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

PROTOCOL FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTS

Brassica napus L. emend. Metzg.

RAPE SEED

UPOV Species Code: BRASS_NAP

Adopted on 16/11/2011

Entered into force on 01/08/2011

I SUBJECT OF THE PROTOCOL

The protocol describes the technical procedures to be followed in order to meet the requirements of Council Regulation 2100/94 on Community Plant Variety Rights. The technical procedures have been agreed by the Administrative Council and are based on general UPOV Document TG/1/3 and UPOV Guideline TG/36/6 dated 18h October 1996 for the conduct of tests for Distinctness, Uniformity and Stability. This protocol applies to all varieties of ***Brassica napus* L. emend. Metzg**.

II SUBMISSION OF SEED AND OTHER PLANT MATERIAL

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

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

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

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

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

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

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

3. Seed requirements

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

   Quality of seed: ..... The minimum requirements for germination capacity, analytical purity and seed health should not be less than the standards laid down in EC Directive 2002/57/EC

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

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

III CONDUCT OF TESTS

1. Variety collection

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

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

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

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

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

2. Material to be examined

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

3. Characteristics to be used

The characteristics to be used in DUS tests and preparation of descriptions shall, be those referred to in the table of characteristics. Without prejudice to the provisions in the following paragraph, all the characteristics shall be used provided that the observation of a characteristic is not rendered impossible by the expression of any other characteristic, or the expression of a characteristic is prevented by the environmental conditions under which the test is conducted. In the latter case, the CPVO should be informed. In addition the existence of some other regulation e.g. plant health, may make the observation of the characteristic impossible.

The 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, 18, 19, 21. 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° 1239/95, to insert additional characteristics and their expressions in respect of a variety.

4. Grouping of varieties

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

a) Seed: erucic acid (characteristic 1)

b) Leaf: lobes (characteristic 8)

c) Time of flowering (characteristic 11)

d) Production of pollen (characteristic 15)

e) Plant: total length including side branches (characteristic 16)

5. Trial designs and growing conditions

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

The test design is as follows:

If not otherwise indicated, each test should include at least 200 plants which should be divided between two or more replicates.

The assessment for the characteristic 'Tendency to form inflorescences' should be carried out on at least 100 plants.

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

Unless otherwise stated, all observations for the assessment of distinctness on individual plants should be made on 45 plants or parts of 45 plants.

The assessment on characteristics referring to cotyledons should be made on 40 plants or parts of plants.

6. Special tests

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

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

7. Standards for decisions

a) **Distinctness**

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

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

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

(ii) check of 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 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.

Qualitative characteristics:

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

Quantitative characteristics:

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

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

In the case of observations on individual plants or parts of plants (VS, MS), the combined over years analysis (COYD) should be applied for the assessment of distinctness. The probability level for the COYD criterion should be 1% or less ($p<0.01$) in a test over either two or three growing cycles.

In the assessment of distinctness the GAIA method may be used for the selection of similar varieties. Details of the specific application of the GAIA method are explained in annex II.3 hereto.

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

b) **Uniformity**

In case of visual observation, uniformity is assessed on the basis of off-types.

In case of measurements, uniformity should be assessed by using COYU or other appropriate statistical methods.

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% with an acceptance probability of at least 95% should be applied. In the case of hybrids, the population standard should be 10% with the same acceptance probability of at least 95%.

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

If uniformity is assessed by the combined over years uniformity method (COYU), the candidate variety can be accepted as uniform when its combined over years uniformity (COYU) is not significantly greater than that of the appropriate reference varieties at the 0.1% (P=0.001) significance level after a test of two or three years.

c) **Stability**

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

IV REPORTING OF RESULTS

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

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

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

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

V LIAISON WITH THE APPLICANT

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

VI ENTRY INTO FORCE

The present protocol enters into force on **01.08.2011.** Any ongoing DUS examination of candidate varieties started before the aforesaid date will not be affected by the approval of the revised Technical Protocol. Technical examinations of candidate varieties are carried out according to the TP in force when the DUS test starts. The starting date of a DUS examination is considered to be the due date for submitting of plant material for the first test period.

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

****

ANNEXES TO FOLLOW

ANNEX I

Legend:
°) Assessment of characteristic not obligatory in case the GAIA method (see annex II) is used
(+) See explanations on the Table of characteristics
G Grouping characteristic
0 -89 Decimal Code for the Growth Stages
C Special test

Types of expression of characteristics:

QL – Qualitative characteristic
QN – Quantitative characteristic
PQ – Pseudo-qualitative characteristic

Type of 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

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

The second differentiation deals with the number of observations the expert attributes to each variety, thus the attribution of either G or S.
If a single observation of a group consisting of an undefined number of individual plants is appropriate to assess the expression of a variety, we talk about a visual observation or a measurement made on a group of plants, thus we attribute the letter G (either VG or MG). If the expert makes more than one observation on that group of plants, the decisive part is that we have at the end only one data entry per variety which means that we have to deal with G (e.g. measurement of plant length on a plot – MG, visual observation of green colour of leaves on a plot – VG).
If it is necessary to observe a number of individual plants to assess the expression of a variety, we should attribute the letter S (thus either VS or MS). Single plant data entries are kept per variety for further calculations like the variety mean (e.g. measurement of length of ears – MS, visual observation of growth habit of single plants in grasses – VS). The number of individual plants to be observed in such cases is stated in section III.5.

ANNEX II

Annex II.1: Explanation and methods
Annex II.2: Description of the SGE method for the analysis of isoenzymes of *Brassica napus*
Annex II.3: Application of the GAIA method

ANNEX III

Technical Questionnaire

ANNEX I

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

CPVO N°	UPOV N°	Characteristics	Stage[1] Method	Examples[2]	Note
1.	**1.**	**Seed: erucic acid**	00		
(+) PQ	*	absent	**C**	King 10; Jerome	1
G		present		Zeruca; Erucola	9
2. °)		**Cotyledon: ratio length/width**	15-17		
(+)		small	**MS**	PR46W14; Tamarin	3
QN		medium		PR46W15; Jerome	5
		large		NK Festivo; Sheik	7
3. °)		**Cotyledon: saddle depth**	15-17		
(+)		small	**MS**	Contact; Dorothy	3
QN		medium		King 10; Jerome	5
		large		BU1015105; Senator	7
4. °)		**Cotyledon: ratio lobe separation/width**	15-17		
(+)		small	**MS**	Catalina; Campino	3
QN		medium		Mendel; Calida	5
		large		Ontario; Sheik	7
5. °)		**Cotyledon: ratio lobe separation/ saddle depth**	15-17		
		small		Cigal; Magma	3
(+)		medium	**MS**	Komando; Pauline	5
QN		large		Zeruca; Calida	7

[1] The optimum stage of development as well as method of observation for the assessment of each characteristic is indicated by numbers and letters. Explanations are given in Annex II.1 in 'Explanations and Methods'.

[2] Example varieties, separated by a semicolon, are indicated for winter rape seed before the semicolon, for spring rape seed they follow the semicolon. Example varieties are given as an indication, others may be used.

CPVO N°	UPOV N°	Characteristics	Stage1 Method	Examples2	Note
6.	**4.**	**Leaf: green colour**	15-17		
QN	*	light	**VG**	Campari; Calida	3
		medium		King 10; Lyside	5
		dark		Capitol; -	7
7.		**Leaf: glaucosity**	23-27		
QL		absent	**VG**		1
		present			9
8.	**5.**	**Leaf: lobes**	23-27		
(+)	*	absent	**VG**	Akela; Calida	1
PQ, G		present		King 10; Dorothy	9
9.	**6.**	**Leaf: number of lobes (fully developed leaf)**	23-27		
(+)	*	few	**MS**	DK Cabernet; SW Svinto	3
QN		medium	**VG**	PR45D04; Dorothy	5
		many		Dante; Tamarin	7
10.	**7.**	**Leaf: dentation of margin**	23-27		
(+)	*	weak	**VG**	Vision; Pauline	3
QN		medium		Mohican; Heros	5
		strong		-; -	7
11.	**11.**	**Time of flowering**	61-62		
(+)	*	very early	**MS**	Cavalcade;	1
QN		early	**MG**	ES Alegria; Campino	3
		medium		Komando; Liaison	5
		late		MSL007C; Jerome	7
G		very late		Greenland; -	9

CPVO N°	UPOV N°	Characteristics	Stage1 Method	Examples2	Note
12.	**12.**	**Flower: colour of petals**	62-63		
PQ	*	white	**VG**	-;-	1
		cream		Greenland; Lyside	2
		yellow		King 10; Jerome	3
		orange-yellow		-;-	4
13.	**13.**	**Flower: length of petals**	62-63		
QN		very short	**MS**	-;-	1
		short	**VG**	MSL004C; MSL523C	3
		medium		King 10; Heros	5
		long		Mohican; Osorno	7
14.	**14.**	**Flower: width of petals**	62-63		
QN		narrow	**MS**	NK Rapster; Magma	3
		medium	**VG**	King 10; SW Landmark	5
		broad		Appolon; Tamarin	7
15.	**15.**	**Production of pollen**	62-63		
QL		absent	**VG**	MSL007C; MSL523C	1
G		present		King 10; Jerome	9
16.	**17.**	**Plant: total length including side branches**	70-80		
QN	*	very short	**MS, MG**	NW 4193 BC; -	1
		short		PR45D03; Cadoma	3
		medium		King 10; Heros	5
		long		PR46W15; Palmiro	7
G		very long		Appolon; -	9
17.	**18.**	**Siliqua: length (between peduncle and beak)**	75-89		
QN		very short	**MS**	Imola; R 1385	1
		short		Belcanto; Calida	3
		medium		NK Diamond; Heros	5
		long		Mohican; SW Landmark	7

CPVO N°	UPOV N°	Characteristics	Stage1 Method	Examples2	Note
18. °)		**Siliqua: width**	75-89		
QN		narrow	**MS**	NK Passion; Calida	3
		medium		Mohican; Tamarin	5
		broad		Capitol; Jerome	7
		very broad		MO13392; -	9
19. °)		**Siliqua: ratio length/width**	75-89		
QN		narrow	**MS**	MSL301C; Jerome	3
		medium		Mendel; Heros	5
		broad		Charly; Magma	7
		very broad		-; -	9
20.	**19.**	**Siliqua: length of beak**	75-89		
(+)		very short	**MS**	DK Cabernet; -	1
QN		short		NK Diamond; Calisto	3
		medium		ES Astrid; Calida	5
		long		Tosca; Liaison	7
		very long		BL643196; -	9
21. °)	**20.**	**Siliqua: length of peduncle**	75-89		
(+)		short	**MS**	DK Cabernet; Calida	3
QN		medium		King10; Campino	5
		long		Cindi CS; SW Landmark	7
22.	**21.**	**Tendency to form inflorescences in year of sowing for <u>spring</u> sown trials**			
(+)		absent or very weak	**MG**	Akela; -	1
QN		weak	**VG**	Mohican; -	3
		medium		Zenith; -	5
		strong		Cavalcade; -	7
		very strong		Tenor; -	9

CPVO N°	UPOV N°	Characteristics	Stage1 Method	Examples2	Note
23.	**22.**	**Tendency to form inflorescences in year of sowing for <u>late summer</u> sown trials**			
(+)		absent or very weak	**MG**	-; C 999031	1
QN		weak	**VG**	-; Forte	3
		medium		-; Calida	5
		strong		-; -	7
		very strong		-; -	9

ANNEX II.1

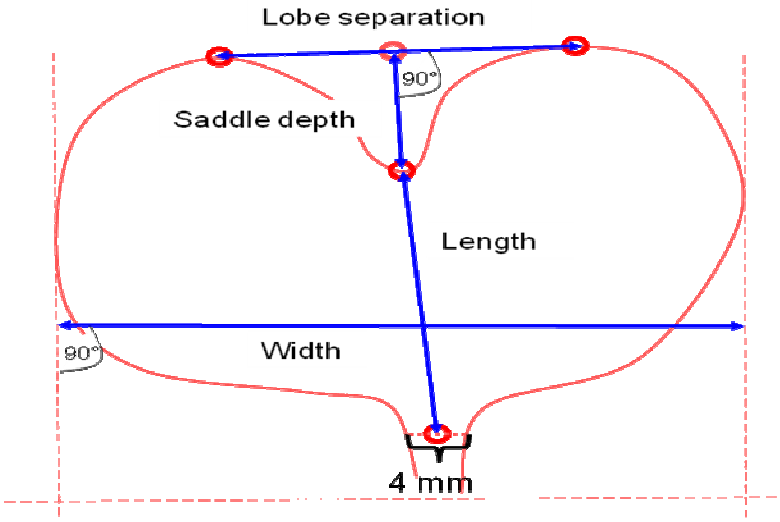
EXPLANATIONS AND METHODS

Ad. 1: Seed: erucic acid

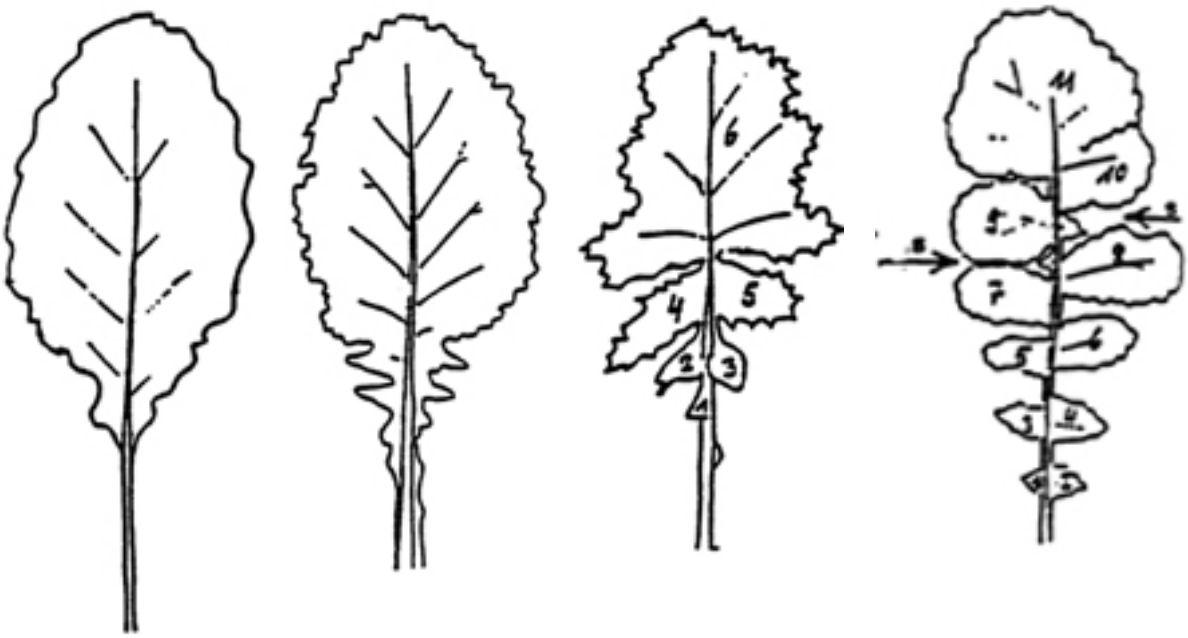
The erucic acid content should be observed on seed sent in by the applicant. It should be expressed as a percentage by mass of methyl esters in accordance with the ISO standard in document 5508, paragraph 6.2.2.1. Seed containing 2% or less of erucic acid will be classified as "absent."

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

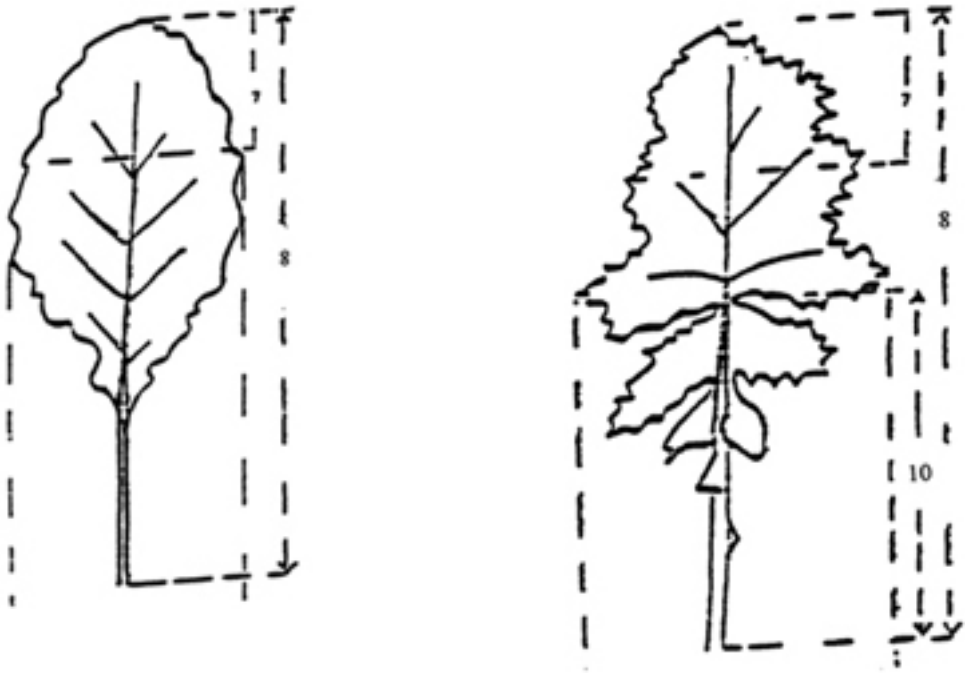


1
absent

9
present

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. 17 - 21: Siliqua

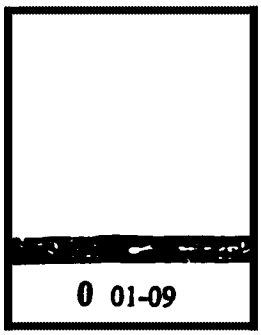
All observations on the siliqua should be recorded in the mid-part of the inflorescence of the main stem.

Ad. 22 - 23: 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.

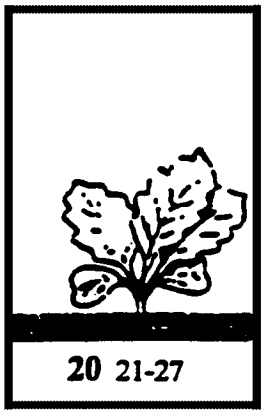
KEY FOR THE GROWTH STAGES

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

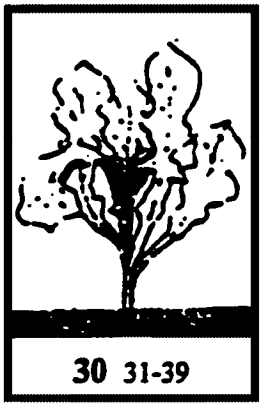


germination

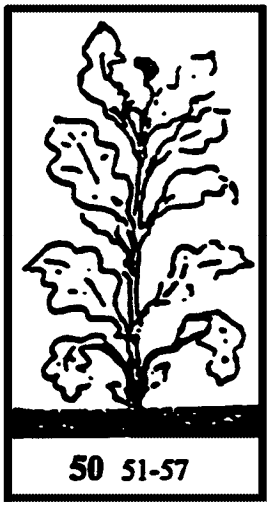
seedling growth



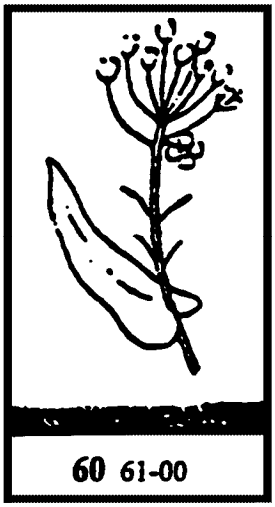
rosette



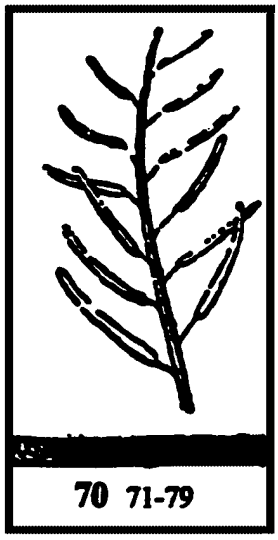
stem elongation



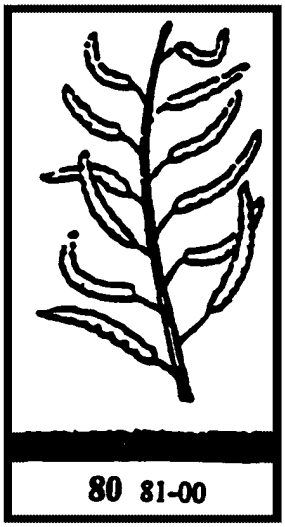
bud formation



flower



silique



maturation

ANNEX II.2

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
Variety	Continent	Type	PGI	LAP	6-PGD	MDH	TPI	ACO	ShDH	ACP
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

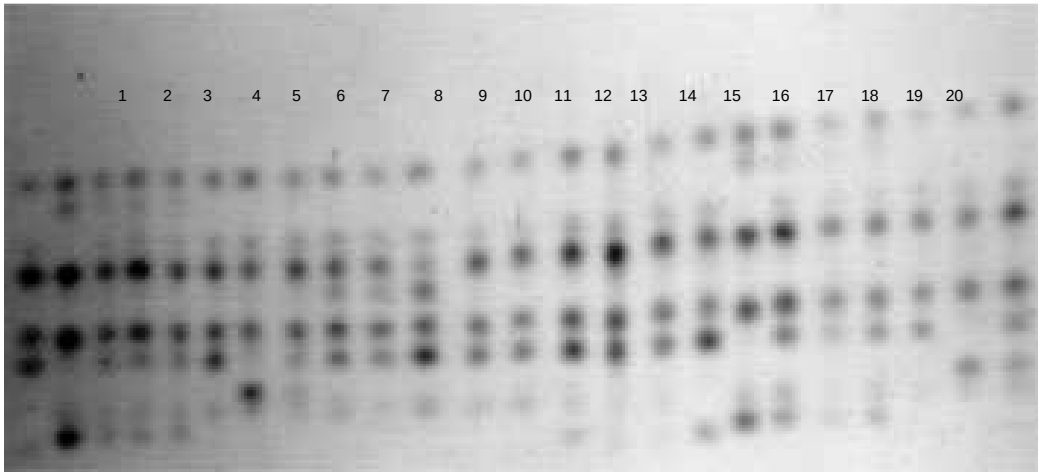
6.5.1.1 Genetics

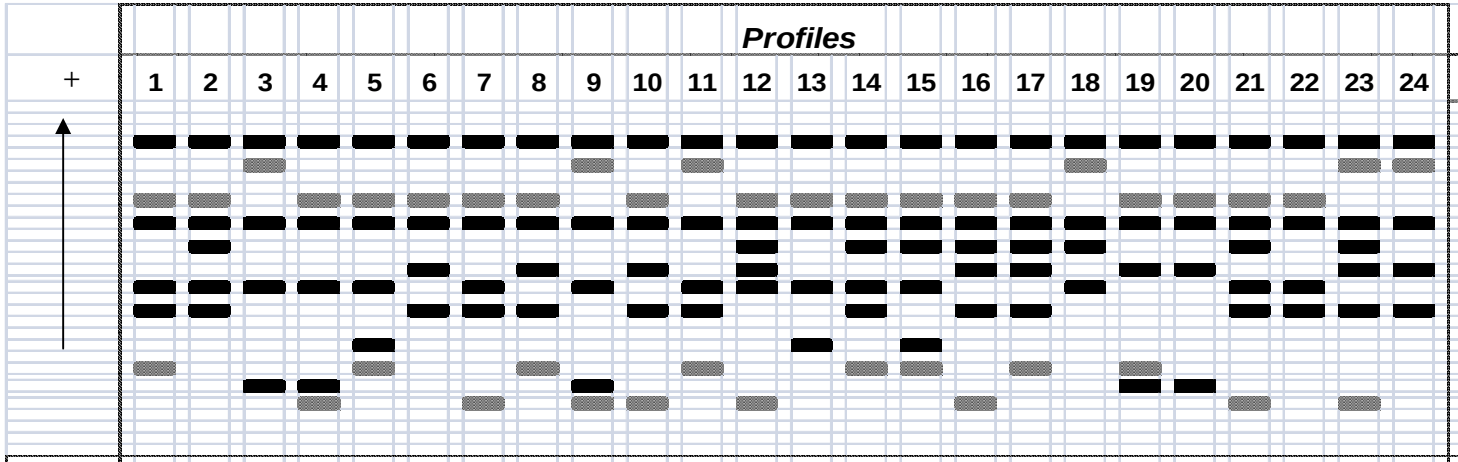
Four regions of activity are detected (ACO-1, ACO-2, ACO-3, ACO-4) each corresponding to one locus expressed in the three diploid species B. oleracea, B. rapa and B. nigra (Arus, 1989; Chèvre et al., 1995). Aconitase is present in the cytosolic, mitochondrial and glyoxysomal fractions (Wendel et al., 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.

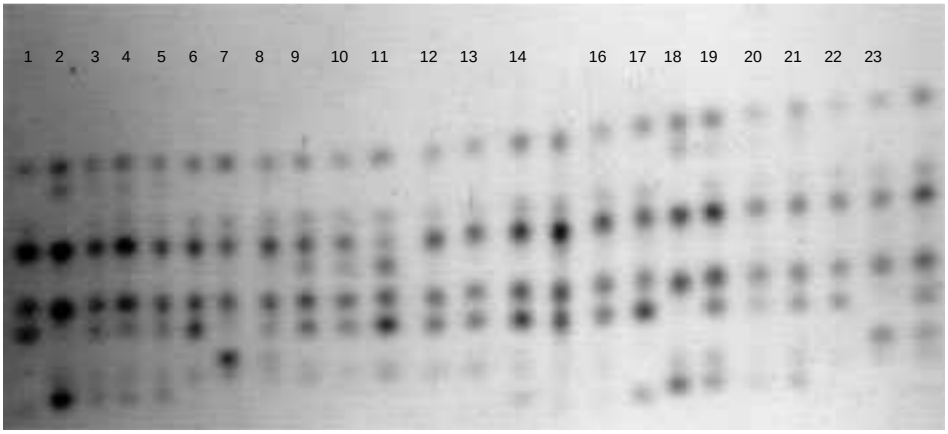
6.5.1.2 Zymograms

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.

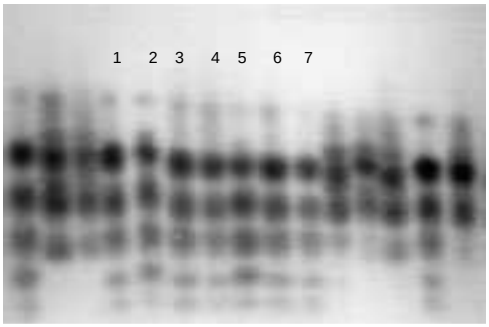
No reference is available for this enzyme. The study of several oilseed rape crosses has made it possible to deduce the genetics of only three polymorphic genes:

- 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.

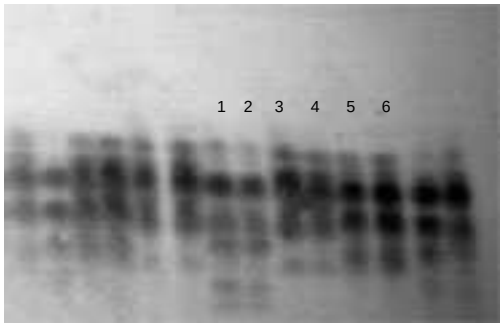
Two alleles have been identified for each of these genes (Table IV).

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

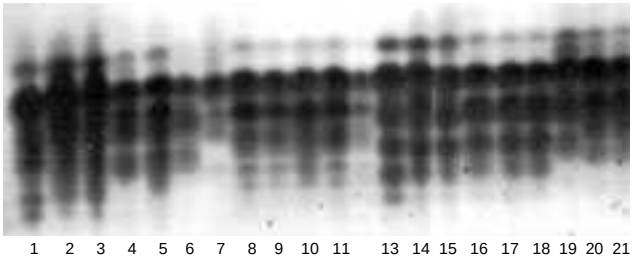
Figure 5: Zymograms of malate dehydrogenases (MDH) from oilseed rape



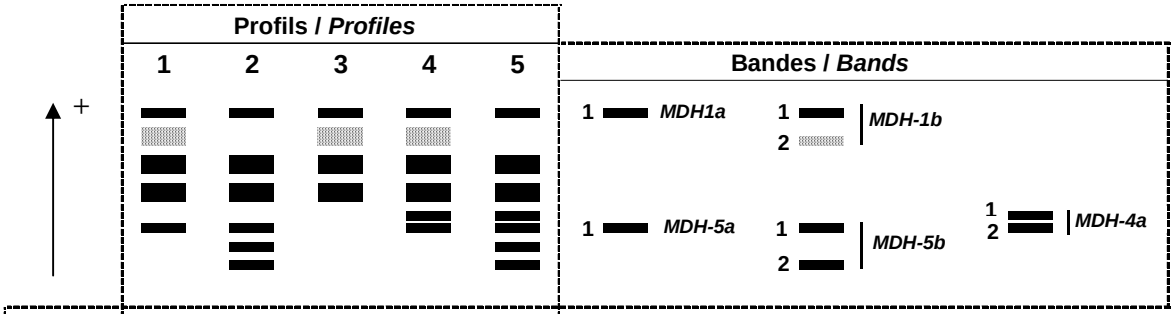
Lanes	Genotypes
1, 3, 4, 6, 7------	Profile 2
2, 5----------	Profile 5



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.

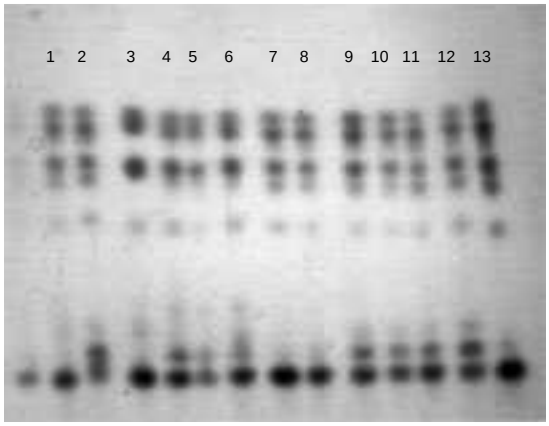
6.5.3.2 Zymograms

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

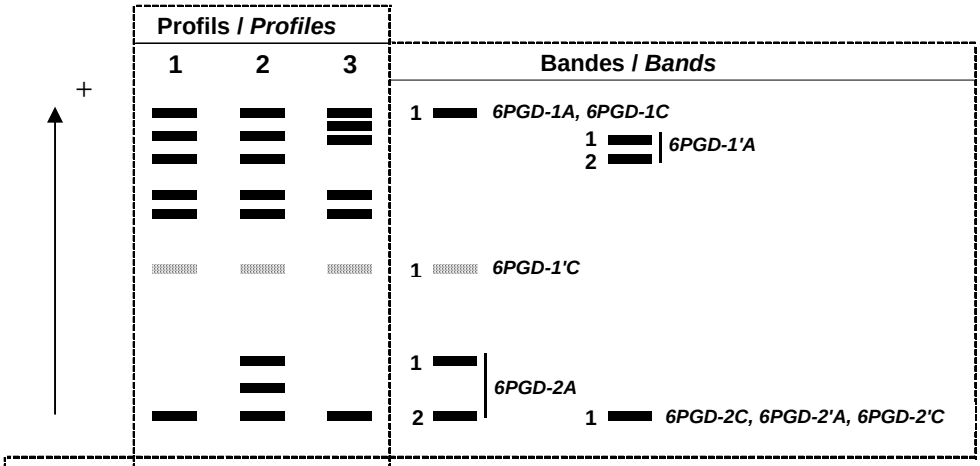
The enzyme is dimeric. All oilseed rape genotypes generate six bands in the 6-PGD-1 region. These bands correspond to the homodimers for each gene (6-Pgd-1A, 6-Pgd-1C, 6-Pgd-1'A, 6-Pgd-1'C) and heterodimers corresponding to interactions between the duplicated genes of the two genomes, A and C, expressed 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).

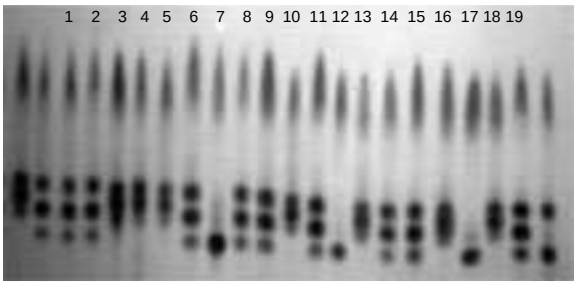
In oilseed rape too, only the genes corresponding to the PGI-2 region are polymorphic.

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).

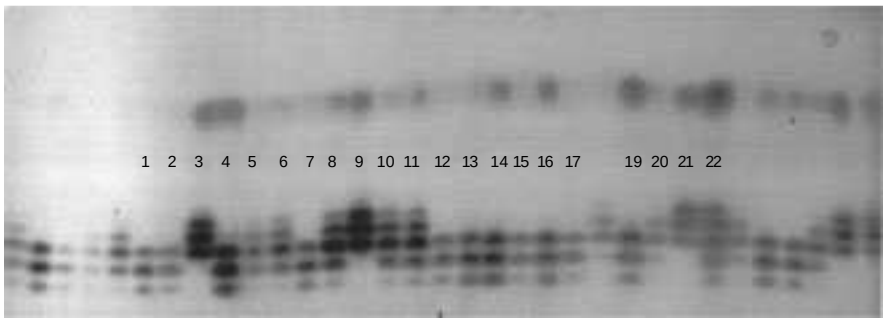
Three alleles have been identified at the Pgi-2A locus, with allele 1 the most frequent. A null allele has been observed in some genotypes, such as the variety Topas.

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).

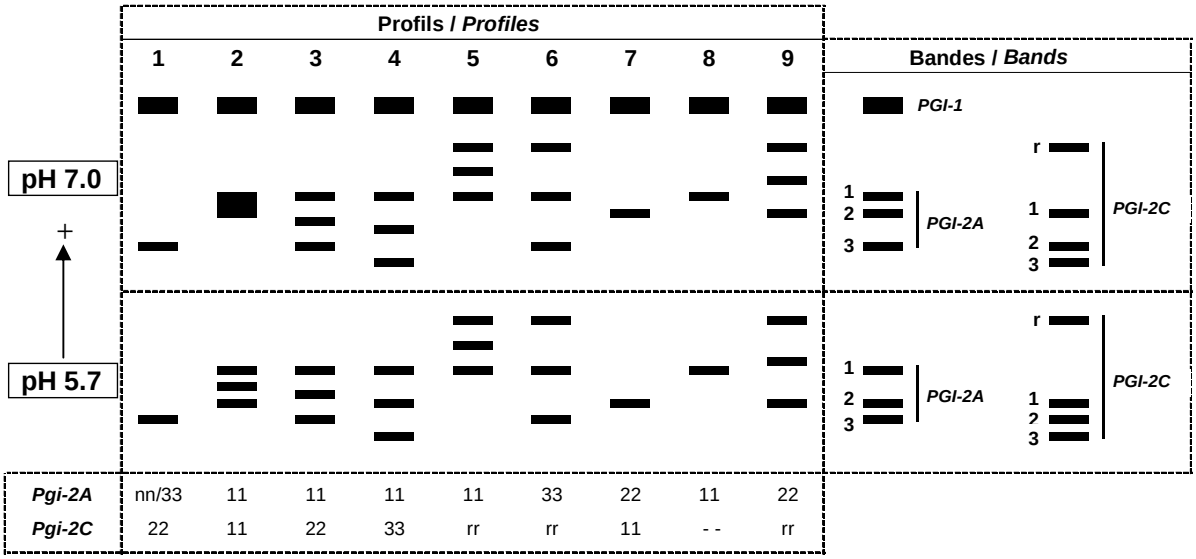
Figure 7: Zymograms of phosphoglucoisomerases (PGI) from oilseed rape



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).

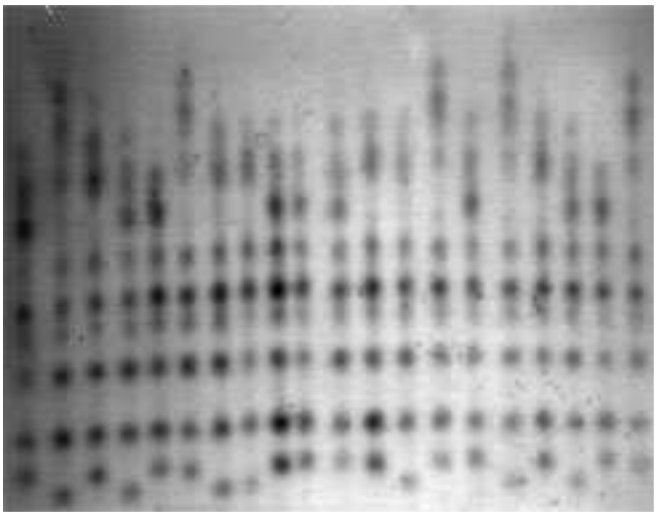
However, this polymorphism is most frequently encountered in Asian genotypes. Profile 1 is typical of European genotypes.

6.5.5.2 Zymograms

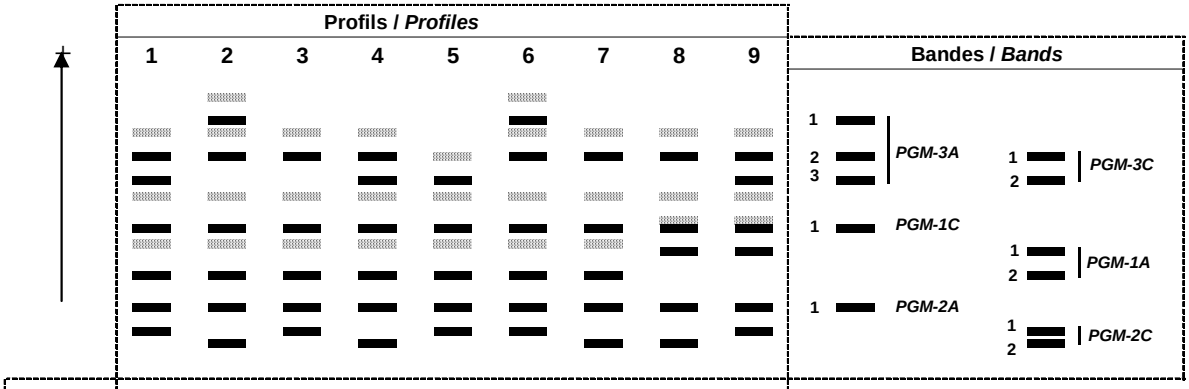
Electrophoretic separation is carried out in the pH 5.7 buffer system. The pH 6.5 and pH 7.0 buffer systems may also be used.

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.

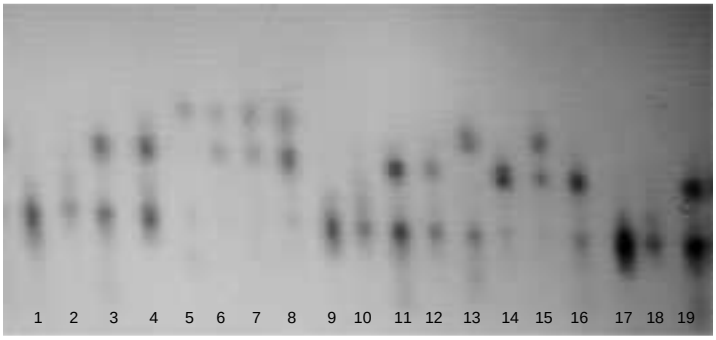
In the PGM-1 and PGM-2 regions, all the heterozygous genotypes are readily distinguished from the homozygous genotypes.

6.5.6 Shikimate dehydrogenase (SHDH) E.C.1.1.1.25

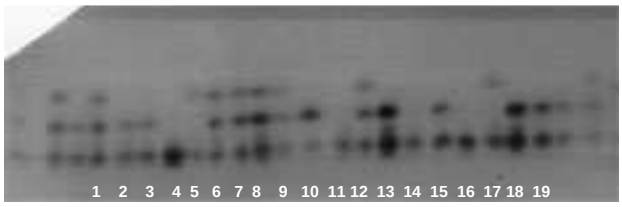
6.5.6.1 Genetics

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.

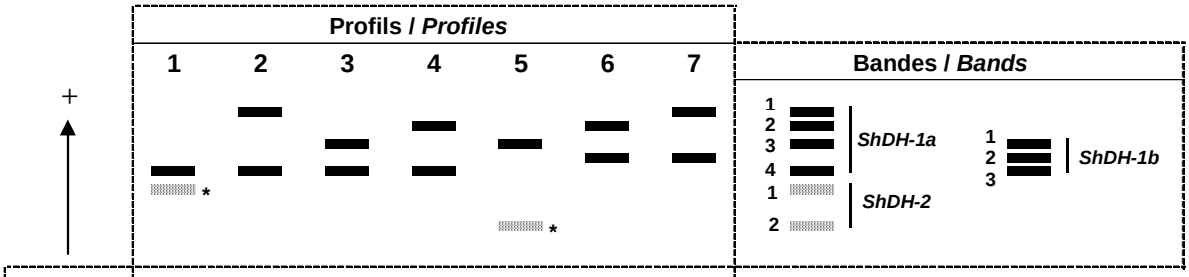
Figure 9: Zymograms of shikimate dehydrogenases (ShDH) from oilseed rape



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.

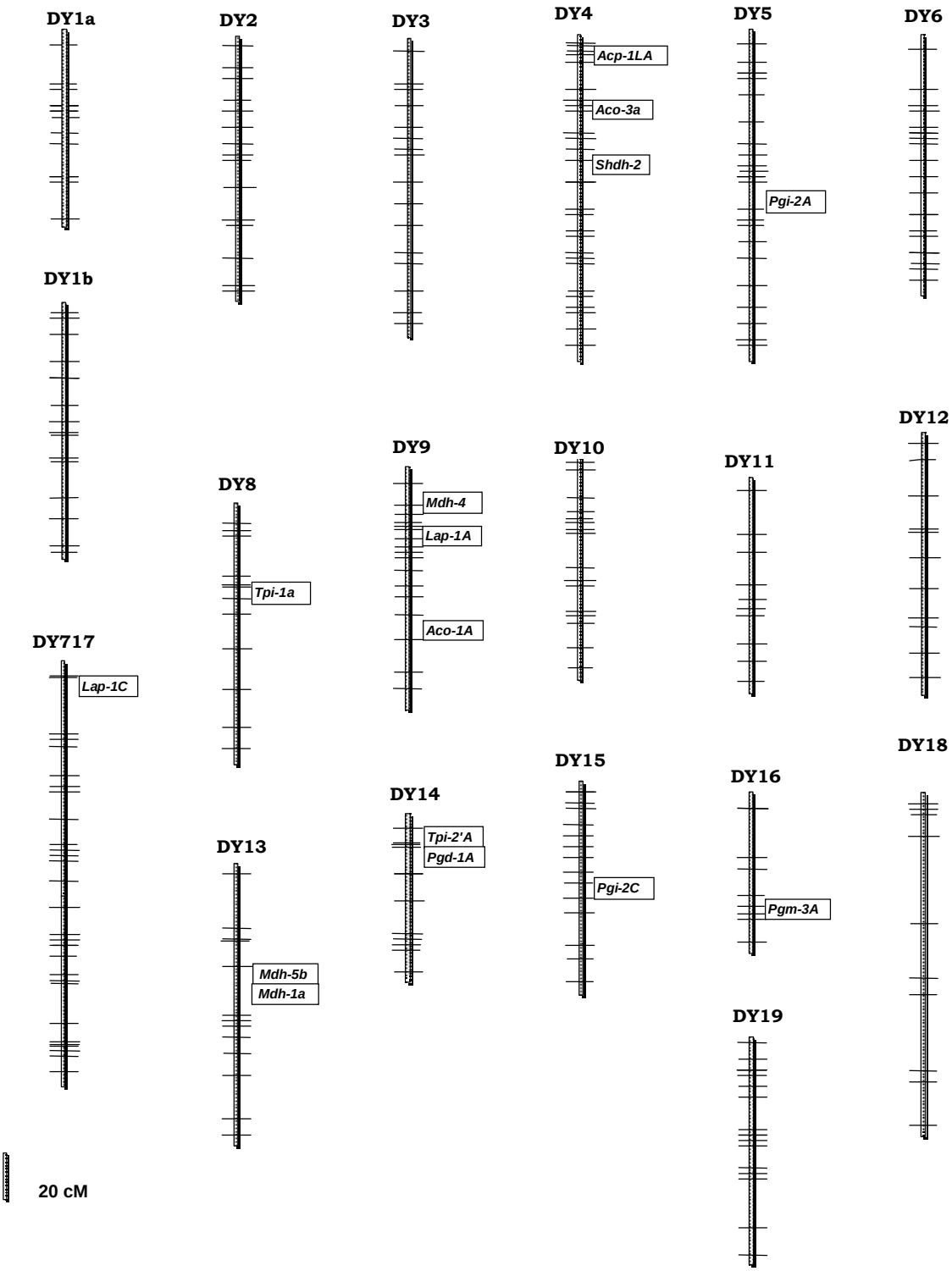
Shikimate dehydrogenase has strong activity in leaves but much weaker activity in seedlings.

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 heterozygote 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'



ANNEX II.3

GAIA methodology in 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/9, here section 2.4 "Characteristics in combination" 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

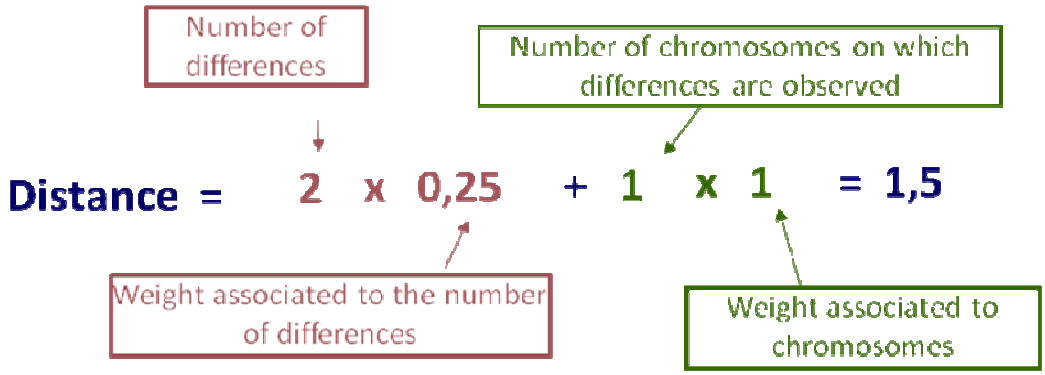
16: Length: 15 cm: weight 3
16: Length: 20 cm: weight 6

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	Pgi2C-1	Pgi2C-2	Pgi2C-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.

ANNEX III

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

TECHNICAL QUESTIONNAIRE

to be completed in connection with an application for Community Plant Variety Rights
Please answer all questions. A question without any answer will lead to a non-attribution of an application date. In cases where a field / question is not applicable, please state so.

1. **Botanical taxon:** Name of the genus, species or sub-species to which the variety belongs and common name

***Brassica napus* L.**

RAPE SEED

2. **Applicant(s):** Name(s) and address(es), phone and fax number(s), Email address, and where appropriate name and address of the procedural representative

3. **Variety denomination**

a) Where appropriate proposal for a variety denomination:

b) Provisional designation (breeder's reference):

4. Information on origin, maintenance and reproduction of the variety

4.1 Type of material

(a) line

- male fertile line ..... [ ]

use (only for male fertile line)

[ ] as component

[ ] as commercial variety

- male sterile line ..... [ ]

(b) hybrid

- male sterile hybrid ..... [ ]
- male fertile hybrid ..... [ ]
- self incompatible hybrid ..... [ ]

(c) other (please indicate)

4.2 Formula (if applicable, for each component in separate sheets, the information according to the following chapter 5 or 7 to be added)

Single hybrid

– Denomination or breeder's reference of female parental line

.....

– Denomination or breeder's reference of male parental line

.....

Three-way hybrid

Denomination or breeder's reference of:

– single hybrid used .....

  – female parental line of the single hybrid .....

  – male parental line of the single hybrid.....

  – female parent of the three-way hybrid.....

– male parental line of the three-way hybrid.....

NB: In case of use of male sterility system, indicate the name of the maintainer line of the female parental line

.....

In case of use of self-incompatibility system, indicate if applicable the name of the self-compatible lines

.....

4.3 Other information on genetic origin and breeding method

4.4 Geographical origin of the variety: the region and the country in which the variety was bred or discovered and developed

4.5 Shall the information on data relating to components of hybrid varieties including data related to their cultivation be treated as confidential?

[ ] YES [ ] NO

If yes, please give this information on the attached form for confidential information.

If no, please give information on data relating to components of hybrid varieties including data related to their cultivation:

Breeding scheme (indicate female component first)

5. Characteristics of the variety to be indicated (the number in brackets refers to the corresponding characteristic in the CPVO Protocol; please mark the state of expression which best corresponds).

Characteristics	Example varieties	Note
5.1 (1) Seed: erucic acid		
absent	Cadoma; Express	1 [ ]
present	Rabelais; Zeruca	9 [ ]
5.2 (8) Leaf: lobes		
absent	Calida; Akela	1 [ ]
present	Dorothy; Express	9 [ ]
5.3 (11) Time of flowering (quote main date of flowering of variety as well as of two well-known comparable varieties)		
.....		
.....		
.....		
5.4 (16) Plant: total length including side branch (quote length of variety as well as of two well-known comparable varieties)		
.....		
.....		
.....		

6. Similar varieties and differences from these varieties:

Denomination of similar variety	Characteristic in which the similar variety is different[1)]	State of expression of similar variety	State of expression of candidate variety

1) In the case of identical states of expressions of both varieties, please indicate the size of the difference

7. Additional information which may help to distinguish the variety

7.1 Resistance to pests and diseases

7.2 Special conditions for the examination of the variety

(a) Group

– Spring oilseed rape [ ]

– Winter oilseed rape [ ]

– Spring forage rape [ ]

– Winter forage rape [ ]

(b)

– Low glucosinolate content [ ]

– High glucosinolate content [ ]

(c) Other conditions

[ ] YES, please specify .....

[ ] NO

7.3 Other information

[ ] YES, please specify

[ ] NO

8. GMO-information required

The variety represents a Genetically Modified Organism within the meaning of Article 2(2) of Council Directive EC/2001/18 of 12/03/2001.

[ ] YES [ ] NO

If yes, please add a copy of the written attestation of the responsible authorities stating that a technical examination of the variety under Articles 55 and 56 of the Basic Regulation does not pose risks to the environment according to the norms of the above-mentioned Directive.

9. Information on plant material to be examined

9.1 The expression of a characteristic or several characteristics of a variety may be affected by factors, such as pests and disease, chemical treatment (e.g. growth retardants or pesticides), effects of tissue culture, different rootstocks, scions taken from different growth phases of a tree, etc.

9.2 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 the plant material has undergone such treatment, full details of the treatment must be given. In this respect, please indicate below, to the best of your knowledge, if the plant material to be examined has been subjected to:

(a) Microorganisms (e.g. virus, bacteria, phytoplasma)	[ ] Yes	[ ] No
(b) Chemical treatment (e.g. growth retardant or pesticide)	[ ] Yes	[ ] No
(c) Tissue culture	[ ] Yes	[ ] No
(d) Other factors	[ ] Yes	[ ] No

Please provide details of where you have indicated "Yes":

10. Possible place of the technical examination

In case the CPVO needs to arrange a technical examination for this candidate variety, there might be more than one examination office entrusted by the CPVO suitable to grow your variety. In this case, the Office will decide on the place of the technical examination but you might wish to express here a preference in respect of an examination office. The available entrusted examination offices for that species can be found in the S2 Gazette under http://www.cpvo.europa.eu/main/en/home/documents-and-publications/s2-gazette

I/we hereby declare that to the best of my/our knowledge the information given in this form is complete and correct.

Date Signature Name

[End of document]