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

PROTOCOL FOR TESTS ON DISTINCTNESS, UNIFORMITY AND STABILITY

Trifolium pratense L.

RED CLOVER

UPOV Code: TRFOL_PRA

Adopted on 13/02/2025

Entry into force on 15/02/2025

TABLE OF CONTENTS

CPVO-TP/005/1-Rev

1. SUBJECT OF THE PROTOCOL AND REPORTING

1.1 Scope of the technical protocol

This Technical Protocol applies to all varieties of *Trifolium pratense* L.

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/5/8 dated 17/12/2020 (https://www.upov.int/edocs/tgdocs/en/tg005.pdf) for the conduct of tests for Distinctness, Uniformity and Stability.

1.2 Entry into Force

The present protocol enters into force on **15.02.2025.** 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 pertinent agreement, on matters of particular urgency, 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 https://public.plantvarieties.eu/publication in the special issue S2/S3 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

3.1.1 The minimum duration of tests should normally be two independent growing cycles.

3.1.2 The two independent growing cycles should be in the form of two separate plantings.

3.1.3 The testing of a variety may be concluded when the competent authority can determine with certainty the outcome of the test.

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 8.3.

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: special test

3.4 Test design

3.4.1 Spaced plants: Each test should be designed to result in a total of at least 60 plants, which should be divided between at least 3 replicates.

Row plots: Each test should be designed to result in a total of at least 3000 plants, which should be divided between at least 2 replicates.

3.4.2 The design of the tests should be such that plants or parts of plants may be removed for measurement or counting without prejudice to the observations which must be made up to the end of the growing cycle.

3.5 Special tests for additional characteristics

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 characteristics listed in the protocol.

3.6 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.

3.6.1 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.

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

3.6.3 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.

3.6.4 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.

3.6.5 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

4.1.1 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.

4.1.2 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.

4.1.3 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.

If distinctness is assessed using the 2 x 1% criterion, the difference between two varieties is clear if the respective characteristics are significantly different in the same direction at the 1% level in at least two out of three. The tests in each year are based on Student's two-tailed 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 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 significance level or statistical methods prescribed are not appropriate the method used should be clearly described.

4.1.4 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 60 plants or parts taken from each of 60 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.

4.1.5 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. colour 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.

With respect to the use of protein electrophoresis, the Office follows the actual UPOV approach as laid down under point 9 of this protocol. 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. Electrophoretic characteristics with a lack of uniformity shall not be taken into account for the assessment of distinctness.

4.2 Uniformity

4.2.1 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:

4.2.2 This Technical Protocol has been developed for the examination of cross-pollinated 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.

The assessment of uniformity should be according to the recommendations for cross-pollinated varieties in the UPOV-General Introduction to DUS.

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 consecutive cycles.

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 ($p<0.0001$) in a test over three consecutive cycles.

If uniformity is assessed by the relative variance method the candidate variety is sufficiently uniform in the respective characteristic if the variance does not exceed the average variance of comparable varieties in at least two out of three years. The threshold of F (at P = 0.01) for a sample size of 60 is 1.47.

4.3 Stability

4.3.1 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.

a) Plant: ploidy (characteristic 1)

b) Time of flowering (characteristic 15)

c) Stem: length (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

States of expression are given for each characteristic to define the characteristic and to harmonize descriptions. Each state of expression is allocated a corresponding numerical note for ease of recording of data and for the production and exchange of the description. All relevant states of expression are presented in the characteristic.

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':

A, B, C		- see Chapter 3.3
MG, MS, VG, VS		- see Chapter 4.1.5
(a)-(b)	Explanations covering several Characteristics	- see Chapter 8.1
00-69	Explanations on growth stages	- see Chapter 8.3

7. TABLE OF CHARACTERISTICS

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
1. (+)	**1. (*)**	**MG|C**	**Plant: ploidy**		
QL			diploid	Start	2
G			tetraploid	Titus	4
2. (+)	**2.**	**MS|C**	**Cotyledon: length**		
QN		**11**	short		1
			short to medium		2
			medium	Agil, Temara	3
			medium to long		4
			long	Atlantis, Maro	5
3. (+)	**3.**	**MS|C**	**Cotyledon: width**		
QN		**11**	narrow	Lemmon, Vltavín	1
			narrow to medium		2
			medium	Renegade, Temara	3
			medium to broad		4
			broad	Maro	5
4.	**4. (*)**	**VG|C**	**Petiole: density of hairs**		
QN		**13-19**	sparse	Lucrum	1
			sparse to medium		2
			medium	Formica	3
			medium to dense		4
			dense	Grasslands Pawera	5

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
5.	**5.**	**MG|B/ VG|B**	**Plant: natural height <u>without</u> vernalization**		
QN		**29**	very short		1
			very short to short		2
			short		3
			short to medium		4
			medium	Lucrum	5
			medium to tall		6
			tall	Formica	7
			tall to very tall		8
			very tall		9
6.	**6.**	**VG|B**	**Leaf: intensity of green colour <u>without</u> vernalization**		
QN		**29**	very light		1
			very light to light		2
			light	Kenland	3
			light to medium		4
			medium	Rotra	5
			medium to dark		6
			dark	Tedi	7
			dark to very dark		8
			very dark		9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
7. (+)	**7. (*)**	**VS|A**	**Plant: growth habit**		
QN		**29**	erect		1
			erect to semi-erect		2
			semi-erect		3
			semi-erect to intermediate		4
			intermediate		5
			intermediate to semi-prostrate		6
			semi-prostrate	Formica, Rotra	7
			semi-prostrate to prostrate		8
			prostrate	Montana	9
8. (+)	**8.**	**VG|B/ VS|A**	**Plant: tendency to flower <u>without</u> vernalization**		
QN			very weak		1
			very weak to weak		2
			weak	Rajah	3
			weak to medium		4
			medium	Cyklon, Podjavorina	5
			medium to strong		6
			strong	Formica	7
			strong to very strong		8
			very strong		9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
9.	**9. (*)**	**VG|B/ VS|A**	**Leaf: conspicuousness of marking**		
QN		**29**	absent or very weak		1
			very weak to weak		2
			weak		3
			weak to medium		4
			medium	Lucrum	5
			medium to strong		6
			strong	Astur, Temara	7
			strong to very strong		8
			very strong		9
10.	**10. (*)**	**MG|B/ MS|A/ VG|B**	**Plant: natural height <u>after</u> vernalization**		
QN		**31-39**	very short		1
			very short to short		2
			short		3
			short to medium		4
			medium	Lucrum	5
			medium to tall		6
			tall	Manuela, Tedi	7
			tall to very tall		8
			very tall		9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
11.	**11. (*)**	**VG|B**	**Leaf: intensity of green colour <u>after</u> vernalization**		
QN		**31-39**	very light		1
			very light to light		2
			light	Renegade	3
			light to medium		4
			medium	Freedom, Montana	5
			medium to dark		6
			dark	Astur, Grasslands Turoa, Lucrum	7
			dark to very dark		8
			very dark		9
12. (+)	**12.**	**MS|A**	**Leaf: length of petiole**		
QN		**31-69**	very short		1
		(b)	short		2
			medium	Metis	3
			long	Formica	4
			very long		5
13.	**13. (*)**	**MS|A**	**Middle leaflet: length**		
QN		**31-69**	very short		1
		(b)	very short to short		2
			short	Tuscan	3
			short to medium		4
			medium	Astur, Vltavín	5
			medium to long		6
			long		7
			long to very long		8
			very long		9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
14.	**14. (*)**	**MS|A**	**Middle leaflet: width**		
QN		**31-69**	very narrow		1
		(b)	very narrow to narrow		2
			narrow		3
			narrow to medium		4
			medium	Lemmon, Merviot	5
			medium to broad		6
			broad	Ostro, Rotra	7
			broad to very broad		8
			very broad		9
15. (+)	**15. (*)**	**MS|A**	**Time of flowering**		
QN			very early		1
			very early to early		2
			early	Astur, Formica	3
			early to medium		4
			medium	Agil, Margot	5
			medium to late		6
			late	Lucrum	7
			late to very late		8
G			very late	Rajah	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
16. (+)	**16. (*)**	**MS|A**	**Stem: length**		
QN		**39-69**	very short		1
		(a)	very short to short		2
			short	Aberchianti	3
			short to medium		4
			medium	Slavin, Tempus	5
			medium to long		6
			long		7
			long to very long		8
G			very long	Jogeva 205	9
17. (+)	**17.**	**MS|A**	**Stem: thickness**		
QN		**39-69**	thin		1
		(a)	thin to medium		2
			medium	Astur, Noe	3
			medium to thick		4
			thick		5
18.	**18. (*)**	**MS|A**	**Stem: number of internodes**		
QN		**39-69**	very few		1
		(a)	very few to few		2
			few		3
			few to medium		4
			medium	Polana, Tedi	5
			medium to many		6
			many	Lucrum, Titus	7
			many to very many		8
			very many	Jogeva 205	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
19. (+)	**19.**	**MG|B/ VG|B**	**Plant: natural height in aftermath**		
QN			very short		1
			very short to short		2
			short	Ilte	3
			short to medium		4
			medium	Lemmon, Tornado	5
			medium to tall		6
			tall	Formica, Tempus	7
			tall to very tall		8
			very tall		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) Observations should be done on the longest stem excluding side branches.

b) To be assessed on the longest stem on the third leaf back from the growing tip.

8.2 Explanations for individual characteristics

Ad. 1: Plant: ploidy

Ploidy should be assessed by standard cytological methods.

Ad. 2: Cotyledon: length

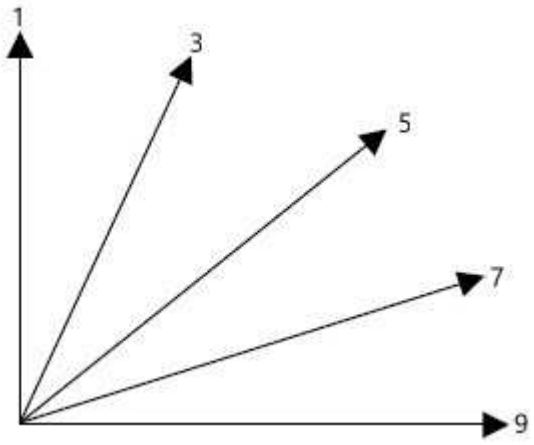
Observations should be made 12-14 days after sowing in the greenhouse, when the first leaf is fully developed. If the two cotyledons differ in size, the biggest one should be measured.

Ad. 3: Cotyledon: width

Observations should be made 12-14 days after sowing in the greenhouse, when the first leaf is fully developed. If the two cotyledons differ in size, the biggest one should be measured.

Ad. 7: Plant: growth habit

A visual estimate is taken of the angle that the outer shoots make with the horizontal axis.



1 = erect
3 = semi-erect
5 = intermediate
7 = semi-prostrate
9 = prostrate

Ad. 8: Plant: tendency to flower without vernalization

The number of plants showing inflorescences should be recorded for each variety. To be assessed at one occasion on the whole trial when the development stagnates before vernalization.

Ad. 12: Leaf: length of petiole

Length of the petiole should be measured from the base of the middle leaflet to the point of attachment to the stem.

Ad. 15: Time of flowering

Time of flowering is reached when 3 inflorescences per plant are showing colour.

Ad. 16: Stem: length

Stem length should be measured from the base of plant to the base of the terminal inflorescence.

Ad. 17: Stem: thickness

Stem thickness should be measured 2 to 4 cm above tillering node.

Ad. 19: Plant: natural height in aftermath

Observations should be made within 4 to 6 weeks after the summer cut.

8.3 Explanations on growth stages

Phenological growth stage based on the general BBCH-scale (Meier, 2001) adjusted for Red Clover

Principal growth stage 0: Germination
00: Dry seed

Principal growth stage 1: Leaf development
11: First leaf unfolded
13: 3 leaves unfolded

Principal growth stage 2: Formation of side shoots/tillering
29: 9 or more shoots visible

Principal growth stage 3: Stem elongation
31: Stem 10% of final length
39: Maximum stem length reached

Principal growth stage 6: Flowering
69: End of flowering

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 *Trifolium pratense* 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 routine characteristics,
- 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 based on seed protein polymorphisms alone.

9.2 Characteristic derived by using protein 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 grouped in the following band ranges allowing a description of the characteristic:

Band range	Description of the banding pattern
J	1 - 3
K	35 - 4
L	50 - 8
M	87 - 1

9.3 Description of the SDS-PAGE method

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

Laboratory mixer mill
Vortex mixer
Platform shaker
Centrifuge (min. 6000 rpm)
Water bath or thermal mixer
Laboratory circulators
Magnetic stirrer
Multiple syringe
Power supply with a capacity of at least 400 V and 150 mA providing constant current and constant voltage output

Any suitable vertical electrophoresis apparatus with a cooling system and power supply may be used. A minimum running length of the gels of 10 cm is necessary. A gel thickness of not more than 1.5 mm is recommended.

9.3.3 Chemicals

All chemicals should be of "Analytical Reagent grade" or better. Some of the chemicals could be toxic and should only be used in compliance with good laboratory practice considering the respective safety data sheet. In brackets the CAS-No. can be found.

9.3.3.1 Chemicals for extraction of seed proteins

Glycerine (56-40-6)
2-Mercaptoethanol (60-24-2)
Sodium dodecyl sulfate SDS (151-21-3)
Hydrochloric acid (HCl) (7647-01-0)
Tris-(hydroxymethyl)-aminomethane (TRIS) (77-86-1)

9.3.3.2 Chemicals for electrophoresis

40 % Acrylamide solution (w/v), ready to use (79-06-1)
Ammonium peroxodisulfate (7727-54-0)
2 % Bisacrylamide solution (w/v), ready to use (110-26-9)
Bromphenole blue (BPB) (115-39-9)
Ethanol (64-17-5)
Glycine (56-40-6)
Sodium dodecyl sulfate SDS (151-21-3)
Hydrochloric acid (HCl) (7647-01-0)
Sucrose (57-50-1)
N,N,N'N'-Tetramethylendiamine (TEMED) (110-18-9)

9.3.3.3 Chemicals for staining of proteins

Coomassie blue G250 (6104-58-1)
Coomassie Blau R250 (6104-59-2)
80 % Acetic acid, ready to use (64-19-7)
Methanol (67-56-1)
Trichloro acetic acid (TCA) (76-03-9)
Glycerine (56-40-6)

9.3.4 Solutions

9.3.4.1 Extraction solutions

No.	Solution	Ingredients	Amount	Remarks
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 Glycerine	8,0 g 25,0 ml 124,5 ml 40,0 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) Mercaptoethanol	22,0 ml 1,5 ml	Prepare daily.

9.3.4.2 Electrophoresis buffers and gel preparation solutions

No.	Solution	Ingredients	Amount	Remarks
9.3.4.2.1	Resolving gel buffer	TRIS Distilled water	75 g ad 1000 ml	Adjust to pH 8,9 with HCl. Stored in a refrigerator 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. Stored in a refrigerator stable for 4 month.
9.3.4.2.3	40 % Acrylamide solution	Acrylamide Distilled water	40 g ad 100 ml	There are ready to use solutions available.
9.3.4.2.4	2 % BIS solution	Bisacrylamide Distilled water	2 g ad 100 ml	There are ready to use solutions available.
9.3.4.2.5	10 % SDS-solution	SDS Distilled water	10 g ad 100 ml	Stable for month.
9.3.4.2.6	2 % APS-solution	Ammonium peroxodisulfat Distilled water	1 g ad 50 ml	Prepare daily.
9.3.4.2.7	20 % Ethanol	Ethanol Distilled water	20 ml ad 100 ml	At room temperature stable for month.
9.3.4.2.8	Stacking gel preparing solution	Stacking gel buffer (9.3.4.2.2) 40 % Acrylamide solution (9.3.4.2.3) 2 % Bisacrylamide solution (9.3.4.2.4) 10 % SDS (9.3.4.2.6) Distilled water Sucrose	280 ml 45 ml 73 ml 6 ml 150 ml 80 g	Stored in a refrigerator stable for about one month.
9.3.4.2.9	Electrophoresis buffer stock solution	TRIS SDS Glycine Distilled water	108 g 21 g 71 g ad 1000 ml	Stable for month.
9.3.4.2.10	Electrophoresis buffer	Electrophoresis buffer stock solution (9.3.4.2.9) Distilled water	50 ml ad 1000 ml	Prepare daily.

9.3.4.3 Staining Solutions

No.	Solution	Ingredients	Amount	Remarks
9.3.4.3.1	Coomassie blue stock solution	Coomassie Blau G 250 Coomassie Blau R 250 Distilled water	0,25 g 0,75 g ad 100 ml	Stirred for at least 1 h; shake very well before use
9.3.4.3.2	TCA solution	TCA Distilled water	1 kg 400 ml	Dissolve over night
9.3.4.3.3	Staining solution	Dissolved TCA (9.x.x.x.) 80 % Acetic acid Tap water Methanol Coomassie blue stock solution (9.3.4.3.1)	240 ml 520 ml 3200 ml 600 ml 90 ml	
9.3.4.3.4	Glycerine solution	Glycerine Tap water	50 g ad 1000 ml	

9.3.5 Procedure

9.3.5.1 Preparation of seeds and extraction of proteins

1.5 g seeds are grinded for 2 minutes by 2000 rpm with a swing mill and an appropriate amount of steel balls. The ground material is stored in an appropriate vessel.

0.08 g of the well mixed ground material is weighed into 2 ml reaction tubes. After addition of 1 ml extraction solution A (9.3.4.1.2) the suspension is mixed by using a vortex mixer. The samples are left at room temperature for 1 hour, then vortexed again and heated for 20 minutes at 75°C (water bath or thermal mixer). After cooling in a water bath or a thermal mixer, the tubes are centrifuged at 10000 rpm 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 can be stored frozen until application of the SDS-PAGE.

9.3.5.2 Preparation of the gels

The SDS-PAGE is a discontinuous electrophoresis technique. Each gel consists of a resolving gel and a stacking gel. The clean and dry gel cassettes are assembled according to the type of equipment in use.

To make about 100 ml resolving gel solution, the following ingredients are mixed and stirred gently:

60,0 ml resolving gel buffer (9.3.4.2.1)
8,3 ml distilled water,
25,0 ml 40 % acrylamide solution (9.3.4.2.3),
6,3 ml 2 % bisacrylamid solution (9.3.4.2.4) and
1,0 ml 10 % SDS solution (9.3.4.2.5)

Polymerisation is started by addition of:
0,1 ml TEMED and
5,0 ml 2 % APS solution (9.3.4.2.6).

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 overlayed with 20 % ethanol solution (9.3.4.2.7) using a syringe. The gel needs to polymerise 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 ingredients are mixed by gentle stirring:

15,0 ml stacking gel preparing solution (9.3.4.2.8)
0,06 ml TEMED
0,375 ml 2 % APS-solution (9.3.4.2.6)

The gels are carefully poured, avoiding the formation of bubbles. The height of the stacking gel should be about 20 mm. 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 from the stacking gel.

9.3.5.3 Sample loading

The wells of the gel are carefully rinsed using electrophoresis buffer (9.3.4.2.10).
For separation of the seed proteins, to each well an appropriate volume of protein extract (see 9.3.5.1) is injected using a multiple syringe. The volume depends on the gel thickness and the size of the wells.

9.3.5.4 Electrophoresis

If a vertical electrophoresis apparatus with two gels is used, the conditions for the electrophoresis are the following:

Electrophoresis buffer:	Solution (9.3.4.2.10), fill up chamber
Voltage:	120 V (for 20 minutes), then 230 V
Current:	120 mA
Temperature:	5°C to 15°C
Running distance:	when the marker line of bromophenol blue drains out at the bottom of the gel

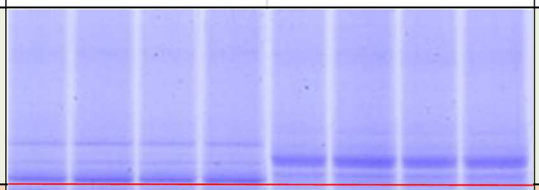
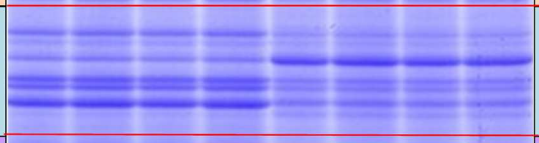
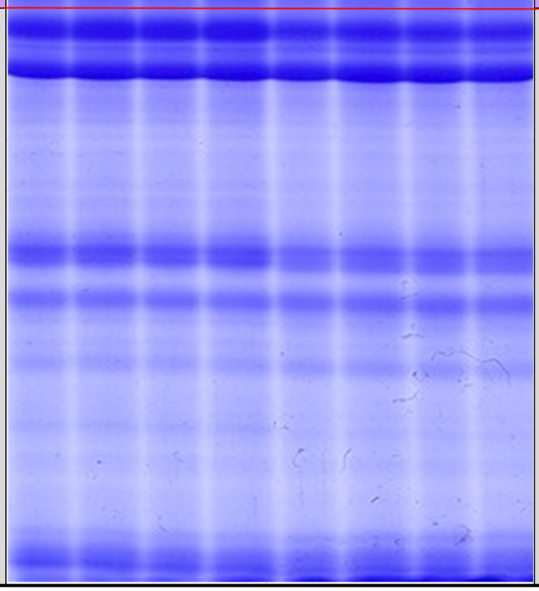
9.3.5.5 Staining

Two gels can be stained simultaneously. The gels are transferred to a container filled with 250 ml staining solution (9.3.4.3.3) 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 water for 30 min twice. Finally, the gels are incubated on the shaker in 5 % glycerine solution (9.3.4.3.4) for 5 min.

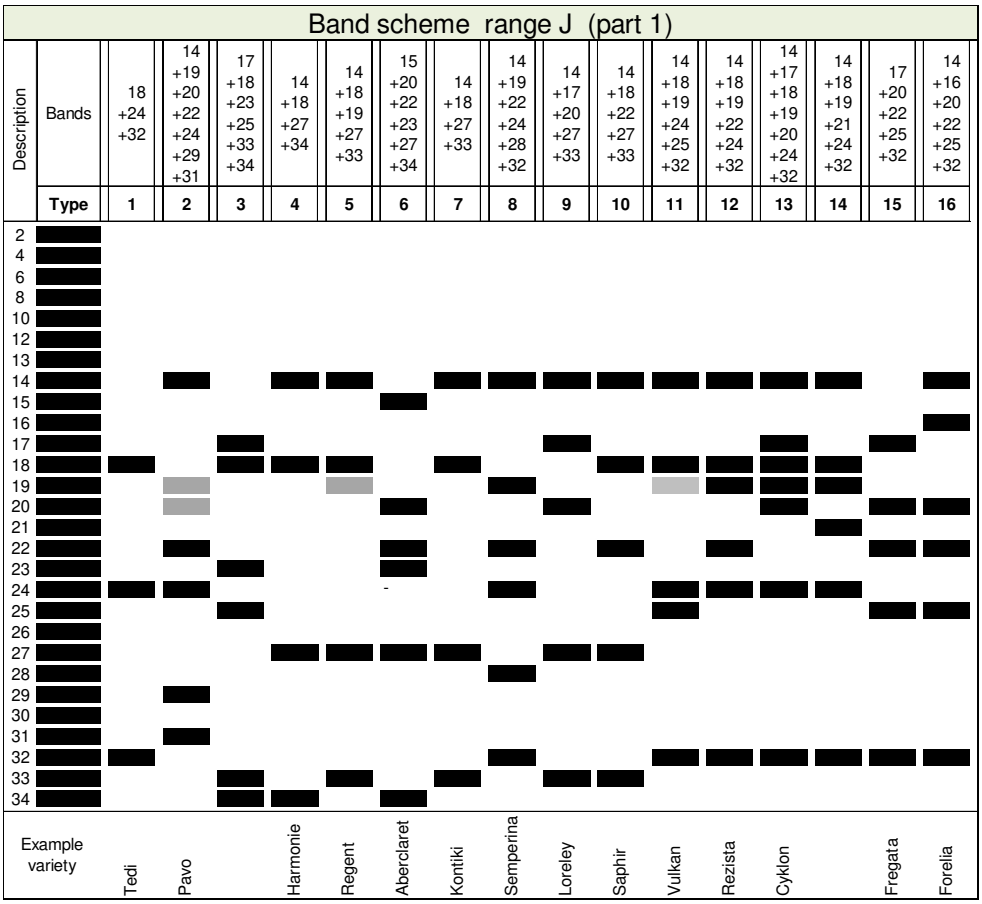
Note: For long-term storage, they gels can be preserved by wrapping them between two cellophane films (soaked in 7 % glycerine solution, stretched on glass plates) and then dried.

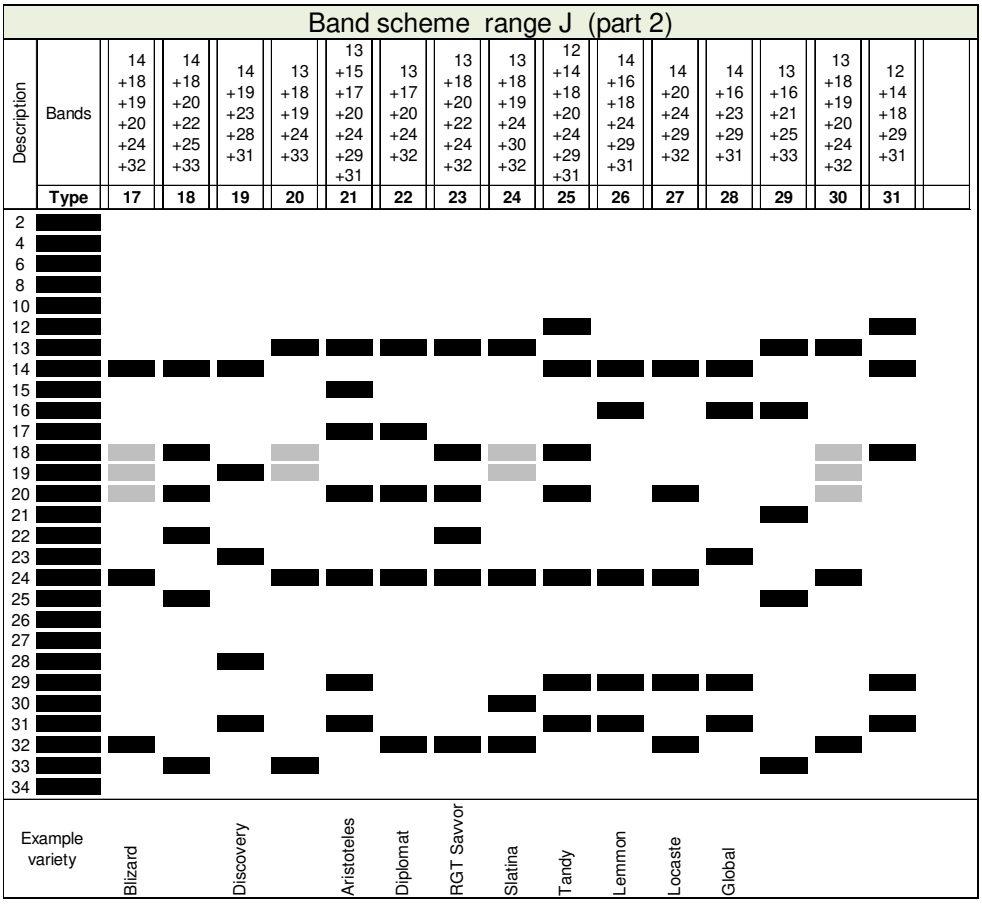
9.4 Interpretation of the gels

Band positions on 10 % SDS-PAGE

direction of migration	Band range	example variety ´Tedi´	example variety ´Pavo´	Band-No.
-	J			1 - 34
	K			35 - 49
	L			50 - 86
	M			87 - 102
+	grey area: not analyzed			

9.4.1 Band scheme range J



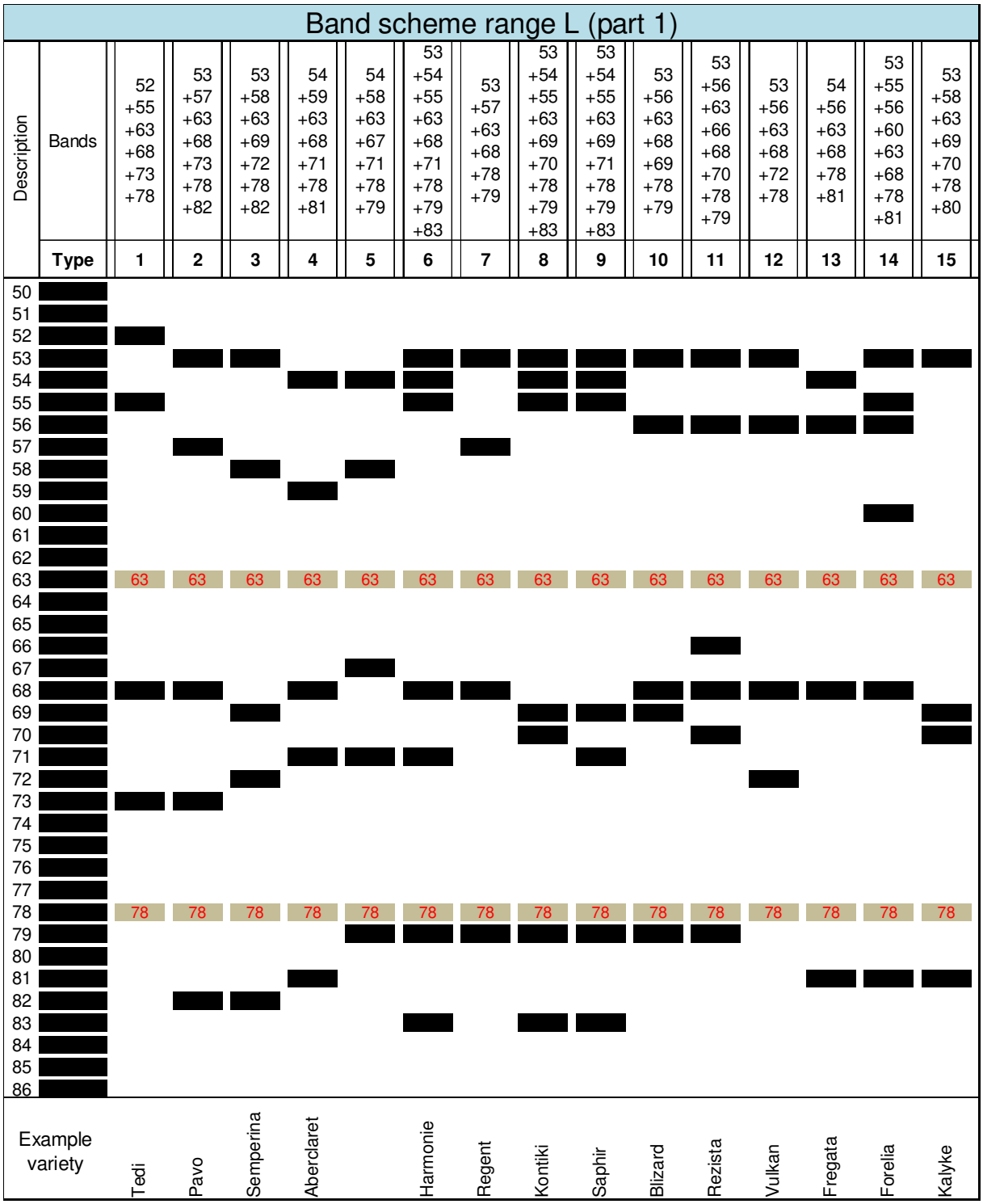


9.4.2 Band scheme range K

Band scheme range K

Description	Band	37 +39 +48	37 +40 +48	37 +41 +48	37 +42 +48	37 +43 +48	35 +38 +39 +48 +49	37 +39 +40 +49	35 +37 +39 +41 +48 +49	35 +38 +41 +49 +48 +49	37 +41 +48 +49	36 +40 +41 +44 +48 +49	36 +37 +38 +42 +44 +48 +49	37 +42 +48 +49	36 +37 +42 +44 +48	35 +37 +42 +46 +48 +49
	Type	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
35							■		■	■						■
36	■											■	■		■	
37	■	■	■	■	■	■		■	■		■		■	■	■	■
38	■						■			■			■			
39	■	■					■	■	■							
40	■		■					■				■				
41	■			■					■	■	■	■				
42	■				■								■	■	■	■
43	■					■										
44	■											■	■		■	
45	■															
46	■															■
47	■															
48	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■
49	■						■	■	■	■	■	■	■	■		■
Example variety		Tedi	Aberclaret	Harmonie	Pavo	Kontiki	Vulkan	Fregata	Tandy			Diadem	Discovery	RGT Savvor		Global

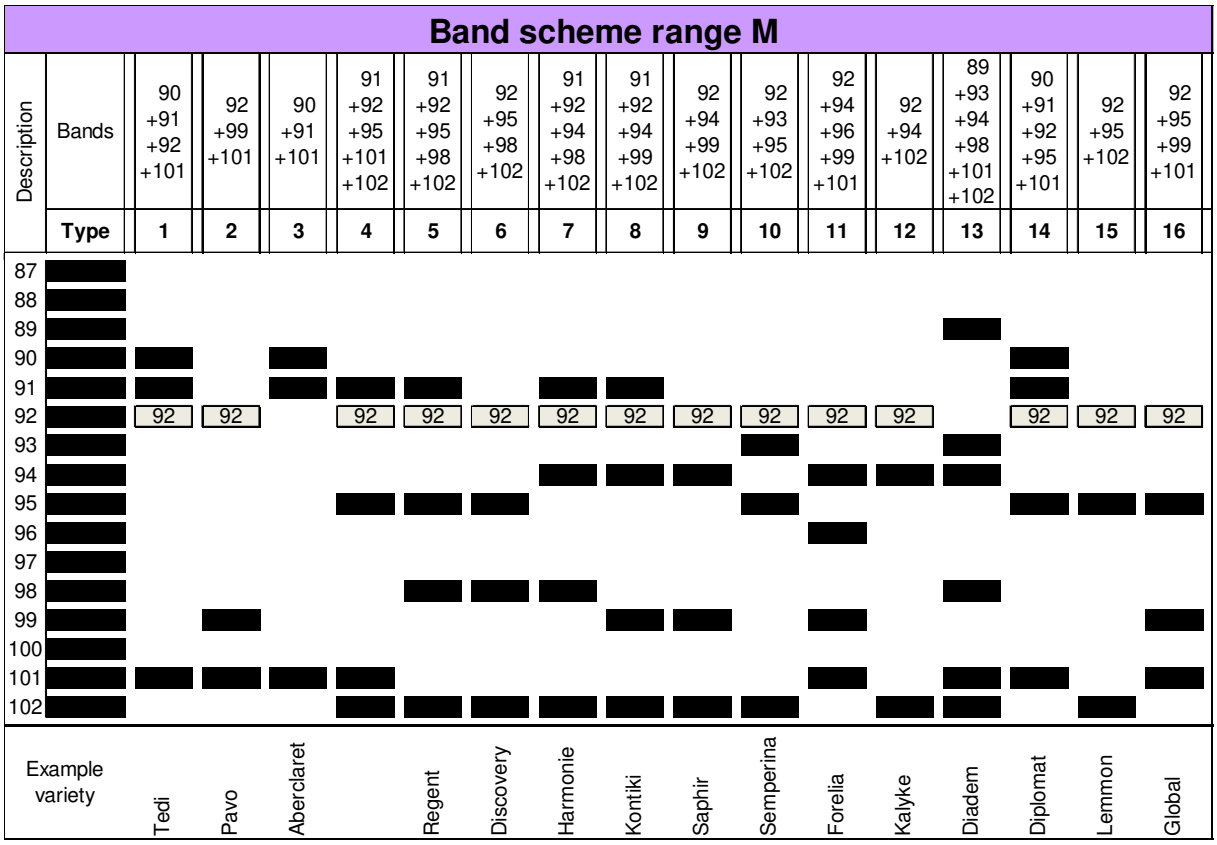
9.4.3 Band scheme range L



Band scheme range L (part 2)

Description	Bands	54 +58 +62 +63 +67 +73 +79 +86	54 +58 +62 +63 +68 +73 +79 +86	53 +55 +63 +68 +73 +78 +79	54 +63 +65 +68 +72 +78 +79 +83	54 +57 +63 +65 +68 +72 +78 +79 +83	53 +63 +64 +72 +78 +83	53 +57 +63 +73 +78 +82	54 +56 +63 +68 +72 +78 +79 +83	63 +70 +78 +82						
	Type	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**						
	50															
	51															
	52															
	53															
	54															
	55															
	56															
	57															
	58															
	59															
	60															
	61															
	62															
	63	63	63	63	63	63	63	63	63	63						
	64															
	65															
	66															
	67															
	68															
	69															
	70															
	71															
	72															
	73															
	74															
	75															
	76															
	77															
	78	78		78	78	78	78	78	78	78						
	79															
	80															
	81															
	82															
	83															
	84															
	85															
	86															
Example variety		Diadem	Discovery		RGT Savvor	Diplomat										

9.4.4 Band scheme range M



10. LITERATURE

Meier, U., 2001: Growth stages of mono- and dicotyledonous plants. BBCH-Monograph, German Federal Biological Research Centre for Agriculture and Forestry.

Mousset-Déclas, C., 1992: Le Trèfle Violet. In "Amélioration des espèces végétales cultivées, objectif et critères de sélection," ed. Gallais et Bannerot, INRA ed., pp.339-348.

Mousset-Déclas, C., 1995: Les trèfles ou le genre Trifolium. In "Ressources génétiques des plantes fourragères et à gazon. Prosperi, Guy, Balfourier Coord. Coéd. BRG-INRA, pp. 177-211.

Taylor, N.L., 1985: Clover science and technology, Agronomy nr. 25 in the series American Society of Agronomy, Inc., Crop Science Society.

Taylor, N.L., Quesenberry, K.H., 1996: Red Clover Science, Kluwer Academic Publishers, 228 pp.

11. TECHNICAL QUESTIONNAIRE

The Technical Questionnaire is available on the CPVO website under the following reference:
CPVO-TQ/005/1-Rev – *Trifolium pratense* L. – red clover