



EUROPEAN UNION
COMMUNITY PLANT VARIETY OFFICE

PROTOCOL FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTS

Helianthus annuus L.

SUNFLOWER

UPOV Species Code: HLNTS_ANN

Adopted on 31/10/2002

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/81/6 dated 5th April 2000 for the conduct of tests for Distinctness, Uniformity and Stability. This protocol applies to all varieties of *Helianthus annuus* L. with the exception of ornamental varieties.

II - SUBMISSION OF SEED AND OTHER PLANT MATERIAL

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

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

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

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

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

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

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

3. Seed requirements

Final dates for request for technical examination and sending of Technical Questionnaire by the CPVO as well as submission date of plant material by the applicant (the seed requirements can be subject to changes, actual information is on the CPVO web site www.cpvo.europa.eu).

Examination Office in	Request of examination	Plant material	Seed requirements
FRANCE	15/02	01/03	Hybrid: 1kg seeds + 5000 grains of each component (male sterile line, maintainer, restorer and in case of 3-way hybrid the single hybrid) Line: 5000 grains, in case of male sterile line additional 5000 grains of the maintainer
SPAIN	01/12	01/01	Hybrid: 1kg seeds + 500 g of each component (male sterile line, maintainer, restorer and in case of 3-way hybrid the single hybrid) Line: 500 g, in case of male sterile line additional 500 g of the maintainer

Cited are closing and submission dates and seed requirements for national applications

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 69/208/EEC

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

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

III - CONDUCT OF TESTS

1. Variety collection

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

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

- varieties listed or protected at the EU level or at least in one of the EEA Member States;
- varieties protected in other UPOV Member States;

- any other variety in common knowledge.
- In case of hybrids, all components of hybrid varieties in common knowledge must be considered as part of the reference collection.

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

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

2. Material to be examined

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

3. Characteristics to be used

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

In cases where the distinction of hybrid varieties is based on the distinction of parental lines, the description of the hybrid must at least include those characteristics of Annex 1 which are indicated by an H.

The list of characteristics derived from electrophoresis as in Annex 2 should only be used as a complement to other differences in morphological or physiological characteristics.

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

4. Grouping of varieties

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

- a) Leaf: green colour (characteristic 4)
- b) Leaf: blistering (characteristic 5)
- c) Time of flowering (characteristic 14)
- d) Ray floret: colour (characteristic 19)
- e) Plant: natural height (characteristic 28)
- f) Plant: branching (excluding environmental branching) (characteristic 29)
- g) Seed: main colour (characteristic 38)
- h) Seed: stripes on margin (characteristic 39)
- i) Seed: stripes between margins (characteristic 40)

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.

If not otherwise indicated, each test should include at least 40 plants which should be divided between two or more replicates.

All observations for the assessment of distinctness and uniformity should be made on at least 40 plants or parts taken from each of the 40 plants in each testing place and growing cycle.

All observations should be made on the main stem.

All observations on the leaf should be made on fully developed leaves at the 2/3 height of the plant, after bud stage but before the flowering stage. The bud should reach a size of about 5 cm.

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) verification of the originality of the parental lines in comparison with the reference collection, based on the characteristics in the table of characteristics in order to screen the closest inbred lines;
- (iii) verification of the originality of the hybrid formula in comparison with those of the hybrids in common knowledge taking into account the closest inbred lines;
- (iv) assessment of the distinctness at the hybrid level of varieties with a close formula.

Qualitative characteristics:

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

Quantitative characteristics:

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

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

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

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

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

b) Uniformity

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

For the assessment of uniformity of inbred lines, a population standard of 2% with an acceptance probability of at least 95% should be applied. In addition, the same population standard and acceptance probability should apply for the assessment of uniformity regarding out-crosses and isogenic male fertile plants in a male sterile line. For the assessment of uniformity of single hybrids, a population standard of 5% with an acceptance probability of at least 95% should be applied. For three-way hybrids and open-pollinated varieties, the variability within the variety should not exceed the variability of comparable varieties already known.

Number of off-types allowed for different cases and sample sizes	Off-types	Number of plants observed	Number of off-types allowed
<u>Male sterile inbred line¹</u> (population standard: 2%, acceptance probability: 95%)	Out-crossed plants and isogenic fertile plants	19 - 41	2
		42 - 69	3
		70 - 99	4
		100 - 131	5
	Other off-types	19 - 41	2
		42 - 69	3
		70 - 99	4
		100 - 131	5
<u>Male fertile inbred line</u> (population standard: 2%, acceptance probability: 95%)	All types of off-types	19 - 41	2
		42 - 69	3
		70 - 99	4
		100 - 131	5
<u>Single-cross hybrid</u> (population standard: 5%, acceptance probability: 95%)	All types of off-types	17 - 28	3
		29 - 40	4
		41 - 53	5
		54 - 67	6
		68 - 81	7
		82 - 95	8
		96 - 110	9
		111 - 125	10

With respect to the use of enzyme electrophoresis, the Office follows the actual UPOV approach as laid down in part I of Annex 2 hereto. If enzyme electrophoresis is used for testing distinctness, the same population standard and the same acceptance probability as for other characteristics should be applied. However, a sequential analysis approach could be applied to reduce the workload. All plants within an inbred line with one locus or more loci being heterozygous with one allele in each locus coming from the inbred line (e.g. AX) should be considered out-crosses. All other cases of heterozygosity as well as cases where one foreign allele is present in one locus with homozygous status should be considered off-types.

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

¹ For example, a male sterile inbred line with 2 out-crossed plants and 2 off-types for characteristics of leaves on 40 plants observed is accepted. On the other hand, a male sterile inbred line with 3 off-types for the characteristics of the leaves on 40 plants observed is refused.

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

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
1.	1.	Hypocotyl: anthocyanin coloration	A2		
		absent	VG	HA 850	1
		present		RHA 271	9
2.	2.	Hypocotyl: intensity of anthocyanin coloration	A2		
		weak	VG	H 52.6.3	3
		medium		HA 290	5
		strong		RHA 271	7
3. H ³	3.	Leaf: size	E4		
		small	VG	HA 124	3
		medium		HA 821	5
		large		DK 3790	7
4. H G	4.	Leaf: green colour	E4		
		light	VG	H 52.9.1.1	3
		medium		HA 821	5
		dark		HA 303	7
5. H G	5.	Leaf: blistering	E4		
		absent or very weak	VG		1
		weak		HA 342, RHA 273	3
		medium		HA 291	5
		strong		HA 303, RHA 361	7

¹ The optimum stage of development for the assessment of each characteristic is indicated by numbers and letters. Explanations are given in Annex1 'Explanations and Methods'.

² Example varieties are given as an indication. Others may be used.

³ H = In cases where the distinction of hybrid varieties is based on the distinction of parental lines, the description of the hybrid must at least include those characteristics

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
6. (+) ⁴ H	6.	Leaf: serration	E4		
		isolated or very fine	VG	HA 393	1
		fine		HA 124	3
		medium		RHA 271	5
		coarse		RHA 299	7
		very coarse			9
7. (+) H	7.	Leaf: shape of cross section	E4		
		strongly concave	VG		1
		weakly concave		RHA 273	2
		flat		H 55.9.2.1.1	3
		weakly convex		HA 303	4
		strongly convex			5
8. (+) H	8.	Leaf: shape of distal part	E4		
		lanceolate	VG		1
		lanceolate to narrow triangular			2
		narrow triangular		RHA 855	3
		narrow triangular to broad triangular			4
		broad triangular		HA 821	5
		broad triangular to acuminate			6
		broad triangular to rounded		HA 303	7
		acuminate		HA 124	8
		rounded		HA 234	9

⁴ See in Annex 1 in 'Explanations and Methods'

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
9. (+) H	9.	Leaf: auricles	E4		
		none or very small	VG	HA 290	1
		small			3
		medium		HA 852	5
		large		HA 89	7
		very large		HA 303	9
10. (+)	10.	Leaf: wings	E4		
		none or very weakly expressed	VG	HA 89	1
		weakly expressed			2
		strongly expressed		RHA 274, RHA 348	3
11. (+) H	11.	Leaf: angle of lowest lateral veins	E4		
		acute	VG	HA 290, RHA 295	1
		right angle or nearly right angle		HA 89	2
		obtuse		HA 303	3
12. (+)	12.	Leaf: height of the tip of the blade compared to insertion of petiole (at 2/3 height of plant)	E4		
		low	VG	RHA 275	3
		medium		RHA 274	5
		high		RHA 400	7
13. H	13.	Stem: hairiness at the top (last 5 cm)	F1		
		absent or very weak	VG		1
		weak		RHA 271	3
		medium		RHA 273	5
		strong		HA 303	7
		very strong			9

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
14.	14.	Time of flowering	MS or MG		
(+)		very early		HA 302, RHA 381	1
		early		RHA 273	3
H		medium		RHA 274	5
		late		RHA 271	7
G		very late		RHA 361	9
15.	15.	Ray florets: density	F3.2		
		sparse	VG	HA 385	3
		medium		HA 89	5
		dense			7
16.	16.	Ray floret: shape	F3.2		
(+)		fusiform	VG	H 52.9.1.1	1
H		narrow ovate		RHA 274	2
		broad ovate		HA 821	3
		rounded			4
17.	17.	Ray floret: disposition	F3.2		
		flat	VG	HA 89	1
		longitudinal recurved		HA 850	2
		undulated		HA 234	3
		strongly recurved to back of head		CM 592	4
18.	18.	Ray floret: length	F3.2		
H		short	VG	RHA 361	3
		medium		HA 89	5
		long		H 52.6.3	7

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
19. H	19.	Ray floret: colour	F3.2		
		yellowish white	VG		1
		light yellow			2
		medium yellow		HA 89	3
		orange yellow		RHA 361	4
		orange		CM 587, RHA 295	5
		purple			6
		reddish brown			7
G		multicoloured			8
20.	20.	Disk flower: colour	F3.2		
		yellow	VG		1
		orange		HA 89	2
		purple			3
21. (+) H	21.	Disk flower: anthocyanin coloration of stigma	F3.2		
		absent	VG	HA 89	1
		present		H 55.9.2.1.1, HA 348	9
22. (+) H	22.	Disk flower: intensity of anthocyanin coloration of stigma	F3.2		
		weak	VG	HA 290, HA 394	3
		medium		HA 60, HA 291	5
		strong		RHA 348	7
23.	23.	Disk flower: production of pollen	F3.2		
		absent	VG		1
		present			9
24. (+)	24.	Bract: shape	F3.2		
		clearly elongated	VG	HA 379	1
		neither clearly elongated nor clearly rounded		HA 292	2
		clearly rounded		RHA 801	3

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
25. (+) H	25.	Bract: length of tip	F3.2		
		short	VG	RHA 273, RHA 361	3
		medium		HA 302	5
		long		HA 292, HA55.9.2.1.1	7
		very long		H 52.6.3	9
26. H	26.	Bract: green colour of outer side	F3.2		
		light	VG	H 52.9.1.1	3
		medium		HA 850	5
		dark		HA 303	7
27.	27.	Bract: attitude in relation to head	M0		
		not embracing or very slightly embracing	VG	H 52.9.1.1	1
		slightly embracing		HA 337, HA 343	2
		strongly embracing		RHA 234	3
28. (+) H G	28.	Plant: natural height	M0		
		very short	MS or MG	HA 379	1
		short		HA 291	3
		medium		RHA 801	5
		tall		H 52.9.1.1	7
29. G	29.	Plant: branching (excluding environmental branching)	M0-M2		
		absent	VG	HA89	1
		present		RHA 271	9
30. (+)	30.	Plant: type of branching (as for 29)	M0-M2		
		only basal	VG	RHA 295	1
		predominantly basal			2
		overall		RHA 273	3
		predominantly apical		RHA 271	4
		only apical		RHA 294	5

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
31.	31.	Plant: natural position of highest lateral head to the central head	M0-M2		
		below	VG	RHA 361	1
		same level		RHA 857	2
		above		RHA 274	3
32. (+) H	32.	Head: attitude	M3		
		horizontal	VG		1
		inclined			2
		vertical		RHA 395	3
		half-turned down with straight stem			4
		half-turned down with curved stem			5
		turned down with straight stem			6
		turned down with slightly curved stem			7
		turned down with strongly curved stem			8
		over turned			9
33. H	33.	Head: size	M3		
		small	VG	RHA 273	3
		medium		RHA 271	5
		large		H 52.9.1.1	7
34. (+) H	34.	Head: shape of grain side	M3		
		strongly concave	VG		1
		weakly concave			2
		flat		RHA 273	3
		weakly convex		HA 89	4
		strongly convex		CM 400	5
		deformed		RHA 271	6

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
35. H	35.	Seed: size	M4		
		small	VG	RHA 801	3
		medium		HA 89	5
		large		HA 292	7
		very large		HA 316	9
36. (+) H	36.	Seed: shape	M4		
		elongated	VG	HA 60	1
		narrow ovoid		RHA 271	2
		broad ovoid		HA 89	3
		rounded		CM 447	4
37.	37.	Seed: thickness relative to width	M4		
		thin	VG	RHA 274	3
		medium		RHA 271	5
		thick			7
38. (+) H G	38.	Seed: main colour	M4		
		white	VG		1
		whitish grey			2
		grey		TRISUN 860	3
		light brown			4
		medium brown		RHA 273	5
		dark brown			6
		black		HA 89	7
		purple			8
39. (+) H G	39.	Seed: stripes <u>on</u> margin	M4		
		none or very weakly expressed	VG	RHA 273	1
		weakly expressed		H 52.9.1.1	2
		strongly expressed		HA 89	3

CPVO N°	UPOV N°	Characteristics	Stage ¹	Examples ²	Note
40.	40.	Seed: stripes <u>between</u> margins	M4		
(+)		none or very weakly expressed	VG	RHA 273	1
H		weakly expressed		RHA 293	2
G		strongly expressed		HA 89	3
41.	41.	Seed: colour of stripes	M4		
		white	VG	RHA 295	1
		grey		HA 89	2
		brown		HA 292	3
		black		Narval 30	4

ANNEXES TO FOLLOW

ANNEX I	<u>PAGE</u>
Explanations and methods,	20
Growth stage of sunflower	26
Electrophoresis	29

ANNEX II

Technical Questionnaire

ANNEX I

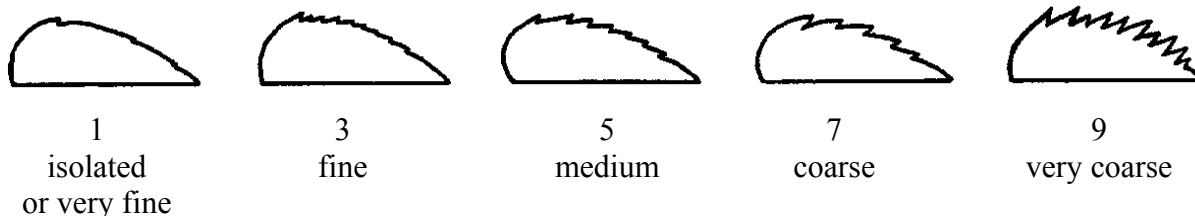
EXPLANATIONS AND METHODS

Method of observation of characteristics

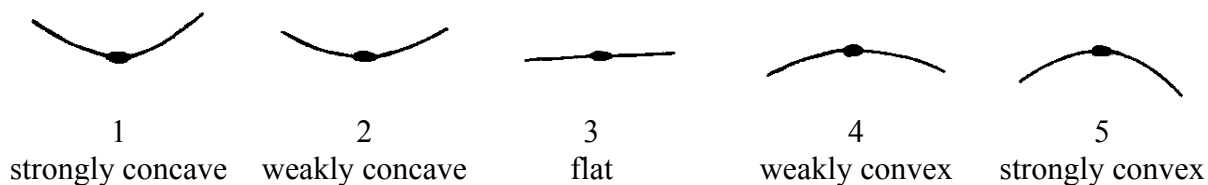
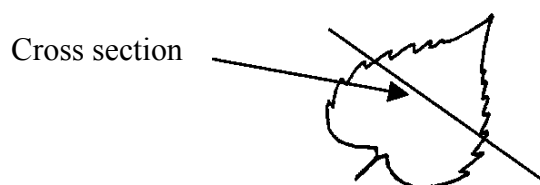
Letters indicate the relevant method for the assessment of distinctness

MG	Measurement of a group of plants or plant parts for the assessment of distinctness
MS	Measurement of single plants or plant parts for the assessment of distinctness
VG	Visual assessment by a single observation of a group of plants or plant parts for the assessment of distinctness

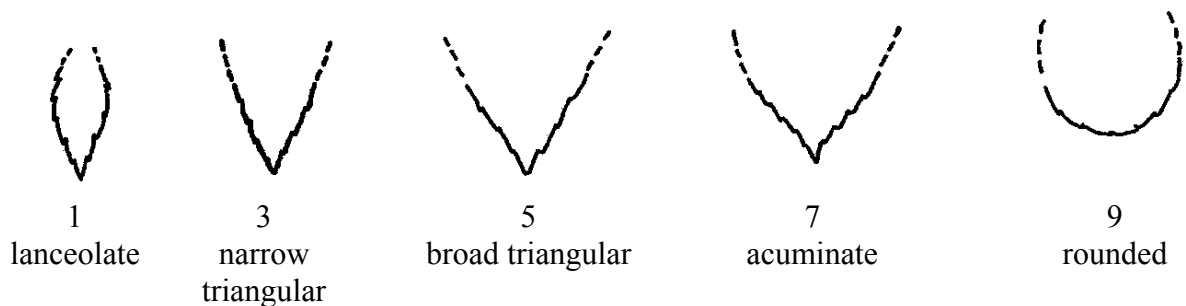
Ad. 6: Leaf: serration



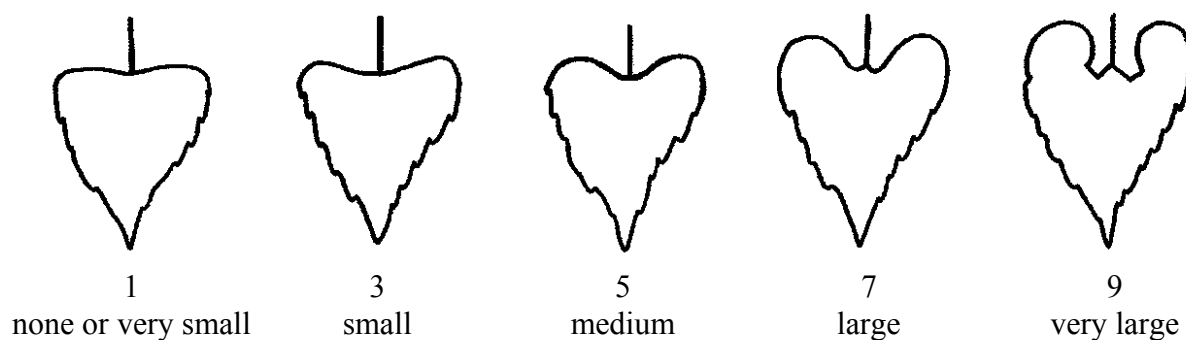
Ad. 7: Leaf: shape of cross section (through the middle of the leaf)



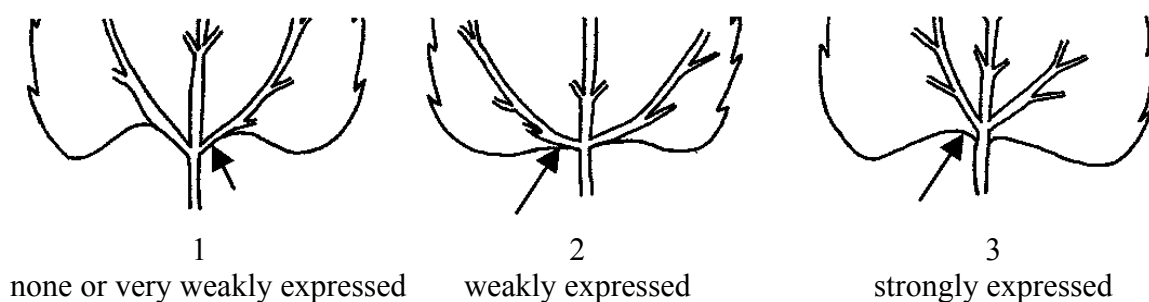
Ad. 8: Leaf: shape of distal part



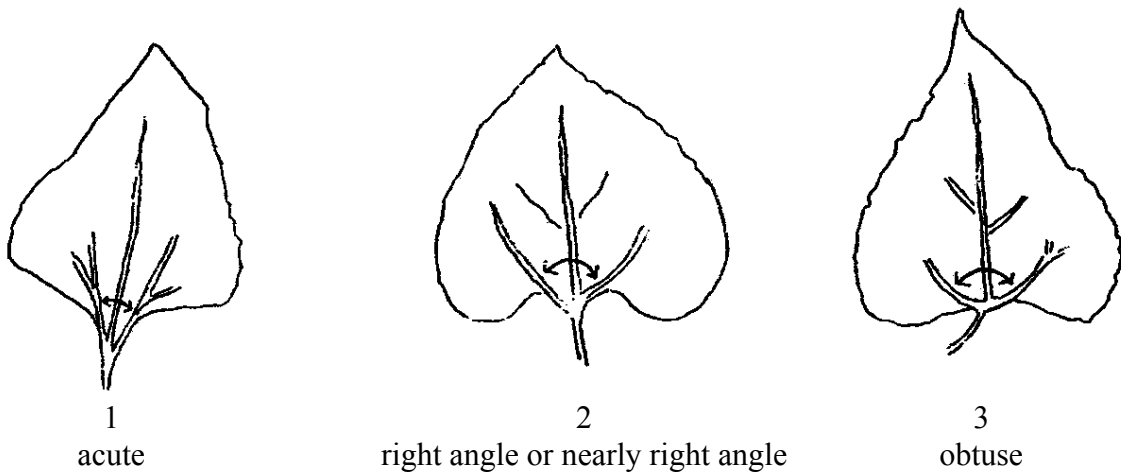
Ad. 9: Leaf: auricles



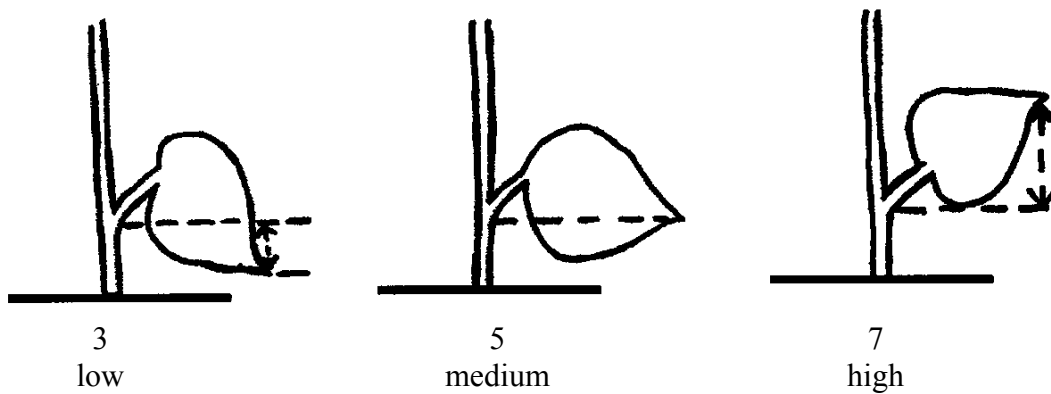
Ad. 10: Leaf wings
(parenchym at base of lateral veins)



Ad. 11: Leaf: angle of lowest lateral veins



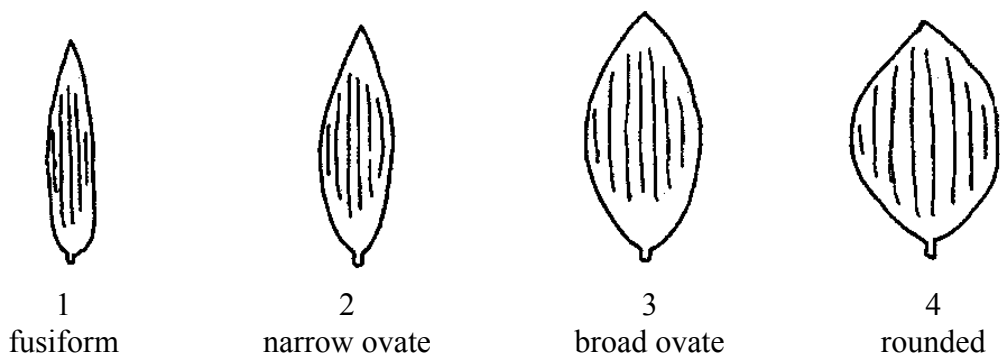
Ad. 12: Leaf: height of the tip of the blade compared to insertion of petiole



Ad. 14: Time of flowering

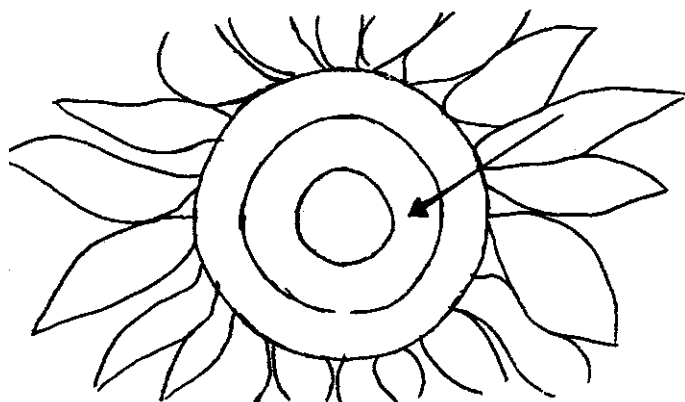
The time of flowering is reached when 50% of the plants are in flower. A plant is considered in flower when it shows at least one ray floret erected and coloured.

Ad. 16: Ray floret: shape



Ad. 21 and 22: disk flower: anthocyanin coloration of stigma

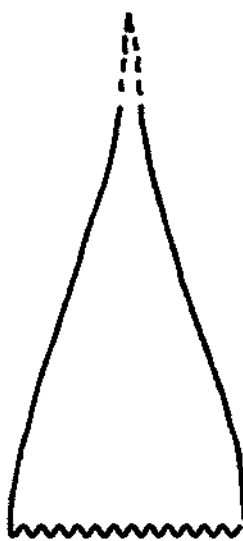
The anthocyanin coloration should be recorded on the stigma from the central third of the head just after the pollen appears at the top of the anthers.



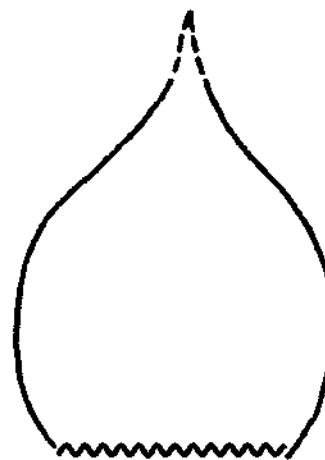
Ad. 24: Bract: shape



1
clearly elongated

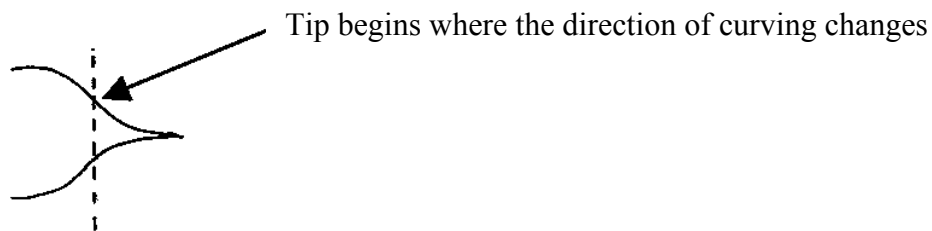


2
neither clearly elongated
nor clearly rounded



3
clearly rounded

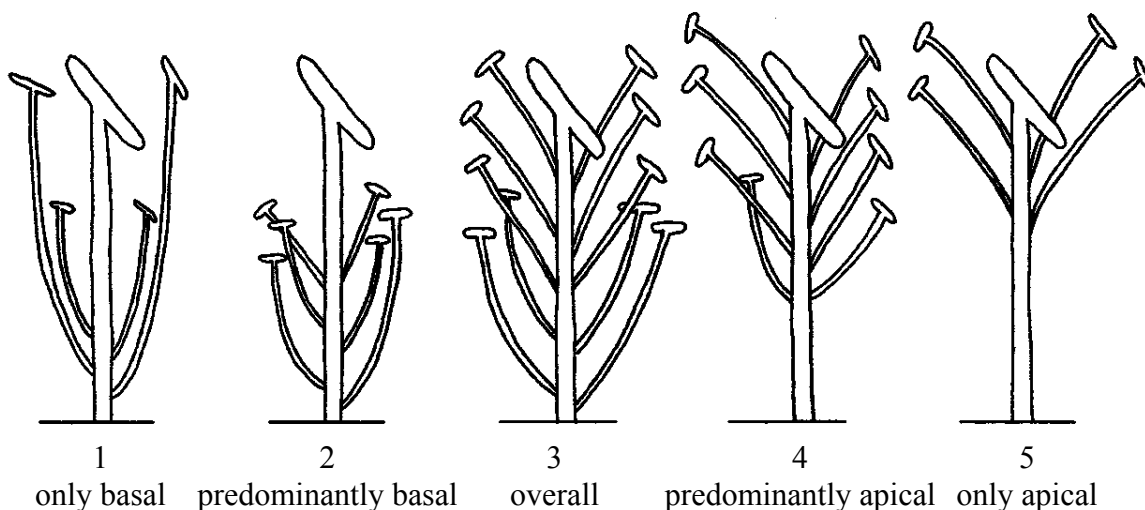
Ad. 25: Bract: length of the tip



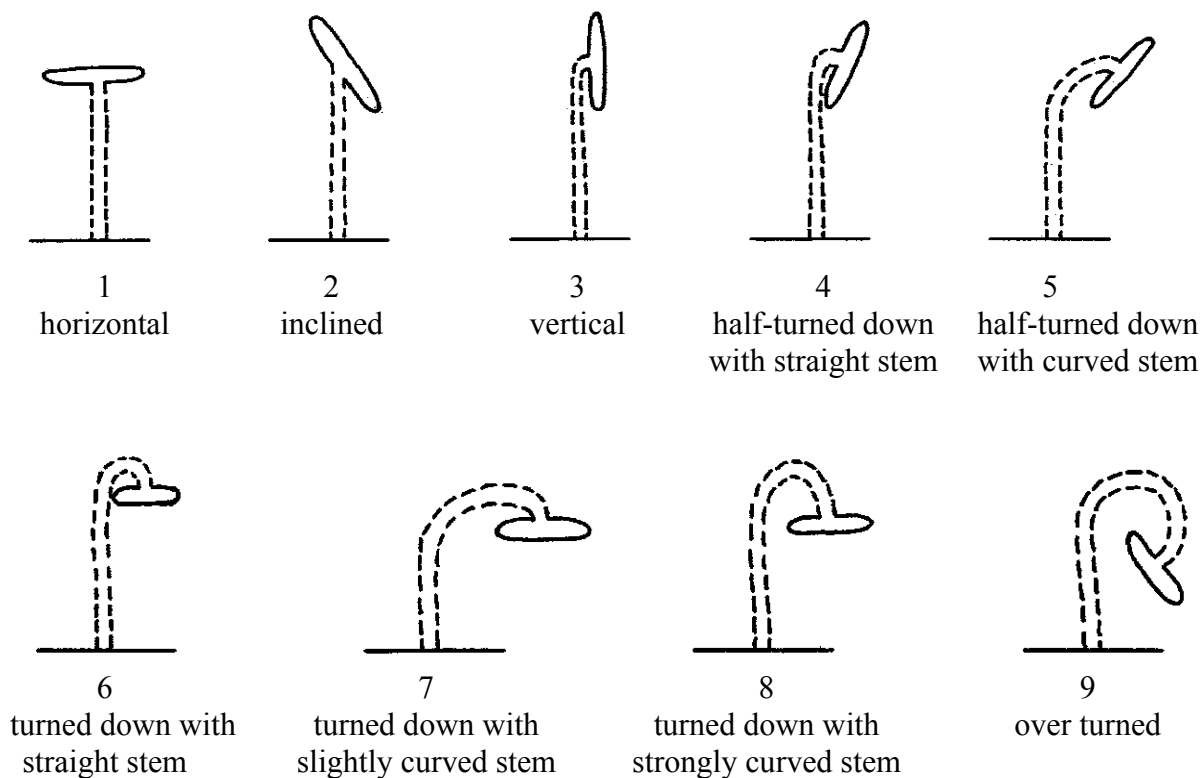
Ad. 28: Plant: natural height

Different environmental conditions may require separate scales for lines, hybrids and open pollinated varieties.

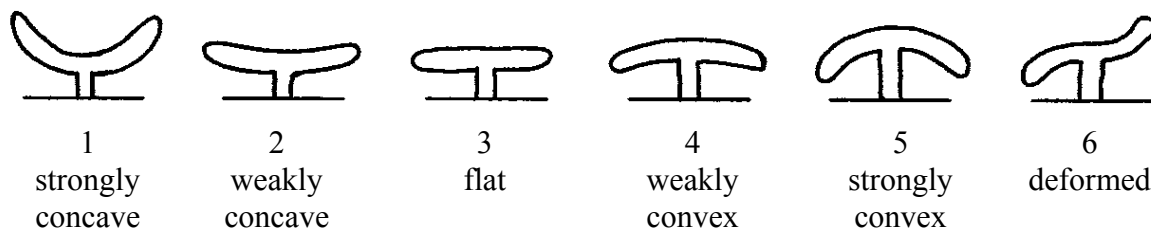
Ad. 30: Plant type of branching
(excluding environmental branching)



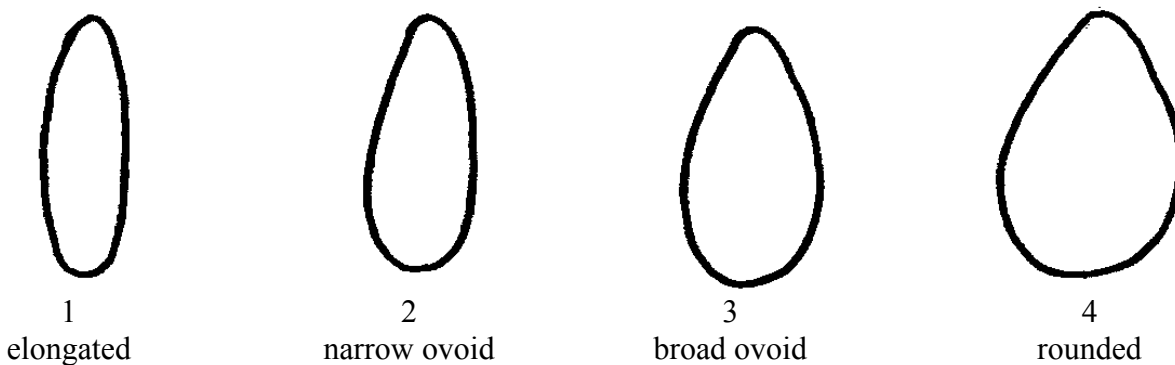
Ad. 32: Head: attitude



Ad. 34: Head: shape of grain side



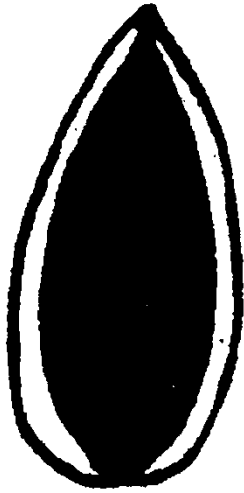
Ad. 36: Seed: shape



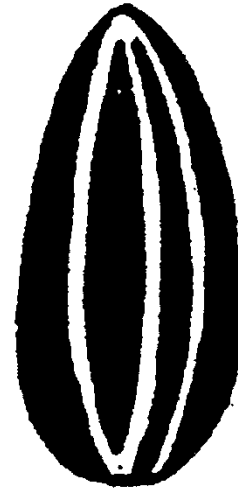
Ad. 38: Seed: main colour

The main colour of the seed is the colour with the largest area. In case of doubt which is the largest area, the darkest colour is the main colour.

Ad. 39 and 40: Seed: stripes



on margin



between margins

GROWTH STAGES OF SUNFLOWER¹

(A stage is reached when 50% of the plants show that stage)

Germination - Emergence (A)**Vegetative Phase (B)**

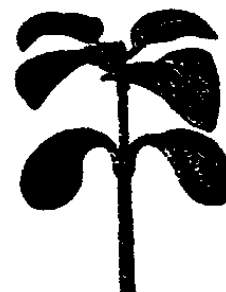
A 1

Appearance of hypocotyl
as a cross.



A 2

Emergence of cotyledons and
first leaves visible.



B 3 – B 4

The second pair of opposed
leaves appears and has
about 4 cm of length; the
petioles are visible from below.

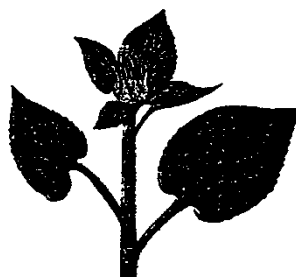
Flower Bud Phase (E)

E 1



Appearance of leaf bud
narrowly inserted in the
middle of young leaves: stage
of star bud.

E 2



The bud detaches from the
leaf crown, the bracts are
clearly distinguishable from
the leaves. The diameter
varies from 0.5 to 2 cm.

E 4



The bud is clearly free from
the leaves, its diameter varies
from 5 to 8 cm, it remains
horizontal. One part of the
bracts is unfolded.

¹ Reproduced with the kind permission of CETIOM (France)

Flowering (F)

Maturity (M)



F 1

The flower bud bends; the ray florets are outwards of the disc.



F 3.2

The three most outer rows of disc florets have their anthers visible and detached and their stigmas unfolded.



M 0

Falling of ray florets. The back of the head is still green.

Maturity (M)



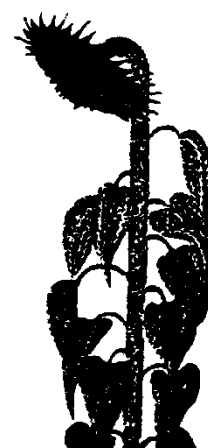
M 2

The back of the head is yellow. The bracts are at 3/4 brown. The humidity of the seed is about 20 to 25%.



M 3

The back of the head is brown marbled. The bracts are brown. The stem dries out. The humidity of the seed is near 15%.



M 4

All organs of the plant are dark brown. The humidity of the seed is near 10%.

ELECTROPHORESIS

Additional Useful Explanations

	<u>TABLE OF CONTENTS</u>	<u>PAGE</u>
Part I	Introduction.....	30
Part II	Characteristics derived by using electrophoresis	31
Part III	Description of the method to be used.....	32

Part I

Introduction

The following Annex contains a list of characteristics derived by using electrophoresis and a description of the method to be used. UPOV decided to place these characteristics in an Annex to the Test Guidelines, thereby creating a special category of characteristic, because the majority of the UPOV member States is of the view that it is not possible to establish distinctness solely on the basis of a difference found in a characteristic derived by using electrophoresis. Such characteristics should therefore only be used as a complement to other differences in morphological or physiological characteristics. UPOV has reconfirmed that these characteristics are considered useful but that they might not be sufficient on their own to establish distinctness. They should not be used as a routine characteristic but at the request or with the agreement of the applicant of the candidate variety.

Part II

Characteristics Derived by Using Electrophoresis

	Characteristics	Example Varieties	Note
43. (+)	Allele expression at locus Me1		
	Genotype 2/2	HA 89	1
	Genotype 4/4	RHA 274	2
	Genotype 2/4	Florence	3
44. (+)	Allele expression at locus Pgd1		
	Genotype 2/2	RHA 274	1
	Genotype 4/4	HA 850	2
	Genotype 2/4	Santafe	3
45. (+)	Allele expression at locus Pgi2		
	Genotype 2/2	RHA 274	1
	Genotype 4/4	H559211	2
	Genotype 2/4	Santafe	3
46. (+)	Allele expression at locus Shdh1		
	Genotype 2/2	HA 89	1
	Genotype 4/4	RHA 856	2
	Genotype 2/4	Florence	3
47. (+)	Allele expression at locus Pgm4		
	Genotype 2/2	RHA 274	1
	Genotype 4/4	HA 89	2
	Genotype 2/4	Florence	3

Part III

Description of the Method to be Used

Description of the SGE Method for the Analysis of Isoenzymes from *Helianthus annuus L.*

1. Number of seedlings per test:

- For checking formula:
 - 10 seedlings each of inbred lines
 - 4 seedlings of single hybrids
 - 10 seedlings of three-way hybrids
- For distinctness, uniformity and stability test:
 - at least 40 seedlings for inbred lines, hybrids and open-pollinated varieties

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:

Tris- (hydroxymethyl) aminomethane (Tris)
Hydrochloric acid
β-Mercaptoethanol

3.2 Chemicals for electrophoresis

Bromophenol blue
Citric acid monohydrate
L-Histidine
Starch hydrolysed, for electrophoresis, (Sigma S-4501 or equivalent)

3.3 Chemicals for staining enzymes

95% Ethanol
Ethylenediamine tetra-acetic acid, disodium salt (EDTA Na₂)
D-Fructose 6-phosphate, disodium salt
α-D-Glucose 1-phosphate, monohydrate, disodium salt
Glucose 6-phosphate dehydrogenase (Sigma G5885)
Hydrochloric acid (HCl)
Magnesium chloride hexahydrate (MgCl₂, 6H₂O)
DL-Malic acid, monosodium salt

Dimethylthiazol diphenyl tetrazolium (MTT)
 β -Nicotinamide adenine dinucleotide phosphate (NADP)
Nitro-blue tetrazolium (NBT)
6-phosphogluconic acid, trisodium salt dihydrate
Phenazine methosulfate (PMS)
Shikimic acid
Sodium hydroxide (NaOH)
Tris- (hydroxymethyl) aminomethane (Tris)

4. Solutions

4.1 Extraction solution: 0.1M Tris HCl (pH 7.2) + 0.2 % 2-mercaptoethanol (v/v). In order to improve the results on PGD, Polyvenyl-Pyrrolidone (PVP) of 2% (p/v) can be added to the solution.

4.2. Electrophoresis buffers

4.2.1 Buffers for SGE pH 6.5

4.2.1.1 Stock solution: 0.364 M L-histidine-citrate

50.44 g L-histidine
8.34 g Citric acid monohydrate
made up to 1 l with de-ionised water

4.2.1.2 Running buffer: 0.072 M L-histidine-citrate pH 6.5 (Stock solution diluted 1 in 5)

400 ml stock solution (4.2.1.1)
made up to 2 l with de-ionised water

4.2.1.3 Gel buffer: 0.024 M L-histidine-citrate (Stock solution diluted 1 in 15)

80 ml stock solution (4.2.1.1)
made up to 1200 ml with de-ionised water

4.2.2 Buffers for SGE pH 5.7

4.2.2.1 Running buffer: 0.067 M L-histidine-citrate pH 5.7:

20.18 g L-histidine
8.34 g Citric acid monohydrate
made up to 2 l with de-ionised water

4.2.2.2 Gel buffer: 0.011 M L-histidine-citrate (Running buffer diluted 1 in 6):

100 ml running buffer (4.2.2.1) made up to 1200 ml 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 7.5

121.1 g Tris, made up to 1 l with de-ionised water and adjusted to pH 7.5 with 50 % HCl

4.3.1.2 1 M Tris-HCl pH 8.5

121.1 g Tris, made up to 1 l with de-ionised water and adjusted to pH 8.5 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 Sodium malate solution

2.5 g DL-malic acid
made up to 50 ml with de-ionised water and adjusted to pH 8.0 with 1M NaOH.

4.3.2 Staining solutions

4.3.2.1 ME staining solution

100 ml 0.1 M Tris HCl, pH 7.5 (4.3.1.1 diluted 1 in 10)
4 ml Sodium malate solution (4.3.1.7.)
1 ml NBT solution (4.3.1.4.)
1 ml PMS solution (4.3.1.5.)
1,8 ml MgCl₂ solution (4.3.1.6.)
17.5 mg NADP

4.3.2.2 PGD + PGI staining solution

100 ml 0.1 M Tris HCl, pH 7.5 (4.3.1.1. diluted 1 in 10)
100 mg D-Fructose 6-phosphate Na₂ salt
60 mg 6-Phosphogluconic acid Na₃ salt
10 mg NADP
1 ml MTT solution (4.3.1.3.)
1.5 ml PMS solution (4.3.1.5.)
1 ml MgCl₂ solution (4.3.1.6.)
40 units of Glucose-6-phosphate dehydrogenase (SIGMA G 5885)

To stain PGI only, do not include 6-phosphogluconic acid.

To stain PGD only, do not include either fructose 6-phosphate disodium salt or glucose 6-phosphate dehydrogenase.

4.3.2.3 ShDH staining solution

100 ml 0.2 M Tris HCl, pH 8.5 (4.3.1.2 diluted 1 in 5)
50 mg shikimic acid
1 ml MTT solution (4.3.1.3)
1.25 ml PMS solution (4.3.1.5)
12 mg NADP

4.3.2.4 PGM staining solution

100 ml 0.1 M Tris HCl, pH 8.5 (4.3.1.2. diluted 1 in 10)
150 mg α -D-Glucose 1-phosphate 1H₂O, Na₂ salt
150 mg EDTA, Na₂
10 mg NADP
1.5 ml MTT solution (4.3.1.3)
1 ml PMS solution (4.3.1.5)
4 ml MgCl₂ solution (4.3.1.6)
40 units of Glucose 6-phosphate dehydrogenase

5. **Procedure**

5.1. Enzyme extraction

Seedlings are grown on moistened germination paper or in moistened sterile sand, at 25°C, in darkness, for 2 to 3 days. Seed coats are removed and cotyledons are crushed at 4°C, with a pestle in 1.5 ml microtubes containing 300 μ l extraction buffer (4.1).

The extracts can be stored at -30°C or at -80°C.

5.2 Preparation of the gel

Prepare the gels the day before migration.

To make two 12.5 % starch gels (18 x 18 x 1 cm) the following is required: 128 g starch are mixed in 1020 ml gel buffer (4.2.1.3 or 4.2.2.2) in a 1000 ml Büchner flask and heated at 78°C. The mixture is degassed with a water jet aspirator for 30 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 45 min, then placed in a refrigerator for 1 h. The gels are wrapped with polyethylene film for overnight storage. And cooled to 4°C for 1 h before migration.

5.3 Electrophoresis

- 5.3.1 Each electrode tank is filled with the appropriate volume of running buffer (4.2.1.2 or 4.2.2.1) pre-cooled to 4°C. The polyethylene film is lifted up and two transversal slits are cut in the gel 3 cm and 4 cm from the edge (cathode side) of the mould.

The 1 cm gel slice is removed and the extracts are loaded as follows:

The enzyme extracts are thawed from 5.1, and absorbed on a filter paper wick (1.5 mm x 20 mm, Whatman N° 3).

The wicks are inserted into the gel, tightly against the first slit.

One wick soaked with bromophenol blue solution (4.2.2.3) (migration dye marker) is placed on each side of the gel.

The gel slice is cautiously replaced. Each gel is covered with polyethylene film.

The two gels, with the extracts on the cathodal side, are placed on the two electrode buffer tanks, in a refrigerated cabinet at 4°C.

The electrophoresis is carried out at 4°C, towards the anode. After 15 min of migration at the first voltage, the wicks are removed and the voltage is increased. Constant voltage should be maintained during each phase.

The electrophoretic conditions are indicated in the following table.

Buffer systems	Constant voltage	Distance run by bromophenol blue	Duration of migration
Histidine citrate pH 5.7	260 V for 15 min then 290 V	13 cm	5 h
Histidine citrate pH 6.5	240 V for 15 min then 280 V	11 cm	5 h

Voltage should depend on the size of the gel, at a rate of 15 - 16 V/cm². Distance run by bromophenol blue (dye front) is also depending on the size of the gel, but the Duration of migration (time of migration) is a fixed value.

SGE at pH 5.7 should be used for detecting ME, PGD and PGI. The isoenzymes PGM and ShDH should be analysed by SGE pH 6.5.

5.4 Enzyme staining

After switching off the current, the gel is cut horizontally in 1 mm thick slices with a very fine steel wire or a fishing line. The upper slice is discarded. Individual gel slices are stained by incubation at 37°C, in darkness in the following solutions:

for ME:	solution 4.3.2.1,	incubation time: 15 h
for PGD and PGI:	solution 4.3.2.2,	incubation time: 1 h
for SHDH:	solution 4.3.2.3,	incubation time: 1 h
for PGM:	solution 4.3.2.4,	incubation time: 1/2 h

After staining the gel slices are rinsed in de-ionised water and fixed in 40% ethanol solution. The following procedures for long time storing can be successfully used: e.g. drying of the gels between two cellophane sheets soaked in a 5% glycerol solution, or storing in sealed polyethylene bags.

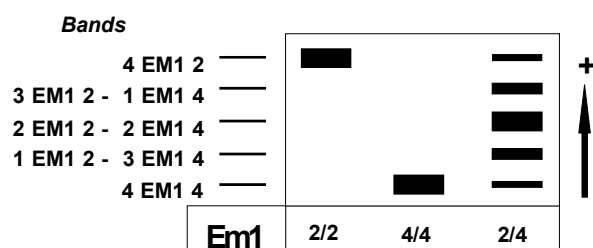
6. Recognition of the alleles encoding isoenzymes

6.1 Recognition of the alleles encoding ME

6.1.1 Genetic interpretation of the zymogrammes

Enzyme	Quaternary structure	Locus	Alleles
Malic enzyme (ME)	Tetrameric	Me1	2 4

6.1.2 Schematization of the zymogrammes

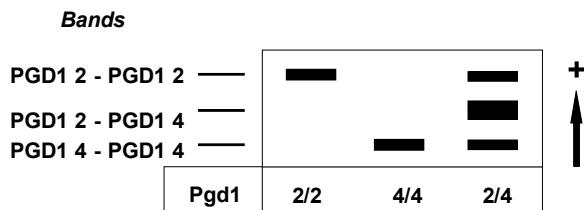


6.2 Recognition of the alleles encoding PGD

6.2.1 Genetic interpretation of the zymogrammes

Enzyme	Quaternary structure	Locus	Alleles
6-phosphogluconate dehydrogenase (PGD)	Dimeric	Pgd1	2 4

6.2.2 Schematization of the zymogrammes



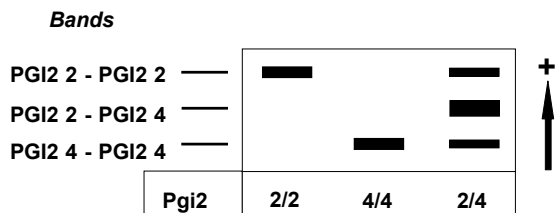
Two migration zones can be observed; only the slowest migrating bands are polymorphic.

6.3 Recognition of the alleles encoding PGI

6.3.1 Genetic interpretation of the zymogrammes

Enzyme	Quaternary structure	Locus	Alleles
Phosphoglucoisomerase (PGI)	Dimeric	Pgi2	2 4

6.3.2 Schematization of the zymogrammes



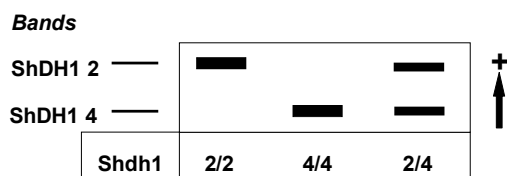
There are two migration zones; only the slowest migrating bands are scored.

6.4 Recognition of the alleles encoding ShDH

6.4.1 Genetic interpretation of the zymogrammes

Enzyme	Quaternary structure	Locus	Alleles
Shikimate dehydrogenase (ShDH)	Monomeric	Shdh1	2 4

6.4.2 Schematization of the zymogrammes

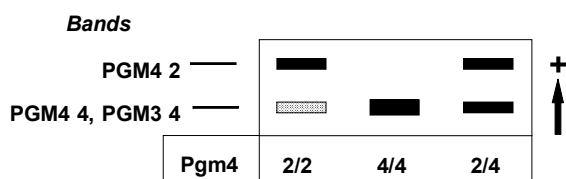


6.5 Recognition of the alleles encoding PGM

6.5.1 Genetic interpretation of the zymogrammes

Enzyme	Quaternary structure	Locus	Alleles
Phosphoglucomutase	Monomeric	Pgm4	2 4

6.5.2 Schematization of the zymogrammes



Several migration zones can be observed; only the fastest zone is polymorphic.

There is another gene which has not been considered. This has been designated Pgm3, encoding an enzyme which comigrates with PGM4 4.

So, the genotypes Pgm4 2/2 and Pgm4 2/4 give a two-band zymogramme. These both genotypes differ only by relative band intensities.

ANNEX II



European Union
Community Plant Variety Office

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.

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

Helianthus annuus L.

SUNFLOWER

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

3.1 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) inbred line []
 - male sterile line []
 - male fertile line B (maintainer of sterility) []
 - male fertile line R (restorer of fertility) []
- (b) hybrid []
 - male sterile single hybrid []
 - male fertile single hybrid []
 - three-way hybrid []
- (c) open-pollinated variety []
- (d) other (please indicate) []

4.2 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 this information as follows:

Formula

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

Single hybrid

() X ()
 female line male line

Three-way hybrid

() X ()
 female parental line male parental line

of single hybrid used as female X ()
 male parental line of 3-way hybrid

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

4.3 Other information on genetic origin and breeding method (to be filled for each component of an hybrid variety)

Component name	Genetic origin	Parentage with any other components already known
----------------	----------------	---

4.4 Origin

- (e) Seedling (indicate parent varieties) []
- (f) Mutation (indicate parent variety) []
- (c) Discovery (indicate where, when and how the variety has been developed):..... []
- (d) Other (please specify)..... []

4.5 Method of propagation

- (a) Cuttings []
- (b) *In vitro* propagation []
- (c) Seed []
- (d) Other (please specify): []

4.6 Other information:

In the case of seed propagated varieties: method of production:

- (a) Self-pollinated []
- (b) Cross-pollinated (please give details)..... []

- (c) Hybrid (please give details)..... []

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

4.8 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 (4)	Leaf: green colour		
	light	H 52.9.1.1	3 []
	medium	HA 821	5 []
	dark	HA 303	7 []
5.2 (5)	Leaf: blistering		
	absent or very weak		1 []
	weak	HA 342, RHA 273	3 []
	medium	HA 291	5 []
	strong	HA 303, RHA 361	7 []
	very strong		9 []
5.3 (14)	Time of flowering		
	very early	HA 302, RHA 381	1 []
	early	RHA 273	3 []
	medium	RHA 274	5 []
	late	RHA 271	7 []
	very late	RHA 361	9 []
5.4 (19)	Ray floret: colour		
	yellowish white		1 []
	light yellow		2 []
	medium yellow	HA 89	3 []
	orange yellow	RHA 361	4 []
	orange	CM 587, RHA 295	5 []
	purple		6 []
	reddish brown		7 []
	multicoloured		8 []

	Characteristics	Example varieties	Note
5.5 (28)	Plant: natural height		
	very short	HA 379	1 []
	short	HA 291	3 []
	medium	RHA 801	5 []
	tall	H 52.9.1.1	7 []
	very tall		9 []
5.6 (29)	Plant: branching (excluding environmental branching)		
	absent	HA 89	1 []
	present	RHA 271	9 []
5.7 (38)	Seed: main colour		
	white		1 []
	whitish grey		2 []
	grey	TRISUN 860	3 []
	light brown		4 []
	medium brown	RHA 273	5 []
	dark brown		6 []
	black	HA 89	7 []
	purple		8 []
5.8 (39)	Seed: stripes <u>on</u> margin		
	none or very weakly expressed	RHA 273	1 []
	weakly expressed	H 52.9.1.1	2 []
	strongly expressed	HA 89	3 []
5.9 (40)	Seed: stripes <u>between</u> margins		
	none or very weakly expressed	RHA 273	1 []
	weakly expressed	RHA 293	2 []
	strongly expressed	HA 89	3 []

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

(a) Downy Mildew []
 (precise the races)

(b) rust []

(c) other pests or diseases (please specify) []

7.2 Use

(a) oil and cake []

(b) birds and direct human consumption []

(c) ornamental []

(d) other use (please specify) []

7.3 Special conditions for the examination of the variety

YES, please specify

NO

7.4 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.

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]