CPVO · OCVV
Community Plant Variety Office
Office Communautaire des Variétés Végétales

PROTOCOL FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTS

Triticum aestivum L.

WHEAT

UPOV Species Code: TRITI_AES

Adopted on 16/02/2011

Entry into force on 01/08/2010

I SUBJECT OF THE PROTOCOL

The protocol describes the technical procedures to be followed in order to meet the requirements of Council Regulation 2100/94 on Community Plant Variety Rights. The technical procedures have been agreed by the Administrative Council and are based on general UPOV Document TG/1/3 and UPOV Guideline TG/3/11 dated 4th November 1994 for the conduct of tests for Distinctness, Uniformity and Stability. This protocol applies to all varieties of *Triticum aestivum* L.

II SUBMISSION OF SEED AND OTHER PLANT MATERIAL

1. The Community Plant Variety Office (CPVO) is responsible for informing the applicant of

- the closing date for the receipt of plant material;
- the minimum amount and quality of plant material required;
- the Examination Office to which material is to be sent.

A sub-sample of the material submitted for test will be held in the variety collection of the Examination Office as the definitive sample of the candidate variety.

The applicant is responsible for ensuring compliance with any customs and plant health requirements.

2. Final dates for receipt of documentation and material by the Examination Office

The final dates for receipt of requests, technical questionnaires and the final date or submission period for plant material will be decided by the CPVO and each Examination Office chosen (hereunder point 3).

The Examination Office is responsible for immediately acknowledging the receipt of requests for testing, and technical questionnaires. Immediately after the closing date for the receipt of plant material the Examination Office should inform the CPVO if no plant material has been received. However, if unsatisfactory plant material is submitted the CPVO should be informed as soon as possible.

3. Seed requirements

Information with respect to closing dates and submission requirements of plant material for the technical examination of varieties can be found on the CPVO web site (www.cpvo.europa.eu) and in the special Issue S2 of the Official Gazette of the Office published yearly at the month of September.

Quality of seed:.....The minimum requirements for germination capacity, analytical purity and seed health should not be less than the standards laid down in EC Directive 66/402/EEC

Seed Treatment: .....The plant material must not have undergone any treatment unless the CPVO and the Examination Office allow or request such treatment. If it has been treated, full details of the treatment must be given.

Labelling of sample:.....- Species
- File number of the application allocated by the CPVO
- Breeder's reference
- Examination Office reference (if known)
- Name of applicant
- The phrase "On request of the CPVO".

III CONDUCT OF TESTS

1. Variety collection

A variety collection will be maintained for the purpose of establishing distinctness of the candidate varieties in test. A variety collection may contain both living material and descriptive information. A variety will be included in a variety collection only if plant material is available to make a technical examination.

Pursuant to Article 7 of Council Regulation No. 2100/94, the basis for a collection should be the following:

- varieties listed or protected at the EU level or at least in one of the EEA Member States;
- varieties protected in other UPOV Member States;
- any other variety in common knowledge.
- In case of hybrids, all components of hybrid varieties in common knowledge must be considered as part of the reference collection.

The composition of the variety collection in each Examination Office depends on the ecological conditions in which the Examination Office is located.

Variety collections will be held under conditions which ensure the long term maintenance of each accession. It is the responsibility of Examination Offices to replace reference material which has deteriorated or become depleted. Replacement material can only be introduced if appropriate tests confirm conformity with the existing reference material. If any difficulties arise for the replacement of reference material Examination Offices must inform the CPVO. If authentic plant material of a variety cannot be supplied to an Examination Office the variety will be removed from the variety collection.

2. Material to be examined

Candidate varieties will be directly compared with other candidates for Community plant variety rights tested at the same Examination Office, and with appropriate varieties in the variety collection. When necessary an Examination Office may also include other candidates and varieties. Examination Offices should therefore make efforts to co-ordinate the work with other offices involved in DUS-testing of wheat. There should be at least an exchange of technical questionnaires for each candidate variety, and during the test period, Examination Offices should notify each other and the CPVO of candidate varieties which are likely to present problems in establishing distinctness. In order to solve particular problems Examination Offices may exchange plant material.

3. Characteristics to be used

The characteristics to be used in DUS tests and preparation of descriptions shall be those referred to in the table of characteristics. All the characteristics shall be used, providing that observation of a characteristic is not rendered impossible by the expression of any other characteristic, or the expression of a characteristic is prevented by the environmental conditions under which the test is conducted. In the latter case, the CPVO should be informed. In addition the existence of some other regulation e.g. plant health, may make the observation of the characteristic impossible.

The list of characteristics derived from electrophoresis as in Annex 3 should only be used as a complement to other differences in morphological or physiological characteristics.

The Administrative Council empowers the President, in accordance with Article 23 of Commission Regulation N° 1239/95, to insert additional characteristics and their expressions in respect of a variety.

4. Grouping of varieties

The varieties and candidates to be compared will be divided into groups to facilitate the assessment of distinctness. Characteristics which are suitable for grouping purposes are those which are known from experience not to vary, or to vary only slightly, within a variety and which in their various states of expression are fairly evenly distributed throughout the collection. In the case of continuous grouping characteristics overlapping states of expression between adjacent groups is required to reduce the risks of incorrect allocation of candidates to groups. The characteristics that could be used for grouping are the following (CPVO numbering; G for grouping in table of characteristics)

a) Straw: pith in cross section (characteristic 10)

b) Awns or scurs: presence (characteristic 14)

c) Ear: colour (characteristic 16)

d) Seasonal type (characteristic 25)

5. Trial designs and growing conditions

The minimum duration of tests will normally be two independent growing cycles. Tests will be carried out under conditions ensuring normal growth. The size of the plots will be such that plants or parts of plants may be removed for measuring and counting without prejudice to the observations which must be made up to the end of the growing cycle.

The test design is as follows:

Each test should include about 2000 plants which should be divided between two or more replicates. The assessment for the characteristic 'Seasonal type' should be carried out on at least 500 plants.

If ear rows are used, tests should be conducted on 100 ears.

In case of hybrids, the parent lines have to be included in the test and should be tested and assessed as any other self-pollinating variety. The observations on the hybrid variety itself should be made on at least 200 plants.

All observations for the assessment of distinctness on individual plants should be made on 20 plants or parts of 20 plants.

6. Special tests

In accordance with Article 83(3) of Council Regulation No. 2100/94 an applicant may claim either in the Technical Questionnaire or during the test that a candidate has a characteristic which would be helpful in establishing distinctness. If such a claim is made and is supported by reliable technical data, a special test may be undertaken providing that a technically acceptable test procedure can be devised.

Special tests will be undertaken, with the agreement of the President of CPVO, where distinctness is unlikely to be shown using the characters listed in the protocol.

7. Standards for decisions

a) **Distinctness**

A candidate variety will be considered to be distinct if it meets the requirements of Article 7 of Council Regulation No. 2100/94.

To assess distinctness of hybrids, a pre-screening system on the basis of the parental lines and the formula may be established according to the following recommendations:

(i) description of parental lines according to the Test Guidelines;

(ii) check of the originality of the parental lines in comparison with the reference collection, based on the characteristics in the table of characteristics in order to screen the closest inbred lines;

(iii) check of the originality of the hybrid formula in comparison with those of the hybrids in common knowledge, taking into account the closest inbred lines;

(iv) assessment of the distinctness at the hybrid level of varieties with a similar formula.

Qualitative characteristics:

In the case of characteristics which show discrete discontinuous states of expression, a difference between two varieties is clear if the respective characteristics have expressions which fall into two different states.

Quantitative characteristics:

Characteristics which show a continuous range of expression from one extreme to the other may be either measured or visually observed.

In the case of visually observed characteristics, a difference between two varieties is clear if the expression of the respective characteristics differs by at least the span of one note, taking into account the variability observed within the varieties.

If distinctness is assessed using the t-test least significant difference the difference between two varieties is clear if it occurs with the same sign at the 1% significance level or less ($p \leq 0.01$) in two consecutive or two out of three growing cycles.

If distinctness is assessed by the combined over years distinctness analysis (COYD) the difference between two varieties is clear if the respective characteristics are different at the 1% significance level or less ($p \leq 0.01$) in a test over either two or three years.

If the significance level or statistical methods proposed are not appropriate the method used should be clearly described.

b) **Uniformity**

Uniformity is assessed by visual observation and the detection of off-types.

The number of off-types in a sample size of 2000 plants or parts of plants should not exceed 10 in 2000 (Population standard of 0.3% with an acceptance probability of ≥ 95%).
Characteristics which should be observed on a sample size of 2000 plants are indicated by a "B" in the table of characteristics

For hybrids, the number of off-types in a sample size of 200 plants should not exceed 27 in 200 (Population standard of 10% with an acceptance probability of ≥ 95%).

The number of off-types in a sample size of 100 ear-rows, plants or parts of plants should not exceed 3 in 100 (Population standard of 1% with an acceptance probability of ≥ 95%).

An ear row is considered as off-type if there is more than 1 off-type plant within that ear row.

For all varieties, except hybrid varieties, a re-submission of plant material may be allowed for the second growing cycle if in the first growing cycle the number of off-types did not exceed 18 plants in a sample size of 2000 plants (Population standard of 0.6% with an acceptance probability of ≥ 95%) or 9 plants, parts of plants or ear rows in a sample size of 100 (Population standard of 5% with an acceptance probability of ≥ 95%).

Characteristics which should be observed on a sample size of 100 plants are indicated by an "A" in the table of characteristics. For these "A" characteristics, with the exception of characteristics 1 and 24, the assessment of uniformity can be done in 2 steps. In a first step, 20 plants or parts of plants are observed. If no off-types are observed, the variety is declared to be uniform. If more than 3 off-types are observed, the variety is declared not to be uniform. If 1 to 3 off-types are observed, an additional sample of 80 plants or parts of plants must be observed.

With respect to the use of enzyme electrophoresis, the Office follows the actual UPOV approach as laid down in part I of Annex 3 hereto. If electrophoresis is used for testing distinctness, the same population standard and the same acceptance probability as for other characteristics should be applied. However, a sequential analysis approach could be applied to reduce the workload.

Electrophoresis characteristics with a lack of uniformity shall not be taken into account for the assessment of distinctness.

c) **Stability**

A candidate will be considered to be sufficiently stable when there is no evidence to indicate that it lacks uniformity.
Seed samples of further submissions included in any test must show the same expression of characteristics as the material originally supplied.

IV REPORTING OF RESULTS

After each recording season the results will be summarised and reported to the CPVO in the form of a UPOV model interim report in which any problems will be indicated under the headings distinctness, uniformity and stability. Candidates may meet the DUS standards after two growing cycles but in some cases three growing cycles may be required. When tests are completed the results will be sent by the Examination Office to the CPVO in the form of a UPOV model final report.

If it is considered that the candidate complies with the DUS standards, the final report will be accompanied by a variety description in the format recommended by UPOV. If not the reasons for failure and a summary of the test results will be included with the final report.

The CPVO must receive interim reports and final reports by the date agreed between the CPVO and the Examination Office.

Interim reports and final examination reports shall be signed by the responsible member of the staff of the Examination Office and shall expressly acknowledge the exclusive rights of disposal of CPVO.

V LIAISON WITH THE APPLICANT

If problems arise during the course of the test the CPVO should be informed so that the information can be passed on to the applicant. Subject to prior permanent agreement, the applicant may be directly informed at the same time as the CPVO particularly if a visit to the trial is advisable.

VI ENTRY INTO FORCE

The present protocol enters into force on **01.08.2010**. Any ongoing DUS examination of candidate varieties started before the aforesaid date will not be affected by the approval of the partially revised Technical Protocol. Technical examinations of candidate varieties are carried out according to the TP in force when the DUS test starts. The starting date of a DUS examination is considered to be the due date for submitting of plant material for the first test period.

In cases where the Office requests to take-over a DUS report for which the technical examination has either been finalized or which is in the process to be carried out at the moment of this request, such report can only be accepted if the technical examination has been carried out according to the CPVO TP which was in force at the moment when the technical examination started.

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VI TABLE OF CHARACTERISTICS TO BE USED IN DUS-TEST AND PREPARATION OF DESCRIPTION

CPVO N°	UPOV N°	Characteristics	Stage, [1] Method	Examples[2]	Note
1.	**1.**	**Coleoptile: anthocyanin coloration**	**09-11**		
(+)[3]		absent or very weak	**A; VG**	Herzog; Delos	1
		weak		Niklas; Baldus	3
		medium		Andros; Planet	5
		strong		Obelisk; Briscard	7
		very strong		Albatros; -	9
2.	**2.**	**Plant: growth habit**	**25-29**		
(+)		erect	**B; VG**	Castan; -	1
		semi-erect		Frandoc; Remus	3
		intermediate		Obelisk; Troll	5
		semi-prostrate		Boss; -	7
		prostrate		Beaver; -	9
3.	**4.**	**Plant: frequency of plants with recurved flag leaves**	**47-51**		
(+)		absent or very low	**B; VG**	Apollo	1
		low		Recital; Axona	3
		medium		Obelisk; Filou	5
		high		Frandoc; Prinqual	7
		very high		Capitole; -	9

[1] The optimum stage of development as well as method of observation for the assessment of each characteristic are indicated by numbers and letters. Explanations are given in Annex 1 in 'Explanations and Methods'.
[2] Example varieties, separated by a semicolon, are indicated for winter wheat before the semicolon, for spring wheat they follow the semicolon. Example varieties are given as an indication, others may be used.
[3] See explanations in Annex 1 in 'Explanations and Methods'.

CPVO N°	UPOV N°	Characteristics	Stage, [1] Method	Examples[2]	Note
4.	**5.**	**Time of ear emergence (first spikelet visible on 50% of ears)**	**50-52**		
		very early	**B; MG**	Britta; Florence Aurore	1
		early		Recital; Remus	3
		medium		Astron; Paros	5
		late		Moulin; Vitus	7
		very late		Beaver; -	9
5.	**6.**	**Flag leaf: glaucosity of sheath**	**60-65**		
(+)		absent or very weak	**B; VG**	Cargo; Adonis	1
		weak		Heiduck; Ventura	3
		medium		Agent; Hanno	5
		strong		Orestis; Prinqual	7
		very strong		Haven; Wim	9
6.		**Flag leaf: glaucosity of blade (lower side)**	**60-65**		
		absent or very weak	**A; VG**	Shamrock; -	1
		weak		Valoris; Josselin	3
		medium		Pauillac; Tecnico	5
		strong		Cezanne; Torka	7
		very strong		Charger;	9
7.	**7.**	**Ear: glaucosity**	**60-69**		
		absent or very weak	**B; VG**	Soissons; Adonis	1
		weak		Garant; Ventura	3
		medium		Contra; Paros	5
		strong		Niklas; Combi	7
		very strong		Boxer; Wim	9

CPVO N°	UPOV N°	Characteristics	Stage, [1] Method	Examples[2]	Note
8.	**8.**	**Culm: glaucosity of neck**	**60-69**		
		absent or very weak	**B; VG**	Goelent; Adonis	1
		weak		Soissons; Ventura	3
		medium		Haven; Attis	5
		strong		Herzog; Nandu	7
		very strong		Quotador; Wim	9
9.	**9.**	**Plant: length (stem, ear, awns and scurs)**	**75-92**		
		very short	**B; MG**	Courtot; Briscard	1
		short		Konsul; Remus	3
		medium		Sideral; Ventura	5
		long		Boxer; Adonis	7
		very long		Aladin; Vitus	9
10.	**10.**	**Straw: pith in cross section (halfway between base of ear and stem node below)**	**80-92**		
(+)		absent or very thin	**A; VG**	Boregar; SW Kadrilj	1
		medium		Provinciale; Tybalt	2
G		very thick or filled		Camp Remy; Azurite	3
11.	**11.**	**Ear: shape in profile**	**92**		
(+)		tapering	**A; VG**	Slejpner; Filou	1
		parallel sided		-; -	2
		semi-clavate		Pane 247; -	3
		clavate		Beauchamp; Prinqual	4
		fusiform		Declic; Nandu	5

CPVO N°	UPOV N°	Characteristics	Stage, [1] Method	Examples[2]	Note
12.	**12.**	**Ear: density**	**80-92**		
(+)		very lax	**A; VG**	Demar 4; -	1
		lax		Castan; Ventura	3
		medium		Soissons; Hanno	5
		dense		Forby; Combi	7
		very dense		-; -	9
13.	**13.**	**Ear: length (excluding awns and scurs)**	**80-92**		
(+)		very short	**A; MS**	-; -	1
		short		Carat; -	3
		medium		Ritmo; Arkas	5
		long		Forby; Prinqual	7
		very long		Amifort; -	9
14.	**14.**	**Awns or scurs: presence**	**80-92**		
(+)		both absent	**B; VG**	Futur; Axona	1
		scurs present		Festival; Furio	2
G		awns present		Soissons; Ventura	3
15.	**15.**	**Awns or scurs at tip of ear: length**	**80-92**		
		very short	**B; MS**	Herzog; -	1
		short		Andros; Combi	3
		medium		Pagode; Hanno	5
		long		Fidel; -	7
		very long		Gaucho; -	9
16.	**16.**	**Ear: colour**	**80-92**		
		white	**B; VG**	Herzog; Furio	1
G		coloured		Gallo; Prinqual	2

CPVO N°	UPOV N°	Characteristics	Stage, [1] Method	Examples[2]	Note
17.	**17.**	**Apical rachis segment: hairiness of convex surface**	**80-92**		
(+)		absent or very weak	**A; VG**	Soissons; -	1
		weak		Slejpner; Furio	3
		medium		Beaver; Rock	5
		strong		Apollo; Axona	7
		very strong		Carat; -	9
18.	**18.**	**Lower glume: shoulder width (spikelet in midthird of ear)**	**80-92**		
(+)		absent or very narrow	**A; VG**	Courtot; -	1
		narrow		Soissons; Wim	3
		medium		Sideral; Furio	5
		broad		Castan; Filou	7
		very broad		Abo	9
19.	**19.**	**Lower glume: shoulder shape (as for 18)**	**80-92**		
(+)		sloping	**A; VG**	Courtot; -	1
		slightly sloping		Forby; Ventura	3
		straight		Herzog; Prinqual	5
		elevated		Beaver; Adonis	7
		strongly elevated with 2nd point present		Farnese; -	9
20.	**20.**	**Lower glume: beak length (as for 18)**	**80-92**		
		very short	**A; VG**	Aladin; Sunnan	1
		short		Sideral; Axona	3
		medium		Recital; Furio	5
		long		Soissons; Tejo	7
		very long		Courtot; Prinqual	9

CPVO N°	UPOV N°	Characteristics	Stage, [1] Method	Examples[2]	Note
21.	**21.**	**Lower glume: beak shape (as for 18)**	**80-92**		
(+)		straight	**A; VG**	Festival; Lobo	1
		slightly curved		Slejpner; Furio	3
		moderately curved		Courtot; Rock	5
		strongly curved		Arum; -	7
		geniculate		-; -	9
22.	**22.**	**Lower glume: extent of internal hair (as for 18)**	**80-92**		
(+)		weak	**A; VG**	Slejpner; Prinqual	3
		medium		Sideral; Furio	5
		strong		Declic; Tejo	7
23.	**24.**	**Grain: colour**	**92**		
		white	**A; VG**	Recital; Florence Aurore	1
		red		Soissons; Ventura	2
24.	**25.**	**Grain: coloration with phenol**	**92**		
(+)		none or very light	**A; VG**	-; -	1
		light		Soissons; -	3
		medium		Orestis; Prinqual	5
		dark		Slejpner; Rock	7
		very dark		Sideral; Ventura	9
25.	**26.**	**Seasonal type**	**-**		
(+)		winter type	**B; VG**	Slejpner; -	1
		alternative type		Fidel; -	2
G		spring type		-; Nandu	3

ANNEXES TO FOLLOW

ANNEX I PAGE

ANNEX II

ANNEX III

ANNEX I

EXPLANATIONS AND METHODS

Method of observation of characteristics

Letters indicate the relevant method for the assessment of uniformity and distinctness

A	Sample size of 100 plants to be observed for the assessment of uniformity
B	Sample size of appr. 2000 plants to be observed in a plot for the assessment of uniformity
MG	Single measurement of a group of plants or parts of plants for the assessment of distinctness
MS	Measurement of a number of individual plants or parts of plants for the assessment of distinctness
VG	Visual assessment by a single observation of a group of plants or parts of plants for the assessment of distinctness
VS	Visual assessment by observation of individual plants or parts of plants for the assessment of distinctness

How to apply the above mentioned assessment methods in practice:

1. Assessment of uniformity

When attributing the letter A or B for the assessment of uniformity of a certain characteristic, the expert should address himself to single plants (A) or to all plants of the plot (B). The population standards as defined for observations made on either A or B need to be applied accordingly.

2. Assessment of distinctness

When a method of observation is attributed to a certain characteristic, the first differentiation is made depending if the action taken is a visual observation (V) or a measurement (M).

The second differentiation deals with the number of observations the expert attributes to each variety, thus the attribution of either G or S.
If a single observation of a group consisting of an undefined number of individual plants is appropriate to assess the expression of a variety, we talk about a visual observation or a measurement made on a group of plants, thus we attribute the letter G (either VG or MG). If the expert makes more than one observation on that group of plants, the decisive part is that we have at the end only one data entry per variety which means that we have to deal with G (e.g. measurement of plant length on a plot – MG, visual observation of green colour of leaves on a plot – VG).

If it is necessary to observe a number of individual plants to assess the expression of a variety, we should attribute the letter S (thus either VS or MS). Single plant data entries are kept per variety for further calculations like the variety mean (e.g. measurement of length of ears – MS, visual observation of growth habit of single plants in grasses – VS). The number of individual plants to be observed in such cases is stated in section III.5.

Ad 1: Coleoptile: anthocyanin coloration

Method for the Determination of Anthocyanin Coloration

Number of grains per test ..... 100 grains

Preparation of grains ..... Set up non-dormant grains on moistened filter paper covered with a Petri dish lid during germination.

Place ..... Laboratory or greenhouse.

Light ..... After the coleoptiles have reached a length of about 1 cm in darkness, they are placed in artificial light (daylight equivalent), at 15,000 lux continuously for 3 - 4 days.

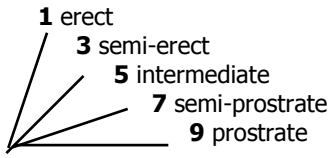
Temperature ..... 15 to 20°C.

Time of recording ..... Coleoptiles fully developed (about 1 week) at stage 09-11.

Scale of recording ..... See characteristic 1.

Note ..... At least two of the example varieties should be included as a control when testing for distinctness.

Ad 2: Plant: growth habit



The growth habit should be assessed visually from the attitude of the leaves and tillers. The angle formed by the outer leaves and the tillers with an imaginary vertical axis should be used.

Ad 3: Plant: frequency of plants with recurved flag leaves

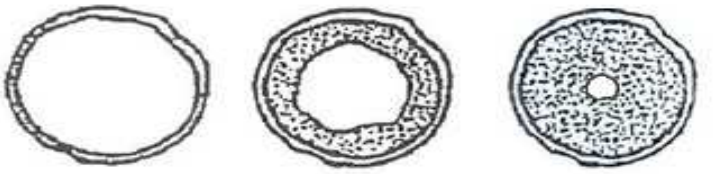
1. ..... all flag leaves are rectilinear
3. .....about 1/4 of the plants with recurved flag leaves
5. .....about 1/2 of the plants with recurved flag leaves
7. .....about 3/4 of the plants with recurved flag leaves
9. .....all flag leaves are recurved

Ad 5: Flag leaf: glucosity of sheath

The strongest expression on the sheath should be observed.

Ad 10: Straw: pith in cross section (half way between base of ear and stem node below)

All stems of the plant should be checked and the strongest expression per plant recorded.

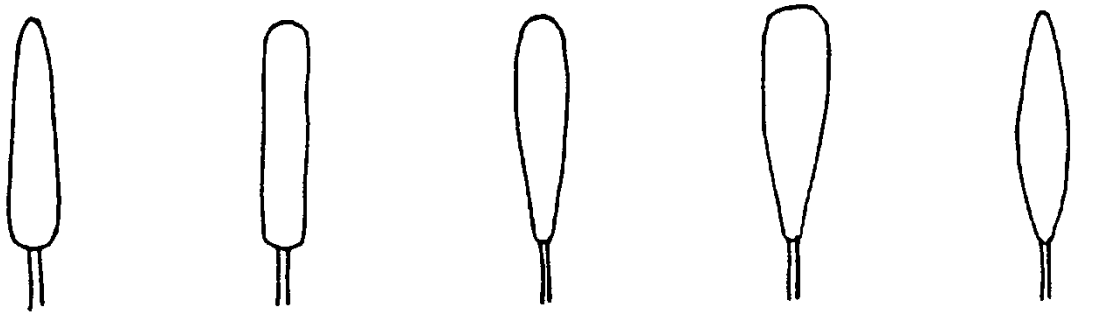


1
absent or very thin

2
medium

3
very thick or filled

Ad 11: Ear: shape in profile



1
tapering

2
parallel-sided

3
semi-clavate

4
clavate

5
fusiform

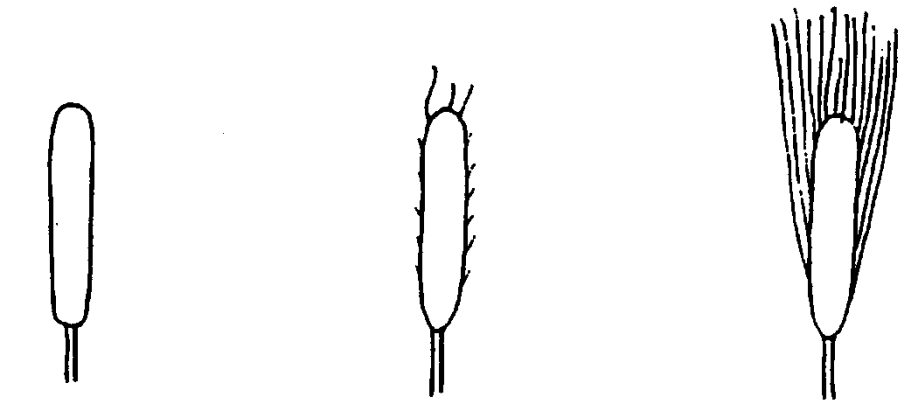
Ad 12: Ear: density

The density can be assessed either visually or as measurement of the ratio of the number of spikelets/ear length.

Ad 13: Ear: length (excluding awns and scurs)

Single ear of the main stem to be measured.

Ad 14: Awns or scurs: presence

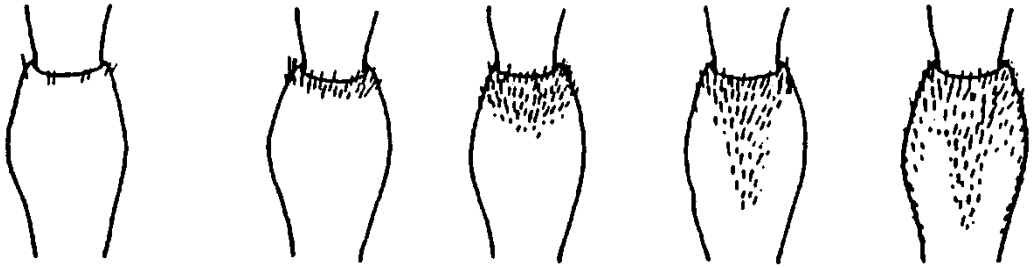


1
both absent

2
scurs present

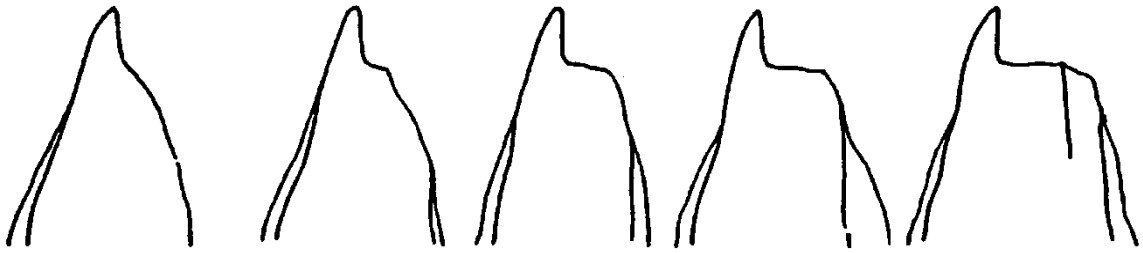
3
awns present

Ad 17: Apical rachis segment: hairiness of convex surface



1 absent or very weak | 3 weak | 5 medium | 7 strong | 9 very strong

Ad 18: Lower glume: shoulder width (spikelet in mid-third of ear)



1 absent or very narrow | 3 narrow | 5 medium | 7 broad | 9 very broad

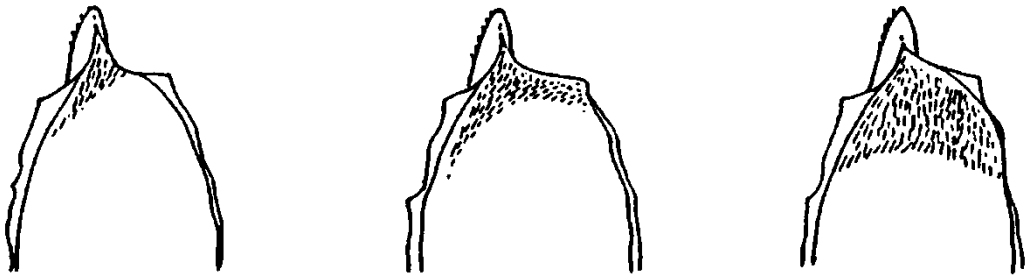
Ad 19: Lower glume: shoulder shape (spikelet in mid-third of ear)

1 sloping | 3 slightly sloping | 5 straight | 7 elevated | 9 strongly elevated with 2nd point present

Ad 21: Lower glume: beak shape (spikelet in mid-third of ear)

1 straight | 3 slightly curved | 5 moderately curved | 7 strongly curved | 9 geniculate

Ad 22: Lower glume: extent of internal hairs (spikelet in mid-third of ear)



3
weak

5
medium

7
strong

Ad 24: Grain: coloration with phenol

Method for Determination of Phenol Reaction

Number of grains per test ..... 100 grains. The grains should not have been treated chemically

Preparation of grains ..... Soak in tap water for 16 to 20 hours, drain and remove surface water, place the grains with crease downwards, cover dish with lid

Concentration of solution ..... 1 per cent Phenol-solution (freshly made up)

Amount of solution ..... The grains should be about 3/4 covered

Place ..... Laboratory

Light ..... Daylight - out of direct sunshine

Temperature ..... 18 to 20°C

Time of recording ..... 4 hours (after adding solution)

Scale of recording ..... See characteristic 25 in the Table of Characteristics

Note ..... At least two of the example varieties should be included as a control

Ad 25: Seasonal type

The seasonal type should be assessed on one or several plots sown in springtime. Example varieties should always be included in the plots. When the example varieties behave according to this description, the varieties under study can be described. At the time when the latest spring type variety is fully mature (stage 91/92 of the Eucarpia decimal code), the growth stage reached by the respective variety should be assessed. The states of expression are defined as follows:

Winter type: ..... The plants have reached stage 45 of the Eucarpia decimal code (boots swollen) at maximum.

Alternative type: ..... The plants have exceeded stage 45 of the Eucarpia decimal code---as a rule they have exceeded stage 75---and have reached stage 90 at maximum.

Spring type: ..... The plants have exceeded stage 90 of the Eucarpia decimal code.

DECIMAL CODE FOR THE GROWTH STAGE[4]

2- digit Code	General description	Feekes' Scale	Additional remarks on Wheat, Barley, Rye, Oats and Rice
	Germination		
00	Dry seed		
01	Start of imbibition		
02	-		
03	Imbibition complete		
04	-		
05	Radicle emerged from caryopsis		
06	-		
07	Coleoptile emerged from caryopsis		
08	-		
09	Leaf just at coleoptile tip		
	Seedling growth		
10	First leaf through coleoptile	1 - Second leaf visible (less than 1 cm)	
11	First leaf unfolded (1)		
12	2 leaves unfolded	50% of laminae unfolded	
13	3 leaves unfolded		
14	4 leaves unfolded		
15	5 leaves unfolded		
16	6 leaves unfolded		
17	7 leaves unfolded		
18	8 leaves unfolded		
19	9 or more leaves unfolded		
	Tillering		
20	Main shoot only		
21	Main shoot and 1 tiller	2	This section to be used to supplement records from other sections of the table: "Concurrent codes".
22	Main shoot and 2 tillers		
23	Main shoot and 3 tillers		
24	Main shoot and 4 tillers		
25	Main shoot and 5 tillers	3	
26	Main shoot and 6 tillers		
27	Main shoot and 7 tillers		
28	Main shoot and 8 tillers		
29	Main shoot and 9 or more tillers		
	Stem elongation		
30	Pseudo stem erection (2)	4-5	In rice: vegetative lag phase
31	1st node detectable	6	Jointing stage
32	2nd node detectable	7	
33	3rd node detectable		Above crown nodes
34	4th node detectable		
35	5th node detectable		
36	6th node detectable		
37	Flag leaf just visible	8	
38	-		Pre-boot stage
39	Flag leaf ligule / collar just visible	9	In rice: Opposite auricle

[4] Reproduced from EUCARPIA Bulletin No. 7, 1974, pp.49 - 52, with the kind permission of the authors. For further information, see J.C. Zadoks, T.T. Chang and C.F. Konzak, EUCARPIA Bulletin No. 7, 1974, pp. 42 - 52. The French translation has been kindly furnished by Mrs. R. Cassini, Mr. R. Cassini and Mr. R. Marie. The German translation has been kindly furnished by Mr. A.O. Klomp and Mrs. I. Volk.

2- digit Code	General description	Feekes' Scale		Additional remarks on Wheat, Barley, Rye, Oats and Rice
	Booting			Little enlargement of the
40	-			inflorescence, early-boot stage
41	Flag leaf sheath extending			
42	-			
43	Boots just visibly swollen			
44	-			Mid-boot stage
45	Boots swollen	10		Late-boot stage
46	-			
47	Flag leaf sheath opening			
48	-			
49	First awns visible		10.1	In awned forms only
	Inflorescence emergence			
50	First spikelet of inflorescence just	N		
51	visible	S		
52	1/4 of inflorescence emerged	N	10.2	N = non-synchronous crops
53		S		S = synchronous crops
54	1/2 of inflorescence emerged	N	10.3	
55		S		
56	3/4 of inflorescence emerged	N	10.4	
57		S		
58	Emergence of inflorescence	N	10.5	
59	completed	S		
	Anthesis			
60	Beginning of anthesis	N	10.51	Not easily detectable in barley.
61		S		In rice: usually immediately
62	-			following heading
63	-			
64	Anthesis half-way	N	10.52	
65		S		
66	-			
67	-			
68	Anthesis complete	N	10.53	
69		S		
	Milk development			
70	-			
71	Caryopsis watery ripe		10.54	
72	-			
73	Early milk			Increase in solids of liquid
74	-		11.1	endosperm notable when
75	medium milk			crushing the caryopsis between
76	-			fingers.
77	Late milk			
78	-			
79	-			
	Dough development			
80	-			
81	-			
82	-			
83	Early dough			
84	-			
85	Soft dough		11.2	Fingernail impression not held.
86	-			
87	Hard dough			Fingernail impression held,
88	-			inflorescence losing chlorophyll.
89	-			

2- digit Code	General description	Feekes' Scale	Additional remarks on Wheat, Barley, Rye, Oats and Rice
	Ripening		
90	-		In rice: terminal spikelets ripened.
91	Caryopsis hard (difficult to divide by thumb-nail) (3)	11.3	
92	Caryopsis hard (can no longer be dented by thumb-nail) (4)	11.4	In rice: 50% of spikelets ripened
93	Caryopsis loosening in daytime		In rice: over 90% of spikelets ripened
94	Over-ripe, straw dead and collapsing		
95	Seed dormant		Risk of grain loss by shedding
96	Viable seed giving 50% germination		
97	Seed not dormant		
98	Secondary dormancy induced		
99	Secondary dormancy lost		
	Transplanting and recovery (rice only)		
T1	Uprooting of seedlings		
T2	-		
T3	Rooting		
T4	-		
T5	-		
T6	-		
T7	Recovery of shoots		
T8	-		
T9	Resumption of vegetative growth		

Notes on the Table of the Decimal Code for the Growth Stages or Cereals

(1) Stage of seedling inoculation with rust in the greenhouse.

(2) Only applicable to cereals with a prostrate or semi-prostrate early growth habit.

(3) Ripeness for binder (ca. 16% water content). Chlorophyll of inflorescence largely lost.

(4) Ripeness for combine harvester (less than 16% water content).

(5) Optimum harvest time.

ANNEX II

ELECTROPHORESIS

Additional Useful Explanations

TABLE OF CONTENTS PAGE

Part I

Introduction

The following Annex contains a list of characteristics derived by using 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 member States is of the view that it is not possible to establish distinctness solely on the basis of a difference found in a characteristic derived by using electrophoresis. Such characteristics should therefore only be used as a complement to other differences in morphological or physiological characteristics. UPOV has reconfirmed 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 high molecular weight (HMW) glutenins, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS PAGE) should be used. Glutenins are encoded by three compound loci, known as Glu-A1, Glu-B1 and Glu-D1 on the long arms of the group 1 chromosomes (Payne, 1987). There are a number of alleles at each locus and the analysis of HMW glutenins 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 alleles are described by band numbers according to the definition given to them by Payne and Lawrence, 1983 (see Chapter IX, Literature). The corresponding letters and apparent molecular weights are reproduced in the description of the method used.

Part II

Characteristics Derived by Using Electrophoresis

	Characteristics	Stage[1)]		Example Varieties	Note
27. (+)	Glutenin composition: allele expression at locus Glu-A1				
			band 1	Kadett	1
			band 2*	Courtot	2
			no band	Talent	3
28. (+)	Glutenin composition: allele expression at locus Glu-B1				
			bands 6 + 8	Norman	1
			bands 7 + 8	Courtot	2
			bands 7 + 9	Kadett	3
			band 7 (or 7 + 9 in the presence of bands 5 + 10 of char. 29)	Okapi	4
			bands 13 + 16	Carala	5
			bands 14 + 15	Troll	6
			bands 17 + 18	Moulin	7
			band 20	Figaro	8
			bands 6.1 + 22	Schwabenkorn	9
29. (+)	Glutenin composition: allele expression at locus Glu-D1				
			bands 2 + 12	Courtot	1
			bands 3 + 12	Norman	2
			bands 4 + 12	Talent	3
			bands 5 + 10	Kadett	4

Part III

Description of the Method to be Used

Glutenin composition: allele expression at loci Glu-A1 (27), Glu-B1 (28) and Glu-D1 (29)
SDS PAGE Method for Analysis of HMW Glutenins from T. aestivum

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.

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 Y (or G)
Glycerol (d = 1.256)
Methanol or ethanol
Coomassie Brilliant Blue R-250 (or equivalent)
Coomassie Brilliant Blue G-250 (or equivalent)

3. Solutions
3.1 Extraction solution
3.1.1 Extraction of glutenins only

Stock solution:
6.25 ml 1M TRIS HCl buffer, PH 6.8 (see 3.3.2)
12.05 ml distilled water
2g SDS
10 mg Pyronin Y (or G)
10 ml glycerol
This solution can be stored for two months at 4°C.
Immediately before use, extraction solution is prepared as follows:
4.25 ml stock solution (above) plus 0.75 ml 2-mercaptoethanol made up to 10.0 ml with distilled water. This solution must be prepared immediately prior to use and cannot be stored.

3.1.2 Extraction of glutenins following gliadins

Solution A - 25 ml 2 - chloroethanol + 50 mg Pyronin Y/G, made up to 100 ml with distilled water.
Solution B - 27.0 g urea, 3.0 ml 2 - mercaptoethanol + 10.0 g SDS, made up to 100 ml with distilled water.

3.2 Electrophoresis (running) buffer

Stock solution:

141.1 g glycine
30.0 g TRIS
10.0 g SDS
made up to 1 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.

3.3 Gel preparation solutions

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 l with distilled water. This buffer can be stored at 4°C for 2 months.

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 l with distilled water. This buffer can be stored at 4°C for 2 months.

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.

3.3.4 1% (w/v) ammonium persulphate solution

1g of APS dissolved in distilled water and made up to 100 ml. This solution must be prepared immediately prior to use.

3.3.5 Stock acrylamide solution

40.02g acrylamide made up to 100 ml with distilled water.

3.3.6 Stock bisacrylamide solution

0.5198g bisacrylamide made up to 130 ml with distilled water.

3.4 Staining solutions

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.

3.4.2 55g TCA, 65 ml glacial acetic acid, 180 ml methanol or ethanol plus 25 ml solution 3.4.1, made up to 11 with distilled water.

4. Procedure

4.1 Protein extraction

4.1.1 Glutenins only

Individual seeds are ground using a hammer (or other device). Ground seed meal is mixed with diluted sample extraction buffer (3.1.1) in a 3 ml polypropylene hemolyse or similar tube with a screw-on or fitted 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 18000g for 5 minutes.

4.1.2 Glutenins following gliadins

If desired, glutenins and gliadins can be analyzed from the same grain. Gliadins are extracted first by adding 0.25 ml of Solution A (3.1.2) to a crushed grain (or half-grain) in a microtiter plate or micro-centrifuge tube and incubating overnight at room temperature. Following this, glutenins are extracted by adding 0.5 ml of Solution B (3.1.2) to the crushed grain and incubating overnight at room temperature.

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.

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.

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 (3.3.5)
- 26 ml stock bisacrylamide solution (3.3.6),
- 30 ml stock gel buffer (3.3.1).

These should be at room temperature. The mixture is degassed in a 100 ml Büchner flask for 2 - 3 minutes. To this is added:

2 ml APS (3.3.4),
0.8 ml SDS (3.3.3),
40 μl TEMED (use straight from bottle).

The gels are then carefully poured, avoiding the formation of air bubbles, and polymerization 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 polymerization is finished (about 30 min.), the gel surface is carefully rinsed with distilled water and dried with filter paper.

4.2.2 Resolving (main) gel (7% acrylamide, pH 8.8)

To resolve the sub-units 2 and 2*, it is necessary to use main gels of 7% acrylamide concentration.

To make two slab gels of 180 x 160 x 1.5 mm, the following is required:

- 14 ml stock acrylamide solution (3.3.5)
- 6 ml distilled water
- 26 ml stock bisacrylamide solution (3.3.6),
- 30 ml stock gel buffer (3.3.1).

These should be at room temperature. The mixture is de-gassed in a 100 ml Büchner flask for 2 - 3 minutes. To this is added:

- 2 ml APS (3.3.4),
- 0.8 ml SDS (3.3.3),
- 40 μ TEMED (use straight from bottle).

The gels are then carefully poured, avoiding the formation of air bubbles, and polymerization 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 polymerization is finished (about 30 min.), the gel surface is carefully rinsed with distilled water and dried with filter paper.

4.2.3 Stacking gel (3% acrylamide, pH 6.8)

In a 50 ml Büchner flask, mix:

- 1.50 ml stock acrylamide solution (3.3.5),
- 2.15 ml stock bisacrylamide solution (3.3.6)
- 2.50 ml stock gel buffer (3.3.2) and
- 13.15 ml distilled water.

Following de-gassing add:

- 0.75 ml APS (3.3.4),
- 0.2 ml SDS (3.3.3),
- 15 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 polymerize for about 2 hours at room temperature. The "combs" are then removed carefully from the gel cassettes and the wells rinsed using diluted electrophoresis running buffer (3.2).

4.3 Electrophoresis

The tank is filled with the appropriate volume of running buffer (3.2), cooled to 15°C. Following sample loading, electrophoresis is carried out at a constant current of 8 mA/cm^2 (cross-sectional area) of gel until the pyronin Y/G has moved through the stacking gel, and then at 16 mA/cm^2 of gel (maximum voltage 300V) until the marker is at the bottom of the gel. The temperature should be maintained at 15°C.

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 (3.4.2) at room temperature. Destaining 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.

Recognition of Glutenin Alleles

This Table is designed to illustrate the alleles described above and to assist in the recognition of the different bands. It depicts the position and molecular weight of all of the glutenin bands from each locus, compared to those found in the Example Variety Courtot, along with the band numbers using the nomenclature of Payne; the letter given to each allele following Payne and Lawrence (1983) is also given.

Sub-Units of HMW Glutenins: nomenclature of the individual bands and recognition of the corresponding alleles

Characteristic 27: **Glu-A1** locus

		Example Variety (Courtot)	**Note**		
			1 (a)	2 (b)	3 (c)
1	(113)---		1---		
2/2*	(108)---	2/2*---		2*---	n (no band)
3	(107)---				
4	(106)---				
5	(105)---				
6	(100)---				
6.1	(99)---				
7	(98)---	7 ---			
13/14/ 20	(94)---				
15	(91)---				
16/ 17/18	(90/--- 89.5)				
22	(87)---				
8	(86)---	8 ---			
9/10	(83)---				
12	(80)---	12 ---			

Characteristic 28: **Glu-B1** locus

		Example variety (Courtot)	**Note**								
			1 (d)	2 (b)	3 (c)	4 (a)	5 (f)	6 (h)	7 (i)	8 (e)	9
1	(113)---										
2/2*	(108)---	2/2*---									
3	(107)---										
4	(106)---										
5	(105)---										
6	(100)---		6---								
6.1	(99)---										6.1---
7	(98)---	7 ---		7---	7---	7---					
13/14/ 20	(94)---						13---	14---		20---	
15	(91)---							15---			
16/ 17/18	(90/--- 89.5)						16---		17/18---		
22	(87)---										22---
8	(86)---	8 ---	8---	8---							
9/10	(83)---				9---						
12	(80)---	12 ---									

Characteristic 29: **Glu-Dl** locus

		Example variety (Courtot)	**Note**					
			1 (a)		2 (b)	3 (c)	4 (d)	
1	(113)---							
2/2*	(108)---	2/2*---	2---					
3	(107)---				3---			
4	(106)---					4---		
5	(105)---						5---	
6	(100)---							
6.1	(99)---							
7	(98)---	7 ---						
13/14/ 20	(94)---							
15	(91)---							
16/ 17/18	(90/--- 89.5)							
22	(87)---							
8	(86)---	8 ---						
9/10	(83)---						10---	
12	(80)---	12 ---	12---	12---	12---			

Note: Certain bands (e.g. bands 9 and 10) have similar molecular weights. This leads to the fact that in the presence of bands 5 + 10 of characteristic 29 two states of expression of characteristic 28, band 7 and bands 7 + 9, cannot be differentiated from one another. Therefore, in the presence of bands 5 + 10 of characteristic 29, Note 4 of characteristic 28 could be either band 7 or bands 7 + 9. Other bands having similar molecular weights can be differentiated from one another by their known association with other bands. For characteristic 28, band 13 is always associated with band 16 and band 14 with band 15 while band 40 remains alone.

ANNEX III

The Technical Questionnaire is available on the CPVO website under the following reference: CPVO-TQ/003/4 Rev.2