**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)<br>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)<br>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** |
|---|---|---|---|
| Male sterile inbred line[1] (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 |
| Male fertile inbred line (population standard: 2%, acceptance probability: 95%) | **All types of off-types** | 19 - 41 | 2 |
| | | 42 - 69 | 3 |
| | | 70 - 99 | 4 |
| | | 100 - 131 | 5 |
| Single-cross hybrid (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.** | **3.** | **Leaf: size** | E4 | | |
| **H**[3] | | small | VG | HA 124 | 3 |
| | | medium | | HA 821 | 5 |
| | | large | | DK 3790 | 7 |
| **4.** | **4.** | **Leaf: green colour** | E4 | | |
| **H** | | light | VG | H 52.9.1.1 | 3 |
| | | medium | | HA 821 | 5 |
| **G** | | dark | | HA 303 | 7 |
| **5.** | **5.** | **Leaf: blistering** | E4 | | |
| **H** | | absent or very weak | VG | | 1 |
| | | weak | | HA 342, RHA 273 | 3 |
| | | medium | | HA 291 | 5 |
| **G** | | 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.** | **6.** | **Leaf: serration** | E4 | | |
| (+)[4] | | isolated or very fine | VG | HA 393 | 1 |
| **H** | | fine | | HA 124 | 3 |
| | | medium | | RHA 271 | 5 |
| | | coarse | | RHA 299 | 7 |
| | | very coarse | | | 9 |
| **7.** | **7.** | **Leaf: shape of cross section** | E4 | | |
| (+) | | strongly concave | VG | | 1 |
| **H** | | weakly concave | | RHA 273 | 2 |
| | | flat | | H 55.9.2.1.1 | 3 |
| | | weakly convex | | HA 303 | 4 |
| | | strongly convex | | | 5 |
| **8.** | **8.** | **Leaf: shape of distal part** | E4 | | |
| (+) | | lanceolate | VG | | 1 |
| **H** | | 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.** | **9.** | **Leaf: auricules** | E4 | | |
| (+) | | none or very small | VG | HA 290 | 1 |
| **H** | | 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.** | **11.** | **Leaf: angle of lowest lateral veins** | E4 | | |
| (+) | | acute | VG | HA 290, RHA 295 | 1 |
| **H** | | 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.** | **13.** | **Stem: hairiness at the top (last 5 cm)** | F1 | | |
| **H** | | 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.** | **19.** | **Ray floret: colour** | F3.2 | | |
| **H** | | 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.** | **21.** | **Disk flower: anthocyanin coloration of stigma** | F3.2 | | |
| (+) | | absent | VG | HA 89 | 1 |
| **H** | | present | | H 55.9.2.1.1, HA 348 | 9 |
| **22.** | **22.** | **Disk flower: intensity of anthocyanin coloration of stigma** | F3.2 | | |
| (+) | | weak | VG | HA 290, HA 394 | 3 |
| **H** | | 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.** | **25.** | **Bract: length of tip** | F3.2 | | |
| (+) | | short | VG | RHA 273, RHA 361 | 3 |
| **H** | | medium | | HA 302 | 5 |
| | | long | | HA 292, HA55.9.2.1.1 | 7 |
| | | very long | | H 52.6.3 | 9 |
| **26.** | **26.** | **Bract: green colour of outer side** | F3.2 | | |
| **H** | | 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.** | **28.** | **Plant: natural height** | M0 | | |
| (+) | | very short | MS or MG | HA 379 | 1 |
| **H** | | short | | HA 291 | 3 |
| | | medium | | RHA 801 | 5 |
| **G** | | tall | | H 52.9.1.1 | 7 |
| **29.** | **29.** | **Plant: branching (excluding environmental branching)** | M0-M2 | | |
| | | absent | VG | HA89 | 1 |
| **G** | | 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.** | **32.** | **Head: attitude** | M3 | | |
| (+) | | horizontal | VG | | 1 |
| **H** | | 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.** | **33.** | **Head: size** | M3 | | |
| **H** | | small | VG | RHA 273 | 3 |
| | | medium | | RHA 271 | 5 |
| | | large | | H 52.9.1.1 | 7 |
| **34.** | **34.** | **Head: shape of grain side** | M3 | | |
| (+) | | strongly concave | VG | | 1 |
| **H** | | 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.** | **35.** | **Seed: size** | M4 | | |
| **H** | | small | VG | RHA 801 | 3 |
| | | medium | | HA 89 | 5 |
| | | large | | HA 292 | 7 |
| | | very large | | HA 316 | 9 |
| **36.** | **36.** | **Seed: shape** | M4 | | |
| (+) | | elongated | VG | HA 60 | 1 |
| **H** | | 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.** | **38.** | **Seed: main colour** | M4 | | |
| (+) | | white | VG | | 1 |
| **H** | | whitish grey | | | 2 |
| | | grey | | TRISUN 860 | 3 |
| | | light brown | | | 4 |
| | | medium brown | | RHA 273 | 5 |
| | | dark brown | | | 6 |
| | | black | | HA 89 | 7 |
| **G** | | purple | | | 8 |
| **39.** | **39.** | **Seed: stripes on margin** | M4 | | |
| (+) | | none or very weakly expressed | VG | RHA 273 | 1 |
| **H** | | weakly expressed | | H 52.9.1.1 | 2 |
| **G** | | strongly expressed | | HA 89 | 3 |

| CPVO N° | UPOV N° | Characteristics | Stage[1] | Examples[2] | Note |
|---|---|---|---|---|---|
| **40.** | **40.** | **Seed: stripes between 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

PAGE

Explanations and methods, .......... 20

Growth stage of sunflower .......... 26

Electrophoresis .......... 29

## ANNEX II

Technical Questionnaire

# ANNEX I

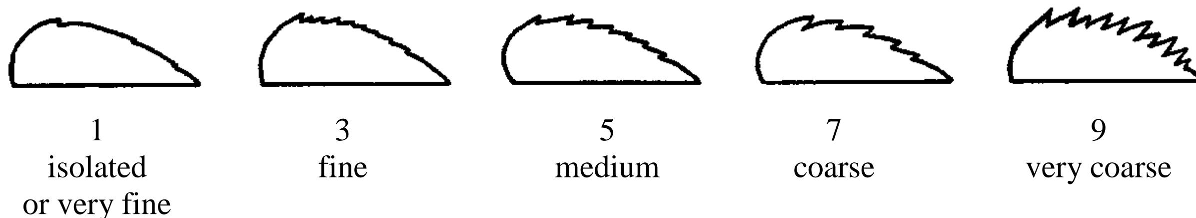
## EXPLANATIONS AND METHODS

<u>Method of observation of characteristics</u>

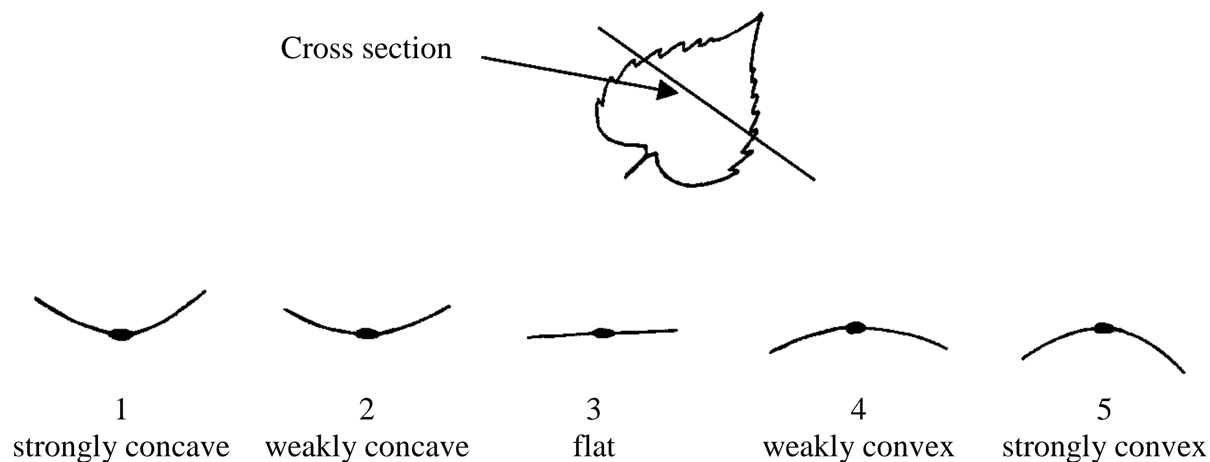
Letters indicate the relevant method for the assessment of distinctness

| | |
|---|---|
| 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 |

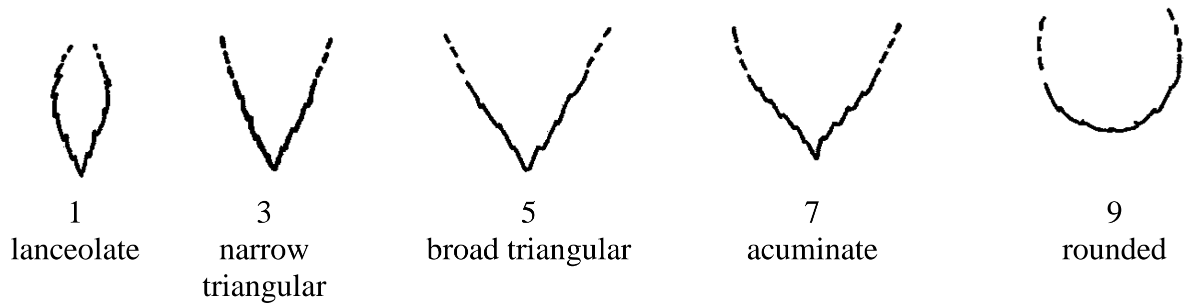
<u>Ad. 6: Leaf: serration</u>



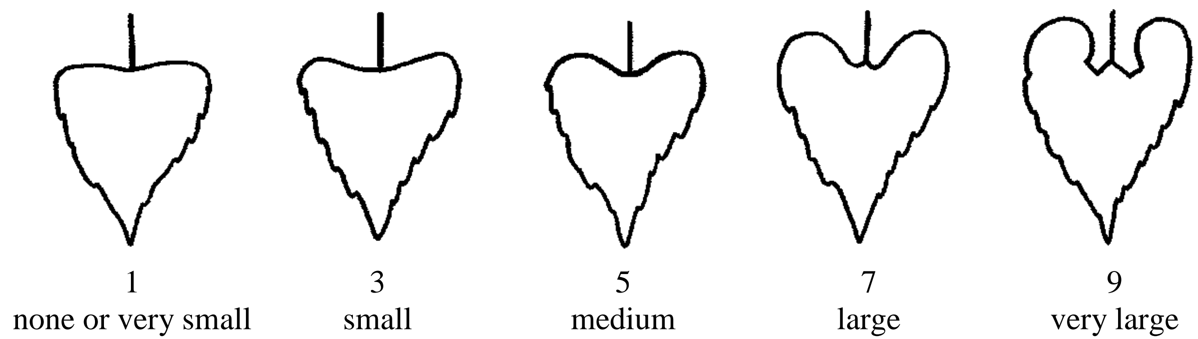

<u>Ad. 7: Leaf: shape of cross section</u>
(through the middle of the leaf)



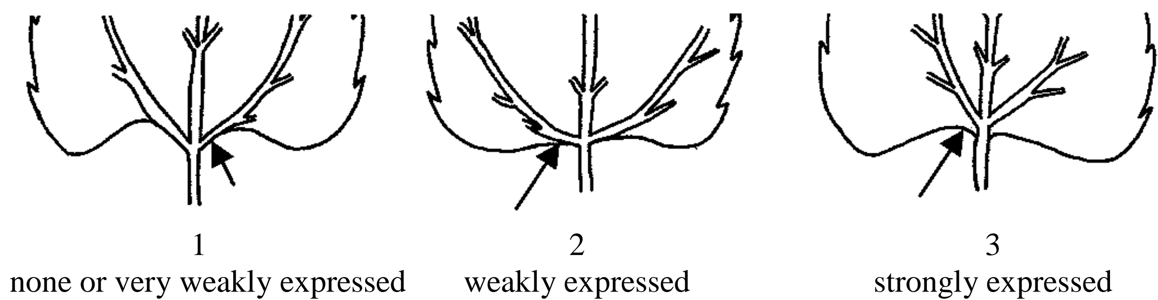
Ad. 8: Leaf: shape of distal part



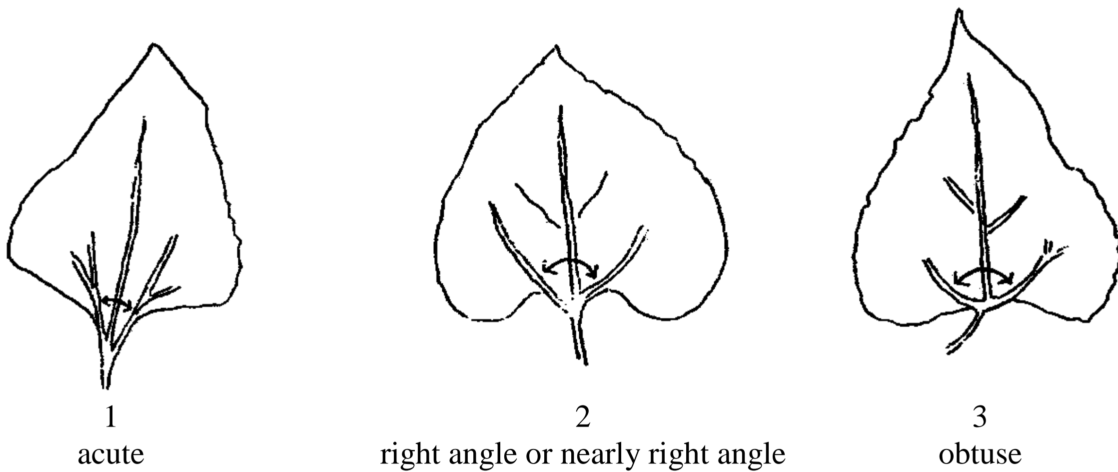

Ad. 9: Leaf: auricles




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

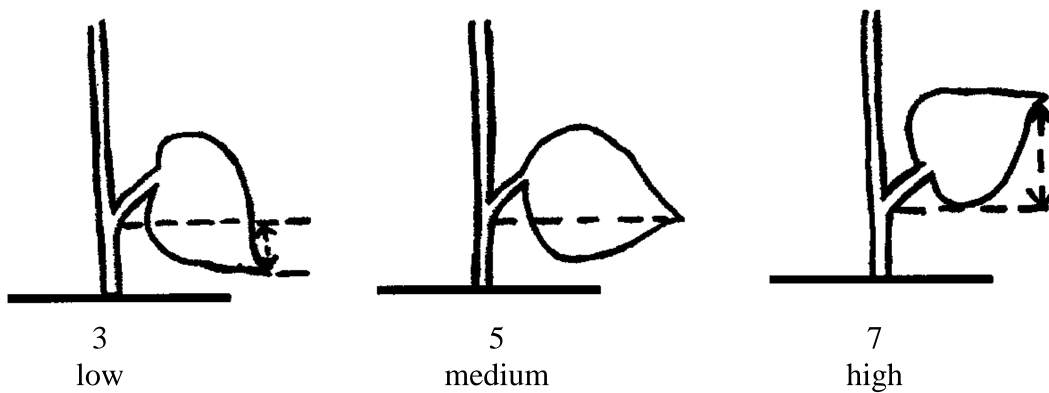


Ad. 11: Leaf: angle of lowest lateral veins



| 1 | 2 | 3 |
|---|---|---|
| acute | right angle or nearly right angle | obtuse |

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

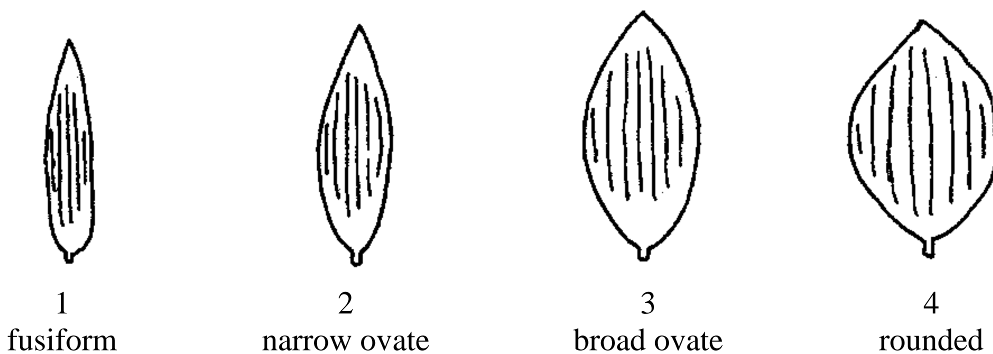


| 3 | 5 | 7 |
|---|---|---|
| low | medium | high |

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.

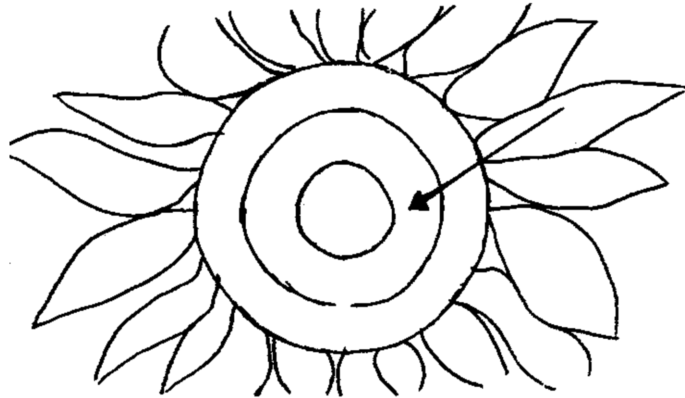
Ad. 16: Ray floret: shape



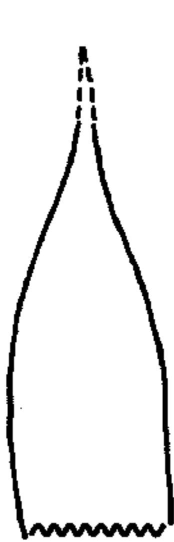
| 1 | 2 | 3 | 4 |
|---|---|---|---|
| fusiform | narrow ovate | broad ovate | rounded |

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

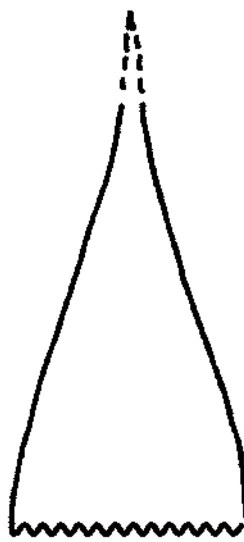
The anthocyanin coloration should be recorded on the stigma from the central third of the head just after the pollen appears at the top of the anthers.



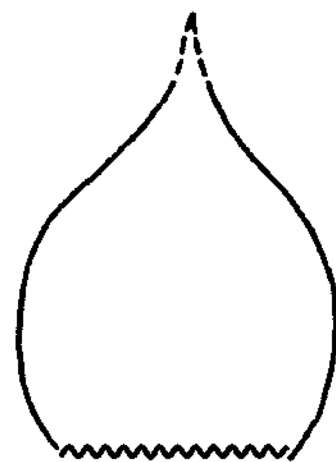
Ad. 24: Bract: shape



1
clearly elongated

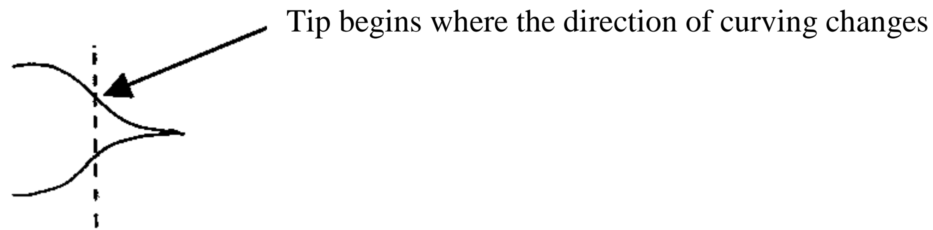


2
neither clearly elongated nor clearly rounded



3
clearly rounded

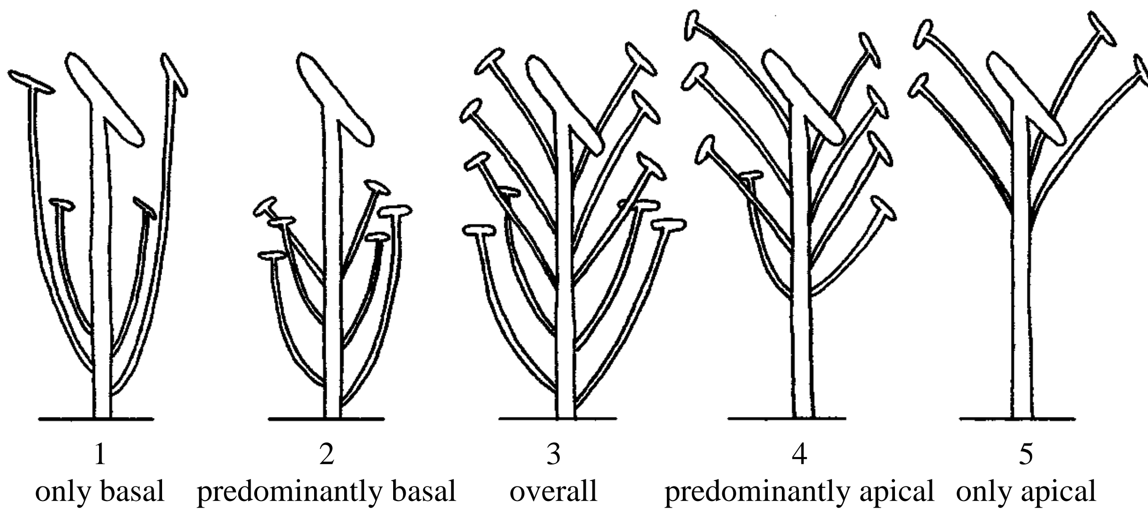
Ad. 25: Bract: length of the tip



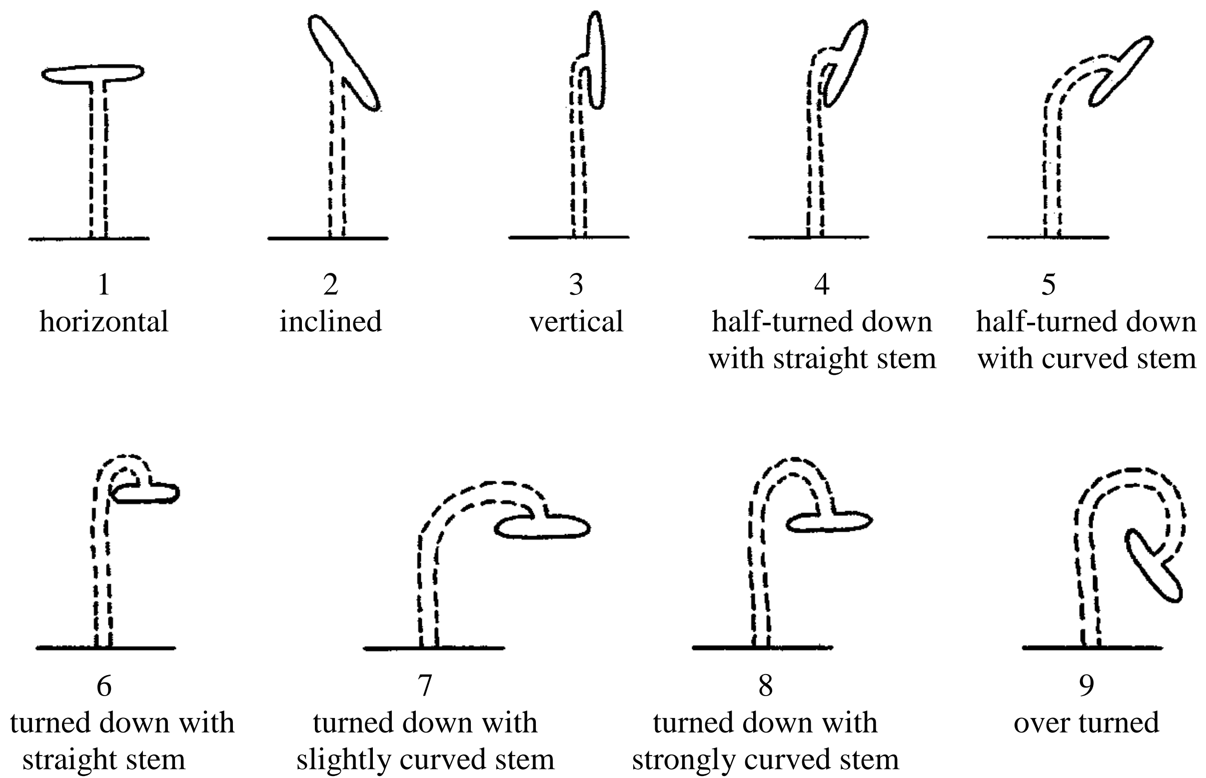

Ad. 28: Plant: natural height

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

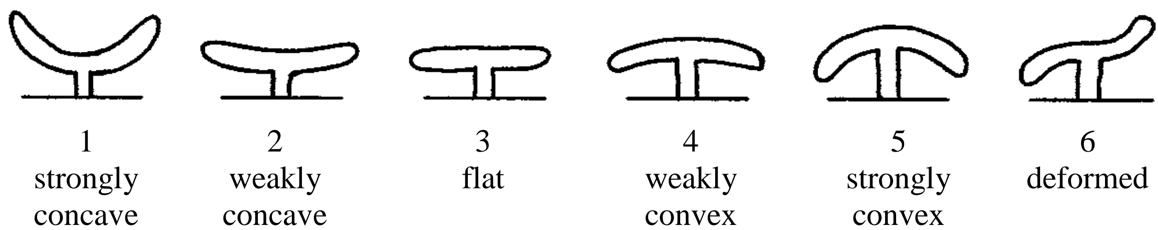
Ad. 30: Plant type of branching
(excluding environmental branching)



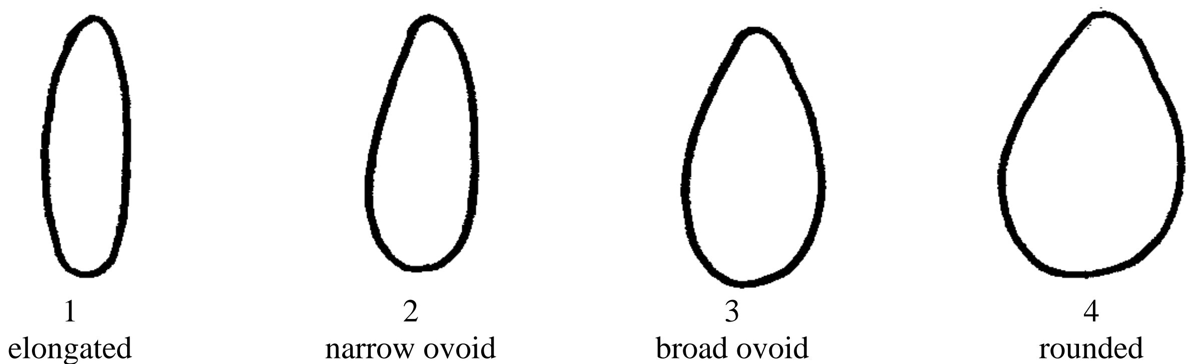
Ad. 32: Head: attitude




Ad. 34: Head: shape of grain side



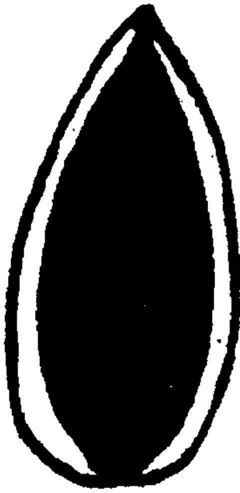

Ad. 36: Seed: shape




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 SUNFLOWER[1]

(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 stigmates 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

TABLE OF CONTENTS PAGE

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 $Na_2$)
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 ($MgCl_2$, $6H_2O$)
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 $MgCl_2$ 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 $Na_2$ salt
60 mg 6-Phosphogluconic acid $Na_3$ salt
10 mg NADP
1 ml MTT solution (4.3.1.3.)
1.5 ml PMS solution (4.3.1.5.)
1 ml $MgCl_2$ 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 $1H_2O$, $Na_2$ salt
150 mg EDTA, $Na_2$
10 mg NADP
1.5 ml MTT solution (4.3.1.3)
1.ml PMS solution (4.3.1.5)
4 ml $MgCl_2$ 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 µl 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/cm$^2$ . 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.

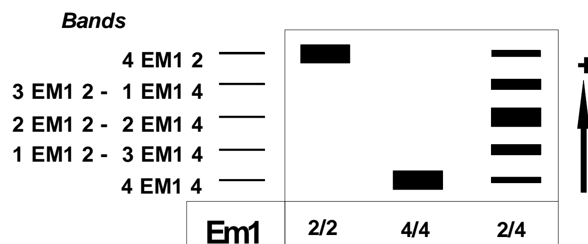
## 6. **Recognition of the alleles encoding isoenzymes**

### 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<br>4 |

#### 6.1.2 Schematization of the zymogrammes

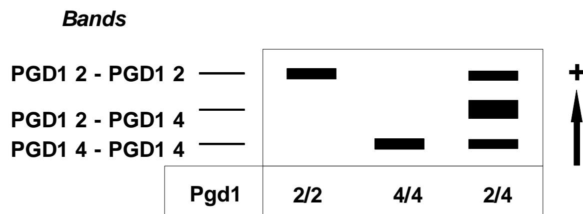



### 6.2 Recognition of the alleles encoding PGD

#### 6.2.1 Genetic interpretation of the zymogrammes

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

#### 6.2.2 Schematization of the zymogrammes



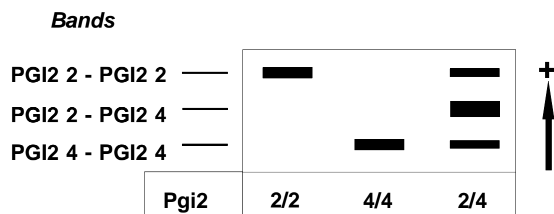

Two migration zones can be observed; only the slowest migrating bands are polymorphic.

### 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<br>4 |

#### 6.3.2 Schematization of the zymogrammes



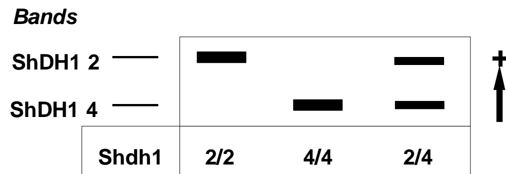

There are two migration zones; only the slowest migrating bands are scored.

### 6.4 Recognition of the alleles encoding ShDH

#### 6.4.1 Genetic interpretation of the zymogrammes

| Enzyme | Quaternary structure | Locus | Alleles |
|---|---|---|---|
| Shikimate dehydrogenase (S**h**DH) | Monomeric | Shdh1 | 2<br>4 |

6.4.2 Schematization of the zymogrammes

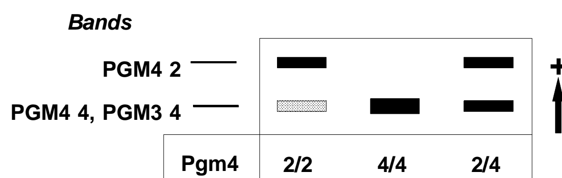



6.5 Recognition of the alleles encoding PGM

6.5.1 Genetic interpretation of the zymogrammes

| Enzyme | Quaternary structure | Locus | Alleles |
|---|---|---|---|
| Phosphoglucomutase | Monomeric | Pgm4 | 2<br>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

The Technical Questionnaire is available on the CPVO website under the following reference: CPVO-TQ/081/1