



TG/81/6

INTERNATIONAL UNION  
FOR THE PROTECTION  
OF NEW VARIETIES OF  
PLANTS

UNION INTERNATIONALE  
POUR LA PROTECTION  
DES OBTENTIONS  
VÉGÉTALES

INTERNATIONALER  
VERBAND ZUM SCHUTZ  
VON PFLANZEN-  
ZÜCHTUNGEN

UNIÓN INTERNACIONAL  
PARA LA PROTECCIÓN  
DE LAS OBTENCIONES  
VEGETALES

**GUIDELINES**  
**FOR THE CONDUCT OF TESTS**  
**FOR DISTINCTNESS, UNIFORMITY AND STABILITY**

**SUNFLOWER**

*(Helianthus annuus L.)*

**GENEVA**  
**2000**

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## **GUIDELINES**

### **FOR THE CONDUCT OF TESTS**

#### **FOR DISTINCTNESS, UNIFORMITY AND STABILITY**

### **SUNFLOWER**

*(Helianthus annuus L.)*

These Guidelines should be read in conjunction with document TG/1/2, which contains explanatory notes on the general principles on which the Guidelines have been established.

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ANNEX

## I. Subject of these Guidelines

These Test Guidelines apply to all varieties of *Helianthus annuus* L., comprising inbred lines, hybrids and open-pollinated varieties.

## II. Material Required

1. The competent authorities decide when, where and in what quantity and quality the plant material required for testing the variety is to be delivered. Applicants submitting material from a State other than that in which the testing takes place must make sure that all customs formalities are complied with. The minimum quantity of seed to be supplied by the applicant in one or several samples should be:

5000 grains for inbred lines  
1 kg for hybrid and open-pollinated varieties

In the case of hybrid varieties, an additional 5000 grains of each component (e.g. for a single hybrid, the female line (male sterile line) and the male line) should be submitted. In the case of male sterile lines, an additional 5000 grains of the maintainer line should be submitted. The seed should at least meet the minimum requirements for germination capacity, moisture content and purity for marketing certified seed in the country in which the application is made. The germination capacity should be as high as possible.

2. The plant material must not have undergone any treatment unless the competent authorities allow or request such treatment. If it has been treated, full details of the treatment must be given.

## III. Conduct of Tests

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

2. The tests should normally be conducted at one place. If any important characteristics of the variety cannot be seen at that place, the variety may be tested at an additional place.

3. The field tests should be carried out under conditions ensuring normal growth. The size of the plots should be such that plants or parts of plants may be removed for measurement and counting without prejudice to the observations which must be made up to the end of the growing period. Each test should include at least 40 plants which should be divided between two or more replicates. Separate plots for observation and for measuring can only be used if they have been subject to similar environmental conditions.

4. Additional tests for special purposes may be established, e.g. head-row tests in the event of the competent authority accepting results carried out by the applicant before the date of application.

5. In the event of the formula of hybrids being checked with the help of electrophoresis of enzymes, a test should be carried out on at least 10 seedlings from each inbred line. At least

4 seedlings should be analyzed for single hybrids and at least 10 seedlings for three-way hybrids. In case of doubt, additional seedlings should be analyzed.

#### IV. Methods and Observations

1. The characteristics described in Chapter VII should be used for the testing of distinctness of inbred lines, hybrids and open-pollinated varieties.
2. However, to assess distinctness of hybrids, a prescreening system on the basis of the parental lines and the formula may be established according to the recommendations:
  - (a) description of the parental lines according to the Test Guidelines;
  - (b) verification of the originality of those parental lines in comparison with the reference collection based on the characteristics in Chapter VII in order to screen the closest inbred lines;
  - (c) 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;
  - (d) assessment of the distinctness at the hybrid level of varieties with a close formula.
3. All observations for the assessment of distinctness and uniformity, including electrophoretic characteristics if any, should be made on at least 40 plants or parts taken from each of the 40 plants in each testing place and growing period.
4. All observations should be made on the main stem.
5. 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. 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 those countries which foresee difficulties with too large a change to adjust their system to the newly adopted rules, a possible interim period of 2 years from the adoption of the Test Guidelines would be acceptable before they change to the new rules. 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

	<b>Off-types</b>	<b>Number of plants observed</b>	<b>Number of off-types allowed</b>
<u>Male sterile inbred line<sup>1</sup></u> (population standard: 2%, acceptance probability: 95%)	<b>Out-crossed plants and isogenic fertile plants</b>	19 - 41	2
		42 - 69	3
		70 - 99	4
		100 - 131	5
	<b>Other off-types</b>	19 - 41	2
		42 - 69	3
		70 - 99	4
		100 - 131	5
<u>Male fertile inbred line</u> (population standard: 2%, acceptance probability: 95%)	<b>All types of off-types</b>	19 - 41	2
		42 - 69	3
		70 - 99	4
		100 - 131	5
<u>Single-cross hybrid</u> (population standard: 5%, acceptance probability: 95%)	<b>All types of off-types</b>	17 - 28	3
		29 - 40	4
		41 - 53	5
		54 - 67	6
		68 - 81	7
		82 - 95	8
		96 - 110	9
		111- 125	10

7. If enzyme electrophoresis is used for testing distinctness, the same population standard and the same acceptance probability as for other characteristics should be applied. 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.

<sup>1</sup> 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.

## V. Grouping of Varieties

1. The collection of varieties to be grown should 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. Their various states of expression should be fairly evenly distributed throughout the collection.
2. In the first instance, the varieties should be separated into inbred lines and other varieties. Within each group it is recommended that the competent authorities use the following characteristics for grouping varieties:
  - (a) Leaf: green color (characteristic 4)
  - (b) Leaf: blistering (characteristic 5)
  - (c) Time of flowering (characteristic 14)
  - (d) Plant: natural height (characteristic 28)
  - (e) Plant: branching (excluding environmental branching) (characteristic 29)
  - (f) Seed: stripes on margin (characteristic 39)
  - (g) Seed: stripes between margins (characteristic 40)

## VI. Characteristics and Symbols

1. To assess distinctness, uniformity and stability, the characteristics and their states as given in the Table of Characteristics should be used.
2. Notes (numbers), for the purposes of electronic data processing, are given opposite the states of expression for each characteristic.
3. Legend
  - (\*) Characteristics that should be used on all varieties in every growing period over which examinations are made and always be included in the variety descriptions, except when the state of expression of a preceding characteristic or regional environmental conditions render this impossible.
  - (+) See Explanations on the Table of Characteristics in Chapter VIII.
  - (1) The optimum stage of development for the assessment of each characteristic is indicated by a number in the second column. The stages of development denoted by each number are described at the end of Chapter VIII.



VII. Table of Characteristics/Tableau des caractères/Merkmalestabelle/Tabla de caracteres

Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota	
1.	A2	<b>Hypocotyl: anthocyanin coloration</b>	<b>Hypocotyle: pigmentation anthocyanique</b>	<b>Hypokotyl: Anthocyanfärbung</b>	<b>Hipocotilo: pigmentación antociánica</b>		
		absent	absente	fehlend	ausente	HA 850	1
		present	présente	vorhanden	presente	RHA 271	9
2.	A2	<b>Hypocotyl: intensity of anthocyanin coloration</b>	<b>Hypocotyle: intensité de la pigmentation anthocyanique</b>	<b>Hypokotyl: Intensität der Anthocyanfärbung</b>	<b>Hipocotilo: intensidad de la pigmentación antociánica</b>		
		weak	faible	gering	débil	H 52.6.3	3
		medium	moyenne	mittel	media	HA 290	5
		strong	forte	stark	fuerte	RHA 271	7
3. (* )	E4	<b>Leaf: size</b>	<b>Feuille: taille</b>	<b>Blatt: Größe</b>	<b>Hoja: tamaño</b>		
		small	petite	klein	pequeña	HA 124	3
		medium	moyenne	mittel	media	HA 821	5
		large	grande	groß	grande	DK 3790	7
4. (* )	E4	<b>Leaf: green color</b>	<b>Feuille: couleur verte</b>	<b>Blatt: Grünfärbung</b>	<b>Hoja: color verde</b>		
		light	claire	hell	claro	H 52.9.1.1	3
		medium	moyenne	mittel	medio	HA 821	5
		dark	foncée	dunkel	oscuro	HA 303	7
5. (* )	E4	<b>Leaf: blistering</b>	<b>Feuille: gaufrure</b>	<b>Blatt: Blasigkeit</b>	<b>Hoja: abullonado</b>		
		absent or very weak	absente ou très faible	fehlend oder sehr gering	ausente o muy débil		1
		weak	faible	gering	débil	HA 342, RHA 273	3
		medium	moyenne	mittel	medio	HA 291	5
		strong	forte	stark	fuerte	HA 303, RHA 361	7

Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>6.</b> (*) (+)	<b>E4</b>	<b>Leaf: serration</b>	<b>Feuille: denture</b>	<b>Blatt: Zähnung</b>	<b>Hoja: dentado</b>	
		isolated or very fine	isolée ou très fine	vereinzelt oder sehr fein	aislado o muy fino	HA 393 1
		fine	fine	fein	fino	HA 124 3
		medium	moyenne	mittel	medio	RHA 271 5
		coarse	grossière	grob	grosero	RHA 299 7
		very coarse	très grossière	sehr grob	muy grosero	9
<b>7.</b> (+)	<b>E4</b>	<b>Leaf: shape of cross section</b>	<b>Feuille: forme de la section transversale</b>	<b>Blatt: Form des Querschnitts</b>	<b>Hoja: forma de la sección transversal</b>	
		strongly concave	fortement concave	stark konkav	fuertemente cóncava	1
		weakly concave	faiblement concave	schwach konkav	débilmente cóncava	RHA 273 2
		flat	droite	gerade	plana	H 55.9.2.1.1 3
		weakly convex	faiblement convexe	schwach konvex	débilmente convexa	HA 303 4
		strongly convex	fortement convexe	stark konvex	fuertemente convexa	5
<b>8.</b> (+)	<b>E4</b>	<b>Leaf: shape of distal part</b>	<b>Feuille: forme de la partie distale</b>	<b>Blatt: Form des distalen Teiles</b>	<b>Hoja: forma de la parte distal</b>	
		lanceolate	lancéolée	lanzettlich	lanceolada	1
		lanceolate to narrow triangular	lancéolée à triangulaire étroite	lanzettlich bis schmal dreieckig	lanceolada a triangular estrecha	2
		narrow triangular	triangulaire étroite	schmal dreieckig	triangular estrecha	RHA 855 3
		narrow triangular to broad triangular	triangulaire étroite à triangulaire large	schmal dreieckig bis breit dreieckig	triangular estrecha a triangular ancha	4
		broad triangular	triangulaire large	breit dreieckig	triangular ancha	HA 821 5
		broad triangular to acuminate	triangulaire large à acuminée	breit dreieckig bis zugespitzt	triangular ancha a acuminada	6
		broad triangular to rounded	triangulaire large à arrondie	breit dreieckig bis rundlich	triangular ancha a redondeada	HA 303 7
		acuminate	acuminée	zugespitzt	acuminada	HA 124 8
		rounded	arrondie	rundlich	redondeada	HA 234 9

	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>9.</b> (* (+)	<b>E4</b>	<b>Leaf: auricles</b>	<b>Feuille: oreillettes</b>	<b>Blatt: Größe der Ohren</b>	<b>Hoja: aurículas</b>		
		none or very small	absentes ou très petites	fehlend oder sehr klein	ausentes o muy pequeñas	HA 290	1
		small	petites	klein	pequeñas		3
		medium	moyennes	mittel	medias	HA 852	5
		large	grandes	groß	grandes	HA 89	7
		very large	très grandes	sehr groß	muy grandes	HA 303	9
<b>10.</b> (+)	<b>E4</b>	<b>Leaf: wings</b>	<b>Feuille: ailes</b>	<b>Blatt: Flügel</b>	<b>Hoja: alas</b>		
		none or very weakly expressed	absentes ou très faiblement marquées	keine oder sehr schwach ausgeprägt	ausentes o muy débilmente definidas	HA 89	1
		weakly expressed	faiblement marquées	schwach ausgeprägt	débilmente definidas		2
		strongly expressed	fortement marquées	stark ausgeprägt	fuertemente definidas	RHA 274, RHA 348	3
<b>11.</b> (* (+)	<b>E4</b>	<b>Leaf: angle of lowest lateral veins</b>	<b>Feuille: angle des nervures latérales plus basses</b>	<b>Blatt: Winkel der untersten Seitennerven</b>	<b>Hoja: ángulo de los nervios laterales inferiores</b>		
		acute	aigu	spitz	agudo	HA 290, RHA 295	1
		right angle or nearly right angle	droit ou presque droit	rechtwinklig oder fast rechtwinklig	ángulo recto o casi ángulo recto	HA 89	2
		obtuse	obtus	stumpf	obtuso	HA 303	3

	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>12.</b>	<b>E4</b>	<b>Leaf: height of the tip of the blade compared to insertion of petiole (at 2/3 height of plants)</b>	<b>Feuille: hauteur de l'extrémité du limbe par rapport à l'insertion du pétiole (aux 2/3 de la tige à partir du sol)</b>	<b>Blatt: Höhe der Blattspitze im Verhältnis zur Ansatzstelle des Stieles (in 2/3 der Pflanzhöhe)</b>	<b>Hoja: altura de la punta del limbo en relación con la inserción del pecíolo (a 2/3 de la altura de la planta)</b>		
(+)		low	basse	niedrig	baja	RHA 275	3
		medium	moyenne	mittel	media	RHA 274	5
		high	haute	hoch	alta	RHA 400	7
<b>13.</b>	<b>F1</b>	<b>Stem: hairiness at the top (last 5 cm)</b>	<b>Tige: pilosité au sommet (5 derniers cm)</b>	<b>Stengel: Behaarung an der Spitze (obere 5 cm)</b>	<b>Tallo: vellosoidad en el extremo (últimos 5 cm)</b>		
		absent or very weak	nulle ou très faible	fehlend oder sehr gering	ausente o muy débil		1
		weak	faible	gering	débil	RHA 271	3
		medium	moyenne	mittel	media	RHA 273	5
		strong	forte	stark	fuerte	HA 303	7
		very strong	très forte	sehr stark	muy fuerte		9
<b>14.</b>	<b>(*)</b> <b>(+)</b>	<b>Time of flowering</b>	<b>Époque de floraison</b>	<b>Zeitpunkt der Blüte</b>	<b>Época de la floración</b>		
		very early	très précoce	sehr früh	muy temprana	HA 302, RHA 381	1
		early	précoce	früh	temprana	RHA 273	3
		medium	moyenne	mittel	media	RHA 274	5
		late	tardive	spät	tardía	RHA 271	7
		very late	très tardive	sehr spät	muy tardía	RHA 361	9
<b>15.</b>	<b>F3.2</b>	<b>Ray florets: density</b>	<b>Fleurs ligulées: densité</b>	<b>Zungenblüten: Dichte</b>	<b>Flores liguladas: densidad</b>		
		sparse	faible	locker	laxas	HA 385	3
		medium	moyenne	mittel	medias	HA 89	5
		dense	forte	dicht	densas		7

	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>16.</b> (+)	<b>F3.2</b>	<b>Ray floret: shape</b>	<b>Fleur ligulée: forme</b>	<b>Zungenblüte: Form</b>	<b>Flor ligulada: forma</b>		
		fusiform	allongée	spindelförmig	fusiforme	H 52.9.1.1	1
		narrow ovate	ovoïde étroite	schmal eiförmig	oval estrecha	RHA 274	2
		broad ovate	ovoïde large	breit eiförmig	oval ancha	HA 821	3
		rounded	arrondie	rundlich	redondeada		4
<b>17.</b>	<b>F3.2</b>	<b>Ray floret: dis- position</b>	<b>Fleur ligulée: dis- position</b>	<b>Zungenblüte: An- ordnung</b>	<b>Flor ligulada: dis- posición</b>		
		flat	plane	eben	plana	HA 89	1
		longitudinal recurved	recourbée longitu- dinalement	längsseits nach außen gebogen	recurvada longitu- dinalmente	HA 850	2
		undulated	ondulée	gewellt	ondulada	HA 234	3
		strongly recurved to back of head	fortement recour- bée vers le dos du capitule	stark gebogen zur Korbrückseite	fuertemente recur- vada hacia la parte posterior del capítulo	CM 592	4
<b>18.</b>	<b>F3.2</b>	<b>Ray floret: length</b>	<b>Fleur ligulée: lon- gueur</b>	<b>Zungenblüte: Länge</b>	<b>Flor ligulada: longitud</b>		
		short	courte	kurz	corta	RHA 361	3
		medium	moyenne	mittel	media	HA 89	5
		long	longue	lang	larga	H 52.6.3	7
<b>19.</b> (*)	<b>F3.2</b>	<b>Ray floret: color</b>	<b>Fleur ligulée: couleur</b>	<b>Zungenblüte: Farbe</b>	<b>Flor ligulada: color</b>		
		yellowish white	blanc jaunâtre	gelblichweiß	blanco amarillento		1
		light yellow	jaune pâle	hellgelb	amarillo pálido		2
		medium yellow	jaune moyen	mittelgelb	amarillo medio	HA 89	3
		orange yellow	jaune orange	orangegelb	amarillo naranja	RHA 361	4
		orange	orange	orange	naranja	CM 587, RHA 295	5
		purple	pourpre	purpur	púrpura		6
		reddish brown	brun rougeâtre	rotbraun	marrón rojizo		7
		multicolored	multicolore	mehrfarbig	multicolor	8	

	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>20.</b>	<b>F3.2</b>	<b>Disk flower: color</b>	<b>Fleuron: couleur</b>	<b>Röhrenblüte: Farbe</b>	<b>Flósculo (flor del disco): color</b>		
		yellow	jaune	gelb	amarillo		1
		orange	orange	orange	naranja	HA 89	2
		purple	pourpre	purpur	púrpura		3
<b>21.</b> (+)	<b>F3.2</b>	<b>Disk flower: anthocyanin coloration of stigma</b>	<b>Fleuron: pigmentation anthocyanique des stigmates</b>	<b>Röhrenblüte: Anthocyanfärbung der Narbe</b>	<b>Flósculo (flor del disco): pigmentación antocianica del estigma</b>		
		absent	absente	fehlend	ausente	HA 89	1
		present	présente	vorhanden	presente	H 55.9.2.1.1, HA 348	9
<b>22.</b> (+)	<b>F3.2</b>	<b>Disk flower: intensity of anthocyanin coloration of stigma</b>	<b>Fleuron: intensité de la pigmentation anthocyanique des stigmates</b>	<b>Röhrenblüte: Intensität der Anthocyanfärbung der Narbe</b>	<b>Flósculo (flor del disco): intensidad de la pigmentación antocianica del estigma</b>		
		weak	faible	gering	débil	HA 290, HA 394	3
		medium	moyenne	mittel	media	HA 60, HA 291	5
		strong	forte	stark	fuerte	RHA 348	7
<b>23.</b>	<b>F3.2</b>	<b>Disk flower: production of pollen</b>	<b>Fleuron: production de pollen</b>	<b>Röhrenblüte: Pollenproduktion</b>	<b>Flósculo (flor del disco): producción de polen</b>		
		absent	absente	fehlend	ausente		1
		present	présente	vorhanden	presente		9
<b>24.</b> (+)	<b>F3.2</b>	<b>Bract: shape</b>	<b>Bractée: forme</b>	<b>Hüllblatt: Form</b>	<b>Bráctea: forma</b>		
		clearly elongated	nettement allongée	deutlich länglich	claramente elongada	HA 379	1
		neither clearly elongated nor clearly rounded	ni nettement allongée ni nettement arrondie	weder deutlich länglich noch deutlich rundlich	ni claramente elongada ni claramente redondeada	HA 292	2
		clearly rounded	nettement arrondie	deutlich rundlich	claramente redondeada	RHA 801	3

	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>25.</b>	<b>F3.2</b>	<b>Bract: length of tip</b>	<b>Bractée: longueur du sommet</b>	<b>Hüllblatt: Länge der Spitze</b>	<b>Bráctea: longitud del acumen</b>		
(+)		short	court	kurz	corta	RHA 273, RHA 361	3
		medium	moyen	mittel	media	HA 302	5
		long	long	lang	larga	HA 292, HA 55.9.2.1.1	7
		very long	très long	sehr lang	muy larga	H 52.6.3	9
<b>26.</b>	<b>F3.2</b>	<b>Bract: green color of outer side</b>	<b>Bractée: couleur verte de la face externe</b>	<b>Hüllblatt: Grünfärbung der Außenseite</b>	<b>Bráctea: color verde de la cara externa</b>		
		light	claire	hell	claro	H 52.9.1.1	3
		medium	moyenne	mittel	medio	HA 850	5
		dark	foncée	dunkel	oscuro	HA 303	7
<b>27.</b>	<b>M0</b>	<b>Bract: attitude in relation to head</b>	<b>Bractée: port par rapport au capitule</b>	<b>Hüllblatt: Haltung zum Korb</b>	<b>Bráctea: porte en relación con el capítulo</b>		
		not embracing or very slightly embracing	non enveloppante ou très faiblement enveloppante	nicht umfassend oder sehr gering umfassend	no envolvente o muy ligeramente envolvente	H 52.9.1.1	1
		slightly embracing	faiblement enveloppante	gering umfassend	ligeramente envolvente	HA 337, HA 343	2
		strongly embracing	fortement enveloppante	stark umfassend	fuertemente envolvente	RHA 234	3
<b>28.</b>	<b>M0</b>	<b>Plant: natural height</b>	<b>Plante: hauteur</b>	<b>Pflanze: Natürliche Höhe</b>	<b>Planta: altura</b>		
(*)		very short	très courte	sehr kurz	muy baja	HA 379	1
(+)		short	courte	kurz	baja	HA 291	3
		medium	moyenne	mittel	media	RHA 801	5
		tall	haute	lang	alta	H 52.9.1.1	7

	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>29.</b> (* (*)	<b>M0 - M2</b>	<b>Plant: branching (excluding environmental branching)</b>	<b>Plante: ramification (à l'exclusion de la ramification due à l'environnement)</b>	<b>Pflanze: Verzweigung (ohne umweltbedingte Verzweigung)</b>	<b>Planta: ramificación (excluyendo la causada por el medio ambiente)</b>		
		absent	absente	fehlend	ausente	HA89	1
		present	présente	vorhanden	presente	RHA 271	9
<b>30.</b> (* (+)	<b>M0 - M2</b>	<b>Plant: type of branching (as for 29)</b>	<b>Plante: type de ramification (comme pour 29)</b>	<b>Pflanze: Art der Verzweigung (wie unter 29)</b>	<b>Planta: tipo de ramificación (como para 29)</b>		
		only basal	uniquement basale	nur basal	sólo basal	RHA 295	1
		predominantly basal	prédominance basale	überwiegend basal	predominantemente basal		2
		overall	totale	vollständig	total	RHA 273	3
		predominantly apical	prédominance apicale	überwiegend apikal	predominantemente apical	RHA 271	4
only apical	uniquement apicale	nur apikal	solo apical	RHA 294	5		
<b>31.</b>	<b>M0 - M2</b>	<b>Plant: natural position of highest lateral head to the central head</b>	<b>Plante: position naturelle du capitule latéral le plus haut par rapport au capitule central</b>	<b>Pflanze: Natürliche Position des höchsten Seitenkorbes zum Hauptkorb</b>	<b>Planta: posición natural del capítulo lateral más alto, en relación con el capítulo central</b>		
		below	au-dessous	unterhalb	debajo	RHA 361	1
		same level	au même niveau	gleiche Höhe	al mismo nivel	RHA 857	2
		above	au-dessus	oberhalb	encima	RHA 274	3



Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielsorten Variedades ejemplo	Note/ Nota
<b>32.</b>	<b>M3</b>	<b>Head: attitude</b>	<b>Capitule: port</b>	<b>Korb: Haltung</b>	<b>Capítulo: porte</b>	
(*)		horizontal	horizontal	waagerecht	horizontal	1
(+)		inclined	incliné	geneigt	inclinado	2
		vertical	vertical	senkrecht	vertical	RHA 395 3
		half-turned down with straight stem	demi-renversé avec tige droite	halbüberhängend mit geradem Sten- gel	semiinvertido con el tallo recto	4
		half-turned down with curved stem	demi-renversé avec tige arquée	halbüberhängend mit gebogenem Stengel	semiinvertido con el tallo arqueado	5
		turned down with straight stem	renversé avec tige droite	überhängend mit geradem Stengel	invertido con el tallo recto	6
		turned down with slightly curved stem	renversé avec tige légèrement arquée	überhängend mit gering gebogenem Stengel	invertido con el tallo ligeramente arqueado	7
		turned down with strongly curved stem	renversé avec tige fortement arquée	überhängend mit stark gebogenem Stengel	invertido con el tallo fuertemente arqueado	8
		over turned	retourné	zurückgebogen	retorcido	9
<b>33.</b>	<b>M3</b>	<b>Head: size</b>	<b>Capitule: taille</b>	<b>Korb: Größe</b>	<b>Capítulo: tamaño</b>	
(*)		small	petit	klein	pequeño	RHA 273 3
		medium	moyen	mittel	medio	RHA 271 5
		large	grand	groß	grande	H 52.9.1.1 7

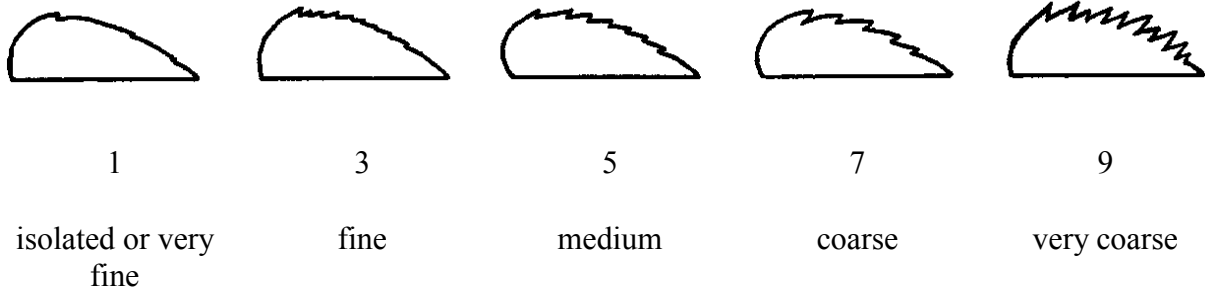
	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>34.</b> (*) (+)	<b>M3</b>	<b>Head: shape of grain side</b>	<b>Capitule: forme de la face portant les grains</b>	<b>Korb: Form der Kornseite</b>	<b>Capítulo: forma de la parte del grano</b>		
		strongly concave	fortement concave	stark konkav	fuertemente cóncavo		1
		weakly concave	faiblement concave	schwach konkav	débilmente cóncavo		2
		flat	plan	gerade	plano	RHA 273	3
		weakly convex	faiblement convexe	schwach konvex	débilmente convexo	HA 89	4
		strongly convex	fortement convexe	stark konvex	fuertemente convexo	CM 400	5
		deformed	difforme	verunstaltet	deformado	RHA 271	6
<b>35.</b>	<b>M4</b>	<b>Seed: size</b>	<b>Grain: taille</b>	<b>Korn: Größe</b>	<b>Semilla: tamaño</b>		
		small	petit	klein	pequeña	RHA 801	3
		medium	moyen	mittel	media	HA 89	5
		large	gros	groß	grande	HA 292	7
		very large	très gros	sehr groß	muy grande	HA 316	9
<b>36.</b> (*) (+)	<b>M4</b>	<b>Seed: shape</b>	<b>Grain: forme</b>	<b>Korn: Form</b>	<b>Semilla: forma</b>		
		elongated	allongé	länglich	elongada	HA 60	1
		narrow ovoid	ovoïde étroit	schmal eiförmig	ovoïde estrecha	RHA 271	2
		broad ovoid	ovoïde large	breit eiförmig	ovoïde ancha	HA 89	3
		rounded	arrondi	rundlich	redondeada	CM 447	4
<b>37.</b>	<b>M4</b>	<b>Seed: thickness relative to width</b>	<b>Grain: épaisseur par rapport à la largeur</b>	<b>Korn: Dicke im Verhältnis zur Breite</b>	<b>Semilla: grosor con relación a la anchura</b>		
		thin	mince	dünn	delgada	RHA 274	3
		medium	moyen	mittel	media	RHA 271	5
		thick	épais	dick	gruesa		7
	Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota

<b>38.</b> (* (+)	<b>M4</b>	<b>Seed: main color</b>	<b>Grain: couleur principale</b>	<b>Korn: Hauptfarbe</b>	<b>Semilla: color principal</b>		
		white	blanc	weiß	blanca		1
		whitish grey	gris-blanchâtre	weißlich grau	gris blancuzca		2
		grey	gris	grau	gris	TRISUN 860	3
		light brown	brun clair	hellbraun	marrón clara		4
		medium brown	brun moyen	mittelbraun	marrón medio	RHA 273	5
		dark brown	brun foncé	dunkelbraun	marrón oscuro		6
		black	noir	schwarz	negra	HA 89	7
	purple	pourpre	purpur	púrpura		8	
<b>39.</b> (* (+)	<b>M4</b>	<b>Seed: stripes <u>on</u> margin</b>	<b>Grain: stries <u>sur</u> les bords</b>	<b>Korn: Streifen <u>am</u> Rand</b>	<b>Semilla: estrías <u>en</u> el borde</b>		
		none or very weakly expressed	aucune ou très faiblement marquées	keine oder sehr schwach ausgeprägt	ninguna o muy débilmente definidas	RHA 273	1
		weakly expressed	faiblement marquées	schwach ausgeprägt	débilmente definidas	H 52.9.1.1	2
	strongly expressed	fortement marquées	stark ausgeprägt	fuertemente definidas	HA 89	3	
<b>40.</b> (* (+)	<b>M4</b>	<b>Seed: stripes <u>between</u> margins</b>	<b>Grain: stries <u>entre</u> les bords</b>	<b>Korn: Streifen <u>zwischen</u> den Rändern</b>	<b>Semilla: estrías <u>entre</u> los bordes</b>		
		none or very weakly expressed	aucune ou très faiblement marquées	keine oder sehr schwach ausgeprägt	ninguna o muy débilmente definidas	RHA 273	1
		weakly expressed	faiblement marquées	schwach ausgeprägt	débilmente definidas	RHA 293	2
	strongly expressed	fortement marquées	stark ausgeprägt	fuertemente definidas	HA 89	3	

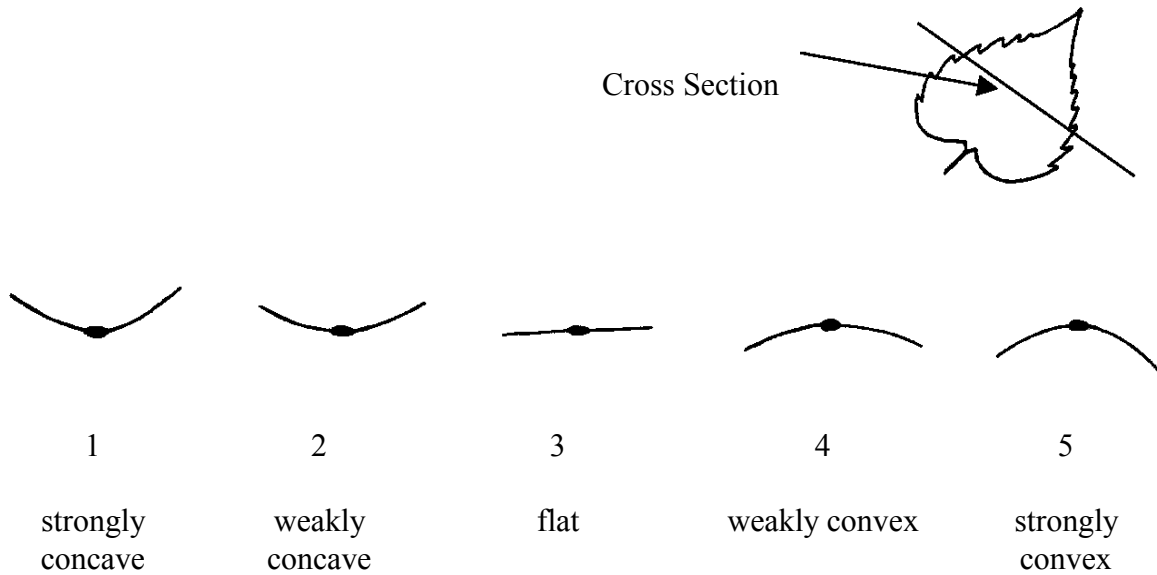
Stage <sup>1)</sup> Stade <sup>1)</sup> Stadium <sup>1)</sup> Estadio <sup>1)</sup>	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
<b>41. M4</b>	<b>Seed: color of stripes</b>	<b>Grain: couleur des stries</b>	<b>Korn: Farbe der Streifen</b>	<b>Semilla: color de las estrias</b>		
	white	blanches	weiß	blanco	RHA 295	1
	grey	grises	grau	gris	HA 89	2
	brown	brunes	braun	marrón	HA 292	3
	black	noires	schwarz	negro	Narval 30	4
<b>42. M4</b>	<b>Seed: spots on pericarp</b>	<b>Grain: tâches sur le péricarpe</b>	<b>Korn: Flecken auf der Samenschale</b>	<b>Semilla: manchas en el pericarpio</b>		
	absent	absentes	fehlend	ausentes		1
	present	présentes	vorhanden	presentes		9

VIII. Explanations on the Table of Characteristics

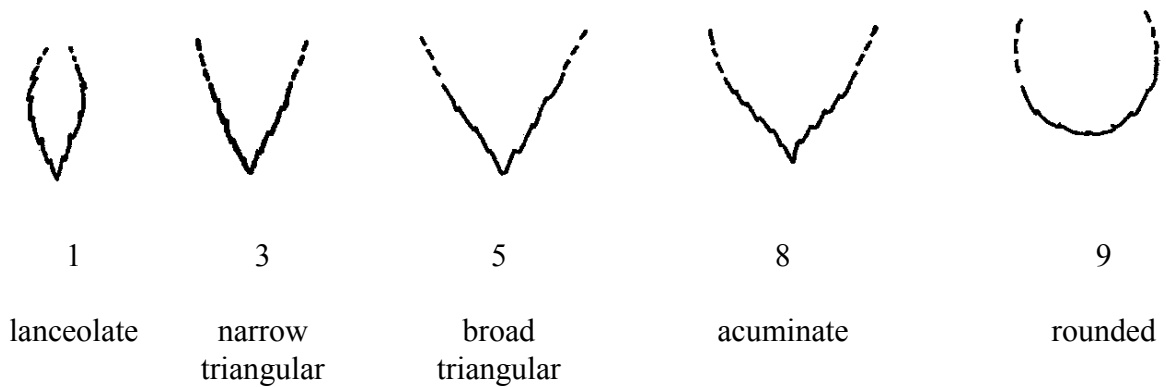
Ad. 6: Leaf: serration



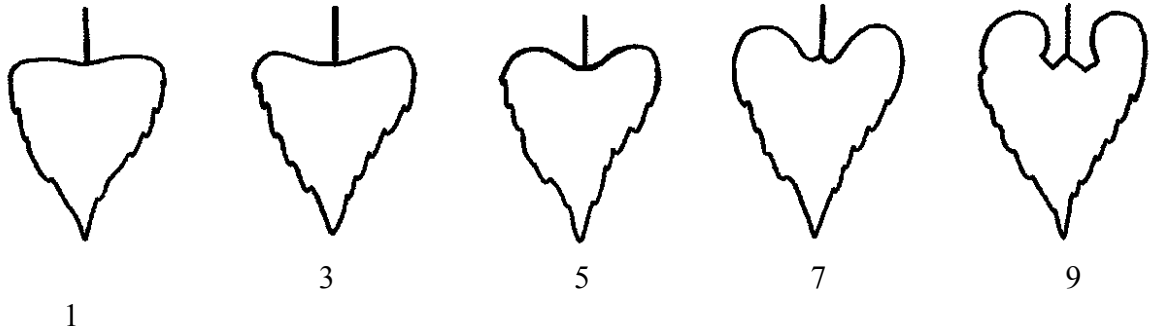
Ad. 7: Leaf: shape of cross section  
 (through the middle of the leaf)



Ad. 8: Leaf: shape of distal part



Ad. 9: Leaf: auricles



1  
none or very small

3  
small

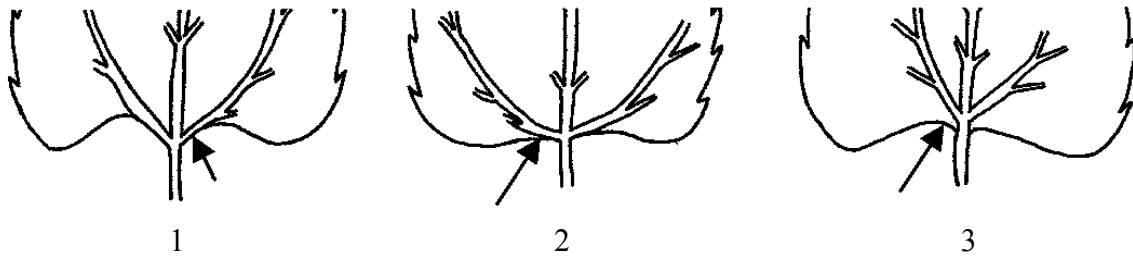
5  
medium

7  
large

9  
very large

Ad. 10: Leaf wings

(parenchyma at base of lateral veins)

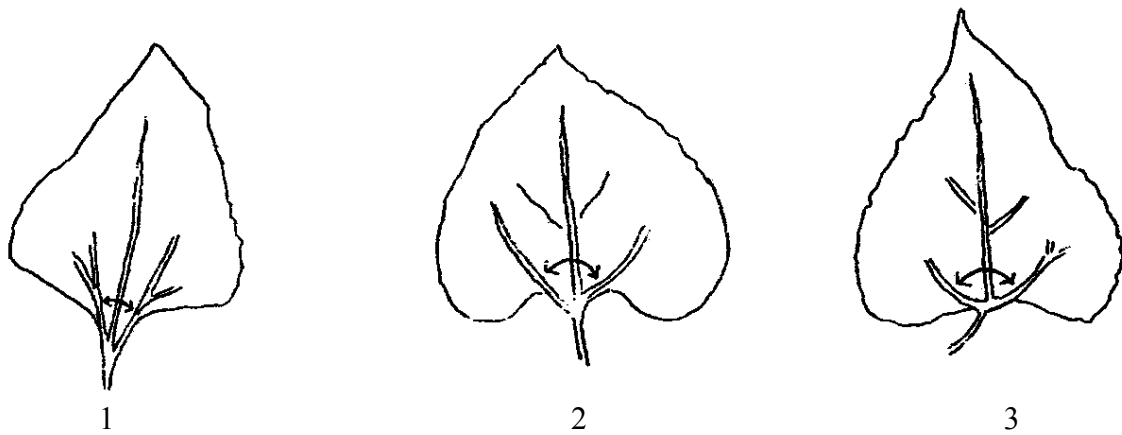


1  
none or very weakly expressed

2  
weakly expressed

3  
strongly expressed

Ad. 11: Leaf: angle of lowest lateral veins

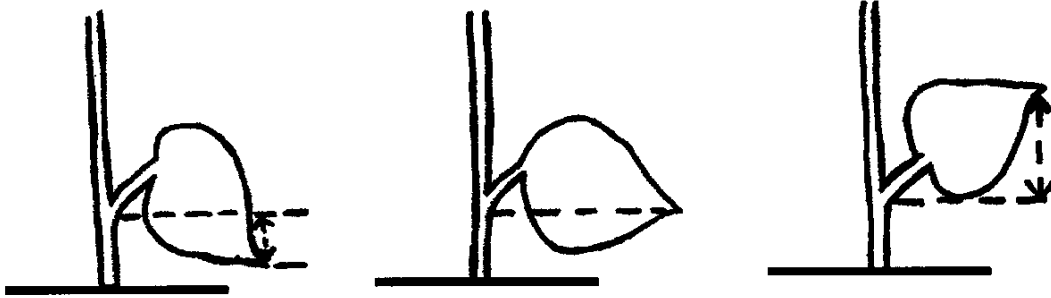


1  
acute

2  
right angle or nearly right angle

3  
obtuse

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



3

5

7

low

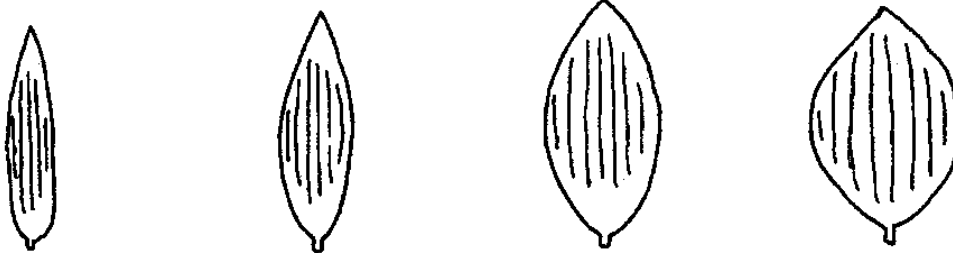
medium

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 colored.

Ad. 16: Ray floret: shape



1

2

3

4

fusiform

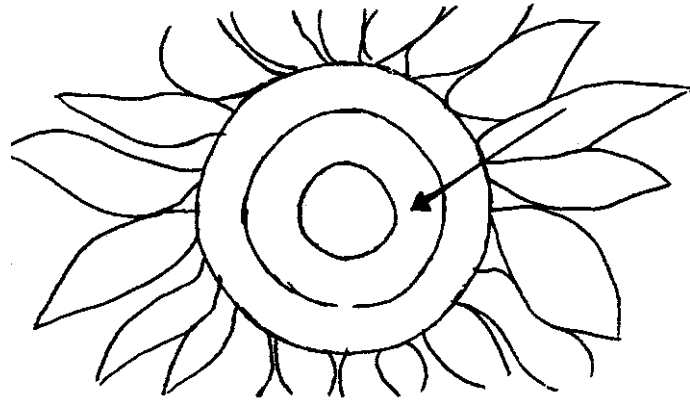
narrow ovate

broad ovate

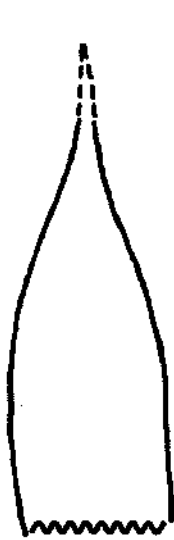
rounded

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.

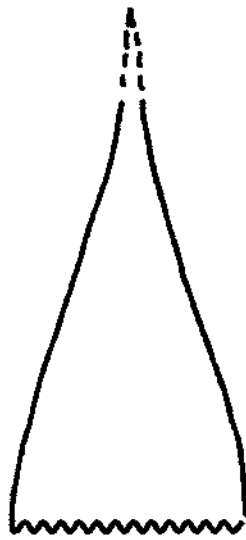


Ad. 24: Bract: shape



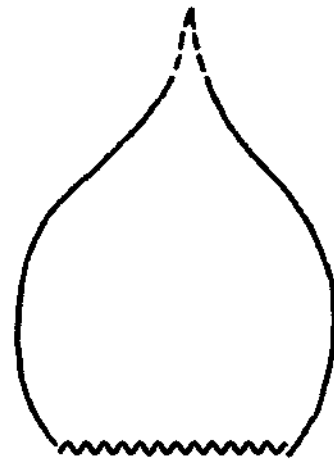
1

clearly elongated



2

neither clearly elongated nor  
clearly rounded

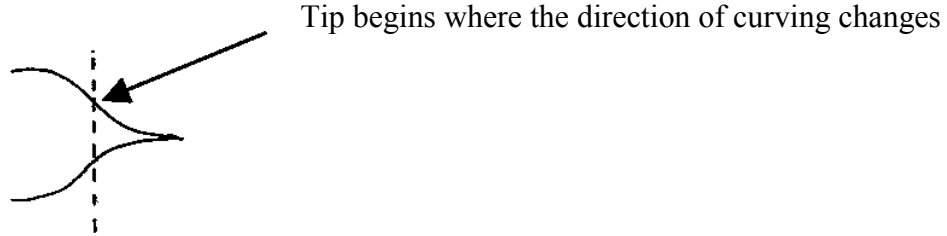


3

clearly rounded



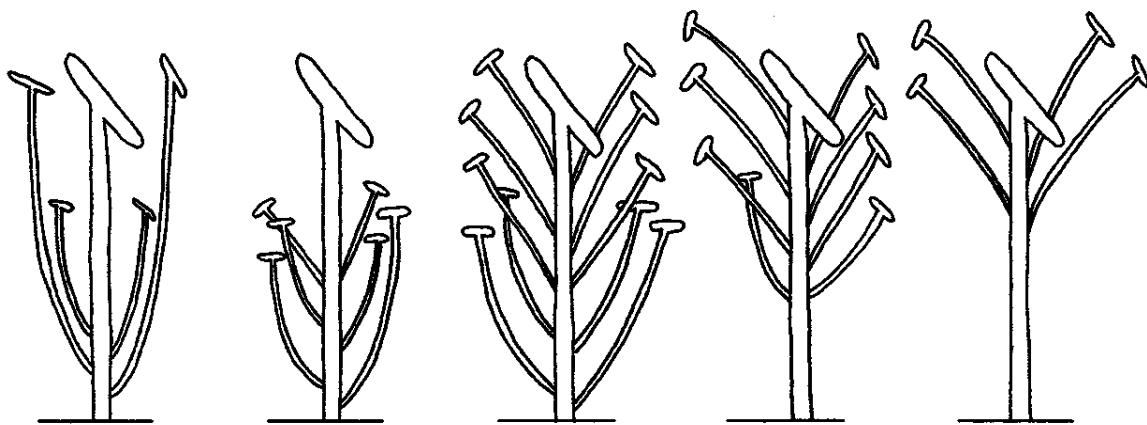
Ad. 25: Bract: length of the tip



Ad. 28: Plant: natural height

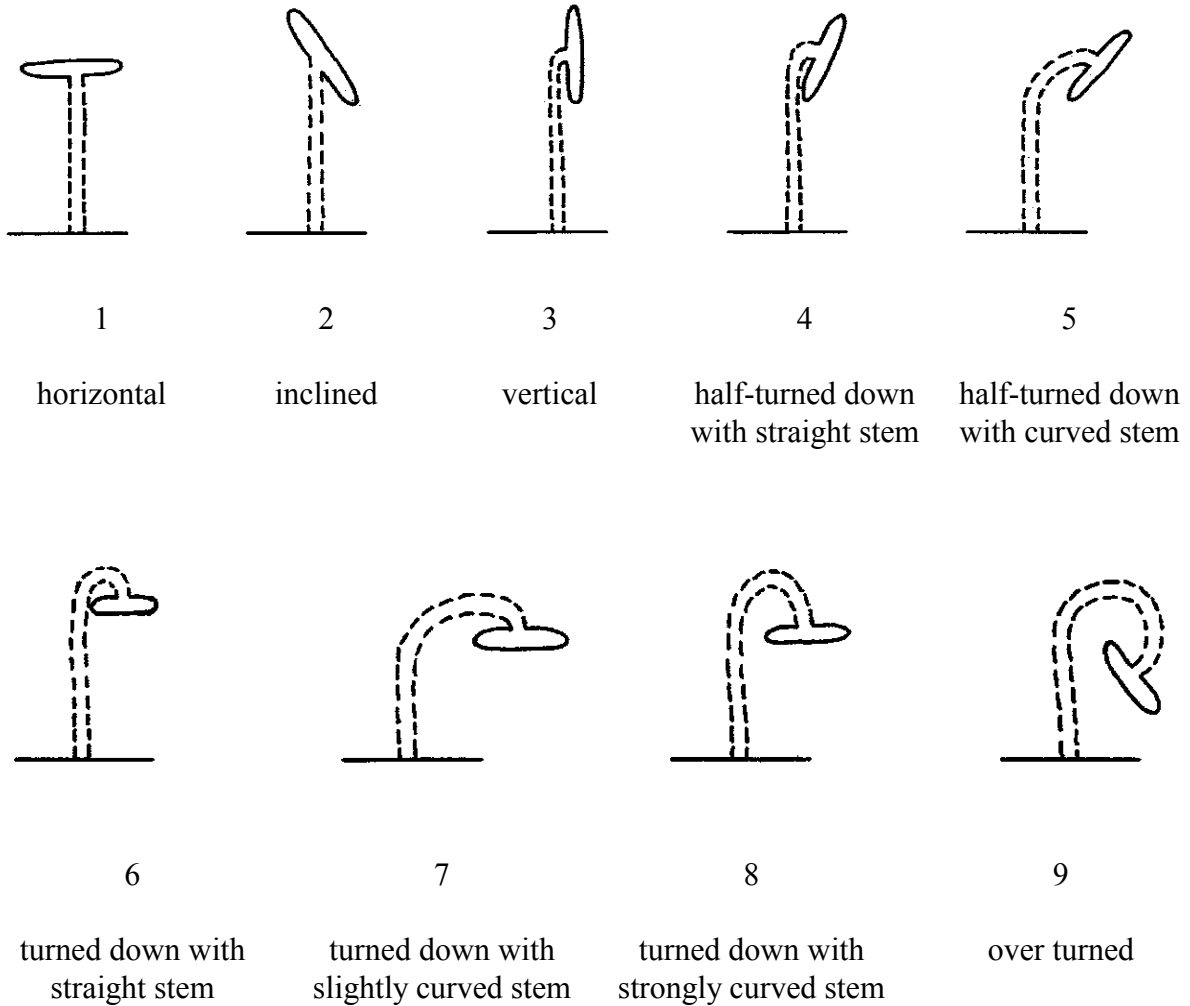
Different environmental conditions may require separate scales for lines, hybrids and open pollinated varieties.

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

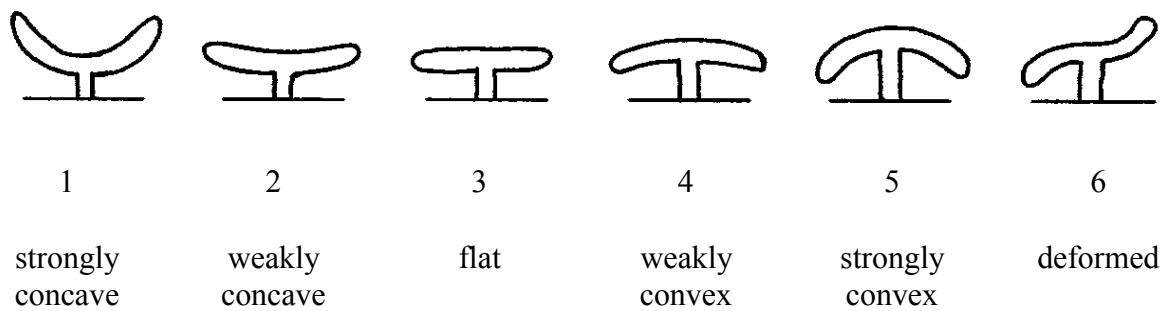


- |            |                     |         |                      |             |
|------------|---------------------|---------|----------------------|-------------|
| 1          | 2                   | 3       | 4                    | 5           |
| only basal | predominantly basal | overall | predominantly apical | only apical |

Ad. 32: Head: attitude



Ad. 34: Head: shape of grain side



Ad. 36: Seed: shape



1

elongated



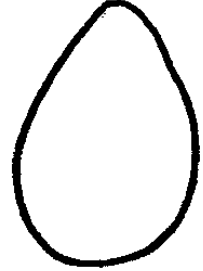
2

narrow ovoid



3

broad ovoid



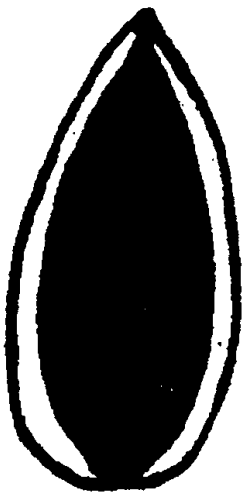
4

rounded

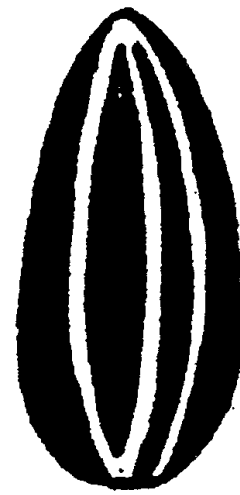
Ad. 38: Seed: main color

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

Ad. 39 and 40: Seed: stripes



on margin



between margins

## GROWTH STAGES OF SUNFLOWER<sup>1</sup>

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

### Germination - Emergence (A)



A 1

Appearance of hypocotyl as a cross.



A 2

Emergence of cotyledons and first leaves visible.

### Vegetative Phase (B)



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)



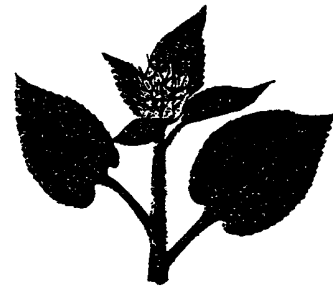
E 1

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.

<sup>1</sup> 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%.

IX. Literature

J.F. Miller: "Update on Inheritance of Sunflower Characteristics," USDA - ARS, Northern Crop Science Laboratory, Fargo, North Dakota 58105, USA

ASFIS, GEVES, GNIS: "Description des géniteurs et variétés de tournesol", édition 2000 (English, French, Spanish) ASFIS, 44, rue du Louvre, 75001 Paris, FR

X. Technical Questionnaire

	Reference Number (not to be filled in by the applicant)
<p>TECHNICAL QUESTIONNAIRE to be completed in connection with an application for plant breeders' rights</p>	
1. Species	<p><i>Helianthus annuus</i> L.  SUNFLOWER</p>
2. Applicant (Name and address)	
3. Proposed denomination or 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) hybrid
  - male sterile hybrid [ ]
  - male fertile single hybrid [ ]
  - three-way hybrid [ ]
- (c) open-pollinated variety [ ]
- (d) other (please indicate) [ ]

.....

4.2 Formula (if applicable, for each component in separate sheets, the information according to the following chapters 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 Genetic origin (to be filled for each component in case of an hybrid variety)

Components name	Genetic origin	Parentage with any other components already known
...	....	....
...	....	....

4.4 Other information



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

Characteristics	Example Varieties	Note
<b>5.1 Leaf: green color (4)</b>		
light	H 52.9.1.1	3[ ]
medium	HA 821	5[ ]
dark	HA 303	7[ ]
<b>5.2 Leaf: blistering (5)</b>		
absent or very weak		1[ ]
weak	HA 342, RHA 273	3[ ]
medium	HA 291	5[ ]
strong	HA 303, RHA 361	7[ ]
<b>5.3 Time of flowering (14)</b>		
very early	HA 302, RHA 381	1[ ]
early	RHA 273	3[ ]
medium	RHA 274	5[ ]
late	RHA 271	7[ ]
very late	RHA 361	9[ ]

Characteristics	Example Varieties	Note
<b>5.4 Ray floret: color (19)</b>		
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[ ]
multicolored		8[ ]
<b>5.5 Plant: natural height (at maturity) (28)</b>		
very short	HA 379	1[ ]
short	HA 291	3[ ]
medium	RHA 801	5[ ]
tall	H 52.9.1.1	7[ ]
<b>5.6 Plant: branching (excluding environmental branching) (29)</b>		
absent	HA89	1[ ]
present	RHA 271	9[ ]
<b>5.7 Seed: stripes on margin (39)</b>		
none or very weakly expressed	RHA 273	1[ ]
weakly expressed	H 52.9.1.1	2[ ]
strongly expressed	HA 89	3[ ]

Characteristics		Example Varieties	Note
<b>5.8 Seed: stripes between margins (40)</b>			
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 <sup>o)</sup>	State of expression of similar variety	State of expression of candidate variety
<p><sup>o)</sup> In the case of identical states of expressions of both varieties, please indicate the size of the difference.</p>			

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  [ ]  
(specify)

.....

7.2 Use

(a) oil and cake  [ ]

(b) birds and direct human consumption  [ ]

(c) ornamental  [ ]

(d) other use (specify)  [ ]

.....

7.3 Special conditions for the examination of the variety

7.4 Other information

8. Authorization for Release

(a) Does the variety require prior authorization for release under legislation concerning the protection of the environment, human and animal health?

Yes  [ ] No  [ ]

(b) Has such authorization been obtained?

Yes  [ ] No  [ ]

If the answer to that question is yes, please attach a copy of such an authorization.

[Annex follows]

ANNEX

Additional Useful Explanations

	<u>TABLE OF CONTENTS</u>	<u>PAGE</u>
Part I	Introduction .....	2
Part II	Characteristics derived by using electrophoresis .....	3
Part III	Description of the method to be used .....	5

## 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 reconfirms 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

	English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
43. (+)	<b>Allele expression at locus Me1</b>	<b>Expression allélique au locus Me1</b>	<b>Allel-Ausprägung im Locus Me1</b>	<b>Expression alélica del locus Me1</b>		
	Genotype 2/2	Génotype 2/2	Genotyp 2/2	Genotipo 2/2	HA 89	1
	Genotype 4/4	Génotype 4/4	Genotyp 4/4	Genotipo 4/4	RHA 274	2
	Genotype 2/4	Génotype 2/4	Genotyp 2/4	Genotipo 2/4	Florence	3
44. (+)	<b>Allele expression at locus Pgd1</b>	<b>Expression allélique au locus Pgd1</b>	<b>Allel-Ausprägung im Locus Pgd1</b>	<b>Expression alélica del locus Pgd1</b>		
	Genotype 2/2	Génotype 2/2	Genotyp 2/2	Genotipo 2/2	RHA 274	1
	Genotype 4/4	Génotype 4/4	Genotyp 4/4	Genotipo 4/4	HA 850	2
	Genotype 2/4	Génotype 2/4	Genotyp 2/4	Genotipo 2/4	Santafe	3
45. (+)	<b>Allele expression at locus Pgi2</b>	<b>Expression allélique au locus Pgi2</b>	<b>Allel-Ausprägung im Locus Pgi2</b>	<b>Expression alélica del locus Pgi2</b>		
	Genotype 2/2	Génotype 2/2	Genotyp 2/2	Genotipo 2/2	RHA 274	1
	Genotype 4/4	Génotype 4/4	Genotyp 4/4	Genotipo 4/4	H559211	2
	Genotype 2/4	Génotype 2/4	Genotyp 2/4	Genotipo 2/4	Santafe	3
46. (+)	<b>Allele expression at locus Shdh1</b>	<b>Expression allélique au locus Shdh1</b>	<b>Allel-Ausprägung im Locus Shdh1</b>	<b>Expression alélica del locus Shdh 1</b>		
	Genotype 2/2	Génotype 2/2	Genotyp 2/2	Genotipo 2/2	HA 89	1
	Genotype 4/4	Génotype 4/4	Genotyp 4/4	Genotipo 4/4	RHA 856	2
	Genotype 2/4	Génotype 2/4	Genotyp 2/4	Genotipo 2/4	Florence	3

English	français	deutsch	español	Example Varieties Exemples Beispielssorten Variedades ejemplo	Note/ Nota
47. <b>Allele expression (+) at locus Pgm4</b>	<b>Expression allélique au locus Pgm4</b>	<b>Allel-Ausprägung im Locus Pgm4</b>	<b>Expression alélica del locus Pgm4</b>		
Genotype 2/2	Génotype 2/2	Genotyp 2/2	Genotipo 2/2	RHA 274	1
Genotype 4/4	Génotype 4/4	Genotyp 4/4	Genotipo 4/4	HA 89	2
Genotype 2/4	Génotype 2/4	Genotyp 2/4	Genotipo 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<sub>2</sub>)

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<sub>2</sub>, 6H<sub>2</sub>O)  
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).

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<sub>2</sub> 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 MgCl<sub>2</sub> 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<sub>2</sub> salt  
60 mg 6-Phosphogluconic acid Na<sub>3</sub> salt  
10 mg NADP  
1 ml MTT solution (4.3.1.3.)  
1.5 ml PMS solution (4.3.1.5.)  
1 ml MgCl<sub>2</sub> 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<sub>2</sub>O, Na<sub>2</sub> salt  
150 mg EDTA, Na<sub>2</sub>  
10 mg NADP  
1.5 ml MTT solution (4.3.1.3)  
1 ml PMS solution (4.3.1.5)  
4 ml MgCl<sub>2</sub> 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, 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

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.

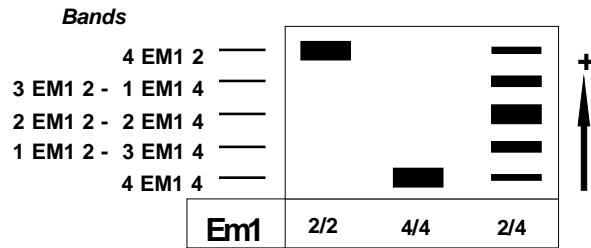
## 6. **Recognition of the alleles encoding isoenzymes**

### 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

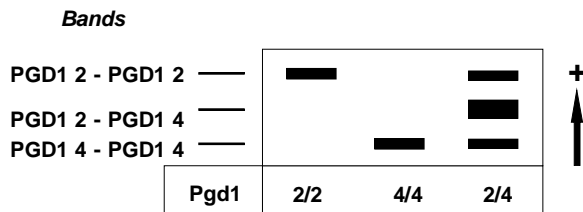


## 6.2 Recognition of the alleles encoding PGD

### 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



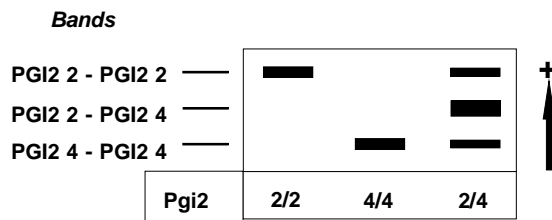
Two migration zones can be observed; only the slowest migrating bands are polymorphic.

## 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



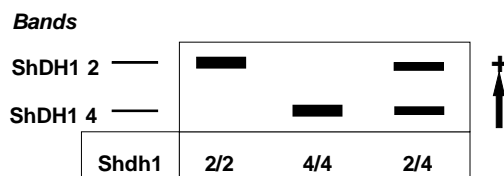
There are two migration zones; only the slowest migrating bands are scored.

### 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



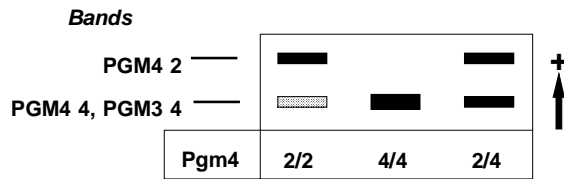
### 6.5 Recognition of the alleles encoding PGM

#### 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.

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