E

UPOV

TG/170/2(proj.)
ORIGINAL: English
DATE: 2001-01-09

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

DRAFT

GUIDELINES

FOR THE CONDUCT OF TESTS

FOR DISTINCTNESS, UNIFORMITY AND STABILITY

SUBTERRANEAN CLOVER

(*Trifolium subterraneum*)

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.

TABLE OF CONTENTS PAGE

I. Subject of these Guidelines

These Test Guidelines apply to all varieties of *Trifolium subterraneum* (ssp. *subterraneum*, spp. *yanninicum* and ssp. *brachycalycinum*).

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:

100g.

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 seed should be inoculated within the appropriate strain of Rhizobium. 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 30 spaced plants and may in addition include 4 meters of row. Separate plots for observation and for measuring can only be used if they have been subject to similar environmental conditions.

4. Plots with spaced plants: Each test should consist of 30 single spaced plants per variety arranged in 2, 3, or 5 replicates, i.e. plots of 15, 10 or 6 plants. More replicates are generally more efficient when fewer varieties are included in the test.

5. Row plots: Each test which includes row plots should consist of at least 4 meters of row arranged in two replicates, each of 2 meters. The size of the plots should be such that plants or parts of plants may be removed for observation without prejudice to the visual assessments which must be made up to the end of the growing period. The density of sowing should be such that about 150 plants per meter should be obtained.

6. Additional tests for special purposes may be established.

IV. Methods and Observations

1. All measurements for assessment of distinctness and stability should be made on 30 plants or parts taken from each of 30 plants.

2. For the assessment of uniformity, a population standard of 1% with an acceptance probability of at least 95% should be applied. In the case of a sample size of 30 plants, the maximum number of off-types allowed would be 1.

3. Unless indicated otherwise, all observations on the leaf should be made on new fully opened leaves at the 50% flowering stage (50% of plants with at least one flower). Observations on flowers should be made 2 weeks after the 50% flowering stage. Observations on the burr and seed should be made on fully mature, senesced plants.

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 place, the collection should be divided according to the subspecies:

- *subterraneum*
- *yanninicum* or
- *brachycalycinum*.

3. It is recommended that the competent authorities use the following characteristics for grouping varieties within each subspecies:

(a) Leaflet: pattern of mark (characteristic 6)
(b) Stipules: degree of anthocyanin coloration (in shaded part of canopy) (characteristic 29)
(c) Time of start of flowering (characteristic 30)
(d) Calyx tube: distribution of coloration (characteristic 34)
(e) Stem (runner): degree of hairiness (internode between the third and fourth nodes on the longest primary branch) (characteristic 36)
(f) Seed: hard seed breakdown over four months (characteristic 43).

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.

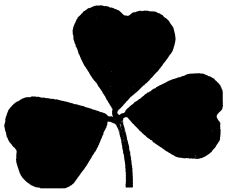
(+) See Explanations on the Table of Characteristics in Chapter VIII.

1) To be observed on: A = spaced plants
B = row plots
C = special test

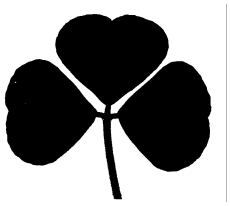
M = actual measurement
VG = visual assessment by a single observation of a group of plants or parts of plants
VS = visual assessment by observation of a number of individual plants or parts of plants

VIII. Explanation on the Table of Characteristics

Ad. 4: Leaflet: general shape



1
triangular



2
triangular to rounded

3
rounded

Ad. 7, 10, 16: Leaflet: pattern of mark

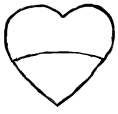
Only for varieties with arms: Leaflet: width of arms (characteristic 7)

3
narrow
(A1)

5
medium
(A2)

7
broad
(A3)

Only for varieties with bands: Leaflet: width of bands (characteristic 10)



3
narrow
(B1)

5
medium

7
broad
(B2)

Only for varieties with crescent: Leaflet: base of crescent (characteristic 16)

1	2	3	4
(C1)	(C2)	(C3)	(C4)

Ad. 26 to 28: Leaf: level of isoflavons (formononetin, genistein, biochanin A) before start of flowering (percentage dry matters)

Estimating levels of isoflavones. The method is essentially as documented in Francis and Millington (1965). In summary, the procedure is as follows:

Samples

Fresh leaf samples are taken from healthy, recently expanded leaves prior to flowering. Twelve leaf discs per variety are sampled for chemical analysis. A duplicate sample of 12 leaf discs is taken for dry weight calculations.

Extraction of isoflavones

Leaf samples for chemical analysis are macerated in test tubes. After leaving for 15 minutes to allow hydrolysis of bound isoflavones, 1 mL of ethanol (commercial grade, absolute) is added. Samples are placed in a shaking water bath at 60^{o} C for 10 minutes and the extract decanted into clean test tubes. The extraction procedure is repeated on the sample residue to extract any further isoflavones. This solution is added to the initial decanted solution and the total volume made up to 2 mL if necessary with ethanol. To concentrate the sample, a 0.5 mL sub-sample is pipetted into small test tubes and placed in a 40^{o}C oven until all ethanol has evaporated. The sub-sample is then re-dissolved in 0.2 mL of commercial grade ethanol.

Chromatography

An aliquot of 5·µL from each sample is spotted onto Silica gel 60 F_{254} thin layer chromatography plates. Standard solutions containing known concentrations of the three isoflavones are also spotted onto each plate. Chromatography is then conducted in a 90:10 solution of chloroform: methanol. Intensity of isoflavone bands is measured under UV light at 254 nm by comparison with the intensity of standard solutions.

Dry weight samples

The duplicate samples taken for dry weight calculations are dried for 48 hours at 60° C and then weighed.

Calculations

The level of each isoflavone is calculated as a percentage of dry weight.

Ad. 43: Seed: rate of hard seed breakdown over six months

Seed samples

Fully formed burrs should be obtained from recently senesced plants. Seed production should have proceeded under adequate but not excessively prolonged irrigation or rainfall. Seeds are gently rubbed out of burrs with care taken not to scratch the seed surface.

Laboratory procedure

Four hundred seeds of each sample are wet with water and placed in a 15° C cabinet for 48 hours. Germinated seeds are counted and discarded. The remaining hard-seeds are used for determining rate of breakdown. They are placed in a cabinet fluctuating between 15° C and 60° C over a 24 hour period for 4 months. Samples are then wet with water and placed in a 15° C cabinet for 48 hours. Germinated seeds are counted. The proportion of hard seeds remaining are calculated as a percentage of the number of hard seeds in the initial sample.

IX. Literature

Dear, B.S. and Sandral, G.A. (1997). Subterranean clover in NSW – identification and use. Agfact P2.5.16, (2nd edition), NSW Agriculture, pp. 36.

Francis, C.M. and Millington, A.J. (1965). Varietal variation in the isoflavone content of subterranean clover: its estimation by a microtechnique. *Aust. J. Agric. Res*. **16**: 557-564.

Nichols, P.G.H., Collins, W.J. and Barbetti, M.J. (1996). Registered cultivars of subterranean clover - their characteristics, origin and identification. Agriculture Western Australia Bulletin No. 4327, pp. 61.

X. Technical Questionnaire

	Reference Number (not to be filled in by the applicant)

TECHNICAL QUESTIONNAIRE
to be completed in connection with an application for plant breeders' rights

1.1	Species	*Trifolium subterraneum* SUBTERRANEAN CLOVER	
1.2	Subspecies	*subterraneum* *yanninicum* *brachycalycinum* other (specify)	[ ] [ ] [ ] [ ]

2. Applicant (Name and address)

3. Proposed denomination or breeder's reference

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

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
5.1 (6)	**Leaflet: pattern of mark**		
	a pair of arms only	Yarloop	1[ ]
	a single transverse band only	Nungarin	2[ ]
	a single, crescent-shaped central mark only	Mt Barker	3[ ]
	a pair of arms and a crescent	Seaton Park	4[ ]
5.2 (29)	**Stipules: degree of anthocyanin coloration (in shaded part of canopy)**		
	absent or very weak	Junne	1[ ]
	weak	Dalkeith, Goulburn	3[ ]
	medium	Denmark, York	5[ ]
	strong	Daliak, Woogenellup	7[ ]
	very strong	Yarloop	9[ ]
5.3 (30)	**Time of start of flowering**		
	very early	Nungarin	1[ ]
	early	Dalkeith	3[ ]
	medium	Riverina, York	5[ ]
	late	Goulburn, Mt Barker	7[ ]
	very late	Tallarook	9[ ]

	Characteristics	Example Varieties	Note
5.4 (34)	**Calyx tube: distribution of coloration**		
	on upper quarter of tube		1
	on upper half of tube	Goulburn	2
	on upper three-quarters of tube	Mt Barker, Nungarin, York	3
	on entire tube	Daliak	4
5.5 (36)	**Stem (runner): degree of hairiness (internode between the third and fourth nodes on the longest primary branch)**		
	absent or very weak	Denmark, Gosse, Goulburn, Riverina	1[ ]
	weak	Nuba	3[ ]
	medium	Daliak, Leura, York	5[ ]
	strong	Dalkeith, Nungarin, Seaton Park	7[ ]
	very strong		9[ ]
5.6 (43)	**Seed: hard seed breakdown over four months**		
	very slow	Geraldton, Northam	1[ ]
	slow	Dalkeith, Nungarin, York	3[ ]
	medium	Junee, Seaton Park	5[ ]
	fast	Gosse, Goulburn	7[ ]
	very fast	Mt Barker, Woogenellup	9[ ]

6. Similar varieties and differences from these varieties

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

[o)] In the case of identical states of expressions of both varieties, please indicate the size of the difference.

7. Additional information which may help to distinguish the variety

7.1 Resistance to pests and diseases

7.2 Special conditions for the examination of the variety

7.3 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.

[End of document]