



TWA/28/4

ORIGINAL: English

DATE: April 28, 1999

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

**TECHNICAL WORKING PARTY
FOR
AGRICULTURAL CROPS**

**Twenty-Eighth Session
Ottawa, June 22 to 25, 1999**

COMMENTS ON THE WORKING PAPER ON DRAFT TEST GUIDELINES FOR
SUBTERRANEAN CLOVER

Document prepared by experts from Australia

THIS DOCUMENT COMMENTS ON THE WORKING PAPER DRAFT UPOV TEST
GUIDELINES FOR SUBTERRANEAN CLOVER (TWA/27/12)

Proposed modifications

II. Material Required

1. 500 grams is seed. much too high. 30 grams would be more than adequate to perform whatever tests would be required.

III. Conduct of Tests

1. A much more appropriate testing regime would be to conduct tests using 2 generations of seed in the one year. The original scheme of testing over two growing periods would prolong the PBR process unnecessarily. This would show up genetic stability between generations more readily without confounding by seasonal variation.

3. There is no need to use rows as well. It would be best to standardize the procedure to spaced plants. Therefore, 4. and 5. could be deleted. Consequently, the 4th sentence of 3. should read “As a minimum, each test should include a total of 30 single plants per variety, spaced 1 m apart and arranged in 2, 3 or 5 replicates, i.e. Plots of 15, 10 or 6 plants”.

IV. Methods and Observations

2. The proposal to measure leaf characters on the 4th true leaf is not appropriate, as some leaf markings do not show up sufficiently then. A more appropriate, and easily definable stage would be to measure leaf characters on new fully opened leaves at the 50% flowering stage. Flower characters would be best measured 2 weeks after the 50% flowering stage. I suggest wording as follows: “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.....”.

V. Grouping of Varieties

2. There are no problems grouping into subspecies.

Other characteristics that could be used to group varieties would be:

Isoflavones: level of formononetin (characteristic 26). This is a very important character and distinguishes oestrogenic from non-oestrogenic varieties.

VII. Table of Characteristics

The following characteristics should be used for all varieties (i.e. given an *):
7, 8, 10, 13, 24, 26, 29, 31, 34, 39, 44 and 46.

1. OK
2. OK
3. OK
4. OK as it stands.
5. OK
6. Arms and crescents can occur on the same leaf. One consideration that could be made would be the ordering of the leafmark characteristics. I suggest placing the crescent characters first, followed by arms and then bands. This would place them in order of the likelihood of encountering them, on the basis of my observations of commercial varieties and a large number of accessions. i.e. most varieties have a crescent, and a fair proportion of them have arms as well, while only a relatively low proportion have bands.

I think the idea of a characteristic for definition of mark is good, as some are faint on some varieties and strong on others. Maybe the term “clarity” is better. However, this characteristic should be used for each of the 3 types of mark: arms, crescent and band. The first state should just be “absent”. I therefore propose deleting 6 and in its place have the following characteristics:

Leaflet: Varieties with arms: Clarity of arms

absent	Uniwager, Mt Barker	1
faint	Denmark	2
clear	Yarloop, Seaton Park	3

Leaflet: Varieties with bands only: Clarity of transverse bands

absent	Uniwager,	1
faint		2
clear	Nungarin, Geraldton	3

Leaflet: Varieties with a crescent: Clarity of central crescent-shaped mark

absent	Uniwager, Yarloop	1
faint	Nuba	2
clear	Yarloop, Seaton Park	3

These characteristics would be best placed following characteristics 8, 10 and 13.

7. Leaflet: pattern of mark. OK, except state 2 (a single transverse band only) should be “types B1 to B3”, as this latter state is possible. This and all the leaf mark attributes are explained in Nichols *et al.* (1996). They are very important characters for distinguishing varieties of subterranean clover and the rating scale is widely used among those working with the species. I suggest following the system in Nichols *et al.* (1996).

8. Leaflet: Varieties with arms: size of arms. This needs to be re-numbered. A better term for this is “width of arms”. I suggest the following:

A1	Yarloop, Dalkeith, Leura	1
A2	Seaton Park, Junee	3
A3		5

9. Add Leura, Junee to state 1.

Transfer Woogenellup and add Rosedale to state 3 (light green).

10. I disagree with referee’s comments. There are accessions with broader bands than Nungarin, so there needs to be an allowance for the possible release of such a type. I suggest the following:

B1	Northam, Geraldton	1
B2	Nungarin	3
B3		5

11. OK.

12. OK.

13. There should be a characteristic for position of the crescent, corresponding with that for bands (characteristic 12). I suggest the following:

Leaflet: Varieties with a crescent: position of central crescent-shaped mark

towards base		1
central	Mt Barker, Seaton Park	2
towards distal end		3

There should be a characteristic for position of arms, relative to crescent. I suggest the following:

Leaflet: Varieties with both a crescent and arms: position of arms relative to central crescent-shaped mark

arms adjacent only to crescent	June, Leura, Trikkala	1
arms both adjacent and beneath crescent	Seaton Park, Karridale	2

14. OK.

15. OK.

16, 19, 23, 29, 33, 36, 38 and 42. I do not see the need for an absence versus presence characteristic for each of these. In all cases, the ‘absence’ state is part of a continuum and should be listed as a state in the characteristic following. e.g. For characteristic 16. ‘absent’ is a state for degree of anthocyanin fleck. The other states would be weak, medium and strong. It is possible that some varieties that would have a state between absent and weak. Having characteristic 16. with absent or present as its states may give a misleading impression.

17. Suggest amalgamating with 16. I am happy with the wording. The referees comments regarding position of flecking are not appropriate. I suggest the following:

Leaflet: Degree of anthocyanin fleck

absent	Seaton Park	1
weak	Dalkeith, Woogenellup	3
medium	Daliak	5
strong	Bacchus Marsh	7

18. OK.

20. Suggest amalgamating with 19. I am happy with the wording. I suggest the following:

Leaflet: Degree of anthocyanin flush

absent	Denmark, Dalkeith	1
weak	Nungarin, Riverina	3
medium	Dinninup, Junee, Trikkala	5
strong	Clare	7

21. This characteristic is important. I suggest the following:

Leaflet: colour of anthocyanin flush

red		1
pinkish brown		2
brown	Dinninup, Yarloop	3
purplish brown	Trikkala, Dwalgnup	4
reddish purple	Gosse, Riverina	5
brownish purple	Clare	6
purple		7

22. OK.

24. Suggest amalgamating with 23. This characteristic should be asterisked. I suggest adding a 'very strong' state to bring it in line with the Nichols *et al.* (1996) bulletin ratings. There are no cultivars with this rating, but other accessions are more pubescent. I suggest the following:

Leaflet: Pubescence of upper surface

absent	Clare, Dinninup, Gosse, Riverina	1
weak	Denmark, Goulburn	3
medium	Dalkeith, Rosedale	5
strong	Leura, Nungarin	7
very strong		9

Reference for this and 27. and 28. is 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.

27 Delete Bacchus Marsh from state 3. Add Mt Barker to state 5. Add Clare to state 9.

28 Add Dwalganup to state 7. Add Bacchus Marsh to state 9.

29 Amalgamate with 30. Remove asterisk, as this is not an imperative character. I do not agree with the wording of pattern of distribution of anthocyanin colouration. It is more the extent of anthocyanin colouration that is important. I suggest the following to make it consistent with characteristics 17. and 20.

Stipules: degree of anthocyanin colouration (in shaded part of canopy)

absent	Junee	1
weak	Dalkeith, Goulburn	3
medium	Denmark, York	5
strong	Daliak, Woogenellup, Yarloop	7

31. Add Riverina in place of Trikkala in state 5. York mis-spelt.

32. OK.

33. Amalgamate with 34. States should then be 1-9. Character should be 'Calyx tube: distribution of anthocyanin colouration', as it does not include the calyx hairs. The categories are wrong as colouration extends from the tip of the calyx down the tube, not from the base. I suggest the following:

Calyx tube: distribution of anthocyanin colouration

Absent	Denmark, Leura, Woogenellup	1
On upper ¼ of tube		3
On upper ½ of tube	Goulburn	5
On upper ¾ of tube	Nungarin, York, Mt Barker	7
On entire tube	Daliak	9

35. This characteristic is important, as a range of colouration can occur. I suggest the following:

Calyx tube: anthocyanin colour

pink		1
pinkish red	Dwalganup, Goulburn	2
brownish pink	Yarloop, Riverina	3
red		4
purplish red	Nungarin, Mt Barker, York	5
purple	Daliak	6
brownish purple		7
brown		8

36. Amalgamate with 37. Use a 1-9 scale in line with Nichols *et al.* (1996). I suggest the following:

Peduncle: pubescence

absent	Denmark	1
weak	Clare, Goulburn, Junee	3
medium	Daliak, Woogenellup, York	5
strong	Dalkeith, Nuba, Seaton Park	7
very strong	Dinninup	9

38. Amalgamate with 39. Use a 1-9 scale in line with Nichols *et al.* (1996). Also best to define a consistent internode on which to rate. I suggest the internode between the 3rd and 4th nodes on the longest primary stem. I suggest the following wording:

Stem (runner): pubescence (internode between the 3rd and 4th nodes on the longest primary stem)

absent	Denmark, Goulburn, Gosse, Riverina	1
weak		3
medium	Daliak, Leura, Nuba, York	5
strong	Dalkeith, Nungarin, Seaton Park	7
very strong		9

40. OK.

41. I disagree with referee's comments. Burr is a well-used and understood term for subterranean clover.

There should be states very small (=1) and very large (=9). I suggest the following:

Burr: size

very small	Daliak, Goulburn	1
small	Geraldton	3
medium	Denmark, Junee	5
large	Clare	7
very large	Dalkeith	9

42. There should be more states for burr burial tendency. I suggest a better term would be 'degree of burial' with states 1-9. I suggest the following:

Burr: degree of burial

absent	Clare, Nuba, Rosedale	1
weak	Mt Barker, Woogenellup	3
medium	Denmark, Gosse, Goulburn	5
strong	Daliak, Geraldton, Seaton Park	7
very strong	Dalkeith	9

43. It is desirable to have seeds per burr in addition to no. of florets per inflorescence (character 32.), as flower abortion rates differ. I suggest the following:

Burr: predominant number of seeds

less than three	Gosse	1
three	Dalkeith	2
four	Denmark, Goulburn	3
more than four		4

45. Add Gosse to state 7.

46. Would 'rapid' be a better term than 'fast'? I would like to modify the variety ratings to as follows:

Seed: rate of hard seed breakdown over 6 months duration

very rapid	Mt Barker, Woogenellup	1
rapid	Gosse, Riverina	3
medium	June, Seaton Park	5
slow	Dalkeith, Nungarin, York	7
very slow		9

Currently, the varieties with a 7 rating are the most hard-seeded among cultivars. However, there are many accessions with higher levels of hard seed.

VIII. Explanations on the Table of Characteristics

Ch. 7. Leaflet: pattern of mark

See figure 4 and explanation on page 24 in Nichols *et al.* (1996).

Ch. 29, 30. Stipules: degree of anthocyanin colouration (in shaded part of canopy)

I have simplified the terminology for this characteristic. We do not use the S ratings any longer. Therefore, there is no need for an explanatory diagram.

IX. Literature

Replace Collins *et al.* (1984) with the updated version:

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.

The Southwood *et al.* (1978) publication is not actually referred to in the document.

However, an updated version of this publication is quite relevant, even though much of the data is taken from the Nichols *et al.* (1996) publication. The updated version is:

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.

The reference for characteristics 26, 27 and 28 is:

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.

X. Protocols

Characters 26, 27 and 28. 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° 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° 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- μ L from each sample is spotted onto Silica gel 60 F₂₅₄ 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.

Characteristic 44. Seed: rate of hard seed breakdown over six months duration.

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 6 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.

[End of document]