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

PROTOCOL FOR TESTS ON DISTINCTNESS, UNIFORMITY AND STABILITY

Brassica napus L. emend. Metzg.

RAPE SEED

UPOV Code: BRASS_NAP

Adopted on 21/04/2020

Entry into force on 01/08/2020

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CPVO-TP/036/3

1. SUBJECT OF THE PROTOCOL AND REPORTING

1.1 Scope of the technical protocol

This Technical Protocol applies to all varieties of ***Brassica napus*** **L. emend. Metz.**

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/036/6 dated 18/10/1996 (https://www.upov.int/edocs/tgdocs/en/tg036.pdf) for the conduct of tests for Distinctness, Uniformity and Stability.

1.2 Entry into Force

The present protocol enters into force on 01/08/2020. 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.

In case the variety is an agricultural hybrid, the variety descriptions of the parental components shall be added as well.

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 greement.

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.

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

3.4 Test design

Each test should be designed to result in a total of at least 200 plants, which should be divided between at least two replicates.

The assessment of the characteristic "Tendency to form inflorescences" should be designed to result in a total of at least 100 plants.

In case of hybrids, components (including the maintainer line) have to be tested and assessed as any other variety.

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 characters 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.

The variety collection shall include all varieties used as components (generally inbred lines) of all the hybrid varieties included in the variety collection, as well as varieties of common knowledge in their own right.

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.

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

(i) description of parental lines according to the Technical Protocols;

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

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

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

Further guidance is provided in documents TGP/9 "Examining Distinctness" and TGP/8 "Trial Design and Techniques Used in the Examination of Distinctness, Uniformity and Stability".

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.

Decision standards

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

In case of observations on individual plants or parts of plants (VS,MS) 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 proposed are not appropriate the method used should be clearly described.

For the assessment of distinctness the GAIA method maybe used. This method is explained in UPOV document TGP/8 Trial design and technicques used in the examination of distinctness, uniformity and stability (https://www.upov.int/edocs/tgpdocs/en/tgp_8.pdf)

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 45 plants or parts taken from each of 45 plants and any other observations made on all plants in the test, disregarding any off-type plants.

The assessment on characteristics referring to cotyledons (Numbering according to the list of CPVO characteristics 2, 3, 4, 5) should be made on 40 plants or parts taken from each of 40 plants.

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. 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

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 In case of visual observations, uniformity is assessed on the basis of off-types. For the assessment of uniformity on the basis of off-types, the number of the aberrant plants or parts of plants should be counted on the total of 200 plants.

For the assessment of uniformity of lines, a population standard of 2% and an acceptance probability of at least 95 % should be applied. In the case of a sample size of 200 plants, 7 off-types are allowed.

For the assessment of uniformity of hybrids, a population standard of 10% and an acceptance probability of at least 95 % should be applied. In the case of a sample size of 200 plants, 27 off-types are allowed.

In the case of observations on individual plants or parts of plants (VS, MS) the combined over years uniformity method (COYU) should be applied.

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 two or three consecutive cycles.

With respect to the use of isoenzyme electrophoresis, the Office follows the actual UPOV approach as laid down in Chapter 9. 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.

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.pd)

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.

Where appropriate, or in cases of doubt, the stability of a hybrid variety may, in addition to an examination of the hybrid variety itself, also be assessed by examination of the uniformity and stability of its parent lines.

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) Seed: erucic acid (characteristic 1)

b) Leaf: lobes (characteristic 8)

c) Time of flowering (characteristic 11)

d) Production of pollen (characteristic 16)

e) Plant: total length including side branches (characteristic 17)

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 use of some quantitative characteristics (QN) where the only type of observation prescribed for that characteristic is the measurement of a number of individual plants or parts of plants (MS) is not obligatory where the GAIA method is used in the assessment of distinctness (see paragraph 7. a) Distinctness).

The characteristics concerned are the following (numbering according to the list of CPVO-characteristics): 2, 3, 4, 5, 15, 19, 20, 22. They are labelled with the symbol °) in the list of characteristics.

If the GAIA method is used isoenzyme characteristics derived from protein electrophoreses may be included in the calculation of distances between variety pairs according to the rules defined in annex II.3. Isoenzyme electrophoresis has to be carried out according to the method specified in annex II.2.

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.

The list of characteristics derived from electrophoresis as 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°':

°)	Assessment of characteristics not obligatory in case GAIA method is used	
G	Grouping characteristic	-see Chapter 5
QL	Qualitative characteristic	
QN	Quantitative characteristic	
PQ	Pseudo-qualitative characteristic	
(+)	Explanations for individual characteristics	-see Chapter 8.1

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':

00-99	Explanations on growth stages	-see Chapter 8.2
MG, MS, VG, VS		-see Chapter 4.1.5
C	Special test	

For column 'Example varieties':
Example varieties are given as an indication, others may be used.

7. TABLE OF CHARACTERISTICS

CPVO N°	UPOV N°	Stage Method	Characteristics	Example varieties Spring	Example varieties Winter	Note
1. (+)	**1. (*)**	**00**	**Seed: erucic acid**			
QL		**C**	absent	Jerome	King 10	1
G			present	Erucola	Zeruca	9
2. °)		**15-17**	**Cotyledon: ratio length/width**			
(+)		**MS**	small	Tamarin	PR46W14	3
QN			medium	Jerome	PR46W15	5
			large	Sheik	NK Festivo	7
3. °)		**15-17**	**Cotyledon: saddle depth**			
(+)		**MS**	small	Dorothy	Contact	3
QN			medium	Jerome	King 10	5
			large	Senator	BU1015105	7
4. °)		**15-17**	**Cotyledon: ratio lobe separation/width**			
(+)		**MS**	small	Campino	Catalina	3
QN			medium	Calida	Mendel	5
			large	Sheik	Ontario	7
5. °)		**15-17**	**Cotyledon: ratio lobe separation/ saddle depth**			
(+)		**MS**	small	Magma	Cigal	3
QN			medium	Pauline	Komando	5
			large	Calida	Zeruca	7
6.	**4. (*)**	**23-27**	**Leaf: green colour**			
QN		**VG**	light	Calida	Campari	3
			medium	Lyside	King 10	5
			dark		Capitol	7

CPVO N°	UPOV N°	Stage Method	Characteristics	Example varieties Spring	Example varieties Winter	Note
7.		**23-27**	**Leaf: glaucosity**			
QL		**VG**	absent			1
			present	PS 8501	Flamingo	9
8. (+)	**5. (*)**	**23-27**	**Leaf: lobes**			
QL		**VG**	absent	Calida	Akela	1
G			present	Dorothy	King 10	9
9. (+)	**6. (*)**	**23-27**	**Leaf: number of lobes (fully developed leaf)**			
QN		**MS/VG**	few	SW Svinto	DK Cabernet	3
			medium	Dorothy	PR45D04	5
			many	Tamarin	Dante	7
10. (+)	**7. (*)**	**23-27**	**Leaf: dentation of margin**			
QN		**VG**	weak	Pauline	Vision	3
			medium	Heros	Mohican	5
			strong			7
11. (+)	**11. (*)**	**61-62**	**Time of flowering**			
QN		**MS/MG**	very early		Cavalcade	1
			early	Campino	ES Alegria	3
			medium	Liaison	Komando	5
			late	Jerome	MSL007C	7
G			very late		Greenland	9
12.	**12. (*)**	**62-63**	**Flower: colour of petals**			
PQ		**VG**	white			1
			cream	Lyside	Greenland	2
			yellow	Jerome	King 10	3
			orange-yellow			4

CPVO N°	UPOV N°	Stage Method	Characteristics	Example varieties Spring	Example varieties Winter	Note
13.	**13.**	**62-63**	**Flower: length of petals**			
QN		**MS/VG**	short	MSL523C	MSL004C	3
			medium	SRR 804186, Heros	Flamingo, King 10	5
			long	Osorno	Mohican	7
14.	**14.**	**62-63**	**Flower: width of petals**			
QN		**MS/VG**	narrow	Magma	NK Rapster	3
			medium	PS 8501, SW Landmark	Flamingo, King 10	5
			broad	Tamarin	Appolon	7
15.		**62-63**	**Flower: length/width ratio of petals**			
°)		**MS**	very small	Tamarin	R4513CA	1
QN			small	SRR804186	NW2239MC	3
			medium	PS 8501	Flamingo	5
			large	D 271310	MSL315C	7
			very large		DM021W11	9
16. QL	**15.**	**62-63**	**Production of pollen**			
		VG	absent	MSL523C	MSL007C	1
G			present	Jerome	King 10	9
17.	**17. (*)**	**70-80**	**Plant: total length including side branches**			
QN		**MS/MG**	very short		NW4193BC	1
			short	Cadoma	PR45D03	3
			medium	Heros	King 10	5
			long	Palmiro	PR46W15	7
G			very long		Appolon	9

CPVO N°	UPOV N°	Stage Method	Characteristics	Example varieties Spring	Example varieties Winter	Note
18. (+)	**18.**	**75-89**	**Siliqua: length (between peduncle and beak)**			
QN		**MS**	very short	R1385	Imola	1
			short	Calida	Belcanto	3
			medium	Heros	NK Diamond	5
			long	SW Landmark	Mohican	7
19. °)		**75-89**	**Siliqua: width**			
(+)		**MS**	narrow	Calida	NK Passion	3
QN			medium	Tamarin	Mohican	5
			broad	Jerome	Capitol	7
			very broad		MO13392	9
20. °)		**75-89**	**Siliqua: ratio length/width**			
(+)		**MS**	narrow	Jerome	MSL301C	3
QN			medium	Heros	Mendel	5
			broad	Magma	Charly	7
			very broad			9
21. (+)	**19.**	**75-89**	**Siliqua: length of beak**			
QN		**MS**	very short		DK Cabernet	1
			short	Calisto	NK Diamond	3
			medium	Calida	ES Astrid	5
			long	Liaison	Tosca	7
			very long		BL643196	9
22. °)	**20.**	**75-89**	**Siliqua: length of peduncle**			
(+)		**MS**	short	Calida	DK Cabernet	3
QN			medium	Campino	King10	5
			long	SW Landmark	Cindi CS	7

CPVO N°	UPOV N°	Stage Method	Characteristics	Example varieties Spring	Example varieties Winter	Note
23. (+)	**21.**		**Tendency to form inflorescences in year of sowing for <u>spring</u> sown trials**			
QN		**MG/VG**	absent or very weak		Akela	1
			weak		Mohican	3
			medium		Zenith	5
			strong		Cavalcade	7
			very strong		Tenor	9
24. (+)	**22.**		**Tendency to form inflorescences in year of sowing for <u>late summer</u> sown trials**			
QN		**MG/VG**	absent or very weak	C 999031		1
			weak	Forte		3
			medium	Calida		5
			strong			7
			very strong			9

8. EXPLANATIONS ON THE TABLE OF CHARACTERISTICS

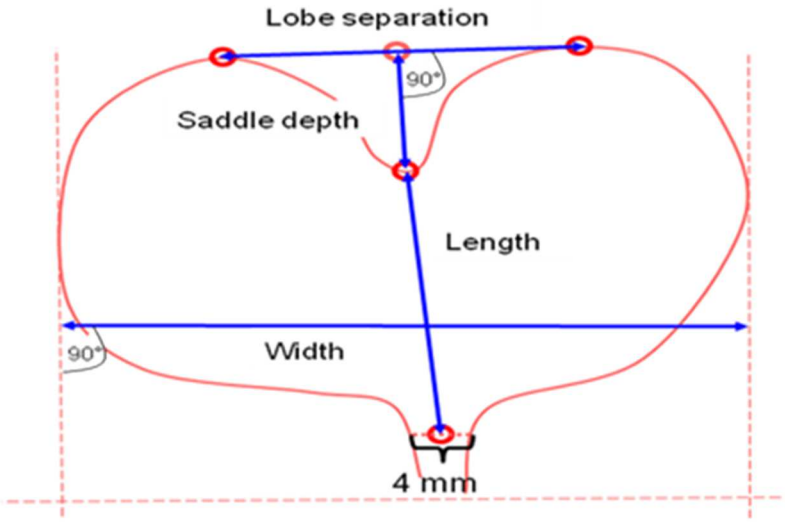
8.1 Explanations for individual characteristics

Ad. 1: Seed: erucic acid

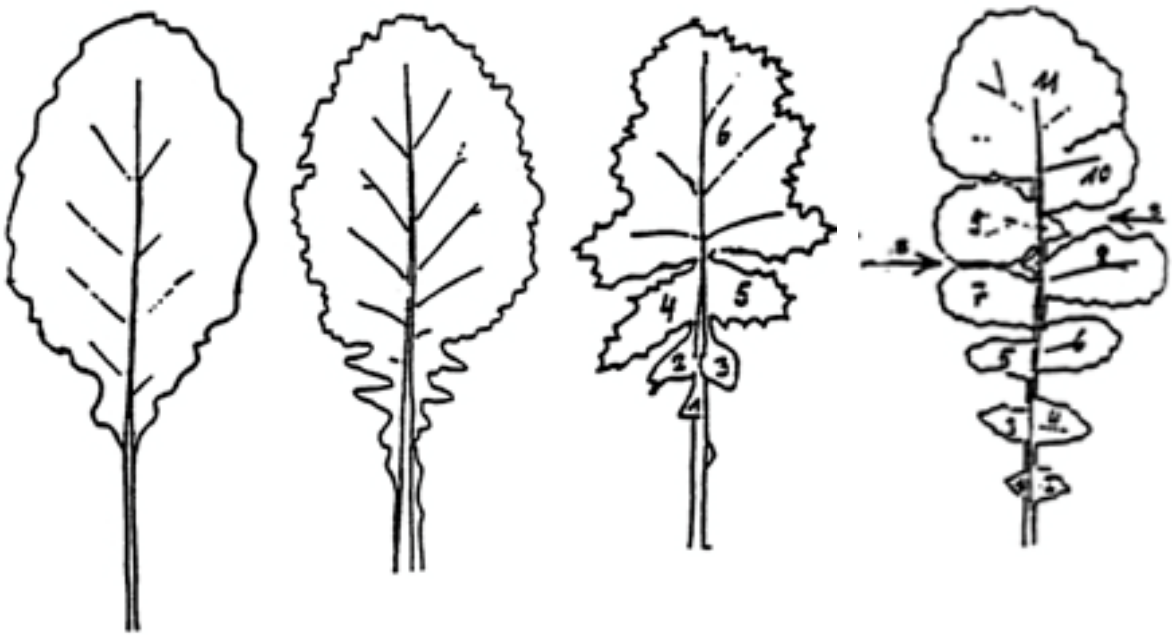
The erucic acid content should be observed on seed sent in by the applicant. It should be expressed as a percentage by mass of methyl esters in accordance with the ISO standard in document 5508, paragraph 6.2.2.1. Seed containing 2% or less of erucic acid will be classified as "absent."

Ad. 2 – 5: Cotyledon characteristics

The measurements should be taken in the glasshouse on cotyledons of 40 seedlings. If the two cotyledons differ in size, the biggest one should be measured. The measurements should be made according to the indications given in the drawing hereunder:

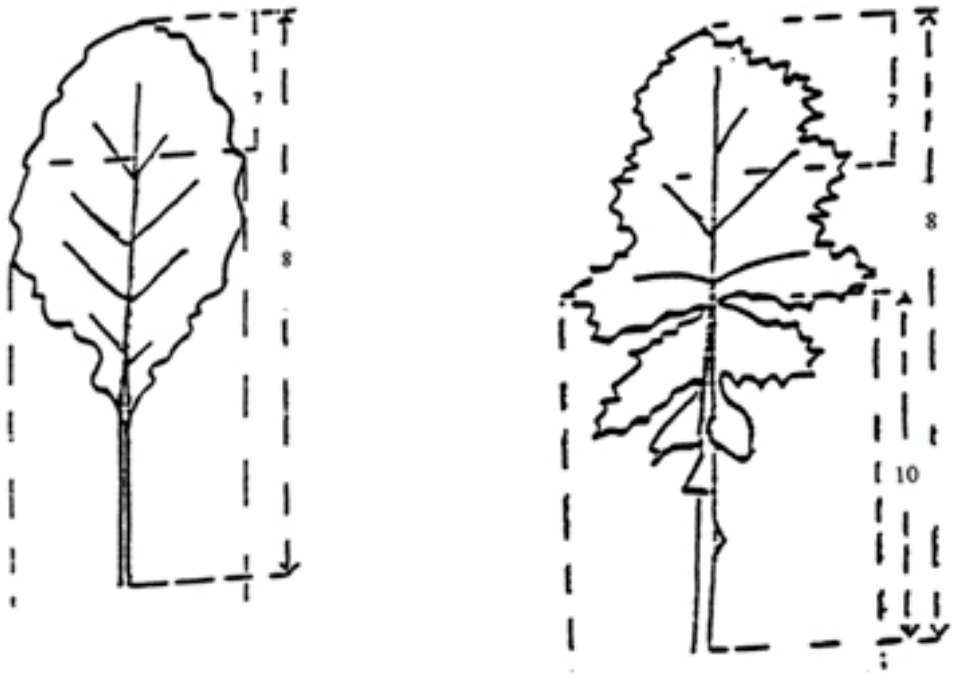


Ad. 8 – 9: Leaf: presence and number of lobes



Absence or presence of lobing should be observed on the whole plant at rosette stage. Parts of the leaf blade are considered as lobes if their length is at least equivalent to the width of the leaf petiole at their point of attachment and if the upper notch of the blade has at least half the length of the lobe itself. Secondary lobes (s) are not counted.

Ad. 10: Leaf: dentation



7 = part on which the dentation should be recorded (characteristic 10)

Ad. 11: Time of flowering

The observation should be done at least three times per week and more frequently if there is any need to do so. When assessed on individual plants, the date should be calculated--if necessary by interpolation--at which 50% of plants show at least one open flower. When assessed on the plot as a whole, the recommended percentage is 10%.

Ad. 18 - 22: Siliqua

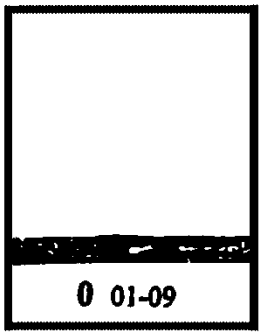
All observations on the siliqua should be recorded in the mid-part of the inflorescence of the main stem.

Ad. 23 - 24: Tendency to form inflorescences in the year of sowing

The tendency to form inflorescences in the year of sowing of winter rape varieties should be recorded in spring sown trials; that of spring rape varieties in late summer sown trials. The observation of the growth stage reached of winter rape varieties should be made in summer when the late spring rape varieties are flowering; that of spring rape varieties in autumn, when their development stagnates.

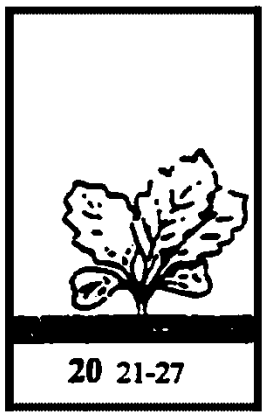
8.2 KEY FOR THE GROWTH STAGES

KEY	GENERAL DESCRIPTION
0	Germination
00	Dry Seed
10	Seedling growth
11	Appearance of cotyledons
13	Cotyledons expanded
15	1 leaf-stage
17	2 leaf-stage
19	3 leaf-stage
20	Rosette
21	4 leaf-stage
22	5 leaf-stage
23	6 leaf-stage
24	7 leaf-stage
25	8 leaf-stage
26	9-11 leaf-stage
27	12 or more leaves are completely developed
30	Stem elongation
31	Distance between cotyledons and vegetation point is more than 5 cm
35	Distance between cotyledons and vegetation point is more than 15 cm
39	Distance between cotyledons and vegetation point is more than 25 cm
50	Bud formation
51	Terminal bud is present, not raised above leaves
53	Terminal bud is raised above level of leaves
57	Pedicels are elongating
59	Buds are yellowing
60	Flower
61	First open bud on terminal raceme
62	Few buds are open on terminal raceme
64	Full flower, lower siliques are elongating
65	Lower siliques are starting to fill, less than 5% of buds are not yet open
67	Seeds in lower siliques are enlarging, all buds are open
70	Siliqua
71	Seeds in lower siliques are in full size translucent
75	Seeds in lower siliques are green, opaque
79	All seeds of siliques on terminal raceme are dark
80	Maturation
81	Seeds in lower siliques on terminal raceme show brown areas
85	Seeds in upper siliques show brown areas
89	Brown siliques are brittle, stems are dry

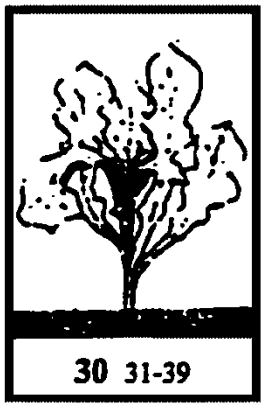


germination

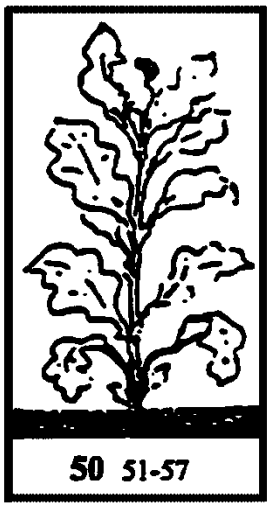
seedling growth



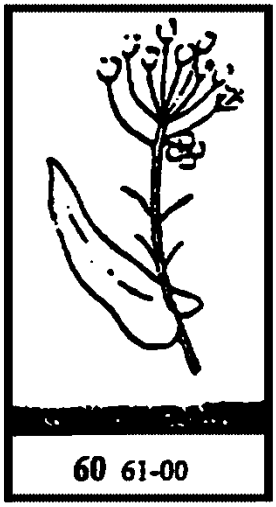
rosette



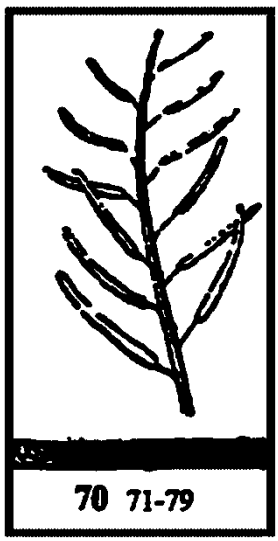
stem elongation



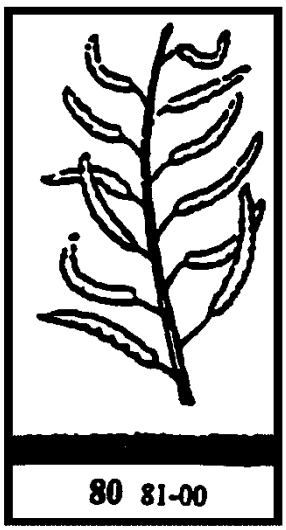
bud formation



flower



silique



maturation

9. ELECTROPHORESIS

Description of the SGE Method for the Analysis of Isoenzymes from *Brassica napus*

1. Number of individuals per test

- for DUS tests:
  10 leaves of each inbred line

2. Apparatus and equipment

Any suitable horizontal electrophoresis system can be used, provided that the gels can be kept at 4°C. A gel thickness of 10 mm is recommended. The power supply used should be capable of delivering constant voltage output.

3. Chemicals

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

3.1 Chemicals for enzyme extraction

Glutathione 2% (reduced)
Tris

3.2 Chemicals for electrophoresis

Bromophenol blue
Citric acid monohydrate
L-Histidine
Starch hydrolyzed, for electrophoresis,)
Trizma Base

3.3 Chemicals for staining enzymes

2,6-Dichlorophenol-indophenol Na salt
Ethanol
Ethylenediamine tetra-acetic acid Na2 Salt (EDTA)
Fast Garnet GBC salt
D-Fructose 6-phosphate Na2 salt
Glucose 1-phosphate dehydrogenase (Serva 22820 or 22822 or Sigma G5885)
Hydrochloric acid (HCl)
DL-Isocitric acid Na3 salt
Magnesium chloride hexahydrate
DL-Malic acid
Dimethylthiazol diphenyl tetrazolium (MTT)
β -Nicotinamide adenine dinucleotide (NAD)
Cis aconitic acid
Isocitric dehydrogenase
β -Nicotinamide adenine dinucleotide phosphate (NADP)
Nitro-blue tetrazolium (NBT)
Sodium hydroxide (NaOH)
6-phosphogluconic acid Na3 salt dihydrate
Phenazine methosulfate (PMS)
Tris-(hydroxymethyl) aminomethane (Tris)
Shikimic acid

4. Solutions

4.1 Extraction solution

400 mg glutathione
made up to 20 ml with de-ionised water and adjusted to pH 7.8 with Tris (2M).

4.2 Electrophoresis buffers

4.2.1 Buffers for SGE pH 5.7

4.2.1.1 Running buffer: -histidine-citrate pH 5.7
20.18 g histidine
+8 g citric acid
made up to 2 1 with de-ionised water

4.2.1.2 Gel buffer
10.088 g histidine
+3.65 g citric acid
made up to 1 1 with de-ionised water

4.2.2 Buffers for SGE pH 7.0

4.2.2.1 Running buffer
31.49 g Trizma base
+16.2 g Citric acid
made up to 2 liters with de-ionised water

4.2.2.2 Gel buffer
2.99 g DL histidine
Adjusted to pH7 with 1M NaOH and made up to 2l with de-ionised water

4.2.2.3 Bromophenol blue solution
50 mg bromophenol blue dissolved in 100 ml de-ionised water

4.3 Staining solutions

4.3.1 Stock solutions

4.3.1.1 1 M Tris-HCL pH 8.0
121.1 g Tris, made up to 1 liter with de-ionised water and adjusted to pH 8.0 with 50% HCl

4.3.1.2 1 M Tris-HCL pH 9.1
121.1 g Tris, made up to 1 liter with de-ionised water and adjusted to pH 9.1 with 50% HCl

4.3.1.3 MTT solution
1.0 g MTT, made up to 100 ml with de-ionised water

4.3.1.4 NBT solution
1.0 g NBT made up to 100 ml with de-ionised water

4.3.1.5 PMS solution
200 mg PMS, made up to 100 ml with de-ionised water

4.3.1.6 MgCl2 solution
10 g Magnesium chloride hexahydrate
made up to 100 ml with de-ionised water

4.3.1.7 Malic acid solution
5 g LL-Malic acid, made up to 100 ml with de-ionised water and adjusted to pH 8.0 with 1 M NaOH
400 mg NADP + 40 ml de-ionised water

4.3.2 Staining solutions (volume: 100 ml)

4.3.2.1 MDH staining solution
10 ml Tris-HCl pH 9.1 (4.3.1.2.)
+ 80 ml de-ionised water
+ 4 ml Malic acid solution (4.3.1.7.)
+ 20 mg NAD
+ 1,5 ml NBT solution (4.3.1.4.)
+ 2,5 ml PMS solution (4.3.1.5.)

4.3.2.2 PGI staining solution
10 ml Tris-HC1 pH 8.0 (4.3.1.1.)
+ 85 ml de-ionised water
+ 40 mg Fructose 6-phosphate Na2 salt
+ 10 mg NADP
+ 1,5 ml NBP solution (4.3.1.4.)
+ 2,5 ml PMS solution (4.3.1.5.)
+ 25 units Glucose 6-phosphate dehydrogenase

4.3.2.3 ACO staining solution
10 ml Tris-HC1 pH 8.0 (4.3.1.1.)
+ 80 ml de-ionised water
+ 115 mg cis aconitic acid
+ 4 ml MgCl2 solution (4.3.1.6.)
+ 10 mg NADP
+ 2 ml MTT solution (4.3.1.3.)
+ 2,5 ml PMS solution (4.3.1.5.)
+ 40 units isocitric dehydrogenase

4.3.2.4 PGM staining solution
20 ml Tris-HC1 pH 8.0 (4.3.1.1.)
+ 180 ml de-ionised water
+ 300 mg Glucose 1-phosphate
+ 10 mg EDTA Na2 salt
+ 2 ml MgCl2 solution (4.3.1.6.)
+ 8 mg NADP
+ 2 ml MTT solution (4.3.1.3.)
+ 3 ml PMS solution (4.3.1.5.)
+ 40 units Glucose 6-phosphate dehydrogenase

4.3.2.5 SHDH staining solution
10 ml Tris-HC1 pH 8 (4.3.1.1.)
+ 82 ml de-ionised water
+ 100 mg shikimic acid
+ 15 mg NADP
+ 2 ml MTT solution (4.3.1.3.)
+ 5 ml PMS solution (4.3.1.5.)

4.3.2.6 6-PGD staining solution
10 ml Tris-HC1 pH 8 (4.3.1.1.)
+ 85 ml de-ionised water
+ 50 mg 6-phospho gluconic water
+ 12 mg NADP
+ 2 ml MTT solution (4.3.1.3.)
+ 2,5 ml PMS solution (4.3.1.5.)

5. Procedure

5.1 Enzyme extraction

Oilseed rape seedlings are grown in a box with sand or vermiculite, at 18°C, in darkness. After 3 weeks, leaves are taken and ground at 4°C, in micro-tubes containing 100 µl extraction solution (4.1). The tubes are then centrifuged at 4°C to obtain a clear supernatant. The extracts can be stored at - 80°C.

5.2 Preparation of the gel

To make two 12.5 % starch gels (18 x 18 x 1 cm) the following is required: 128 g starch are mixed in 1020ml gel buffer (4.2.1.3. or 4.2.2.2.) in a 1000 ml Buchner flask at 80°C. The mixture is degassed for 40 seconds. The gels are poured into gel moulds as described in the user's manual of the equipment used. The formation of air bubbles should be avoided. The gels are allowed to cool at room temperature, for at least two hours, and wrapped with polyethylene film for overnight storage. Before electrophoresis, the gels are cooled at 4°C for at least one hour.

5.3 Electrophoresis

The tanks are filled with the appropriate volume of running buffer (4.2.1.2. or 4.2.2.1.) pre-cooled to 4°C. A slit is cut in the gel at 1 cm from the cathode. The enzyme extracts from 5.1 (30 extracts for on 18 x 18 x 1 cm gel) are absorbed onto 15 x 2 x 1 mm wicks at from Whatman N° 3 chromatography paper. The wicks are placed into the slit. At 1 cm of each edge of the gels, a wick soaked with bromophenol blue solution (4.2.2.3.) is inserted. The electrophoresis is carried out at 4°C. A constant voltage of 200 V (maximum current of 150 mA for two 18 x 18 x 1 cm gels is applied for 20 minutes). The wicks are then removed and the electrophoresis is continued at a constant voltage of 280 V (maximum current of 180 mA for two 18 x 18 x 1 cm gels), until the bromophenol blue marker has migrated 14 cm (4 hours).

5.4 Enzyme staining

After electrophoresis the gel is cut horizontally in 1 mm thick slices. The upper slice is discarded. Individual gel slices are stained by incubation in their respective solutions (4.3.2.) at 37°C in darkness.

The staining durations range between 30 and 120 minutes. After staining the gel, slices are rinsed in distilled water before being stored. The following procedure for long time storing can be successfully used: e.g. drying the gels between two cellophane sheets or storing in sealed polythene bags.

6. Description and interpretation of the zymograms

This section describes the zymograms and specific points for their interpretation. For each enzyme system, the proposed schematic representation of a zymogram corresponds to the electrophoretic separation obtained at the pH suggested.

Genes (loci) and alleles are underlined and written in lower case, genotypes are indicated in square brackets and enzymes and enzyme bands are written in upper case.

A list of reference samples is provided in Table I. Photographs of various zymograms, for all the enzymes considered, are presented. Table II lists the allelic frequencies of registered or protected parental hybrid lines and varieties, in France.

The nomenclature used is that previously described for Brassica (Arus, 1989; Arus and Orton, 1983; Arus and Shields, 1983; Quiros et al., 1987; Chèvre et al., 1995).

Table I: Enzymatic description of reference lines of oilseed rape

Variety	Continent	Type	PGI	LAP	6-PGD	MDH	TPI	ACO	ShDH	ACP
1	Europe	Winter	3	1	1	2	1	1	1	1
2	Europe	Winter	3	2	1	1	2	1	1	2
3	Europe	Winter	3	2	2	5	1	14	1	1
4	Europe	Winter	3	2	1	5	1	17	1	1
5	Europe	Winter	3	2	1	5	6	1	1	4
6	Europe	Winter	3	2	2	5	2	7	3	1
7	Europe	Winter	2	2	1	2	1	6	1	8
8	Europe	Spring	2	3	1	4	3	7	3	1
9	Europe	Spring	2	2	1	4	3	2	2	1
10	Europe	Spring	2	2	1	5	4	8	2	1
11	Europe	Spring	2	3	1	5	1	16	2	1
12	Australia	Spring	1	2	1	1	3	15	4	4
13	Australia	Spring	2	2	2	4	1	-	7	4
14	Australia	Spring	3	2	2	4	4	10	1	4
15	America	Spring	3	2	2	4	4	12	2	1
16	Korea	Spring	3	4	3	5	4	11	5	5
17	Japan	Spring	3	4	1	4	4	4	5	6
18	Japan	Spring	3	5	2	1	4	4	6	5
19	Japan	Spring	3	2	1	4	5	5	3	5
20	Japan	Spring	3	2	3	5	4	3	4	5
21	Japan	Spring	3	2	2	3	4	9	3	4
22	China	Spring	3	2	1	1	7	19	2	2
23	China	Spring	3	2	1	1	4	20	4	2

Table II: Pattern frequency for 196 varieties or parental lines registered or protected since 1993

Enzyme system	Profile	Pattern frequency
ACO	1	0.44
	2	0.01
	3	ε
	4	0.01
	7	0.07
	8	0.03
	10	0.06
	13	0.01
	14	0.07
	16	0.01
	17	0.01
	21	0.02
	22	0.16
LAP	1	0.23
	2	0.7
	3	0.07
PGI	1	ε
	2	0.17
	3	0.71
	5	0.12
	6	ε
ShDH	1	0.67
	2	0.06
	3	0.14
	4	0.11
	5	0.02
	6	ε
	8	ε
MDH	1	0.44
	2	0.19
	4	0.26
	5	0.11
PGM	1	1
	3	ε
	8	ε
PGD	1	0.82
	2	0.18
	3	ε
	5	ε

6.1 Enzyme system

Names, abbreviations and quaternary structures are indicated in Table III.

Table III: Enzyme systems

Enzyme system	Identification	Abbreviation	**Enzyme quaternary structure**
Aconitase	EC 4.2.1.3	ACO	monomeric
Malate dehydrogenase	EC 1.1.1.37	MDH	dimeric
Phosphoglucoisomerase	EC 5.3.1.9	PGI	dimeric
6-Phosphogluconate dehydrogenase	EC 1.1.1.44	PGD	dimeric
Phosphoglucomutase	EC 2.7.5.1	PGM	monomeric
Shikimate dehydrogenase	EC 1.1.1.25	ShDH	monomeric
Acid phosphatase	EC 3.1.3.2	ACP	dimeric

6.2 Region of activity

Gels were divided into regions of activity numbered from anode to cathode (e.g.: ACO-1, ACO-2, ACO-3, ACO-4). In general, each region corresponds to the migration zone of enzymes produced by two homeologous genes. These regions have been defined in diploid species of Brassica as corresponding to enzymes produced by a single gene. For enzyme systems known to involve duplicated genes, the region of activity corresponds to the expression of four homeologous genes.

6.3 Genes

Genes are named according to the region of activity to which their products (enzymes) migrate (ex: Aco-1 for the region ACO-1). As oilseed rape is an amphidiploid generated from a cross between cabbage (B. oleracea, CC) and turnip (B. rapa, AA), two homeologous genes from each parental species are expressed in each region of activity. If a gene is known to originate from the A or C genome, the letter A or C is added to the gene name. If genome assignment is not possible, the letters a and b are used to distinguish the homeologous genes (ex: Aco-1A, Aco-1C, Aco-2a, Aco-2b). For duplicated genes, a special notation is used to distinguish the two genes in the same region of activity (ex: 6Pgd-1A and 6Pgd-1'A).

6.4 Alleles

Alleles are numbered 1, 2, 3 etc., from the fastest to the slowest migrating enzyme. If new alleles are encountered, they can be numbered according to their migration position (e.g.: 1.5, 3.7). Null alleles are identified by the letter "n".

6.5 Genotypes-profiles

Owing to the large number of genes involved in the expression of the isoenzymes, genotypes are translated into profiles, to increase simplicity.

The profiles differ by one or several genes.

Example: Profile 1 of ACO corresponds, for the seven genes of oilseed rape to the following genotype:
[Aco-1A 2/2], [Aco-1C 1/1], [Aco-2a 1/1], [Aco-2b 1/1], [Aco-3a 2/2], [Aco-3b 2/2], [Aco-4 1/1].

The regions of activity, the genes and the alleles observed in oilseed rape are shown in Table IV.

Table IV: Regions of activity, genes and allelic diversity of rapeseed

Enzyme system	Region of activity	Gene	Map(1)	Number of alleles	Name of alleles					
ACO	ACO-1	Aco-1C		1	1					
		Aco-1A	DY9	2		1	2			
	ACO-2	Aco-2a		1	1					
		Aco-2b		2	1	2				
	ACO-3	Aco-3a	SD4	2	1	2				
		Aco-3b		4		1	2	3	4	
	ACO-4	Aco-4		3	1	2	n			
MDH	MDH-1	Mdh-1a	DY13	1	1					
		Mdh-1b		2	1	2				
	MDH-4	Mdh-4a	DS9	2	1	2				
	MDH-5	Mdh-5a		1	1					
		Mdh-5b	DY13	2	1	2				
PGI	PGI-1	Pgi-1A		1	1					
		Pgi-1C		1	1					
	PGI-2	Pgi-2A	DY5	3	1	2	3		n	
		Pgi-2C	SW15	4		1	2	3		r (2)
		6-Pgd-1A		1	1					
6-PGD	6-PGD-1	6-Pgd-1C	DY14	1	1					
		6-Pgd-1'A		2		1	2			
		6-Pgd-1'C		1				1		
		6-Pgd-2A	SD18	2	1	2				
	6-PGD-1	6-Pgd-2C		1		1				
		6-Pgd-2'A		1		1				
		6-Pgd-2'C		1		1				
PGM	PGM-3	Pgm-3A	DY16	3	1	2	3			
		Pgm-3C		2		1	2			
	PGM-1	Pgm-1A		2		1	2			
		Pgm-1C		1	1					
	PGM-2	Pgm-2A		1	1					
		Pgm-2C		2		1	2			
ShDH	ShDH -1	ShDH-1a		4	1	2	3		4	
		ShDH-1b		3			1	2	3	
	ShDH -2	ShDH -2	DY4	2	1	2				
ACP	ACP-1L	Acp-1LA	DY4	3	1	2	3			
		Acp-1LC		2	1	2				

(1) DY = 'Darmor-bzh' x 'Yudal' (Table VI),
DS = 'Darmor' x 'Samourai'
SD = 'Stellar' x 'Drakkar'
SW = 'Stellar' x 'Wesway'

(2) r allele Pgi from radish

For a given region of activity, alleles located in a same column encode isoenzymes of similar electrophoretic mobility.

6.5.1 Aconitase (ACO) E.C.4.2.1.3

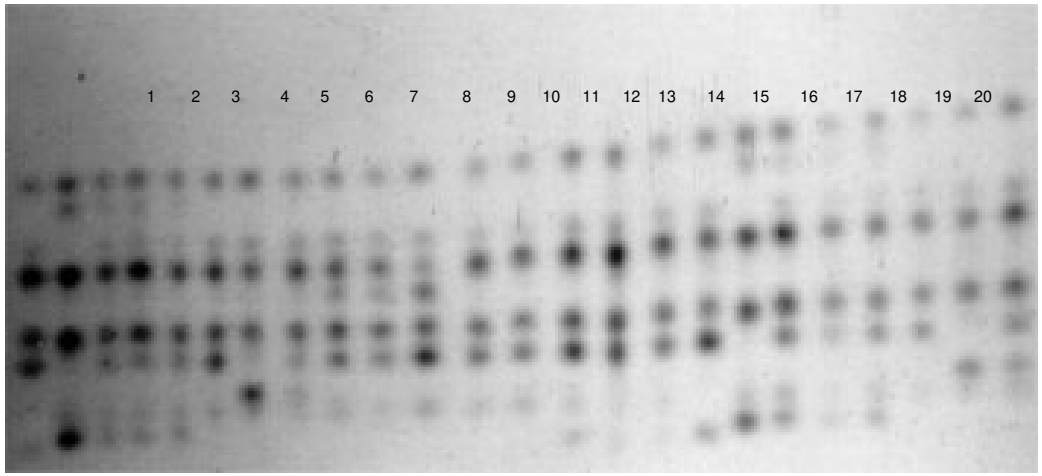
6.5.1.1 Genetics

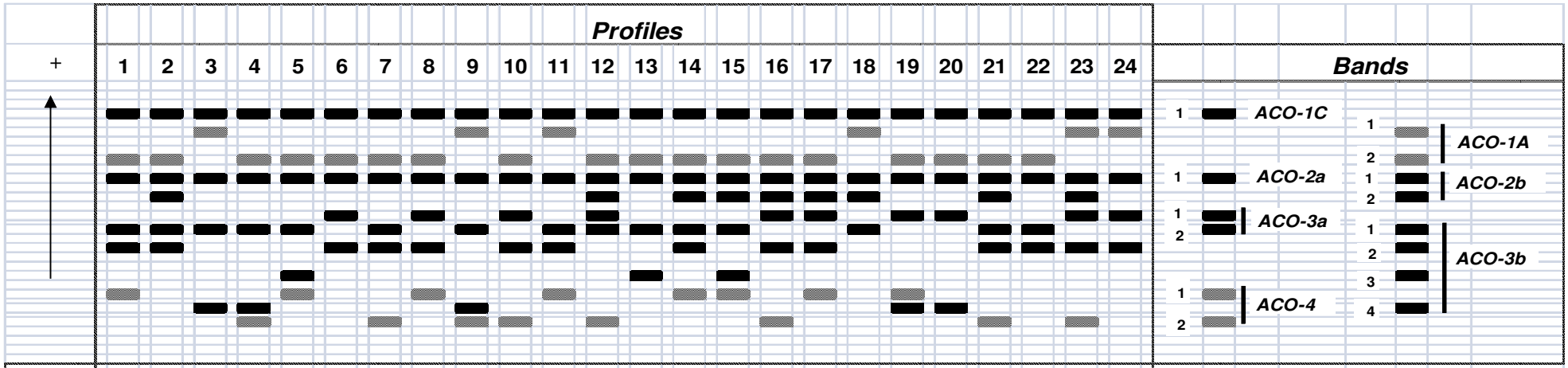
Four regions of activity are detected (ACO-1, ACO-2, ACO-3, ACO-4) each corresponding to one locus expressed in the three diploid species B. oleracea, B. rapa and B. nigra (Arus, 1989; Chèvre et al., 1995). Aconitase is present in the cytosolic, mitochondrial and glyoxysomal fractions (Wendel et al., 1988; Zemlyanukhin et al., 1984). The cellular compartments corresponding to the regions of activity are not known in Brassica.

Each region of activity corresponds to two genes, except for region ACO-4. Only one gene, Aco-4, is expressed in this region, although each of the progenitor species of oilseed rape contains an Aco-4 gene. Only the genes of region ACO-1 have been assigned to genomes A and C.

Figure 3a: Zymograms of aconitases (ACO) from oilseed rape

Lanes	Genotypes
1, 13	Profile 1
3	Profile 3
4	Profile 4
5	Profile 5
6	Profile 6
7	Profile 7
8	Profile 8
9	Profile 9
10	Profile 10
11	Profile 11
12	Profile 12
14	Profile 14
15	Profile 15
16, 20	Profile 16
17	Profile 17
18	Profile 19
19	Profile 20
2	Profile 21





	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Aco-1C	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Aco-1A	22	22	11	22	22	22	22	22	11	22	11	22	22	22	22	22	22	11	22	22	22	22	11	11
Aco-2a	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Aco-2b	11	22	11	11	11	11	11	11	11	11	11	22	11	22	22	22	22	22	11	11	22	11	22	11
Aco-3a	22	22	22	22	22	11	22	11	22	11	22	11	22	22	22	11	11	22	11	11	22	22	11	11
Aco-3b	22	22	44	44	33	22	22	22	44	22	22	11	33	22	33	22	22	11	44	44	22	22	22	22
Aco-4	11	nn	nn	22	11	nn	22	11	22	22	11	22	nn	11	11	22	11	nn	11	nn	22	nn	22	nn

Two genes are monomorphic (Aco-1C and Aco-2a) (Table IV).

Five genes are polymorphic:
- Aco-1A with two codominant alleles, allele 2 is the most frequent.
- Aco-2b with two alleles, allele 1 is more frequent than allele 2.
- Aco-3a with two alleles, allele 2 is more frequent than allele 1.
- Aco-3b with four alleles, allele 2 is the most frequent.
- Aco-4 with three alleles, including one null allele.

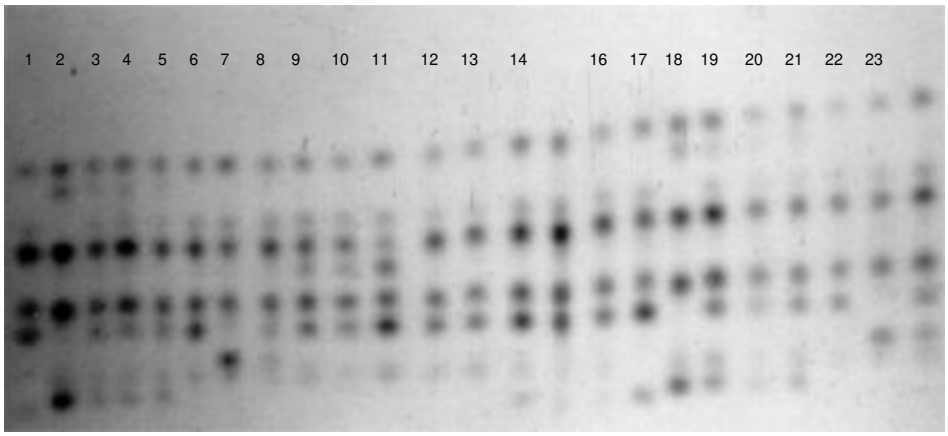
6.5.1.2 Zymograms

Electrophoretic separation of ACO is carried out in the pH 7.0 buffer system.

Buffer system at pH 6.5 can also be used but the enzymes encoded by Aco-3a may be less well separated from those encoded by Aco-3b at this pH.

The activity of aconitases is higher in leaves than in younger tissues, such as 5-day-old seedlings and apices from 5- to 7-day-old seedlings, particularly for Aco-4. Conversely, Aco-3b is much more active than the other genes in 3-day-old seedlings and apices from 5- to 7-day-old seedlings. The activity of Aco-4 is generally low. This is probably the reason why only three zones were described by Mündges et al. (1990), and Ripley et al. (1992), in contrast to the results of Delourme and Foisset (1991), and Chèvre et al. (1994).

Figure 3b: Zymograms of aconitases (ACO) from oilseed rape



Lanes	Genotypes
6, 12, 13, 22	Profile 1
2, 18	Profile 3
7, 23	Profile 5
17	Profile 7
11	Profile 14
3, 4, 5, 19, 20, 21	Profile 1/3
8	Profile 1/5
1, 14, 16	Profile 1/7
9, 10	Profile 1/14

The genes corresponding to each region of activity (ACO-1, ACO-2, ACO-3, ACO-4) number from anode to cathode.

The band encoded by Aco-2a1 migrates to the same position as that encoded by Aco-2b1. The band encoded by Aco-3a2 migrates to the same position as that encoded by Aco-3b1 (figures 3a et 3b).

The zymograms of all homozygous genotypes and of most of the heterozygous genotypes are readily distinguishable. Table VI shows genotypes heterozygous for at least one gene, with the same zymogram. Profile-based notation has been used.

For example, « 1 x 2 » means that the plant has a heterozygous genotype corresponding to a cross between a plant with profile 1 and a plant with profile 2, e.g.. [Aco-1A 2/2], [Aco-1C 1/1], [Aco-2a 1/1], [Aco-2b 1/2], [Aco-3a 2/2], [Aco-3b 2/2], [Aco-4 1/n]. This plant with the profile 1 x 2 is heterozygous for the genes Aco-2b and Aco-4.

The fifth column concerns heterozygous genotypes which could, theoretically, be differentiated by slight differences in band intensity. As not all these genotypes have been analysed, it would be better to consider them to be identical.

Heterozygous genotypes on the same line are identical or should be considered as such (fifth column).

Table V: Crosses giving identical zymograms

				Non-analysed, but considered to be identical
1 x 2	14 x 22			
1 x 6	8 x 22			1 x 8
1 x 9	4 x 11			
1 x 10	7 x 8			
1 x 11				11 x 22
1 x 13	5 x 22			1 x 5
1 x 15	5 x 14			
1 x 16	7 x 17	8 x 21	10 x 14	
1 x 17	8 x 14			
1 x 20	19 x 22			1 x 19
1 x 21	7 x 14			
1 x 23	11 x 16			
1 x 24	6 x 11			
2 x 5	13 x 14	15 x 22		
2 x 7	21 x 22			
2 x 8	6 x 14	17 x 22		
2 x 9	3 x 21			9 x 21
2 x 10	6 x 21	16 x 22		
2 x 19	14 x 20			
2 x 23				21 x 23
3 x 4				4 x 9
3 x 7	9 x 22			7 x 9
3 x 8	11 x 20			11 x 19
3 x 10	4 x 24	6 x 9		9 x 10
3 x 12				9 x 12
3 x 23				9 x 23
4 x 6	7 x 20			4 x 10
4 x 7				4 x 22
4 x 8	7 x 19			
4 x 17	19 x 21			
4 x 23	9 x 16			3 x 16
5 x 6	8 x 13			5 x 8
5 x 11				11 x 13
5 x 16	10 x 15			
5 x 17	8 x 15			
5 x 20	13 x 19			5 x 19
5 x 21	7 x 15			
6 x 7	10 x 22			7 x 10
6 x 12				10 x 12
6 x 15	13 x 17			
6 x 16				10 x 16
6 x 17				8 x 17

6 x 19	8 x 20			10 x 19
6 x 23	16 x 24			10 x 23
7 x 16	10 x 21			
8 x 16	10 x 17			
11 x 14				2 x 11
11 x 17				14 x 24
14 x 16	17 x 21			
17 x 19				17 x 20
21 x 24	22 x 23			7 x 23

6.5.2 Malate dehydrogenase (MDH) E.C.1.1.1.37

6.5.2.1 Genetics

The NAD-dependent malate dehydrogenase is present in three cell compartments: mitochondria, cytosol and peroxysomes (Gottlieb, 1981). However, the zones of activity corresponding to the genes expressed in the various cell compartments are not well separated. The various genes have therefore not been assigned to particular cell compartments.

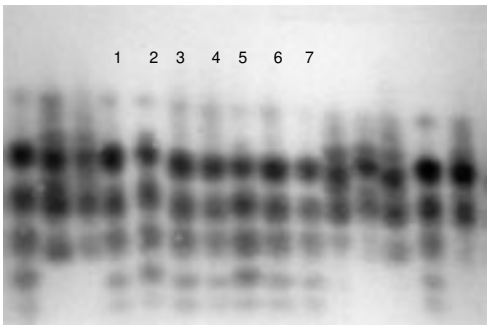
No reference is available for this enzyme. The study of several oilseed rape crosses has made it possible to deduce the genetics of only three polymorphic genes:

- Mdh-1a, the products of which migrate to the region close to the anode and Mdh-5b, the products of which migrate to the region close to the cathode; these two genes are linked;
- Mdh-4a, which encodes enzymes that migrate to a region closer to the anode than those encoded by Mdh-5a.

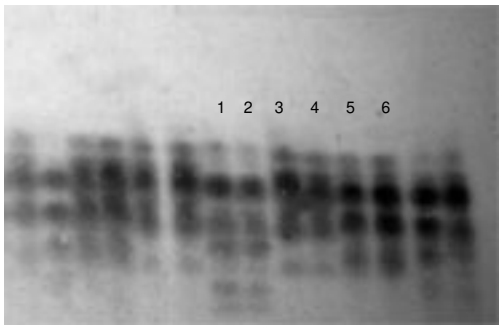
Two alleles have been identified for each of these genes (Table IV).

Profile 3 is found only in the variety "Genkai" and is therefore difficult to interpret genetically.

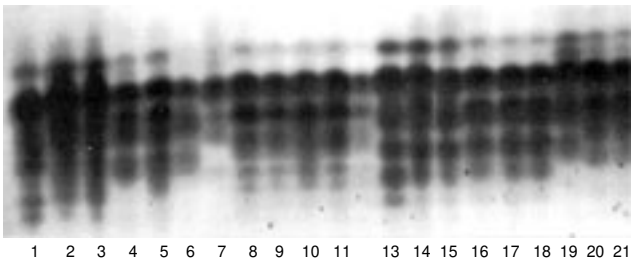
Figure 5: Zymograms of malate dehydrogenases (MDH) from oilseed rape



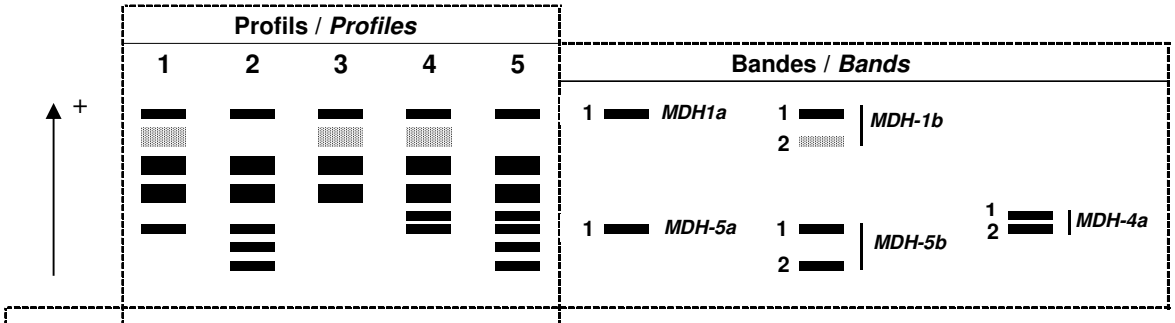
Lanes	Genotypes
1, 3, 4, 6, 7------	Profile 2
2, 5 ----------	Profile 5



Lanes	Genotypes
3, 4 ----------	Profile 1
1, 2 ----------	Profile 2
5, 6 ----------	Profile 4



Lanes	Genotypes
7----------	Profile 1
1----------	Profile 2
4, 6, 19----------	Profile 4
13 ----------	Profile 5
2, 3, 5 ----------	Profile 2/4
8, 9, 10, 11 ----------	Profile 2/1
20, 21----------	Profile 4/1
14, 15, 16, 17, 18 ----------	Profile 5/4



Mdh-1a	11	11	11	11	11
Mdh-1b	22	11	22	22	11
Mdh-4a	22	22	?	11	11
Mdh-5a	11	11	?	11	11
Mdh-5b	11	22	?	11	22

6.5.2.2 Zymograms

Electrophoretic separation of MDH is carried out in buffer systems at pH 5.7 or pH 6.5.

MDH enzymes are active at all physiological stages.

The enzyme is dimeric. This dimeric structure is particularly evident for the Mdh-5 genes, for which profiles 2 and 5 display two additional bands: one corresponds to the homodimer encoded by the allele Mdh-5b2 and the other to the heterodimer formed by the subunits encoded by the two Mdh-5 genes. The complexity of the profiles renders it difficult to distinguish the heterodimers formed by the products of the other genes.

The bands encoded by the alleles Mdh-5a1 and Mdh-5b1 migrate to the same position as the band encoded by the allele Mdh-4a2 (figure 5).

All the homozygous genotypes can be differentiated, but for some electrophoretic separations, profiles 1 and 4 may be confused with profiles 2 and 5, respectively (gene Mdh-4a). The heterozygous genotype [Mdh-5b 1/2] is clearly different from the two homozygous genotypes [Mdh-5b 1/1] and [Mdh-5b 2/2]. The same holds true for the heterozygotes with profiles 1/5, 2/4 and 4/5 which can be distinguished from their corresponding homozygotes. However, it is not possible to differentiate between these hybrid profiles.

6.5.3 6-Phosphogluconate dehygrogenase (6-PGD) E.C.1.1.1.44

6.5.3.1 Genetics

Two regions of activity (6-PGD-1, plastidic, and 6-PGD-2, cytosolic) are observed and each corresponds to four genes because the genes are already duplicated in the progenitor diploid species (Quiros et al., 1987).

Within each region of activity, the duplicated genes are indicated by a "prime" e.g. 6-Pgd-1, 6-Pgd-1', 6-Pgd-2 and 6-Pgd-2'.

6-PGD enzymes have been used to distinguish addition lines in oilseed rape (Chèvre et al., 1991; Struss et al., 1992) and D. erucoides (This et al., 1990) and to describe oilseed rape varieties (Delourme and Foisset, 1991).

Six genes are monomorphic (6-Pgd-1A, 6-Pgd-1C, 6-Pgd-1'C, 6-Pgd-2C, 6-Pgd-2'A, 6-Pgd-2'C).

Only two genes are polymorphic (Table IV):
- 6-Pgd-1'A with two alleles; allele 1 is only present in Asian genotypes;
- 6-Pgd-2A with two alleles.

Profile 3 is particularly frequent among Asian genotypes.

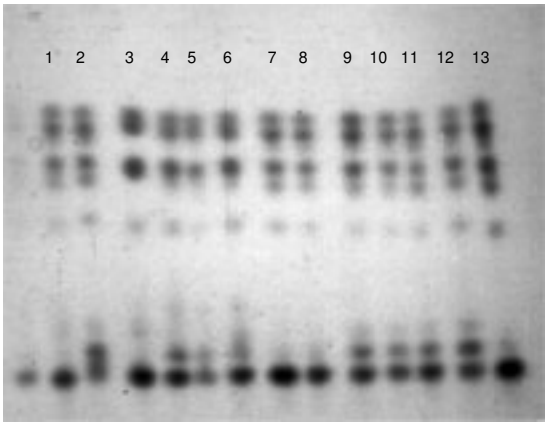
6.5.3.2 Zymograms

Electrophoretic separation is carried out in the pH 5.7 buffer system.

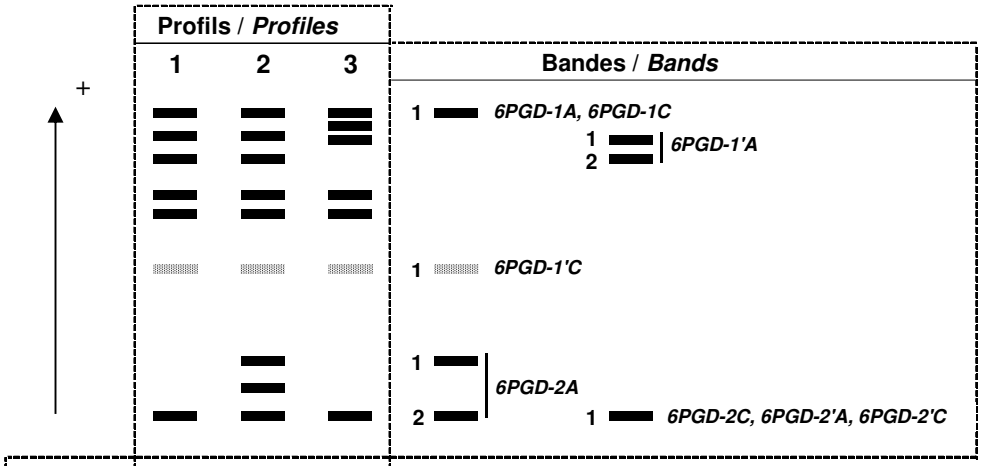
The enzyme is dimeric. All oilseed rape genotypes generate six bands in the 6-PGD-1 region. These bands correspond to the homodimers for each gene (6-Pgd-1A, 6-Pgd-1C, 6-Pgd-1'A, 6-Pgd-1'C) and heterodimers corresponding to interactions between the duplicated genes of the two genomes, A and C, expressed in chloroplasts. All the cytosolic genes (region 6-PGD-2) interact to form a heterodimer in profile 2 plants. In contrast, no heterodimer is formed between the products of genes expressed in different cell compartments.

The enzymes encoded by the alleles 6-Pgd-1A1 and 6-Pgd-1C1 have similar mobility. The same holds true for the alleles 6-Pgd-2C1,6-Pgd-2'A1, 6-Pgd-2'C1 (figure 6).

Figure 6: Zymograms of 6-phosphogluconate dehydrogenases (6-PGD) from oilseed rape



Lanes	Genotypes
1, 7, 8, 13--------	Profile 1
2, 12 ----------	Profile 2
3----------	Profile 3
9, 10, 11 ---------	Profile 1/2
4, 5, 6 ----------	Profile 2/3



6Pgd-1A	11	11	11
6Pgd-1C	11	11	11
6Pgd-1'A	22	22	11
6Pgd-1'C	11	11	11
6Pgd-2A	22	11	22
6Pgd-2C	11	11	11
6Pgd-2'A	11	11	11
6Pgd-2'C	11	11	11

In the region 6-PGD-1, all the homozygotes and heterozygotes [6-Pgd-1'A 1/2] are clearly differentiated. Hybrids with profiles 1/3 or 2/3 can be distinguished from plants with profiles 1, 2 or 3, in this region.

In contrast, in the region 6-PGD-2, the heterozygotes [6-Pgd-2A 1/2] (profiles 1/2 and 2/3) cannot be distinguished from the homozygote [6-Pgd-2A 1/1] (profile 2) and in some cases, are not readily distinguishable from the homozygote [6-Pgd-2A 2/2] (profiles 1 and 3) because artefactual bands may be detected above the band corresponding to genes 6-Pgd-2C, 6-Pgd-2'A and 6-Pgd-2'C, particularly in seedlings.

6.5.4 Phosphoglucoisomerase (PGI) E.C.5.3.1.9

6.5.4.1 Genetics

Two regions of activity (PGI-1, plastid, and PGI-2, cytosolic) are detected and each corresponds to two genes. The PGI-1 region has been described as monomorphic in B. oleracea (Arus and Orton, 1983) and B. rapa (Mündges et al., 1989; Chen et al., 1990; McGrath and Quiros, 1991).

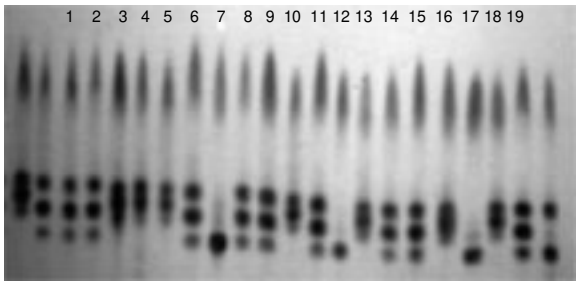
In oilseed rape too, only the genes corresponding to the PGI-2 region are polymorphic.

PGI enzymes have been used to distinguish addition lines in oilseed rape (Quiros et al., 1987; Chèvre et al., 1991; Struss et al., 1992) and to describe oilseed rape varieties (Mündges et al., 1990; Delourme and Foisset, 1991).

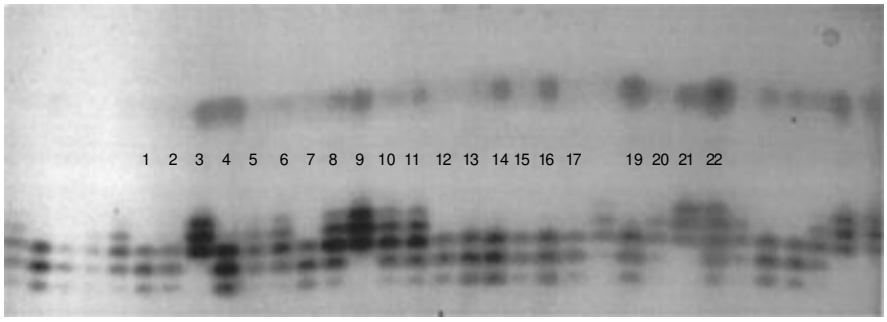
Three alleles have been identified at the Pgi-2A locus, with allele 1 the most frequent. A null allele has been observed in some genotypes, such as the variety Topas.

Four alleles are present at the Pgi-2C locus, including the "r" allele, which was introgressed from radish together with Rfo, the cytoplasmic male sterility restorer gene of the Ogu-INRA system (Delourme and Eber, 1992) (Table IV).

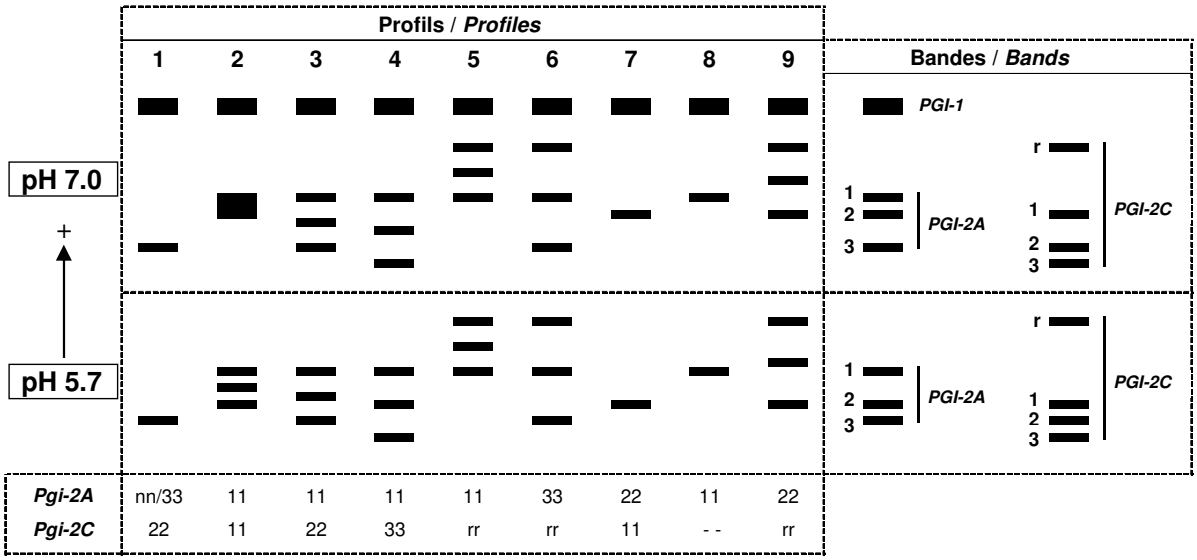
Figure 7: Zymograms of phosphoglucoisomerases (PGI) from oilseed rape



Lanes	Genotype
7, 12, 17 ----------	Profile 1
3, 4, 5, 10, 13, 16, 18 ----------	Profile 2
1, 2, 6, 8, 9, 11, 14, 15, 19 -------	Profile 3



Lanes	Genotypes
1, 2, 4, 7, 12, 13, 14, 15, 16, 17, 19 ------	Profile 3
3, 9, 21 ----------	Profile 5
5, 6, 8, 10, 11, 20, 22----------	Profile 3/5



Profiles 2 and 3 are the most common in oilseed rape. Profile 2 is more frequently found in spring oilseed rape and profile 3 in winter oilseed rape. Profiles 5, 6 and 9 have been observed with the use of restorer lines from the Ogu-INRA system in breeding.

The profile 8 is found in some restorer lines which are homozygous for the gene Rfo but have lost the allele Pgi-2 from radish (Delourme and Eber, 1992).

6.5.4.2 Zymograms

Electrophoretic separation is carried out in buffer systems at pH 7.0 or pH 6.5. It is also possible to use the pH 5.7 buffer system, with which the three bands of profile 2 are better separated.

PGI enzymes are active at all physiological stages, including seeds imbibed for 24 hours.

The enzyme is dimeric. Most varieties of oilseed rape have a three-band zymogram: two homodimers corresponding to Pgi-2A and Pgi-2C and one intergenic heterodimer, resulting from association of the polypeptide subunits encoded by the alleles of the two Pgi-2 genes. No heterodimer is formed between the subunits encoded by the genes of regions PGI-1 and PGI-2, which correspond to different cell compartments.

The enzymes encoded by the Pgi-2A2 and Pgi-2C1 alleles migrate to the same position. The same holds true for the alleles Pgi-2A3 and Pgi-2C2 (figure 7).

All the homozygous and heterozygous genotypes can be differentiated with buffer systems at pH 7.0 and pH 6.5. The heterozygote [Pgi-2C 1/2] (corresponding to hybrid profile 2/3) is difficult to differentiate from the homozygote [Pgi-2C 2/2] (profile 3) at pH 5.7.

6.5.5 Phosphoglucomutase (PGM) E.C.2.7.5.1

6.5.5.1 Genetics

Three regions of activity are observed (PGM-1, PGM-2 and PGM-3), each corresponding to two genes. The PGM-1 and PGM-2 zones have been described by Arus and Orton (1983) from studies with B. oleracea seeds. The third zone, PGM-3, was then detected with leaf extracts from B. oleracea and B. rapa (Arus, 1989; McGrath and Quiros, 1991). These three zones are also observed in B. nigra (Chèvre et al., 1995).

PGM-2 are active in the cytosol and chloroplasts (Weeden and Gottlieb, 1980). According to Quiros et al. (1988), the isozymes of region PGM-2 are active in the cytosol. No information is available for the two other regions.

PGM enzymes have been used to distinguish between addition lines in oilseed rape (Chèvre et al., 1991; Struss et al., 1992) and D. erucoides (Quiros et al., 1988; This et al., 1990).

Two genes are monomorphic (Pgm-1C and Pgm-2A).

Four genes are polymorphic (Pgm-3A, Pgm-3C, Pgm-1A and Pgm-2C) with three alleles for Pgm-3A and two alleles per gene for the others (Table IV).

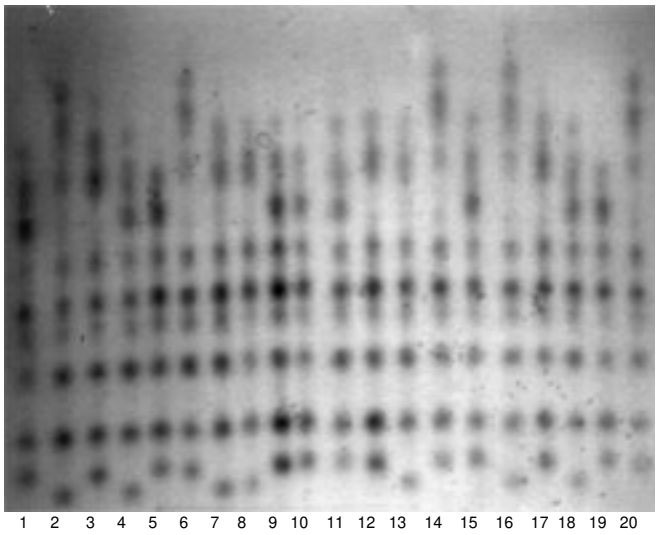
However, this polymorphism is most frequently encountered in Asian genotypes. Profile 1 is typical of European genotypes.

6.5.5.2 Zymograms

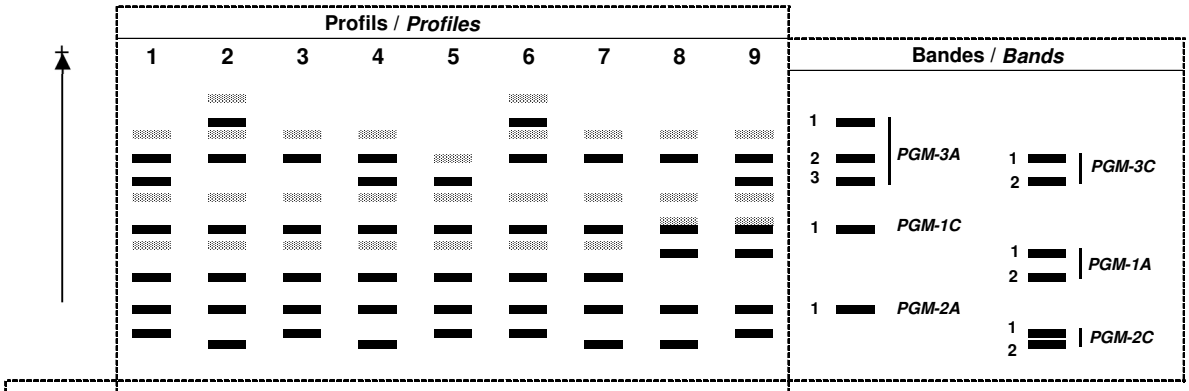
Electrophoretic separation is carried out in the pH 5.7 buffer system. The pH 6.5 and pH 7.0 buffer systems may also be used.

The fastest migrating region is referred to as PGM-3 because it was identified after the two other regions, in studies using leaves (Arus, 1989; McGrath and Quiros, 1991); this region is not observed with seeds and very young seedlings. It is detected only in leaves.

Figure 8: Zymograms of phosphoglucomutases (PGM) from oilseed rape



Lanes	Genotypes
1, 9, 10, 11, 15	Profile 1
2, 16	Profile 2
3, 12, 17	Profile 3
4, 18	Profile 4
5, 19	Profile 5
6, 14, 20	Profile 6
7, 8, 13	Profile 7



	1	2	3	4	5	6	7	8	9
Pgm-3A	33	11	22	33	33	11	22	22	33
Pgm-3C	11	11	11	11	22	11	11	11	11
Pgm-1A	22	22	22	22	22	22	22	11	11
Pgm-1C	11	11	11	11	11	11	11	11	11
Pgm-2A	11	11	11	11	11	11	11	11	11
Pgm-2C	11	22	11	22	11	11	22	22	11

The genes corresponding to the regions PGM-1 and PGM-3 give two bands per allele: one band with strong activity and the other, which migrates closer to the anode, with weaker activity.

The enzymes encoded by the alleles Pgm-3A3 and Pgm-3C2 have the same mobility, as is also the case for the proteins encoded by the alleles Pgm-3A2 and Pgm-3C1 (figure 8).

In the PGM-3 region, all the homozygous genotypes can be differentiated. However, [Pgm-3A 1/3] heterozygotes are the only heterozygotes that can be distinguished from the corresponding homozygotes. Thus, only hybrids between plants having a profile 1, 4, 5 or 9 and plants having a profile 2 or 6 are clearly different from their parental homozygotes.

However, all these hybrids present identical profiles.

The same holds true for hybrids with 3/5, 5/7 or 5/8 profiles: they can be distinguished from the corresponding homozygotes, but not from each other as their profiles are identical, and identical to profile 1.

Lastly, it is not possible to distinguish between the profiles of hybrids between plants with profiles 1, 2, 4, 6 or 9 and plants of profiles 3, 7 or 8 and profiles 1, 2, 4, 6 or 9, respectively.

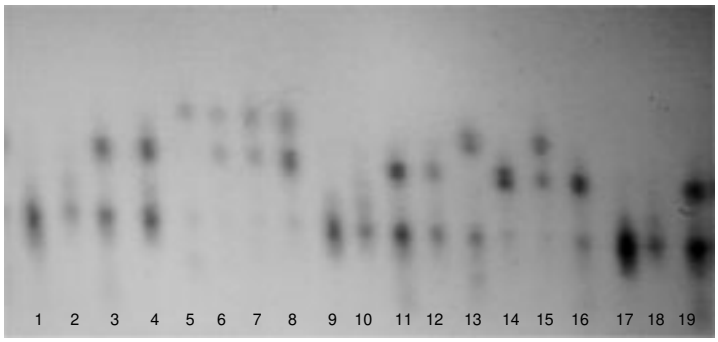
In the PGM-1 and PGM-2 regions, all the heterozygous genotypes are readily distinguished from the homozygous genotypes.

6.5.6 Shikimate dehydrogenase (SHDH) E.C.1.1.1.25

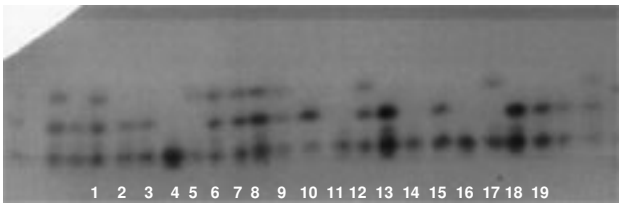
6.5.6.1 Genetics

Two regions of activity have been described in B. rapa (Truco, 1986). In oilseed rape, the first region, ShDH-1, corresponds to two genes and the second, ShDH-2, to only one gene, which has been identified in some genotypes.

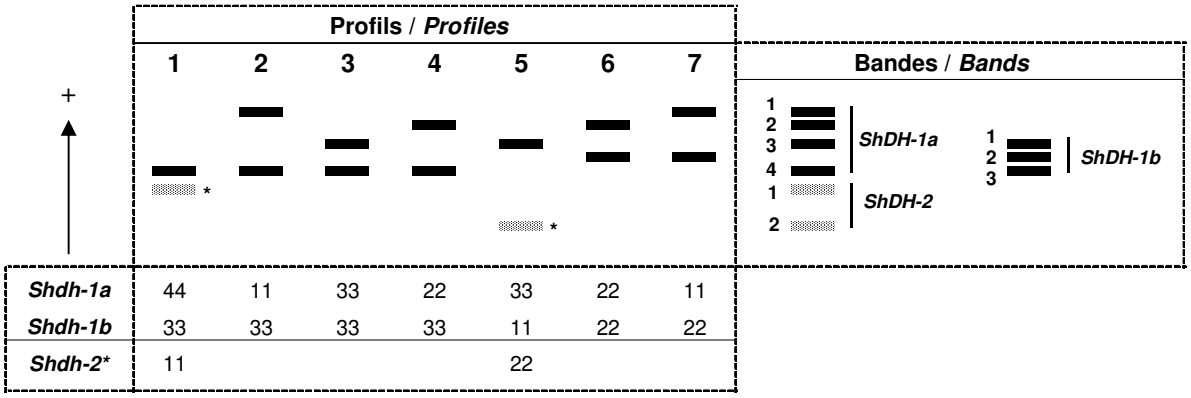
Figure 9: Zymograms of shikimate dehydrogenases (ShDH) from oilseed rape



Lanes	Genotypes
1, 2, 9, 10, 17, 18	Profile 1
3, 4, 11, 12, 19	Profile 3
5 ,13	Profile 4
14	Profile 5
6, 7, 15	Profile 6
8	Profile 7



Lanes	Genotypes
4, 11, 14, 16	Profile 1
2, 3, 10, 13, 15, 18, 19	Profile 3
5, 17	Profile 4
1, 6, 7, 8, 9, 12	Profile 3/4



Shdh-1a	44	11	33	22	33	22	11
Shdh-1b	33	33	33	33	11	22	22
Shdh-2*	11				22		

* non révélé dans tous les génotypes
** not detected in all the genotypes*

ShDH enzymes are active in chloroplasts and the cytosol (Weeden and Gottlieb, 1980; Weeden and Wendel, 1989). The various genes have not been assigned to cell compartments in Brassica.

ShDH enzymes have been used by Mündges et al. (1990) to distinguish oilseed rape varieties.

Four alleles are present at the Shdh-1a locus, three at the Shdh-1b locus and two at the Shdh-2 locus (Table IV).

Profile 1 is the most frequent.

6.5.6.2 Zymograms

Electrophoretic separation of ShDH is carried out in the pH 7.5 buffer system, but also in buffer systems at pH 7.0 or pH 6.5.

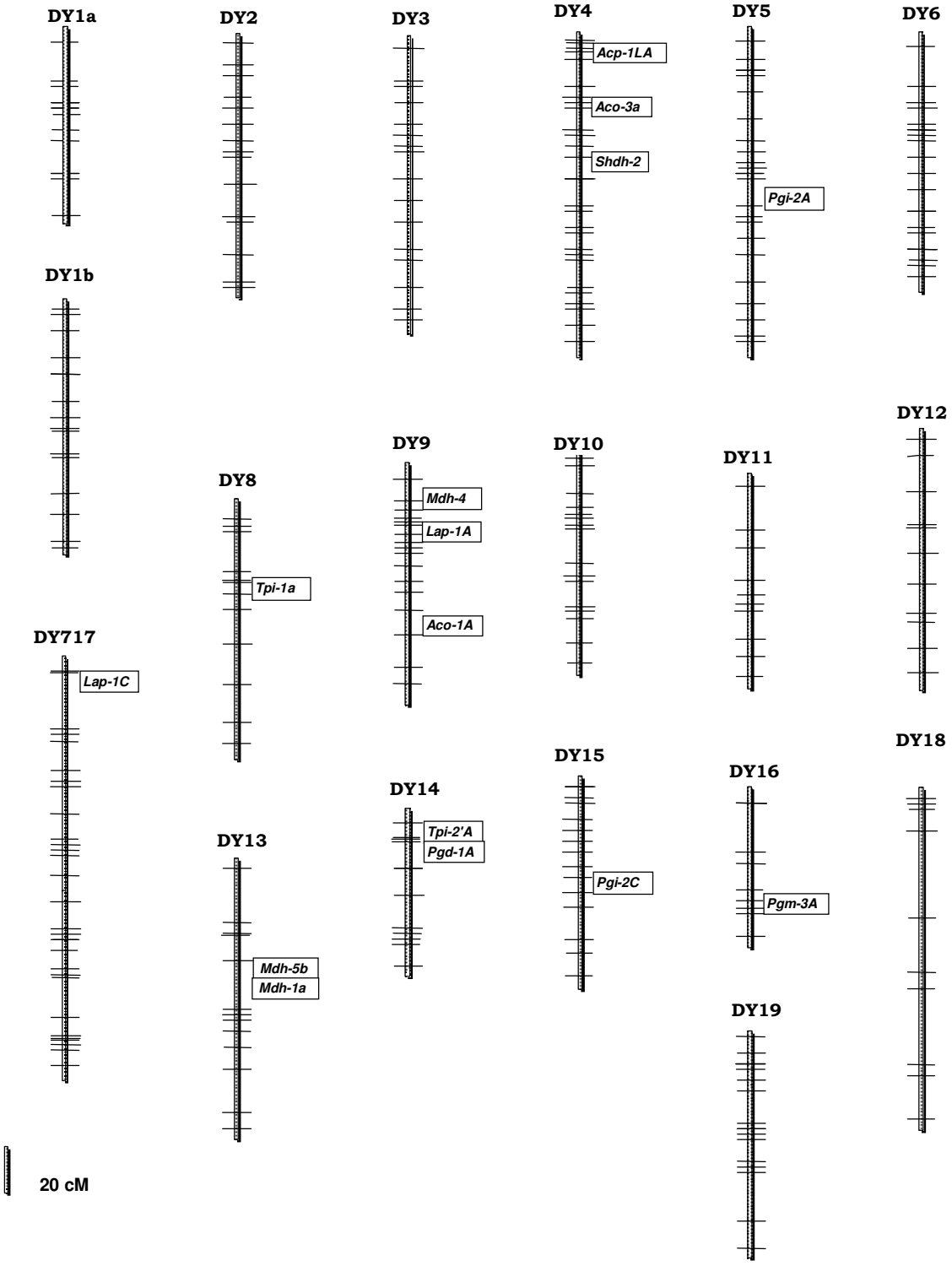
Shikimate dehydrogenase has strong activity in leaves but much weaker activity in seedlings.

The enzyme is monomeric.

The enzymes encoded by the alleles Shdh-1a3 and Shdh-1b1 migrate to the same position, as do the alleles Shdh-1a4 and Shdh-1b3 (figure 9).

All the homozygous genotypes can be differentiated, as can most heterozygous genotypes. Heterozygotes with a 1/2, 1/3 or 1/4 profile may be confused with homozygotes with profiless 2, 3 or 4, respectively. The 3/5 hetero-zygote is also difficult, if not impossible, to differentiate from homozygotes with profile 3.

Table VI: Position of the genes encoding the enzymes on the genetic map for the oilseed rape cross 'Dwarf Darmor' x 'Yudal'



10. DESCRIPTION OF THE GAIA METHOD USED FOR OILSEED RAPE

For general guidance on the conditions and the use of the GAIA methodology reference is made to part II of the UPOV document TGP/8 "Selected techniques used in DUS examination".

Reference is also made to the UPOV document TGP/8, where the principles of the application of GAIA are laid down.

It is underlined that the matrices (which define the weighting for each characteristic) are based on experience gained with the varieties of common knowledge under the agro climatic conditions of a given examination office. They cannot be applied by other examination office without having undergone an adaptation process to the local conditions. Matrices established by other examination offices who wish to use the GAIA methodology will be considered by the relevant expert group and the CPVO in order to verify if its application in oilseed rape would fulfil the general recommendations.

Specific requirements and rules as approved for the French authority GEVES:

Morphological characteristics: the number of plants observed for the attribution of a note is 500, divided between two sites.

Only differences in the same direction at the two sites have a weighting attributed to them.
The weight associated with the larger of the two observed differences is retained.
The weightings attributed to a characteristic can be 2, 3, 4, 5 or 6 as a maximum. Examples of two characteristics illustrate their matrices:

13 – Flower: length of petals

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	0	0	2	3	6	6	6	6	6
2		0	0	2	3	6	6	6	6
3			0	0	2	3	6	6	6
4				0	0	2	3	6	6
5					0	0	2	3	6
6						0	0	2	3
7							0	0	2
8								0	0
9									0

15 – Production of pollen

	1	**9**
1	0	6
9		0

For the characteristics "11: Time of flowering" and "16: Plant: total length including the side branches", a difference of more than the following minimum differences found at least 2 times in 2 different tests and in the same direction, increases the weight attributed as follows:

11: Flowering: 2 days weight 3
11: Flowering: 3 days weight 6

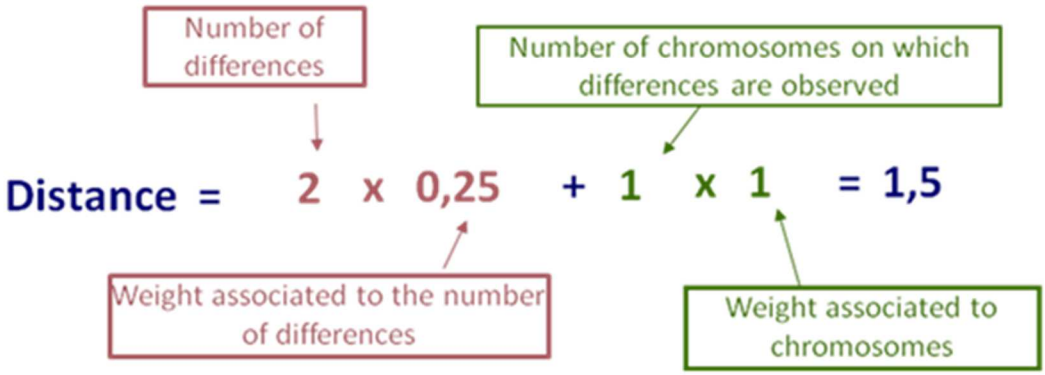
16: Length: 15 cm: weight 3
16: Length: 20 cm: weight 6

Isoenzyme characteristics: The numbers of seeds analyzed to obtain the profile of six iso-enzymatic systems are 10. The uniformity standard applied is 10% with an acceptance probability of at least 95% (7 seeds out of 10 must show the same profile). In case the electrophoretic profile does not fulfil the uniformity requirements, it is not considered in GAIA.

A weighting of 0.25 is associated with the number of differences found and a weight of 1 to the number of chromosomes concerned by these differences. A strictly greater weight than 3 minimum distance shall be evidenced in qualitative analysis in order to take into account the electrophoresis results.

Example:

	Chromosome DY 5			Chromosome SW 5			
Gènes	Pgi2A			Pgi2C			
Allèle	Pgi2A-1	Pgi2A-2	Pgi2A-3	Pgi 2C-1	Pgi 2C-2	Pgi 2C-3	Pgi2C-r
Variété A	1	0	0	1	0	0	0
Variété B	1	0	0	0	0	0	1
Différence	0	0	0	1	0	0	1



Decision rules GAIA:

A variety will be considered clearly distinct to a candidate variety and will be discarded from the direct comparison to the candidate variety if

- the weighting attributed by morphological characteristics is ≥ 6.

If it is below 6, the sum of the weighting will be extended by the weighting resulting from the Isoenzyme characteristics.

A variety will be considered clearly distinct to a candidate variety and will be discarded from the direct comparison to the candidate variety if

- the weighting attributed by morphological characteristics and by isoenzyme characteristics is ≥ 6, whereby the weight attributed by morphological characteristics must contribute with at least 3.

If the combined weighting is below 6, the sum of the weighting will be extended by the weighting resulting from the two characteristics "11: Time of flowering" and "16: Plant: total length including the side branches" using quantitative values instead of qualitative values.

A variety will be considered clearly distinct to a candidate variety and will be discarded from the direct comparison to the candidate variety if

- the weighting attributed by morphological characteristics and by isoenzyme characteristics and by the quantitative values of characteristics "11: Time of flowering" and "16: Plant: total length including the side branches" is ≥ 6, whereby the weight attributed by morphological characteristics must contribute with at least 3.

If the combined weighting is below 6 the candidate variety will be put into the field in the second year of the DUS test for the direct comparison with its similar varieties.

11. LITERATURE

No particular literature.

12. TECHNICAL QUESTIONNAIRE

The Technical Questionnaire is available on the CPVO website under the following reference: CPVO-TQ/036/3