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UPOV

TC/XXV/9
ORIGINAL: English
DATE: August 15, 1989

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

TECHNICAL COMMITTEE

Twenty-fifth Session
Geneva, October 5 and 6, 1989

NEW METHODS, TECHNIQUES AND EQUIPMENT IN THE EXAMINATION OF VARIETIES

Document prepared by the Office of UPOV

1. As a result of the discussions on new methods, techniques and equipment in the examination of varieties in the Technical Committe, the Office of UPOV had prepared document TC/XXV/4, which was presented to the Technical Working Parties for discussion.

2. That document shortly explained the two steps to be taken: (i) to investigate and compile an inventory of the species and new methods in connection with the application of the above new technology and (ii) to prepare clear draft proposals on how to integrate new technology.

3. In the annexes to document TC/XXV/4 the Office of UPOV reproduced the following information:

Annex I A list of species for which member States study the possible use of electrophoresis in the examination of varieties for distinctness: Information received in compliance with the request of the Office of UPOV made in Circular U 1384 of January 12, 1989.

Annex II A list of new methods, other than electrophoresis, under study in the UPOV member States.

Annex III A list of species for which, besides wheat, barley and oats, electrophoresis has been investigated as a means for variety identification, copied from Annex III of document TWA/XVII/9.

Annex IV Proposal for the integration of electrophoresis and machine vision (wheat only) into the Test Guidelines for cereals, prepared by experts from the United Kingdom for the meeting of the Subgroup of Grasses in April 1989 (proposals from NIAB only).

Annex V Proposal for the integration of electrophoresis into the Test Guidelines for Ryegrass and possibly other Test Guidelines, prepared by experts from the United Kingdom (only personal proposal from the expert).

Annex VI Report on the experiences in the use of electrophoresis in Poa pratensis L. made in The Netherlands.

Annex VII Proposal for the incorporation of image analysis into DUS testing of onions, prepared by experts from the United Kingdom (only proposals from NIAB).

Annex VIII Introduction to the various applications of electrophoresis at the biochemistry laboratory of G.E.V.E.S. in connection with variety registration and seed certification, given during the last session of the Technical Working Party for Agricultural Crops (copied from Annex IV of document TWA/XVII/9).

Annex IX Extract from the report on the last session of the Technical Working Party for Agricultural Crops, dealing with electrophoresis (copied from document TWA/XVII/9, paragraphs 21 to 30).

4. Annex I to this document contains an updating to the above information received from South Africa.

5. The results of the discussions in the different Technical Working Parties are reproduced in the following documents:

TWA/XVIII/9 Prov., paragraphs 8 to 14
TWC/VII/20 Prov., paragraphs 29 to 32
TWO/XXII/8 Prov., paragraphs 20 and 21
TWV/XXII/19 Prov., paragraphs 17 to 22

They can be summarized as follows:

Electrophoresis

General Discussions

6. The TWA noted the three possible uses of electrophoresis as explained in document TWA/XVIII/7, as a tool for variety identification, as a tool to assist in taking a decision on distinctness and as a tool to take a decision on distinctness. It further noted Annex V of document TC/XXV/4 on the "Integration of Electrophoresis into UPOV Test Guidelines" with proposals for ryegrass. It highlighted the need to adopt standardized methods and characteristics, to agree on standardized discrimination and minimum distances, to establish ring-test procedures for the checking of results, to consider homogeneity criteria for electrophoresis characteristics and to produce an authenticated data base for common knowledge collection. It agreed to the suggestion that it study in the beginning starch gel electrophoresis on only four isozyme systems, Phosphoglucoisomerase (PGI), Acid Phosphatase (ACP), Isocytrase Dehydrogenase (IDH), and Glutamin Oxalo Transaminase (GOT), together with PAGE on seed globulins. The start should however be made with PGI. In order to use the limited time to best purpose, countries should, however, in the first instance concentrate on cereals.

7. The TWV noted document TWV/XXII/10 containing an inventory of the methods so far studied by the member States. It discussed the usefulness, the need and the possible consequences of the introduction of electrophoresis characteristics as distinctness characteristics for vegetable varieties. At present, the method was only under study and not used for distinctness. Many experts were afraid of possible consequences of allowing too small differences (a difference in 1 band) as sufficient difference for an amended variety to undermine the protection of an existing variety. At present, there was also seen by some experts no need to rush into the new methods as in vegetables the establishing of distinctness in the presence of sufficient traditional characteristics did not pose problems so far. It was also stressed that there was no correlation between a certain electrophoretic band and some morphological change or improvement of the variety. Applicants should really be asked to tell what was the advantage of their candidate over another already existing variety only differing slightly from it.

8. The vegetable breeders following the discussions of the TWV were not yet in a position to tell whether they would be in favor of the introduction of electrophoresis for distinctness for vegetable varieties or not, as at present they did not use these new methods themselves.

9. The TWO had an exchange of the possibilities of new technology in the ornamental species. According to the experience of the experts, very few possibilities were seen. In order to clarify the situation, the experts from The Netherlands will prepare a list of general remarks and all arguments speaking for and against the application of electrophoresis, image analysis and DNA probe for distinctness purposes for circulation to the Working Party for further additions and comments.

Application to Cereals

10. The TWA noted and approved document TWA/XVIII/5 containing the main points agreed in respect of electrophoresis during the meeting of the Subgroup on Cereals held in Hanover, Federal Republic of Germany, in April 1989. That Subgroup recommends adding characteristics obtained with the help of electrophoresis as additional characteristics to the above Test Guidelines for Wheat, Barley and Oats. These characteristics would open the possibility (a) of reducing the DUS tests to one year only and (b) of reducing the number of characteristics in the present Test Guidelines. The next meeting of the Subgroup is foreseen for May 14, 1990, in Wageningen, The Netherlands, the day before the next session of the TWA. It is planned to invite also experts from the professional organizations to these discussions on the inclusion of electrophoresis characteristics in the Test Guidelines for Wheat, Barley and Oats.

11. Characteristics obtained with the help of electrophoresis should be included in the Test Guidelines for Wheat, Barley and Oats as new characteristics without an asterisk. Each band should thereby be considered as a separate characteristic. However, only those bands should be accepted which fulfilled the normal requirements for acceptance valid for any other new characteristic and for which a clear presence or absence could be observed. The proteins and methods to be admitted should be:

species	proteins	methods
barley	B, C and D hordeins	preferably SDS-PAGE, second choice: PAGE pH 3,1
wheat	gliadins, glutelins	PAGE pH 3, 1, SDS-PAGE
oats	prolamins	PAGE pH 3,1

12. More detailed information is included in Annexes II and III to this document, which contains:

Annex II: A summary of the recommendations for the incorporation of electrophoresis into the Guidelines for Wheat, Barley and Oats, including a consideration of how gel interpretation systems might be harmonised;

Annex III: the protocol for the ISTA acid polyacrylamide gel electrophoresis method prepared by Dr. Cooke (GB).

13. The TWA decided that for the introduction of electrophoresis characteristics the normal homogeneity standard of 3 in 100 should be applied when such a characteristic was used as the only distinguishing characteristic for the candidate variety concerned. If the electrophoresis characteristics were not necessary for the distinction of a given candidate variety, for a transitional period twice the tolerance (6 in 100) should be applicable.

Application to Maize

14. The TWA noted that in France studies are made to enquire on the possibilities to incorporate enzyme polymorphism of maize in the distinctness studies for new varieties and the different weighting of characteristics according to the information on their genetic background.

Application to Grasses

15. The TWA noted Annex VI of document TC/XXV/4 on the "Experiences in the Use of Electrophoresis in Poa pratensis L." It highlighted the method of isoelectric focusing on seeds and the PAGE method on plantlets. The whole study was still at the experimental stage and would be continued. For seeds admixtures created problems, for plantlets a good sampling scheme needed to be developed.

Application to Vegetables (Asparagus, Peas, Watermelon)

16. The TWV finally agreed to make some precise studies in order to gain more knowledge on the possible use of electrophoresis. It chose asparagus, peas and watermelon as species for this study. Dr. Habben (DE) would chair the study on asparagus, Mr. Brand (FR) that on peas and Mr. Tabata (JP) (or another expert from Japan) that on watermelon. The Office of UPOV has prepared a circular (U 1473) inviting all member States to join in that study and give more details with respect to each of the species.

Handling of Data by Computer

17. The TWC noted document TWC/VII/2 on the analysis of electrophoresis data by computer, highlighting that the coding of bands should be harmonized, that even the chemicals might have to be standardized, that one single difference would not be sufficient for distinctness, that the decision on the minimum difference depended on each characteristic, and the knowledge on its hereditary effect, whether polygenic or oligogenic, and that the data bases should be harmonized and the minimum fields to be included agreed upon.

18. The TWC noted document TWC/VII/15 on the computer program to store and analyze electrophoretic data in the Varieties Office of the Federal Republic of Germany. It highlighted the three steps leading to the pattern of bands, the identification and coding of the pattern, the data structure and the program functions. For further work, more knowledge was needed, the bands would have to be identified and a harmonized coding had to be agreed upon.

19. A question arose in the TWC as to whether the treatment of electrophoretic data should be handled apart or only in the context of the treatment of data in general, whether a small ad hoc system should be established based on present knowledge or an overall solution awaited. It was finally agreed to set up a small Group with Mr. Grégoire (FR) as leader and Mrs. Campbell (GB), Dr. Laidig (DE) and Mr. Van der Heijden (NL), which would prepare for the next session of the TWC a draft for a data base for electrophoresis data keeping in mind that that draft should not preclude an overall approach.

Methods other than Electrophoresis

20. The TWA agreed that because of the introduction of electrophoresis and the sufficient number of morphological characteristics at present, there was no need to introduce image analysis for cereals. More knowledge was to be gained before rediscussing the possibilities of its introduction.

21. The TWV noted document TWV/XXII/7 giving in its first part the results of the application of image analysis to certain onion varieties. It stressed that so far the method would only be used to observe characteristics already included in the Test Guidelines. However, in future also some new characteristics may be suggested. It will receive another report on further results during the next session of the TWV.

22. The TWC noted especially annexes IV and VII of document TC/XXV/4. It further noted the report from The Netherlands on the new computer and commercial program on image analysis acquired recently (see also Annex IV of TWC/VII/20). Mr. Bar Tel (IL) reported that studies on the use of image analysis on carnations had been stopped in his country, as it was considered too expensive compared with its possible application. Changes were difficult to apply by oneself and would thus have to be paid for each time.

23. The TWC noted that ISTA planned to hold its fourth international symposium on varietal identification in Ames, Iowa, USA, from August 12 to 18, 1990, during which it would discuss the use of new methods in the testing of varieties.

[Three Annexes follow]

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ANNEX I

Republiek van Suid-Afrika

Departement van Landbou-ekonomie en -bemarking

Republic of South Africa

Department of Agricultural Economics and Marketing

Direktoraat Plant- en Drankbeheer
Directorate of Plant and Liquor Control
Privaatsak/Private Bag X179
Pretoria
0001
Tg SATIVA PRETORIA
Tx 3-23264 *Tf* (012) 206-2408
Fax. (012) 206-2786
Navrae/Enquiries D C JOUBERT
Verw./Ref. 10/1/6/1/2

LEB 13/14

1989-06-15

The Vice Secretary-general
U P O V
34 Chemin Des Colombettes - CH - 1211
Genéva 20
SWITZERLAND

Dear Sir

TECHNICAL COMMITTEE : ELECTROPHORETIC TECHNIQUES

We would like to update the document TC/XXV/4 by furnishing the following information :

1. South Africa is currently conducting applied identification studies on sunflowers using seed storage protein, SDS-Page and/or Page, pH 6 to 8,9.

2. Tests are planned or already applied for small scale identification purposes on soja bean, cabbage, beans and sorghum, using the above-mentioned techniques.

D.C. Joubert

pp DIRECTOR

RIG ALLE KORRESPONDENSIE AAN DIREKTEUR: PLANT- EN DRANKBEHEER
DIRECT ALL CORRESPONDENCE TO DIRECTOR: PLANT AND LIQUOR CONTROL

[Annex II follows]

THE USE OF ELECTROPHORESIS FOR DISTINCTNESS TESTING OF WHEAT, BARLEY AND OATS

This paper summarises the main recommendations of the Technical Working Party for Agricultural Crops concerning this topic, following the meeting held in Belfast in June 1989. The TWP was in turn advised by a special sub-group on Cereals, which had met in Hannover in April 1989.

1. Electrophoresis should be included in the revised Test Guidelines for Wheat, Barley and Oats as a character without an asterisk.

2. The proteins to be examined and the methods used should be as follows:

   For wheat - either acid PAGE of gliadins
   or SDS-PAGE of glutelins (glutenin sub-units)

   For barley - either SDS-PAGE
   or acid PAGE of B, C and D-hordeins

   For oats - acid PAGE of avenins (prolamins).

   The acid PAGE method should be that adopted by the International Seed Testing Association as a standard reference method (enclosed; see also Seed Sci. & Technol. 15, 555-575, 1987). The protocol for SDS-PAGE has yet to be defined (F has agreed to draft this for consideration by other member states).

3. The interpretation of the protein banding patterns should be carried out according to a protocol which is being devised by F, D, UK, NL and E. At the moment, member states have exchanged lists of varieties of wheat, barley and oats. D has supplied seed of the reference varieties used at the Bundessortenamt for defining wheat and barley banding patterns to the other countries; UK has done the same for the oats system used at the NIAB. The collaborating member states are to analyse the reference varieties, to see if they include all of the bands/groups of bands currently recognised in their own collections. Details of the systems used for

classification and reference varieties will be exchanged. The exercise should be completed by the end of September. The objectives of this collaborative exercise are:

i) to try to devise a harmonised system for the interpretation and nomenclature of the protein bands for the three species;

ii) to ensure that the harmonised system encompasses the banding patterns necessary to include all varieties presently considered in the different member states;

iii) to establish a collection of reference varieties which will include all of the appropriate protein bands or groups of bands.

3.1 Example - The harmonisation of interpretation systems for barley varieties.

In barley, hordein proteins can be separated electrophoretically into three distinct groups, termed D-, C- and B-hordeins in increasing order of mobility. The genes for these groups of proteins are found at three separate, but linked, loci. The different electrophoretic patterns of D, C and B-hordeins can thus be considered to represent alleles at these loci. Laboratories in different countries have devised and adopted various systems for the nomenclature of these banding patterns (alleles). Consider, for instance the variety Igri. Under the system in use at the NIAB (UK), this is designated as having a hordein composition 2.7, meaning that the D and C-hordein composition is characteristic of (the arbitrarily named) group 2, whilst the B-hordein composition is characteristic of group 7. Other laboratories on the same basis have designated the hordeins of Igri as C2, D6, D4, NC8B8 or Hor 1 Fr/Hor 2 So. The Bundessortenamt (D) have devised a slightly different system which describes the individual bands of the D, C and B-hordeins as being either present (9) or absent (1), whilst still recognising the existence of groups of bands. Thus Igri is described by the formula D 1191 C 9119191119/1191 B 119111111. The problem thus becomes primarily one of nomenclature (see Figure

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below). By exchanging information on classification systems and varieties, member states should be able to establish reference varieties which describe particular patterns. The names given to these patterns would then have to be decided by discussion. It should be relatively straightforward to draw up a table comparing the different names used to describe the patterns of known varieties. Harmonisation might take the form of an agreed standard reference system, for use when exchanging information for instance, whilst still permitting member states to retain their own systems for internal purposes. The patterns of hordeins could be described by the formula devised by the Bundessortenamt, if necessary. Any new patterns of D, C or B-hordeins would be recognised by agreement, the pattern described and a standard reference variety identified.

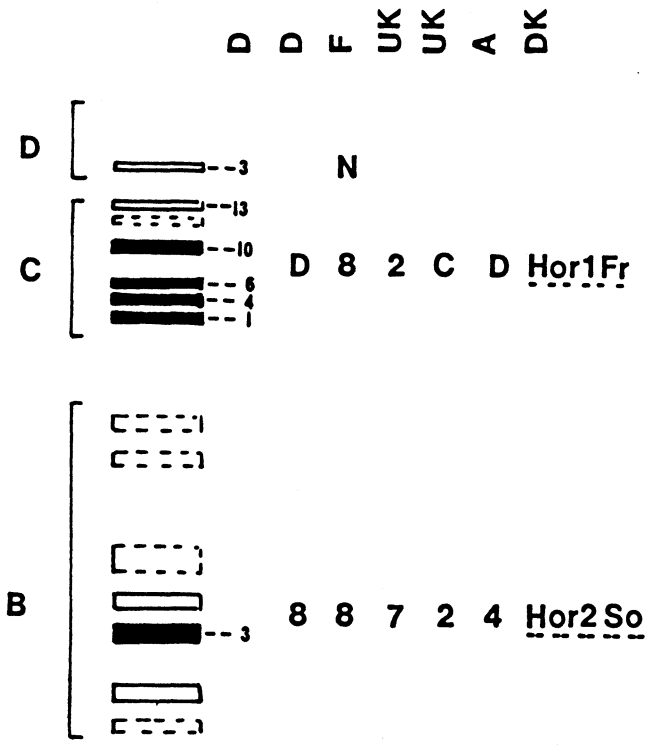

FIGURE - EXAMPLES OF DIFFERENT SYSTEMS USED TO DESCRIBE THE HORDEIN PROTEINS IN THE BARLEY VARIETY IGRI.

In principle, the same kind of system can be envisaged for oats and for wheat gliadins. Again the planned exchange of lists of varieties and nomenclature/classification systems should facilitate harmonisation.

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4. No assessment would be made of the quantitative intensity of protein bands, i.e. they would be described as either absent or present.

5. The criterion for distinctness would be the observation of a clear and repeatable difference in the banding pattern of two varieties, described according to the harmonised nomenclature procedure agreed (see 3 above).

6. The number of individual seeds to be examined would be 10-15, as an indication of distinctness, and 80-100, to study homogeneity.

7. The standard for homogeneity tolerance would initially be twice that contained in TG/1/2, i.e. 4 in 80 (or 6 in 100) unless electrophoresis was the sole criterion for establishing distinctness, in which case the standard would be 2 in 80 (3 in 100).

8. There would be no obligation for existing varieties to be made homogeneous within the above tolerances.

9. At present, some current varieties possess two or more electrophoretic lines or biotypes whilst exhibiting satisfactory homogeneity in respect of other characters. Whilst such varieties with biotypes existed on National Lists, candidate varieties would be compared with all electrophoretic lines. Distinctness would not be demonstrated if a candidate differed from only one line of a given variety.

[Annex III follows]

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ISTA STANDARD REFERENCE METHOD FOR THE IDENTIFICATION OF VARIETIES OF WHEAT AND BARLEY BY ACID POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

1. Principle

The alcohol-soluble proteins (gliadins from wheat, hordeins from barley) are extracted from seeds and separated by PAGE at pH 3.2. The pattern of protein bands produced (electrophoregram) is related to genetic constitution and can be considered as a 'fingerprint' of a variety. The 'fingerprints' can be used to identify unknown samples and mixtures, by single seed analysis.

2. Apparatus and Equipment

2.1 The Pharmacia GE-2/4 electrophoresis apparatus and EPS 400/500 power supply have been successfully used, but any suitable vertical electrophoresis system should give comparable results.

2.2 Chemicals

All chemicals should be of 'Analytical Reagent' grade or equivalent.
Acrylamide ('specially purified for electrophoresis')
Bisacrylamide ('specially purified for electrophoresis')
Urea
Glacial acetic acid
Glycine
Ferrous sulphate
Ascorbic acid
Hydrogen peroxide (or ammonium persulphate and TEMED)
Monothioglycerol (or 2-mercaptoethanol)
Pyronine G (or methyl green)
Trichloroacetic acid
Ethanol
2-chloroethanol
PAGE Blue G-90 (or PAGE Blue 83) (or any similar reagent equivalent to the 'Coomassie Brilliant Blue' series of dyes).

2.3 Solutions

2.3.1 Extraction solution - wheat: pyronine G (or methyl green) (0.05% w/v) in 2-chloroethanol (25% v/v) (keep cold)

- barley: pyronine G (or methyl green) (0.05% w/v) in 2-chloroethanol (20% v/v) containing urea (18% w/v) and monothioglycerol (or 2-mercaptoethanol) (1% v/v) (keep cold or prepare fresh)

2.3.2 Tank buffer solution: glacial acetic acid (4ml) and glycine (0.4g), made up to 1l with water; keep cold.

2.3.3 Gel buffer solution: glacial acetic acid (20ml) and glycine (1.0g), made up to 1l with water; keep cold.

2.3.4 Staining solutions: (1) trichloroacetic acid (100g) in 1l of water, (2) PAGE Blue G-90 (or PAGE Blue 83) (1g) in ethanol (100ml).

3. Procedure

3.1 Extraction

Single seeds are crushed with pliers or similar suitable instrument and transferred to 1.5ml polypropylene centrifuge tubes. Extraction solution (2.3.1) (0.2ml for wheat, 0.3ml for barley) is added, the contents of the tubes are thoroughly mixed and the tubes are allowed to stand overnight at room temperature. The tubes are centrifuged at 18000xg and the supernatants used for electrophoresis.

3.2 Preparation of the gel

Clean and dry gel cassettes are assembled, according to the design of the equipment. Treating the glass plates with a silicon spray prior to assembly can facilitate subsequent removal of the gel. Alternatively, the gel cassettes can incorporate a plastic backing sheet (eg 'Gel Bond PAG', FMC Corporation). This supports the gel during subsequent operations. The volume of gel mixture required will vary depending on the equipment used. To make 100ml of gel medium, gel buffer (2.3.3) (approx. 60ml) is taken and the following added - acrylamide (10g), bisacrylamide (0.4g), urea (6g), ascorbic acid (0.1g), ferrous sulphate (0.005g). The solution is stirred and made up to 100ml with gel buffer solution. Freshly prepared 0.6% (v/v) hydrogen peroxide solution (0.35ml per 100ml of gel medium) is added, mixed quickly and the gel poured. Note that the gel mixture can be cooled to near freezing prior to the addition of the peroxide to slow down the rate of polymerisation, which should be complete in 5-10 minutes. An acrylic 'comb' is placed in the top of the cassette, to make wells in the gel. The gel mixture should over-fill the cassette, or by over-layed with water, to ensure satisfactory polymerisation of the upper surface.

Note that as an alternative to the hydrogen peroxide catalyst, it is possible to use ammonium persulphate (0.1ml of 10% (w/v) solution, freshly prepared) and TEMED (0.3ml, full strength) added to the gel mixture prior to pouring the gel.

3.3 Electrophoresis

The acrylic comb is carefully removed from the gel and the sample wells washed with tank buffer. The electrophoresis tank is filled with an appropriate volume of tank buffer (2.3.2) (depending on the equipment used). Samples (10-20µl of clarified supernatant) are loaded into the wells using a syringe and the gel placed in the tank, ensuring that the sample wells are completely filled. Electrophoresis is carried out at 500V (constant voltage) for twice the time taken for the pyronine G marker dye to leave the gel, or three times if methyl green is used as a tracking dye. Note that the anode (positive electrode) must be at the top of gel. Water or other coolant should be circulated through the buffer tank to maintain the temperature at 15-20°C.

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3.4 Fixing and staining

At the end of the electrophoresis, the power is switched off. The gel cassette is removed from the tank, opened and the gel placed in a plastic box containing 5-10ml of 1% PAGE G90 (or PAGE Blue 83) in 200ml of 10% trichloroacetic acid (2.3.4). The box should be agitated gently. Staining is complete in 1-2 days and de-staining is not usually needed. Precipitated stain should be carefully scraped from the surface of the gel. The gel is washed in water to enhance the stain and can then be examined or photographed. Any blue background in the gel is removed by washing in 10% (w/v) trichloroacetic acid. Gels can be stored in sealed polythene bags at 4°C for many months without deterioration.

[End of Annex III and of document]