several times using a vortex mixer; heated in a boiling water bath for 10 minutes and then allowed to cool. The tubes are centrifuged at 18,000 g for 5 minutes.

According to the gel thickness and the size of the wells; the volume of extract loaded can vary. Between 10 and 25 µl is usually sufficient.

1.4.2 Preparation of the gel

Clean and dry gel cassettes are assembled; according to the design of the equipment used. If tape is used to seal the cassettes; it is advisable to assemble them at least one day in advance of use; to enable the tape to 'age' and adhere better.

1.4.2.1 Resolving (main) gel (10% acrylamide; pH 8.8)

To make two slab gels of 180 x 160 x 1.5 mm; the following is required:
20 ml stock acrylamide solution (1.3.3.5)
26 ml stock bisacrylamide solution (1.3.3.6)
30 ml stock gel buffer (1.3.3.1).

These should be at 4°C. The mixture is de-gassed in a 100 ml Buchner flask for 10 minutes. To this is added:
2 ml APS (1.3.3.4);
0.8 ml SDS (1.3.3.3);
40 µl TEMED (use straight from bottle).

The gels are then carefully poured; avoiding the formation of air bubbles; and polymerisation is allowed to take place at room temperature.

The gel cassettes should not be filled entirely; in order to leave room for a 3-4 cm layer of stacking gel. The gel surface is carefully overlaid with n-butanol (or distilled water) using a syringe. When polymerisation is finished (about 30 min); the gel surface is carefully rinsed with distilled water and dried with filter paper.

1.4.2.2 Stacking gel (3.5% acrylamide; pH 6.8)

In a 50 ml Buchner flask; mix:
1.35 ml stock acrylamide solution (1.3.3.5);
3.17 ml stock bisacrylamide solution (1.3.3.6)
2.50 ml stock gel buffer (1.3.3.2) and
12.30 ml distilled water.

Following de-gassing add:
0.875 ml APS (1.3.3.4);
0.233 ml SDS (1.3.3.3);
17.5 µl TEMED (straight from bottle)

Mix carefully and immediately pour the stacking gels to the top of the gel cassettes. Insert the well-forming "comb"; avoiding air bubbles. Allow to polymerise for about 2 hours. The "combs" are then removed carefully from the gel cassettes and the wells rinsed using diluted electrophoresis running buffer (1.3.2).

1.4.3 Electrophoresis

The tank is filled with the appropriate volume of running buffer (1.3.2); cooled to 15 ^{o}C. Following sample loading; electrophoresis is carried out at a constant current of 8 mA/sq cm (cross-sectional area) of gel until the pyronin G has moved through the stacking gel; and then at 16 mA/sq cm of gel (maximum voltage 300V) until the marker is at the bottom of the gel. The temperature should be maintained at 15 ^{o}C.

1.4.4 Fixing and staining

The gel cassettes are removed from the tank; opened and the gels fixed in 250 ml of 15% (w/v) TCA for at least 30 minutes. The gels are rinsed in distilled water and stained overnight in 250 ml of staining solution (1.3.4.2) at room temperature. Distaining is not usually necessary but gels should be washed in distilled water before being stored in sealed polythene bags.

Other staining procedures can be successfully used (e.g. Coomassie Brilliant Blue G or equivalent in TCA alone). The final quality control criterion; both for gel preparation and gel staining; is to analyze the suggested example varieties on each batch of gels. The separation of the suggested bands; and their relative electrophoretic mobilities (molecular weights) must be clear in order for the procedures to be judged satisfactory.

1.5 Recognition of Hordein Alleles (SDS PAGE)

The band pattern presented in the tables for B-; C- and D-hordeins are schematic and differences in band intensity have been ignored in the presentation.

B-; C- and D-hordeins: nomenclature of the individual bands and recognition of the corresponding alleles (SDS-PAGE)

Characteristic 29: D-Hordein composition: allele expression at locus Hor-3

Band	Example	Note				
	California	1	2	3	4	5
32						--
32.5					--	
33			--			
34	--	--				
35				--		

Characteristic 30: C-Hordein composition: allele expression at locus Hor-1

Band	Example California	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Band
60								--										60
61									--									61
61.5						--								--				61.5
62	--	--	--															62
64											--							64
65	--	--	--	--			--		--				--		--			65
65.5																--		65.5
66			--														--	66
66.5					--	--					--							66.5
67												--						67
67.5								--							--			67.5
68	--	--	--	--					--				--	--				68
68.5								--										68.5
69										--			--					69
70													--					70
70.5																--	--	70.5
71					--	--						--		--				71
72										--								72
73									--									73

Characteristic 31: B-Hordein composition: allele expression at locus Hor-2

Band	Example Quench	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Band
75					--										--	--		--			--			--	--		75
76											--																76
78							--		--					--	--	--		--									78
79	--	--	--	--		--		--		--	--	--	--		--	--		--			--	--	--	--	--	--	79
81															--	--	--	--		--							81
82					--														--								82
83																--		--			--					--	83
84							--										--					--				--	84
85																										--	85
86	--	--				--			--		--	--				--		--									86
87					--																						87
88	--	--	--			--				--	--					--	--		--								88
89												--		--	--						--					--	89
90								--									--								--	--	90
91			--	--	--			--	--	--											--		--	--		--	91
92				--						--		--		--								--	--	--			92
94								--								--									--	--	94
95			--	--			--		--			--	--			--								--		--	95
96																										--	96
97			--	--	--	--																		--			97
99																									--		99
100	--	--						--	--		--					--			--	--							100
101			--	--		--	--			--		--	--	--	--		--							--			101
102																										--	102

2. Acid PAGE Method for Analysis of B- and C-Hordeins from *Hordeum vulgare*

If only B- and C-hordeins are of interest; then acid PAGE can be used. The following method is the standard reference method recommended by the International Seed Testing Association.

2.1. Apparatus and Equipment

Various designs of vertical electrophoresis equipment have been used successfully; including those available from Biometra; Bio-Rad; Desaga and Pharmacia-LKB. The power supply used should be capable of operating at constant voltage and constant current.

2.2. Chemicals

All chemicals should be of "Analytical Reagent" grade or better.

Acrylamide ("specially purified for electrophoresis")
Bisacrylamide ("specially purified for electrophoresis")
Urea
Glacial acetic acid
Glycine
Ferrous sulphate
Ascorbic acid
Hydrogen peroxide
Monothioglycerol
Pyronin G
Trichloroacetic acid (TCA)
Methanol
2-chloroethanol
Coomassie Brilliant Blue G-250 (or equivalent)
Coomassie Brilliant Blue R-250 (or equivalent)

2.3. Solutions

2.3.1 Extraction solution

Pyronin G (0.05%) (w/v) in 2-chloroethanol (20%) (v/v) containing urea (18% w/v) and monothioglycerol (1% v/v) (keep cold or prepare fresh).

2.3.2 Tank buffer solution

Glacial acetic acid (4 ml) and glycine (0.4g); made up to 1 litre with distilled water; keep cold.

2.3.3 Gel buffer solution

Glacial acetic acid (20 ml) and glycine (1.0g); made up to 1 litre with distilled water; keep cold.

2.3.4 Staining solutions

0.25g Coomasie Brilliant Blue G-250 + 0.75g Coomassie Brilliant Blue R-250 in 100 ml water.

55g TCA; 65 ml glacial acetic acid; 180 ml methanol; plus 25 ml solution 2.3.4.1; made up to 1 litre with distilled water.

2.4. Procedure

2.4.1 Protein extraction

Single seeds are crushed with pliers or by similar means and transferred to 1.5 ml polypropylene centrifuge tubes or to micro-titer plates. Extraction solution (2.3.1) (0.3 ml) is added and the tubes or plates are allowed to stand overnight at room temperature. If necessary; the tubes are centrifuged at 18,000xg and the supernatants used for electrophoresis.

2.4.2 Preparation of the gel

Clean and dry gel cassettes are assembled; according to the design of the equipment. Treating the glass plates with silicon prior to assembly can facilitate subsequent removal of the gel. The gel cassettes can incorporate a plastic backing sheet (e.g. "Gel Bond PAG"; FMC Corporation). This supports the gel during subsequent operations. To make 100 ml of gel medium; gel buffer at 4°C (2.3.3) (approximately 60 ml) is taken and the following added: acrylamide (10g); bisacrylamide (0.4g);

urea (6g); ascorbic acid (0.1g); ferrous sulphate (0.005g). The solution is stirred and made up to 100 ml with cold (4°C) stock gel buffer solution (2.3.3). Freshly prepared 0.6% (v/v) hydrogen peroxide solution (0.35 ml per 100 ml of gel medium) is added; mixed quickly and the gel poured. An acrylic "comb" is placed in the top of the cassette; to make wells in the gel. Polymerisation is carried out at room temperature and should be complete in five to 15 minutes. If not; it may be necessary to adjust the volume of hydrogen peroxide added. The gel mixture should over-fill the cassette; or be over-layed with water; to ensure satisfactory polymerisation of the upper surface.

2.4.3 Electrophoresis

The acrylic comb is removed from the gel and the sample wells washed with tank buffer (2.3.2). The tank is filled with an appropriate volume of buffer (2.3.2) (depending on the equipment used). Samples (10-20 ul) are loaded into the wells and the gel placed in the tank, ensuring that the sample wells are completely filled. The temperature of the lower buffer chamber should be kept at 15°C. Electrophoresis is carried out at a constant voltage of not more than 60V/cm^2 (cross-sectional area) of gel (which corresponds to a voltage of 500V for two gels 16 cm wide and 0.15 cm thick) for twice the time taken for the pyronin G marker to leave the gel. It must be remembered that the anode (positive electrode) is at the origin (top of the gel) in this system.

2.4.4 Fixing and staining

The gel cassette is removed from the tank, opened and the gel placed in a plastic box containing 200 ml of staining solution (2.3.4.2). Staining is carried out overnight at room temperature. Destaining if necessary is carried out by placing gels in water for about two to 3 hours at room temperature. Gels can then be dried or stored in sealed polythene bags at 4°C.

It should be noted that other procedures, such as the use of increased temperatures or the use of mixtures of TCA and Coomassie Brilliant Blue G, will give satisfactory staining of gels. The final quality control criterion, both for gel preparation and gel staining, is to analyse the suggested example varieties on each batch of gels. The separation of the designated bands, and their relative electrophoretic mobilities, must be clear and correct in order for the procedures to be satisfactory.

2.5 Recognition of Hordein Alleles (Acid PAGE)

B- and C-Hordeins: nomenclature of the individual bands and recognition of the corresponding alleles: acid PAGE

Characteristic 30: C-Hordein composition: allele expression at locus Hor-1

Band	Example California	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Band
25																25
27	--	--	--	--		--										27
30	--	--	--	--		--					--					30
31														--		31
32	--	--	--	--	--	--	--		--		--				--	32
34			--									--	--	--		34
35								--								35
37	--	--	--	--	--	--	--		--		--	--		--	--	37
38							--	--		--				--		38
39	--	--	--			--			--				--			39
41					--	--			--	--			--	--	--	41
42										--			--			42
43															--	43
		Alleles according to acid PAGE nomenclature														
		10	10A	1	11	17	6	19	2	4	5	18	14	8	3	

Characteristic 31: B-Hordein composition: allele expression at locus Hor-2

Band	Example Quench	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Band
61																					--		61
66					--																		66
67																--							67
69																--		--					69
71	--	--	--		--	--		--		--	--	--	--	--		--	--		--		--		71
72								--	--							--			--	--			72
75								--															75
76				--	--		--		--	--			--					--		--	--	--	76
78						--					--					--							78
79	--	--				--			--	--		--	--		--	--	--	--	--		--		79
81							--															--	81
82			--	--				--															82
83	--	--									--	--			--		--	--			--		83
85								--								--			--				85
86	--	--		--	--			--		--	--	--			--				--				86
88																	--						88
89			--											--		--							89
90						--			--			--			--								90
91																			--				91
93					--													--					93
94	--	--				--	--		--		--					--	--					--	94
97																							97
100	--	--	--	--	--			--			--								--	--		--	100
		3	4	13	14	-	9	1	7	6	-	-	11	16	-	18	-	19	8	15	12	10	

[End of Annex]