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

PROTOCOL FOR TESTS ON DISTINCTNESS, UNIFORMITY AND STABILITY

Botanical name	English name
Lolium perenne L.	Perennial ryegrass
Lolium multiflorum Lam. spp. *italicum* (A. Br.) Vokart; *Lolium multiflorum* Lam. ssp. *non alternativum*	Italian ryegrass
Lolium multiflorum Lam. var. *westerwoldicum* Wittmt; *Lolium multiflorum* Lam. ssp. *alternativum*	Westerwolds ryegrass
Lolium boucheanum Kunth ; *Lolium* x *hybridum* Hausskn.	Hybrid ryegrass
Lolium rigidum Gaudin.	Stiff darnel, Wimmera ryegrass

UPOV Code:
LOLIU_PER; LOLIU_MUL_ITA; LOLIU_MUL_WES; LOLIU_BOU; LOLIU_RIG

Adopted on 19/03/2019

Entry into force on 01/04/2019

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CPVO-TP/004/2

1. SUBJECT OF THE PROTOCOL AND REPORTING

1.1 Scope of the technical protocol

This Technical Protocol applies to all varieties of Lolium perenne L., Lolium multiflorum Lam.ssp. italicum (A. Br.) Volkart, Lolium multiflorum Lam. var. westerwoldicum, Lolium boucheanum Kunth. and Lolium rigidum Gaudin.

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 documents agreed by the International Union for the Protection of New Varieties of Plants (UPOV), such as the General Introduction to DUS (UPOV Document TG/1/3 http://www.upov.int/export/sites/upov/resource/en/tg_1_3.pdf), its associated TGP documents (http://www.upov.int/tgp/en/) and the relevant UPOV Test Guideline TG/4/8 dated 05/04/2006 (https://www.upov.int/edocs/tgdocs/en/tg004.pdf) for the conduct of tests for Distinctness, Uniformity and Stability.

1.2 Entry into Force

The present protocol enters into force on 01.04.2019. Any ongoing DUS examination of candidate varieties started before the aforesaid date will not be affected by the approval of the 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.

1.3 Reporting between Examination Office and CPVO and Liaison with Applicant

1.3.1 Reporting between Examination Office and CPVO

The Examination Office shall deliver to the CPVO a preliminary report ("the preliminary report") no later than two weeks after the date of the request for technical examination by the CPVO.

The Examination Office shall also deliver to the CPVO a report relating to each growing period ("the interim report") and, when the Examination Office considers the results of the technical examination to be adequate to evaluate the variety or the CPVO so requests, a report relating to the examination ("the final report").

The final report shall state the opinion of the Examination Office on the distinctness, uniformity and stability of the variety. Where it considers those criteria to be satisfied, or where the CPVO so requests, a description of the variety shall be added to the report.

If a report is negative the Examination Office shall set out the detailed reasons for its findings.

The interim and the final reports shall be delivered to the CPVO as soon as possible and no later than on the deadlines as laid down in the designation agreement.

1.3.2 Informing on problems in the DUS test

If problems arise during the course of the test the CPVO should be informed immediately 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.

1.3.3 Sample keeping in case of problems

If the technical examination has resulted in a negative report, the CPVO shall inform the Examination Office as soon as possible in case that a representative sample of any relevant testing material shall be kept.

2. MATERIAL REQUIRED

2.1 Plant material requirements

Information with respect to the agreed closing dates and submission requirements of plant material for the technical examination of varieties can be found on http://cpvo.europa.eu/applications-and-examinations/technical-examinations/submission-of-plant-material-s2-publication in the special issue S2 of the Official Gazette of the Office. General requirements on submission of samples are also to be found following the same link.

2.2 Informing the applicant of plant material requirements

The CPVO informs the applicant that

- he is responsible for ensuring compliance with any customs and plant health requirements.
- the plant material supplied should be visibly healthy, not lacking in vigor, nor affected by any important pest or disease.
- the plant material should not have undergone any treatment which would affect the expression of the characteristics of the variety, unless the competent authorities allow or request such treatment. If it has been treated, full details of the treatment must be given.

2.3 Informing about problems on the submission of material

The Examination Office shall report to the CPVO immediately in cases where the test material of the candidate variety has not arrived in time or in cases where the material submitted does not fulfil the conditions laid down in the request for material issued by the CPVO.

In cases where the examination office encounters difficulties to obtain plant material of reference varieties the CPVO should be informed.

3. METHOD OF EXAMINATION

3.1 Number of growing cycles

The minimum duration of tests should normally be two independent growing cycles. The two independent growing cycles should be in the form of separate plantings.

3.2 Testing Place

Tests are normally conducted at one place. In the case of tests conducted at more than one place, guidance is provided in TGP/9 "Examining Distinctness" http://www.upov.int/edocs/tgpdocs/en/tgp_9.pdf.

3.3 Conditions for Conducting the Examination

The tests should be carried out under conditions ensuring satisfactory growth for the expression of the relevant characteristics of the variety and for the conduct of the examination.

The optimum stage of development for the assessment of each characteristic is indicated by a number in the third column of the Table of Characteristics. The stages of development denoted by each number are described in Chapter 8.2.

The recommended type of plot in which to observe the characteristic is indicated by the following key in the second column of the Table of Characteristics:

A: spaced plants
B: row plot
C: greenhouse test

If more than one type of plot is indicated for a specific characteristic, the examination office has to choose the most appropriate plot type under its conditions. The characteristic should not be assessed twice.

3.4 Test design

As a minimum, each test should include at least:
60 spaced plants which should be divided between at least 3 replicates.

In addition the test may include 8 meters of row plot which should be divided between at least 2 replicates. The density of the seed should be such that around 200 plants/meter can be expected and the satisfactory establishment should be checked shortly after emergence.

Unless otherwise indicated, all observations on single plants should be made on 60 plants or parts taken from each of 60 plants and any other observations made on all plants in the test. In the case of observations of parts taken from single plants, the number of parts to be taken from each of the plants should be 1.

The design of the test should 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.

3.6 Special tests for additional characteritics

In accordance with Article 23 of Implementing Rules N° 874/2009 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.

3.7 Constitution and maintenance of a variety collection

The process for the constitution and the maintenance of a variety collection can be summarized as follows:

Step 1: Making an inventory of the varieties of common knowledge

Step 2: Establishing a collection ("variety collection") of varieties of common knowledge which are relevant for the examination of distinctness of candidate varieties

Step 3: Selecting the varieties from the variety collection which need to be included in the growing trial or other tests for the examination of distinctness of a particular candidate variety.

Forms of variety collection
The variety collection shall comprise variety descriptions and living plant material, thus a living reference collection. The variety description shall be produced by the EO unless special cooperation exists between EOs and the CPVO. The descriptive and pictorial information produced by the EO shall be held and maintained in a form of a database.

Living Plant Material
The EO shall collect and maintain living plant material of varieties of the species concerned in the variety collection.

Range of the variety collection
The living variety collection shall cover at least those varieties that are suitable to climatic conditions of a respective EO.

Making an inventory of varieties of common knowledge for inclusion in the variety collection
The inventory shall include varieties protected under National PBR (UPOV contracting parties) and Community PBR, varieties registered in the Common Catalogue, the OECD list, the Conservation variety list and varieties in trade or in commercial registers for those species not covered by a National or the Common Catalogue.

Maintenance and renewal/update of a living variety collection
The EO shall maintain seeds in conditions which will ensure germination and viability, periodical checks, and renewal as required. For the renewal of existing living material the identity of replacement living plant material shall be verified by conducting side-by-side plot comparisons between the material in the collection and the new material.

4. ASSESSMENT OF DISTINCTNESS, UNIFORMITY AND STABILITY

The prescribed procedure is to assess distinctness, uniformity and stability in a growing trial.

4.1 Distinctness

General recommendations
It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 9 'Examining Distinctness' (http://www.upov.int/edocs/tgpdocs/en/tgp_9.pdf° prior to making decisions regarding distinctness. However, the following points are provided for elaboration or emphasis in this Technical Protocol.

Consistent differences
The differences observed between varieties may be so clear that more than one growing cycle is not necessary. In addition, in some circumstances, the influence of the environment is not such that more than a single growing cycle is required to provide assurance that the differences observed between varieties are sufficiently consistent. One means of ensuring that a difference in a characteristic, observed in a growing trial, is sufficiently consistent is to examine the characteristic in at least two independent growing cycles.

Clear differences
Determining whether a difference between two varieties is clear depends on many factors, and should consider, in particular, the type of expression of the characteristic being examined, i.e. whether it is expressed in a qualitative, quantitative, or pseudo-qualitative manner. Therefore, it is important that users of these Technical Protocols are familiar with the recommendations contained in the UPOV-General Introduction to DUS prior to making decisions regarding distinctness.

Decision standards

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 ($p<0.01$) in a test over either two or three years.

If the conditions for the application of the COYD analyses are not fulfilled, distinctness should be assessed using the 2x1% method.

If distinctness is assessed using the 2 x 1% criterion, the varieties need to be significantly different in the same direction at the 1% level in at least two out of three years in one or more measured characteristics. The tests in each year are based on Student's two-tiled t-test of the differences between variety means with standard errors estimated using the residual mean square from the analysis of the variety x replicate plot means.

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

Electrophoresis
In *Lolium perenne* L., the composition of seed proteins can be used as additional characteristic for establishing distinctness, uniformity and stability on request of the applicant under the following conditions:

- in case of distinctness assessment with COYD, if the difference is significant on a level between 1 % and 5 % for at least one of the characteristics mentioned in chapter 7,
- in case of distinctness assessment on the basis of notes, if the difference is at least 1 note in 2 out of 3 years.

Distinctness between two varieties must not be established on the basis of seed protein polymorphisms alone. If electrophoresis is used for testing of distinctness, the same population standard and the same acceptance probability as for other characteristics should be applied for the assessment of uniformity.

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

Number of plants/parts of plants to be examined
Unless otherwise indicated, for the purposes of distinctness, all observations on single plants should be made on 20 plants or parts taken from each of 20 plants and any other observations made on all plants in the test, disregarding any off-type plants.

In the case of observations of parts taken from single plants, the number of parts to be taken from each of the plants should be 1.

Method of observation
The recommended method of observing the characteristic for the purposes of distinctness is indicated by the following key in the third column of the Table of Characteristics (see document TGP/9 "Examining Distinctness", Section 4 "Observation of characteristics"):

MG: single measurement of a group of plants or parts of plants
MS: measurement of a number of individual plants or parts of plants
VG: visual assessment by a single observation of a group of plants or parts of plants
VS: visual assessment by observation of individual plants or parts of plants

Type of observation: visual (V) or measurement (M)
"Visual" observation (V) is an observation made on the basis of the expert's judgment. For the purposes of this document, "visual" observation refers to the sensory observations of the experts and, therefore, also includes smell, taste and touch. Visual observation includes observations where the expert uses reference points (e.g. diagrams, example varieties, side-by-side comparison) or non-linear charts (e.g. color charts). Measurement (M) is an objective observation against a calibrated, linear scale e.g. using a ruler, weighing scales, colorimeter, dates, counts, etc.

Type of record: for a group of plants (G) or for single, individual plants (S)
For the purposes of distinctness, observations may be recorded as a single record for a group of plants or parts of plants (G), or may be recorded as records for a number of single, individual plants or parts of plants (S). In most cases, "G" provides a single record per variety and it is not possible or necessary to apply statistical methods in a plant-by-plant analysis for the assessment of distinctness.

In cases where more than one method of observing the characteristic is indicated in the Table of Characteristics (e.g. VG/MG), guidance on selecting an appropriate method is provided in document TGP/9, Section 4.2.

4.2 Uniformity

It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 10 'Examining Uniformity' (http://www.upov.int/edocs/tgpdocs/en/tgp_10.pdf) prior to making decisions regarding uniformity. However, the following points are provided for elaboration or emphasis in this Technical Protocol:

This Technical Protocol has been developed for the examination of seed propagated varieties. For varieties with other types of propagation the recommendations in the UPOV-General Introduction to DUS and document TGP/13 "Guidance for new types and species", Section 4.5 "Testing Uniformity" should be followed.

If uniformity is assessed by the combined over years uniformity method (COYU) the candidate variety is sufficiently uniform in the respective characteristic if the relative tolerance limit in relation to comparable varieties does not exceed the 1% significance level or less ($p<0.01$) in a test over two years.

If uniformity is assessed by the combined over years uniformity method (COYU) the candidate variety is sufficiently uniform in the respective characteristic if the relative tolerance limit in relation to comparable varieties does not exceed the 0.1% significance level or less ($p<0.001$) in a test over three years.

If the conditions of the COYU analyses are not fulfilled, uniformity should be assessed by the relative variance method for a sample size of 60 plants the threshold level should be 1.6 x variance of the comparable varieties.

4.3 Stability

It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 11 'Examining Stability' (http://www.upov.int/edocs/tgpdocs/en/tgp_11.pdf).

In practice, it is not usual to perform tests of stability that produce results as certain as those of the testing of distinctness and uniformity. However, experience has demonstrated that, for many types of variety, when a variety has been shown to be uniform, it can also be considered to be stable.

Where appropriate, or in cases of doubt, stability may be further examined by testing a new seed stock to ensure that it exhibits the same characteristics as those shown by the initial material supplied.

5. GROUPING OF VARIETIES AND ORGANISATION OF THE GROWING TRIAL

5.1 The selection of varieties of common knowledge to be grown in the trial with the candidate varieties and the way in which these varieties are divided into groups to facilitate the assessment of distinctness are aided by the use of grouping characteristics.

5.2 Grouping characteristics are those in which the documented states of expression, even where produced at different locations, can be used, either individually or in combination with other such characteristics: (a) to select varieties of common knowledge that can be excluded from the growing trial used for examination of distinctness; and (b) to organise the growing trial so that similar varieties are grouped together.

5.3 The following have been agreed as useful grouping characteristics.

Lolium multiflorum Lam. var. westerwoldicum (Lmw) and Lolium rigidum Gaudin (Lr).:

(a) Plant: ploidy (characteristic 1)
(b) Only varieties of Lmw and Lr: Plant: time of inflorescence emergence (without vernalization) (characteristic 8)
(c) Plant: length of longest stem, inflorescence included (when fully expanded) (characteristic 16)

Lolium perenne L.(Lp), Lolium multiflorum Lam. ssp. italicum (A. Br.) Volkart (Lmi) and Lolium boucheanum Kunth (Lb)

(a) Plant: ploidy (characteristic 1)
(b) Only varieties of Lp, Lmi and Lb: Plant: time of inflorescence emergence (after vernalization) (characteristic 10)
(c) Plant: length of longest stem, inflorescence included (when fully expanded) (characteristic 16)

5.4 If other characteristics than those from the Technical Protocol are used for the selection of varieties to be included into the growing trial, the EO shall inform the CPVO and seek the prior consent of the CPVO before using these characteristics.

5.5 Guidance for the use of grouping characteristics, in the process of examining distinctness, is provided through the UPOV-General Introduction to DUS and document TGP/9 "Examining Distinctness".

6. INTRODUCTION TO THE TABLE OF CHARACTERISTICS

6.1 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 or by specific legislation on plant health. In the latter case, the CPVO should be informed.

The Administrative Council empowers the President, in accordance with Article 23 of Commission Regulation N°874/2009, to insert additional characteristics and their expressions in respect of a variety.

Electrophoresis characteristic
The characteristic derived from electrophoresis as described in chapter 9 should only be used as a complement to other differences in morphological or physiological characteristics.

6.2. States of expression and corresponding notes

In the case of qualitative and pseudo-qualitative characteristics, all relevant states of expression are presented in the characteristic. However, in the case of quantitative characteristics with 5 or more states, an abbreviated scale may be used to minimize the size of the Table of Characteristics. For example, in the case of a quantitative characteristic with 9 states, the presentation of states of expression in the Test Guidelines may be abbreviated as follows:

State	Note
small	3
medium	5
large	7

However, it should be noted that all of the following 9 states of expression exist to describe varieties and should be used as appropriate:

State	Note
very small	1
very small to small	2
small	3
small to medium	4
medium	5
medium to large	6
large	7
large to very large	8
very large	9

Further explanation of the presentation of states of expression and notes is provided in UPOV document TGP/7 "Development of Test Guidelines".

6.3 Example Varieties

Where appropriate, example varieties are provided to clarify the states of expression of each characteristic.

6.4 Legend

For column 'CPVO N°':

G	Grouping characteristic	-see Chapter 5
QL	Qualitative characteristic	
QN	Quantitative characteristic	
PQ	Pseudo-qualitative characteristic	
(+)	Explanations for individual characteristics	-see Chapter 8.2

For column 'UPOV N°':

The numbering of the characteristics is provided as a reference to the UPOV guideline.

(*) UPOV Asterisked characteristic – Characteristics that are important for the international harmonization of variety descriptions.

For column 'Stage, method':

MG, MS, VG, VS		-see Chapter 4.1.5
A, B, C		-see Chapter 3.3.3
(a)-(d)	Explanations covering several Characteristics	-see Chapter 8.1
DC10 - DC68	Explanation on growth stages	-see Chapter 8.3

7. TABLE OF CHARACTERISTICS

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
1. (+)	**1.**	**C**	**Plant: ploidy**		
QL			diploid	Bargold (Lp), Barsilo (Lb)	2
G			tetraploid	Tivoli (Lp), Fabio (Lmi)	4
2.	**2.**	**20-29 VS A**	**Plant: vegetative growth habit (without vernalization)**		
QN		**(a)**	erect	Solita (Lmi)	1
			semi-erect	Lemtal (Lmi)	3
			medium	Jeanne (Lmi), Jumbo (Lp)	5
			semi-prostrate	Titus (Lp), Belida (Lp)	7
			prostrate	Citius (Lp)	9
3.	**5.**	**20-29 VG A / VG B**	**Leaf: intensity of green colour (without vernalization)**		
QN			very light		1
			light	Abermont (Lp), Superstar (Lp)	3
			medium	Tivoli (Lp), Barsilo (Lb)	5
			dark	Adeline (Lp), Greenway (Lp)	7
			very dark	Polarstar (Lp)	9
4.	**6.**	**30 MS A/ VS A**	**Plant: width (after vernalization)**		
QN		**(+)**	very narrow	Aberelf (Lp)	1
			narrow	Disco (Lp), Hamlet (Lp)	3
			medium	Jeanne (Lmi)	5
			wide	Lacerta (Lp), Fanal (Lp)	7
			very wide	Pimpernel (Lp)	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
5.	**7.**	**30-39 VS A / VG B**	**Plant: vegetative growth habit (after vernalization)**		
QN		**(a)**	erect	Solita (Lmi)	1
			semi-erect	Grasslands Nui (Lp), Lemtal (Lmi) Lemnos (Lmw), Barsilo (Lb)	3
			medium	Palmer (Lp), Texy (Lb), Lacerta (Lp), Enduro (Lb)	5
			semi-prostrate	Tivoli (Lp),	7
			prostrate	Fabula (Lp)	9
6.	**8.**	**30-39 MS A / VG B**	**Plant: height (after vernalization)**		
QN			very short	Aberelf (Lp)	1
			short	Titus (Lp)	3
			medium	Tivoli (Lp), Fabio (Lmi), Adeline (Lp)	5
			tall	Fox (Lmi), Lacerta (Lp)	7
			very tall		9
7.		**30-39 VG A / VG B**	**Only varieties of Lp, Lmi and Lb: Leaf: intensity of green colour (after vernalization)**		
QN			very light		1
			light	AberDart (Lp)	3
			medium	Vesuve (Lp), Bellem (Lmi), Premiun (Lp), Gemini (Lmi)	5
			dark	Mondora (Lmi), Verdi (Lp) Boxer (Lb)	7
			very dark	Polarstar (Lp)	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
8.	**9.**	**50 MS A**	**<u>Only varieties of Lmw and Lr:</u> Plant: time of inflorescence emergence (without vernalization)**		
QN		**(b)**	very early	Grazer (Lmw)	1
			early	Lifloria (Lmw), Libonus (Lmw)	3
			medium	Elunaria (Lmw)	5
			late	Advance (Lmw), Vivaro (Lmw)	7
G			very late	Koga (Lmw), Telga (Lmw)	9
9.	**10.**	**50 VS A VG B**	**Plant: tendency to form inflorescences (without vernalization)**		
(+)			absent or very weak	Bargold (Lp), Barmultra (Lmi), Enduro (Lb)	1
QN			weak	Arvicola (Lp), Fox (Lmi), Gemini (Lmi)	3
			medium	Faveur (Lp), Ligrande (Lmi) Barsilo (Lb)	5
			strong	Lemtal (Lmi)	7
			very strong	Weldra (Lmw), Arolus (Lp)	9
10.	**11.**	**50 MS A**	**<u>Only varieties of Lp, Lmi and Lb:</u> Plant: time of inflorescence emergence (after vernalization)**		
QN		**(b)**	very early	Ivana (Lp)	1
			early	Lacerta (Lp), Enduro (Lb)	3
			medium	Greenway (Lp), Boxer (Lb)	5
			late	Chagall (Lp)	7
G			very late	Cancan (Lp)	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
11.	**12.**	**50 - 56 MS A**	**Plant: natural height at inflorescence emergence**		
QN		**(c)**	very short	Loretta (Lp)	1
			short	Titus (Lp)	3
			medium	Cancan (Lp), Ligrande (Lmi)	5
			tall	Lemtal (Lmi), Lacerta (Lp)	7
			very tall	Lipo (Lmi)	9
12.		**50 VS A / MS A**	**Plant: growth habit at inflorescence emergence**		
(+)			very erect	Weldra (Lmw)	1
QN			erect	Fabio (Lmi)	3
			medium	Isabel RvP (Lp), Premium (Lp)	5
			prostrate	Carraig (Lp)	7
			very prostrate		9
13.	**14.**	**50 MS A**	**Flag leaf: length**		
QN		**(c)**	very short	Brightstar (Lp)	1
			short	Sauvignon (Lp), Bargold (Lp)	3
			medium	Lipresso (Lp), Fastyl (Lmi)	5
			long	Ibex (Lb), Twins (Lp), Acento (Lp)	7
			very long		9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
14.	**15.**	**50 MS A**	**Flag leaf: width**		
QN		**(c)**	very narrow	Bargold (Lp)	1
			narrow	Loretta (Lp)	3
			medium	Lipresso (Lp), Fennema (Lp) Lemnos (Lmw)	5
			broad	Eurostar (Lp), Lacerta (Lp) Barsilo (Lb)	7
			very broad	Lipo (Lmi)	9
15.	**16.**	**50 MS A**	**Flag leaf: length/width ratio**		
QN		**(c)**	very low	Ivana (Lp)	1
			low	AberElf (Lp), Lacerta (Lp)	3
			medium	Fabio (Lmi), Mondial (Lp)	5
			high	Twystar (Lp)	7
			very high	Cancan (Lp)	9
16.	**17.**	**60-68 MS A**	**Plant: length of longest stem, inflorescence included (when fully expanded)**		
(+)		**(d)**	very short	Brightstar (Lp)	1
QN			short	Loretta (Lp), Grazer (Lmw)	3
			medium	Cancan (Lp)	5
			long	Fox (Lmi), Limbos (Lp)	7
G			very long	Lipo (Lmi), Fleurial (Lb)	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
17. (+)	**18.**	**60-68 MS A**	**Plant: length of upper internode**		
QN		**(d)**	very short	Abersprite (Lp)	1
			short	Adeline (Lp)	3
			medium	Cancan (Lp), Lemtal (Lmi)	5
			long	Montblanc (Lmi), Acrobat (Lb)	7
			very long	Pirol (Lb)	9
18.	**19.**	**60-68 MS A**	**Inflorescence: length**		
QN		**(d)**	very short	Sunbright (Lp)	1
			short	Bellem (Lmi), Bargold (Lp)	3
			medium	Barmega (Lmi), Libonus (Lmw), Vigor (Lp)	5
			long	Lacerta (Lp), Acrobat (Lb)	7
			very long	Fabio (Lmi)	9
19.	**20.**	**60-68 MS A**	**Inflorescence: number of spikelets**		
QN		**(d)**	very few		1
			few	Abersprite (Lp), Bargold (Lp)	3
			medium	Acento (Lp), Fabio (Lmi) Lemtal (Lmi), Barsilo (Lb)	5
			many	Fortimo (Lb)	7
			very many		9

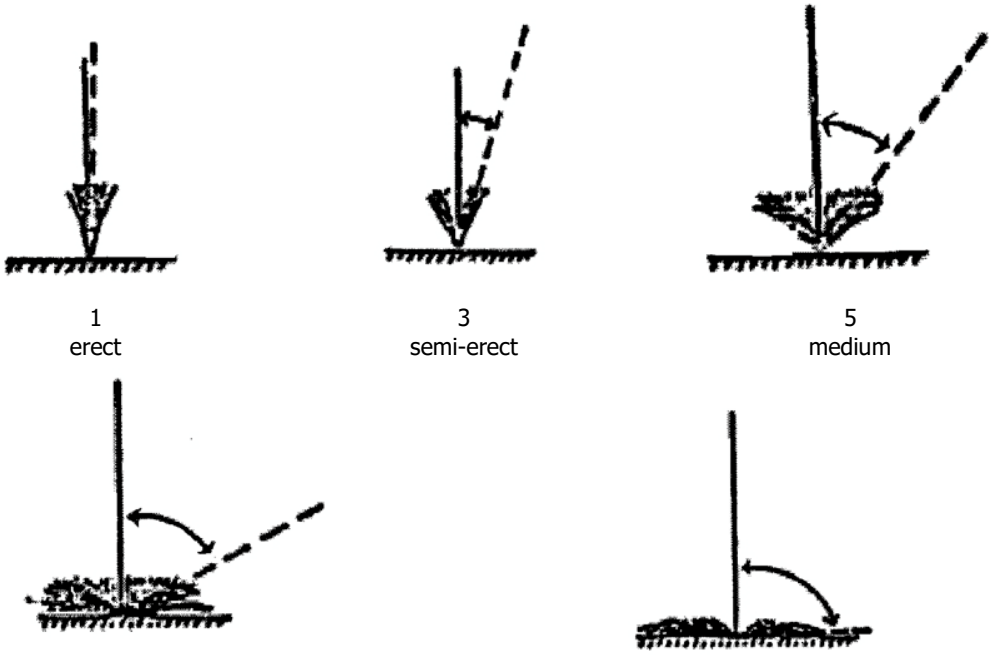
CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
20.	**21.**	**60-68 MS A**	**Inflorescence: density**		
(+)		**(d)**	very lax	Jaran (Lp)	1
QN			lax	Leon (Lp), Ligrande (Lmi)	3
			medium	Meritra (Lmi), Libonus (Lmw)	5
			dense	Lacerta (Lp)	7
			very dense		9
21.	**22.**	**60-68 MS A**	**Inflorescence: length of outer glume on basal spikelet**		
QN		**(d)**	very short	Lema (Lmi)	1
			short	Prestyl (Lmi), Bareuro (Lp)	3
			medium	Fennema (Lp), Ibex (Lb)	5
			long	Meradonna (Lp), Lemnos (Lmw) Texy (Lb)	7
			very long	Litempo (Lp)	9
22.	**23.**	**60-68 MS A**	**Inflorescence: length of basal spikelet excluding awn**		
QN		**(d)**	very short	AberElf (Lp)	1
			short	Sunbright (Lp), Montreux (Lp)	3
			medium	Barprisma (Lmi), Lipresso (Lp)	5
			long	Edda (Lp), Libonus (Lmw), Storm (Lb)	7
			very long	Litempo (Lp)	9

8. EXPLANATIONS ON THE TABLE OF CHARACTERISTICS

8.1 Explanations covering several characteristics

Characteristics containing the following key in the third column of the Table of Characteristics should be examined as indicated below:

(a) Growth habit
Characteristic 2 may be recorded during the growing season in which the trials are planted.
The observations should be made visually from the attitude of the leaves of the plant as a whole. The angle formed by the imaginary line through the region of greatest leaf density and the vertical should be used.



1
erect

3
semi-erect

5
medium

7
semi-prostrate

9
prostrate

(b) Time of inflorescence emergence
Timing of observations will depend upon the time of planting. Spaced plants or row plots should be observed at least twice per week.

A: Plots with spaced plants

The time of inflorescence emergence of each single plant should be observed. A single plant is considered to have headed when the tip of three inflorescences can be seen protruding from the flag leaf sheath (Growth Stage DC 50). From the single plant data, a mean date per plot and a mean date per variety is obtained.

B: Row plots

The time of inflorescence emergence is the date at which the average plot stage DC 54 has been reached. This date should – if necessary– be obtained by interpolation. At each observation date, the average plot stage should be expressed in one of the following growth stages:

DC 50	First spikelet of inflorescence just visible
DC 52	25% of the inflorescence emerged (across all stems)
DC 54	50% of the inflorescence emerged (across all stems)
DC 56	75% of the inflorescence emerged (across all stems)

(c) To be recorded on each individual plant at the time of inflorescence emergence (Growth Stage DC 50), that is at the same time as Characteristic 9 for *Lolium multiflorum* Lam. var. *westerwoldicum* and *Lolium rigidum* Gaudin. and Characteristic 11 for *Lolium perenne* L., *Lolium multiflorum* Lam. ssp. *italicum* (A. Br.) Volkart and *Lolium boucheanum* Kunth.

(d) Observations should be made on the longest stem.

8.2 Explanations for individual characteristics

Ad 1: Plant: ploidy
The ploidy of the plant can be determined either by standard cytological methods or by observing the occurrence of 5-band genotypes (which are present only in tetraploid varieties) in phosphoglucoisomerase (PGI) isoenzyme electrophoresis.

Ad. 4: Plant: width

To allow for irregular plant shapes (for example due to wind shaping effects) the plant width is determined by taking two measurements (MS A) or by making two visual observations (VS A) of the diameter across the plant at right angles to each other and then using the average of these two figures as the plant width.

Ad. 9: Plant: tendency to form inflorescences (without vernalization)

The number of plants showing at least three inflorescences should be recorded for each variety. To be assessed on one occasion on the whole trial when the varieties are judged to have reached their full expression of this characteristic.

Ad. 16: Plant: length of longest stem, inflorescence included (when fully expanded)

To be recorded in the field from ground level, when the inflorescence is fully expanded.

Ad. 17: Plant: length of upper internode

To be measured from the top node to the base of the inflorescence.

Ad. 21: Inflorescence: density

This characteristic is calculated by dividing characteristic 19 (Inflorescence: length) by characteristic 20 (Inflorescence: number of spikelets).

8.3 Growth stages for grasses

All characteristics should be recorded at the appropriate time for the plant concerned. Growth stages of grasses are indicated by decimal codes which are derived from the decimal code for the growth stages of cereals (Zadoks, et al., 1974). This decimal code is in close conformity with the BBCH-code (Meier, 1997).

Seedling growth (seedling: one shoot)

DC 10 First leaf through coleoptile
DC 15 Five leaves unfolded
DC 19 Nine or more leaves unfolded

Tillering

DC 20 Main shoot only (beginning of tillering)
DC 23 Main shoot and 3 tillers
DC 25 Main shoot and 5 tillers
DC 29 Main shoot and 9 or more tillers

Stem elongation

DC 30 Pseudo-stem erection (formed by sheaths of leaves)
DC 31 First node detectable (early stem extension across all stems)
DC 35 Fifth node detectable (50 % extension across all stems)
DC 39 Flag leaf ligula/collar just visible (pre-boot stage)

Booting

DC 41 Flag leaf sheath extending (little enlargement of the inflorescence, early boot-stage)
DC 45 Boots swollen (late-boot stage)
DC 47 First leaf sheath opening
DC 49 first awns visible (in awned forms only)

Inflorescence emergence (mostly non-synchronous)

DC 50 First spikelet of inflorescence just visible
DC 52 25 % of the inflorescence emerged (across all stems)
DC 54 50 % of the inflorescence emerged (across all stems)
DC 56 75 % of the inflorescence emerged (across all stems)
DC 58 Emergence of inflorescence completed

Anthesis (mostly non-synchronous)

DC 60 Beginning of anthesis
DC 64 Anthesis half-way
DC 68 Anthesis complete

9. ELECTROPHORESIS

The following section contains a characteristic derived by using protein electrophoresis and a description of the method to be used.

9.1 Introduction

In *Lolium perenne* L., the composition of seed proteins can be used as additional characteristic for establishing distinctness, uniformity and stability on request of the applicant under the following conditions:

- in case of distinctness assessment with COYD, if the difference is significant on a level between 1 % and 5 % for at least one of the characteristics mentioned in chapter 7,

- in case of distinctness assessment on the basis of notes, if the difference is at least 1 note in 2 out of 3 years.

Distinctness between two varieties must not be established on the basis of seed protein polymorphisms alone.

9.2 Characteristics derived by using electrophoresis

The composition of seed proteins is determined by SDS-polyacrylamide-gel-electrophoresis (SDS-PAGE). The bands are numbered according to their position in the gel. For the purpose of description the bands are combined in groups. The characteristic is described for the following band ranges.

Band range	Description of the banding pattern
O	1 - 20
P	21 - 40
R	61 - 90
U	141 - 154
V	155 - 190

9.3 Description of the SDS-PAGE method for the detection of seed protein polymorphism in Lolium perenne

9.3.1 Number of seeds per test

A bulked sample of 1.5 g seed material is analysed per variety. This weight equals an amount of approximately 1000 seeds.

9.3.2 Equipment

Swing mill
Vortex-mixer
Rocking platform shaker
Tablecentrifuge (min. 6.000 RpM)
Cryostat
Power supply with a capacity of at least 400 V and 150 mA providing constant current and constant voltage output

Any suitable vertical electrophoresis system can be used, provided that the gels can be kept at a constant temperature. A minimum running length of the gels of 10 cm is necessary. A gel thickness of no more than 1.5 mm is recommended.

9.3.3 Chemicals

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

9.3.3.1 Chemicals for extraction of seed proteins

Chemical	Abbr./Synonyme	Formula
Glycerol		$C_3H_8O_3$
2-Mercaptoethanol	ß-ME	C_2H_6OS
Sodium dodecyl sulfate	SDS	$C_{12}H_{25}NaO_4S$
Hydrochloric acid	HCl	HCl
Tris-(hydroxymethyl)-aminomethane	Tris	$C_4H_{11}NaO_{11}S$

9.3.3.2 Chemicals for electrophoresis

Chemical	Abbr./Synonyme	Formula
40% Acrylamide solution(w/v)	AA	C_3H_5NO
Ammonium peroxodisulfat	APS, AP	$(NH_4)_2S_2O_8$
2% Bisacrylamide solution (w/v)	BIS	$C_7H_{10}N_2O_2$
Bromphenol blue		$C_{19}H_{10}Br_4O_5S$
Ethanol	EtOH	C_2H_6O
Glycine		$C_2H_5NO_2$
Dodecyl sulfate Sodium salt	SDS	$C_{12}H_{25}NaO_4S$
Hydrochloric acid		HCl
Sucrose		$C_{12}H_{22}O_{11}$
NNN'N'Tetramethylethylenendiamine	TEMED	$C_6H_{16}N_2$

9.3.3.3 Chemicals for staining of proteins

Chemical	Abbr./Synonyme	Formula
Coomassie blue G250		$C_{47}H_{48}N_3O_7S2$ x Na
Coomassie blue R250		$C_{45}H_{44}N_3O_7S_2$ x Na
Glacial acetic acid		$C_2H_4O_2$
Glycerol		$C_3H_8O_3$
Methanol	MeOH	CH_3OH
Trichloro acetic acid	TCA	$CHCl_3O_2$

9.3.3.4 Solutions

9.3.4.1 Extraction solutions

No.	Solution	Ingredients	Amount	Remark
9.3.4.1.1	Extraction buffer	TRIS Distilled water	12,1 g ad 100,0 ml	Adjust to pH 6,8 with HCl
9.3.4.1.2	Extraction solution A	SDS Extraction buffer (9.3.4.1.1) Distilled water Glycerol	4,0 g 12,5 ml 24,0 ml 20,0 ml	Prepare daily Warming to 30°C bis 40°C to dissolve SDS if necessary
9.3.4.1.3	Extraction solution B	Extraction buffer A (9.3.4.1.2) Distilled water	27 ml 17 ml	Prepare daily
9.3.4.1.4	Extraction solution C	Extraction buffer B (9.3.4.1.3) Mercaptoethanol	22,0 ml 1,5 ml	Prepare daily

9.3.4.2 Electrophoresis buffers and gel preparation solutions

No.	Solution	Ingredients	Amount	Remark
9.3.4.2.1	Resolving gel buffer	TRIS Distilled water	75 g ad 1000 ml	Adjust to pH 8,9 with HCl, at 8°C stable for 4 month
9.3.4.2.2	Stacking gel buffer	TRIS Bromphenol blue Distilled water	16 g 100 mg ad 1000 ml	Adjust to pH 6,7 with HCl, at 8°C stable for 4 month
9.3.4.2.3	Resolving gel preparing solution	Resolving gel buffer (9.3.4.2.1) 40 % Acrylamide solution (9.3.4.2.5) 2 % Bisacrylamide solution (9.3.4.2.6) 10 % SDS-Solution	60 ml 33 ml 8,5 ml 1 ml	Prepare daily
9.3.4.2.4	Stacking gel preparing solution	Stacking gel buffer(9.3.4.2.2) 40% Acrylamide solution (9.3.4.2.5) 2% BIS solution (9.3.4.2.6) Distilled water Sucrose 10% SDS (9.3.4.2.8)	280 ml 45 ml 73 ml 150 ml 80 g 6 ml	At 8°C stable for 1 month
9.3.4.2.5	Acrylamide solution	Acrylamide Distilled water	40 g ad 100 ml	It is strongly recommended to use a ready to use solution
9.3.4.2.6	BIS solution	Bisacrylamide Distilled water	2 g ad 100 ml	It is strongly recommended to use a ready to use solution
9.3.4.2.7	APS-solution	Ammoniumperoxodisulfat Distilled water	1 g ad 50 ml	Prepare daily.
9.3.4.2.8	SDS-solution	SDS Distilled water	10 g ad 100 ml	Stable for month.
9.3.4.2.9	Ethanol 20%	Ethanol Distilled water	20 ml ad 100 ml	At room temperature stable for 6 month.
9.3.4.2.10	Electrophoresis buffer Stock solution	TRIS SDS Glycine Distilled water	103 g 20 g 70 g ad 1000 ml	Stable for month.
9.3.4.2.11	Electrophoresis buffer	Electrophoresis buffer Stock solution (9.3.4.2.10) Distilled water	50 ml ad 1000 ml	Prepare daily.

9.3.4.3 Staining Solutions

No.	Solution	Ingredients	Amount	Remark
9.3.4.3.1	Coomassie Blue Stock solution	Coomassie Blau G 250 Coomassie Blau R 250 Tap water	0,25 g 0,75 g ad 100ml	Stirred for at least 1 h; Shaken very well before use
9.3.4.3.2	Staining solution	TCA Acetic acid 80% Tap water Methanol Stock solution (9.3.4.3.1)	240 ml 520 ml 3100 ml 600 ml 90 ml	
9.3.4.3.3	Glycerol solution	Glycerol Tap water	50 g ad 1000 ml	

9.3.5 Procedure

9.3.5.1 Preparation of samples

1.5 g seeds are grinded for 2 minutes by 2000 RPM with a swing mill and 3 steel balls. The grist is stored in a 5 ml glass tube.

9.3.5.2 Extraction of samples

0.08 g well mixed grist is weighed in 2 ml reaction tubes and mixed with 1 ml extraction solution C (9.3.4.1.4) by using a vortex mixer. The samples are left for 1 hour at room temperature, suspended by using a vortex mixer and heated for 20 minutes in a water bath by a temperature of 75°C. After cooling in a water bath, the tubes are centrifuged at 10,000 x g for 10 minutes at 4°C.
15 µl of the clarified supernatant is diluted with 70 µl extraction solution B (9.3.4.1.3). The sample is stored frozen until SDS-PAGE.

9.3.5.3 Preparation of the gels

9.3.5.3.1 Preparation of the gels for SDS-PAGE

The SDS-PAGE is a discontinuous electrophoresis and each gel consists of resolving gel and stacking gel. Clean and dry gel cassettes are assembled, according to the design of the equipment used.

The resolving gel solution is composed as described in 9.3.4.2.3 and polymerisation is started by addition of:

100 µl TEMED
5 ml APS solution (9.3.4.2.7).

The gels are carefully poured, avoiding the formation of bubbles. The gel should be poured to a height which leaves room for a 20 mm layer of stacking gel. The gel surface is carefully overlapped with 20% ethanol solution (9.3.4.2.9) using a syringe. The gel polymerises at room temperature for at least one hour. When the polymerisation is finished, the ethanol solution is removed. The gel surface is rinsed with distilled water and dried with filter paper.

To make the stacking gels the following is mixed under slow stirring:

15 ml stacking gel preparing solution (9.3.4.2.4)
60 µl TEMED
375 µl APS-solution (9.3.4.2.7)

The gels are carefully poured, avoiding the formation of bubbles. The height of the stacking gel should be about 2 cm. The well-forming combs are inserted into the liquid gel. The gels are allowed to polymerize at room temperature for about 1 hour. The combs should be removed carefully out of the gel.

9.3.5.4 Sample loading

The wells of the gel are carefully rinsed using electrophoresis buffer (9.3.4.2.11).
For separation of the seed proteins each well is filled with 5 µl extract (see 9.3.5.2) using a multiple syringe.

9.3.5.5 Electrophoresis

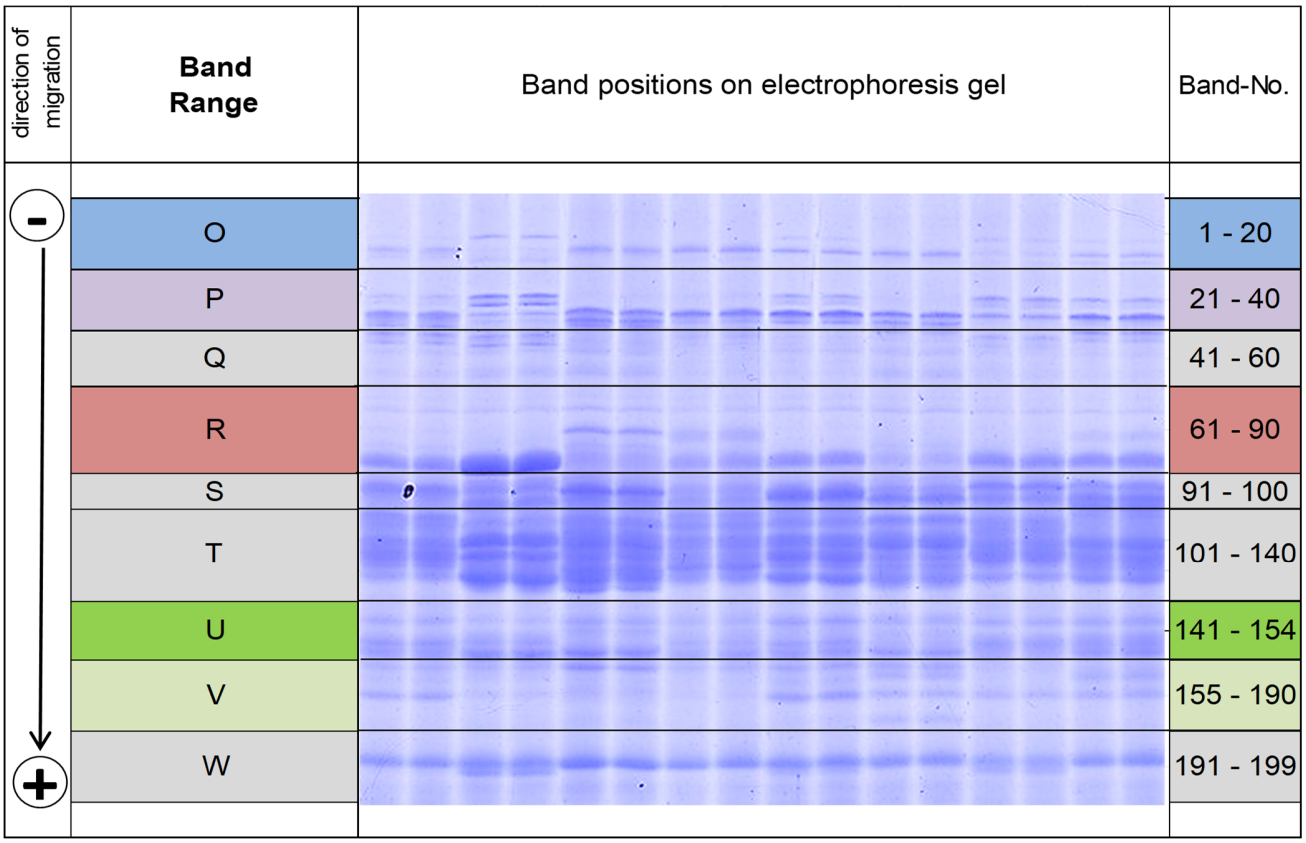
If a vertical dual slab gel instrument for example "Multigel Modell BSA" (Biometra) is used, the conditions for the electrophoresis are the following:

Electrophoresis buffer:	Solution (9.3.4.2.11), fill up chamber
Voltage:	120 V (for 20 minutes), then 230 V
Current:	120 mA
Temperature:	5°C to 15°C
Running distance:	when bromophenol blue runs out at the end of the gel, proceed electrophoresis for another 40 minutes before end of run

9.3.5.6 Staining

2 gels from the SDS-PAGE are marked, e.g. by cutting the gels corner. Then the gels are transferred in a staining container filled with 300 ml staining solution (9.3.4.3.2) and incubated on a rocking platform shaker for 3 hours. The gels remain in the staining solution over night without shaking. For destaining, the gels are incubated on the shaker in tap water for 2 x 30 min. Finally the gels are incubated on the shaker in 5 % glycerol solution (9.3.4.3.3) for 5 min. After this incubation the gels are dried between two layers of cellophane soaked in 5 % glycerol solution (9.3.4.3.3) at room temperature.

9.4 Interpretation of the gels



Range Q, S, T and W are not analysed

Band range - 0

Type	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	26	27	28
Bands	4 8 12	8	8 9	8 12	4 8 10	4 8	2 8	2 8 12	2 8 14	8 14	8 18	8 12 16	4 8 10 20	8 10	6 7	2 8 9	8 20	6 10	1 6 16	7 8 14	8 16 18	8 10 20	8 10 12	8 14 18	1 8 10 14	7 8 9 14	9 10 14	9 10 16
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Example variety	Lilora	Tetramax	Fornido	(none)	Eugenius	Diwan	Bellamini	Henrietta	Traffic	Hugo 1	Expert	Expert	Arsenal	Dressaro	Parcour	Phoenix	Option	Mezquita	Canberra	Ragtime	Twymax	(none)	(none)	Boccacio	Melpaula	Maiko	Resista	Tornado

Band range - P

Type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	22													28		26	24		26		28
	28	28	28		28					30		28	28	32	29	30	30	26	28		30
Bands	30	30	30	28	32	30		34	28	32		32	34	33	34	32	32	32	34	24	32
	36	36	36	36	36	36	36	36	30	36	36	36	36	36	36	36	36	36	36	36	36
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Example variety	Lilora	Henrietta	Rokade	Kelvin	Aut	Abosan 1	Virtuose	Bocardi	Sabor	Defender	Dasher 3	(none)	Kilrea	Indra	(none)	Melpaula	Resista	(none)	Maritim	Bargizmo	Sanova

Band range - R

Type	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	26	27	28
								64							62	64	64	64				64	64		64			
		64			64		64	70				64			64	66	72	70	62	64	64	72	72		68	64	64	64
Bands	64	68	64	64	70	64	70	72	64	64	64	76	64		70	70	78	72	64	72	72	74	76	64	70	76	70	76
	70	70	66	68	76	72	78	78	72	76	76	82	80	64	78	78	80	76	70	80	76	78	80	80	78	80	74	80
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Example variety	Lilora	(none)	Aberdart	Baraudi	Twymax	Cleopatra	Astonhockey	(none)	Henrietta	Dexter 1	Virtuose	Colorado	Cleancut	Traffic	Barnhem	Probat	Arusi	Maggie	Liszt 1	Eiffel	Ventoux	Alfonso	Chouss	Defender	Channi	Barsaxo	Eurosport	(none)

Band range - U

Type	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	26	27	28
Bands	142 148 154	144 148 154	144 150 152 154	148 150 154	148 150 152 154	142 146 148 154	142 146 154	144 150 154	144 150	142 146 150 154	142 148 152 154	142 150 152 154	142 152 154	142 150 154	146 150 154	144 148 150 154	142 148 150 154	142 144 146 154	144 148 152 154	142 144 150 154	142 144 154	142 146 152 154	144 146 154	144 146 150 154	142 144 146 148 154	142 144 146 152 154	144 146 148 154	144 145 146 148 150 154
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Example variety	Ventoux	(none)	Henrietta	Pontiac	Phoenix	(none)	Lilora	Garbor	Sabor	Poseidon	Toulouse	Mezquita	(none)	Cadix	Aberdart	Elegana	Barpasto	Denver5	Eiffel	Vesuvius	Carillon	Mateon 1	(none)	Xanthus	Gemma	(none)	Sures	Sarwal

Band range - V

Type	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	26	27	28
Bands	158 162 170	162 166 168	158 164 166	158 166 176	158 176	158 162 166 176	158 162 168 178	160 166 176	156 158 160 164 166 176	156 160 166	164 168	164 166	164	156 164 170	160 166	156 162 168	158 166 168	158 168	156 164 166 176	160 166 168	158 162 166	168	158 164 170	158 162 164 166 168	156 158 170	156 166	158 166	158 160 168
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Example variety	Lilora	Henrietta	Garbor	Cleancut	Herbal	Akurat	(none)	Montando	(none)	Aberdart	Elegana	Traffic	(none)	Acento	Barnhem	Denver	Ragtime	Greenfair	Arusi	Birtley	Carillon	Neruda	Barmaxima	Masai	(none)	Dressano	Valerio	Ibizal

10. LITERATURE

Meier, U., 1997: Growth stages of mono- and dicotyledonous plants. BBCH-Monograph Blackwell Science. Berlin, Vienna, a.o., 622 pp.

Zadoks, J.C., Chang, T.T., Konzak, C.F., 1974: A decimal code for the growth stages of cereals. Weed Research 14: pp. 415 – 421.

11. TECHNICAL QUESTIONNAIRE

The Technical Questionnaire is available on the CPVO website under the following reference: CPVO-TQ/004/2