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REVISION OF TGP/7: SOURCE OF PROPAGATING MATERIAL

Document prepared by experts from the European Union

1. The purpose of this document is to present a proposal for guidance on source of propagating material for inclusion in a future revision of document TGP/7.

2. The following abbreviations are used in this document:

TC:	Technical Committee
TC-EDC:	Enlarged Editorial Committee
TWA:	Technical Working Party for Agricultural Crops
TWC:	Technical Working Party on Automation and Computer Programs
TWF:	Technical Working Party for Fruit Crops
TWO:	Technical Working Party for Ornamental Plants and Forest Trees
TWPs:	Technical Working Parties
TWV:	Technical Working Party for Vegetables

BACKGROUND

3. The Technical Committee (TC), at its forty-ninth session held in Geneva from March 18 to 20, 2013, noted that information on the influence of the method of vegetative propagation and origin of propagating material, taken from within the plant, on future plant development and characteristic expression and how this might be addressed in Test Guidelines would be presented to the Technical Working Party for Fruit Crops (TWF) and Technical Working Party for Ornamental Plants and Forest Trees (TWO), at their sessions in 2013 by experts from the European Union (see document TC/49/41 "Report on the Conclusions", paragraph 81).

4. The draft of guidance on source of propagating material, prepared by the experts from the European Union, is contained in the Annex to this document.

5. The TWC is invited to consider the proposed guidance on source of propagating material, as presented in Section IV "Guidance for drafting Test Guidelines" of the Annex to this document.

[Annex follows]

ANNEX

SOURCE OF PROPAGATING MATERIAL

I. INTRODUCTION

In horticultural circles a wide range of methods of vegetative propagation are found. On a commercial scale, the most frequently applied methods are the following:

- softwood cuttings,
- hardwood cuttings,
- leaf cuttings,
- division of rhizomes or plant clusters,
- runners,
- daughter bulbs,
- micro-propagation,
- grafting.

Certain methods such as leaf cuttings are limited to only very few crops (e.g. *Gesneriaceae*, *Begonia*, non-variegated *Sanseveria*). In the few crops to which this method can be applied it is usually used for all varieties and it does thus not lead to complications in the DUS test. Therefore, this method shall not be further dealt with. The same counts for crops efficiently propagated by runners (e.g. *Fragaria*) and daughter bulbs (e.g. *Tulipa*). However, where crops are alternatively propagated by softwood or hardwood cuttings or by grafting, this can result in complications in the DUS testing.

Micro-propagation may be applied either as an alternative technique to all or as the only commercially interesting method of propagation to some crops (e.g. *Orchidaceae*, *Bromeliaceae*). Micro-propagation as well as various other *in-vitro* techniques are today common practice in plant breeding (e.g. embryo rescue), in making plants virus free, in plant (mass) propagation and in conditioning for propagation (e.g. rejuvenation). Each technique and method may have both, a direct impact or late effects on the phenotype and thus on the DUS test.

For the purpose of this document, "micro-propagation" shall have the same meaning as the commonly used terms: *in vitro* techniques, "*in vitro* culture", and "tissue culture".

In certain crops the origin of the propagating material from within the mother plant may also have great influence on the further appearance of the daughter plants. This must therefore also be taken into account in addition to the method of vegetative propagation.

The purpose of the document is to investigate how the method of propagation could impact the outcome of the DUS test and how to avoid wrong decisions on the compliance with the DUS requirements.

II. VEGETATIVE PROPAGATION THROUGH CUTTINGS

Cuttings – commonly taken from shoots or branches, rarely from roots – show characteristic behavior for each type: softwood cuttings root and grow on faster than hardwood cuttings. In contrast to tip cuttings, cuttings from central or basal plant parts have their apical dominance broken and the plants resulting from them will be comparable to pinched or cut back plants with multiple shoots whereby a direct correlation between number of adventitious shoots and their size may be expected. When characteristics like "number of shoots", "plant width", "plant density" etc. are assessed the type of cutting used in plant propagation becomes crucial.

As long as all varieties of a given crop are propagated through the same type of cuttings, no effects on the DUS test need to be anticipated. Plants derived from cuttings taken from central parts of the shoot and plants derived from tip cuttings may be comparable if the latter once were pinched.

Whilst for most herbaceous plants, the question of the origin of propagating material from within the mother plant is more of relevance for an efficient (mass) propagation than it is for the comparability of samples in the DUS test this questions may be of crucial importance when dealing with certain woody plants: Topophysis and cyclophysis of the cutting must be taken into account as they do not only affect the ability to root but also their growth habit, their ability to branch and to flower.

Where branches of orthotropic growing plants of a variety are used to produce plagiotropic growing plants (e.g. *Abies*, *Araucaria*, *Picea* and *Pseudotsuga*) these plants must not be treated as new varieties but merely

as different growth of existing varieties. Likewise, where a plagiotropic plant produces occasionally orthotropic growing basal shoots these should not be treated as off-type shoots.

Plagiotropic plants may be of great ornamental value and they may be the only plants on the market. Where orthotropic plants of the variety are not available for comparison and where it is not a variety (e.g. merely a single plant- see UPOV/EXN/VAR, paragraph 5) the DUS test will have to be carried out on the plagiotrop type.

In species where topophysis and cyclophysis effects are known the corresponding Test Guidelines should define the material – including the origin of the propagation material from within the mother plant – to be assessed in the DUS trial. Breeders and providers of reference material should provide full details, possibly by replying to special questions to be formulated in the Technical Questionnaire.

III. EFFECTS OF *IN VITRO* CULTURE

In vitro culture may affect the expression of nearly any characteristic. Morphological and functional alterations have been reported in relation to rooting, growth habit, flowering and fruiting. DUS examiners are commonly confronted with problems like unequal growth of plants, atypical branching (loss of apical dominance), stunned or elongated shoots, loss or appearance of leaf variegation, poor flowering etc.

These alterations may be of temporary or permanent nature. Permanent changes occurring during tissue culture may be due to the activation of transposons, the segregation of chimeras or any “ordinary” mutation as may also happen at any time *ex vitro*. Plants being permanently altered no longer belong to the initial variety and they are therefore to be treated as off-types; reference is made to document TGP10/1 (section 4: Uniformity assessment on the basis of off-types). Likewise, epigenetic variation (e.g. methylation) – often being a side effect of genetic transformation – occurring in tissue culture (or *ex vitro*) results in the expression of characteristics which is not based on the genome. If single plants are concerned these plants could not be treated as off-types; if all plants of a given “variety” are concerned this must – in the absence of a genetic basis for the expression of the characteristics in question – not result in the grant of a title of protection. The difficulty a DUS examiner may be faced with lies in the distinction between these different alterations. In the first case the sample would have to be regarded as not suitable for the conduct of the DUS test; in the second case a possibly wrongly declared compliance with the distinctness requirement will have to be corrected resulting in the nullification of a right granted.

Reasons behind the effects of *in-vitro* culture

When plant material – be it whole plants, parts of plants, single cells or groups thereof (e.g. callus), protoplasts or seeds – is cultivated *in vitro* there is always a certain growing medium used. Growing media contain amongst others growth regulators (plant hormones). The precise composition of the growing medium and the growth regulators added depend on the aim of using *in vitro* culture. In addition to the growth regulators applied by the laboratory, plants themselves are producing these hormones.

Size of the explant

Besides the growth regulators there are other reasons for differences between *in vitro* and conventionally propagated plants. In general, micro-cuttings transferred on a rooting substrate *ex vitro* are considerably smaller than conventional cuttings. In addition, the transplant shock is of more importance. Additional time must be allowed to catch up in growth. Such difference may be of particular importance where micro-propagated plants are compared to plants propagated through division of rhizomes, daughter bulbs or tubers as these organs carry an important reserve of plant nutrients allowing rapid growth after planting. The DUS examiner has to ensure that plants of all varieties in trial really are at the same stage of development and have comparable reserves of plant nutrients (e.g. young plants with tubers vs. merely rooted cuttings before tuber development).

Light regime

Furthermore, plant cultivation *in vitro* is done under artificial light and (usually) under long day conditions. The sometimes observed poor flowering of micro-propagated plants may have its origin here.

Plant hormones

Plant hormones affect nearly all aspects of plant development. The effect (and the direction of the effect as being positive or inhibitory) that plant hormones have does not only depend upon its concentration but also

on the concentration of other hormones. *Ex vitro*, plants produce all hormones themselves which does not hinder them to react on hormones applied externally.

There are five distinct classes of plant hormones: auxin, cytokinin, abscisic acid, gibberellin, and ethylene. The first four classes are composed of many different chemicals being different in structure but similar in their effect on plants; they may vary from one plant species to the next. Additionally, there are numerous synthetic products with comparable effects on plant development.

Number of subcultures

Controversial observations have been made with regard to the effect that the number of subcultures (propagation cycles) *in vitro* will have on the phenotype of the plants after transplanting. Where tissue culture goes along with a rejuvenation of the tissue explant this phenomenon will increase with each subculture until the tissue has been fully rejuvenated. The effect is observed especially in plants with distinct juvenile and adult forms of appearance. In commercial production such rejuvenation may be desired as it allows propagating plants by cuttings instead of grafting. In species where there are no distinct juvenile and adult forms the number of subcultures may have no impact.

Method of in vitro multiplication

Plantlets coming out of tissue culture may behave differently depending on how multiplication took place: *in vitro* tip cuttings are likely to continue growing like regular tip cuttings whereas divided explant clusters are likely to continue growing as multiple shoot plants. In particular where taking of tip cuttings was intended; the cuts however, were made too deep into the cluster or where clusters are divided into sub-clusters with unequal number of shoots the regenerated plants are likely to show unequal growth afterwards whereby the number of shoots may be in inverse correlation with their size.

Transposons

Finally, activation of transposons may occur resulting in varieties which may pass an initial DUS examination; however, their stability cannot be taken for granted. Where the national plant breeders' rights legislation provides for the possibility of a technical verification of protected varieties this should be applied.

Information provided by applicants to be considered in the DUS test

Plant breeders have often outsourced the tissue culture propagation to specialized laboratories. Even though in most cases the classic MS medium (a growing medium after Murashige and Skoog) – modified according to the specific needs of the crop and the laboratory's experience – will be used it is unlikely that commercial laboratories are willing to reveal its precise composition as this is treated as a business secret. The DUS examiner can therefore not expect to receive from the breeder full details on the growth regulators applied to the plants (or to their mother plants) in the DUS trial. Apart from the unreasonable administrative burden the submission of such details would mean for applicants detailed information is of limited use for the examiner as the effects may be very complex and are often circumstantial; the impact of *in vitro* culture is thus hardly predictable for the design of the DUS test. However, information on the fact that the candidate variety was subject to *in vitro* culture may nevertheless be meaningful for the conduct of the DUS test. The following three scenarios may come into play:

Scenario 1: tissue culture is the standard method of propagation meaning that all varieties propagated on a commercial scale are multiplied *in vitro*; the plant material being subject to the DUS test – candidate and reference varieties – came straight out of the flask without intermediate propagation: Even though the influence of *in vitro* culture on the phenotype may be of greatest importance its impact on the DUS test may be regarded as not being any more important than that of comparing plant material coming from different sources.

Example: *Phalaenopsis*

Scenario 2: distant mother or elite plants went through tissue culture meaning that several cycles of *ex vitro* propagation took place before plant material becomes subject to the DUS test: Here it may be assumed that the plant material has grown out of all late effects *in vitro* culture may have had.

Example: *Pelargonium*

Scenario 3: tissue culture is not the only method of propagation on a commercial scale. The plant material in the DUS trial – candidate or reference varieties – may come from different propagations: there are on the one hand the plants coming either straight out of tissue culture or they are direct descendants of such plants and on the other hand plants which were propagated conventionally. A combination of such material in trial may result in an erroneous decision on the compliance with the distinctness requirement as well as in distorted variety descriptions. The consequences for the DUS test are elaborated in section 2.3.

Example: *Rhododendron*

Consequences for the conduct of the DUS technical examination

Variation within the sample

Were variation within the sample is attributed to *in vitro* propagation no decision on the compliance with the uniformity requirement can be taken. It is up to the testing authority to either refuse the sample as being not suitable for the DUS test or to further propagate the plant material to let disappear the impact the *in vitro* culture had. In some cases cutting back the plants may lead to uniform regrowth; however, in some cases it may be sufficient to merely extend the technical examination by another growing cycle.

Rejuvenated plants

Examiners must be aware that certain plant species have morphologically clearly distinct juvenile and adult forms (e.g. leaf shape in *Hedera helix*) which may be transmitted to the daughter plant. Between plants of other species such distinction may be not be made with regard to their morphological characteristics but clearly with regard to their physiological characteristics (e.g. ability to root cuttings of *Syringa vulgaris*) which in turn influences their further growth. Examiners must therefore ensure that all variety descriptions are made on plants having the same (standardized) physiological age.

Late effects of synthetic plant hormones applied during micro-propagation

When tissue culture plantlets are transferred to a growing substrate *ex vitro* they still carry residual amounts of the synthetic plant hormones taken up from the *in vitro* growing medium. These residual amounts of synthetic plant hormones continue to affect plant development. How long such late effects can be observed does not depend on the absolute amount of residual synthetic plant hormones in the plant but on their concentration in the tissue and the on the concentration of hormones produced autonomously by the plant. The concentration will diminish in proportion to further plant growth. Further plant propagation *ex vitro* alone does thus not reduce the late effects of tissue culture; cuttings taken from plants shortly after they were taken out of tissue culture carry residual synthetic plant hormones in much higher concentrations than cuttings taken from plants that came out of tissue culture long time ago (provided that these plants showed important vegetative growth in the meantime). Plant hormones are moved around within the plant and may be temporarily stored and released later. Therefore, a mere cutback of plants with the intention to make the regrowth of different samples comparable is less efficient than producing new plants from cuttings.

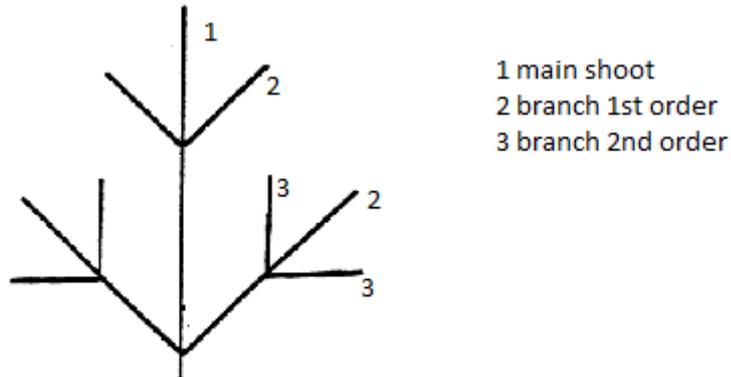
Late effects of other conditions of micro-propagation

Where plant material shows poor or premature flowering, the sample should be considered as not suitable for the conduct of the DUS test. It is up to the testing authority to refuse the sample or to remedy the insufficiency by either cutting back the plants or conducting another growing cycle. If these effects are uniformly found in all plants of a given variety the disturbing influence of *in-vitro* culture on the DUS trial is hard to detect in a single growing cycle.

IV. GUIDANCE FOR DRAFTING TEST GUIDELINES

For varieties conventionally propagated *ex vitro*, Test Guidelines – especially for crops which will not be pruned during the growing trial – may have to define the type of cuttings to be used to produce the sample for the DUS trial. It could be proposed that a chapter dealing with material to be used in DUS testing is added to TGP 9 “Examining Distinctness”: For crops where topophysis and cyclophysis effects are known requirements for the propagation material including their origin from within the mother plant must be particularly prescriptive to ensure comparability of varieties. The Technical Questionnaire should contain questions allowing the examiner to assess whether the plant material propagated by the applicant for the commercial exploitation of the variety is also suitable for the conduct of the DUS test or whether other material has to be provided similar to the example in TG/96/4 (*Picea abies* (L.) Karst.) where it is specified

under point II 'Material Required': "*The plant material supplied should ... preferably not be obtained from in vitro propagation. If the plants are grafted, the rootstock used should be indicated. Scions should be selected in a way that expressions caused by topophysis reactions are avoided.*" The Technical Questionnaire only asks applicants to specify whether the variety originated as a seedling, mutant or if it was discovered. It is proposed that where it was a mutant or a discovery, applicants should be asked to indicate (e.g. by indicating the relevant branch in a drawing) from which part of the mother plants the initial material of the candidate variety was taken. Below is the drawing as used in TG/96/4, chapter VIII modified for this purpose:



With regard to micro-propagation, the Test Guidelines may specify whether such plant material may be used for the DUS test. In crops where tissue culture is not the only applied method of propagation, applicants need to provide precise information – e.g. in the Technical Questionnaire – on which plant generations went through tissue culture rather than detailing the composition of the growing medium. Point 9.2 of the Technical questionnaire could be change into (modifications in *italics*):

“9.2 The plant material should not have undergone any treatment which would affect the expression of the characteristics of the variety, unless the competent authorities allow or request such treatment. If the plant material has undergone such treatment, full details of the treatment must be given. In this respect, please indicate below, to the best of your knowledge, if the plant material to be examined has been subjected to:

- | | | | |
|-----|--|---------|--------|
| (a) | Microorganisms (e.g. virus, bacteria, phytoplasma) | Yes [] | No [] |
| (b) | Chemical treatment (e.g. growth retardant, pesticide) | Yes [] | No [] |
| (c) | Tissue culture (<i>tick the appropriate box</i>): | | |
| | <i>i) plant material to be examined was micro-propagated</i> | | [] |
| | <i>ii) plant material to be examined was propagated ex vitro but its mother plants went through tissue culture</i> | | [] |
| | <i>iii) plant material to be examined as well as its direct mother plants were propagated ex vitro however distant mother plants (e.g. elite material) went through tissue culture</i> | | [] |
| | <i>iv) neither the plant material to be examined nor any of its ancestors were subject to tissue culture</i> | | [] |
| (d) | Other factors | Yes [] | No [] |

Please provide details for where you have indicated “yes”.

.....”

For those crops for which tissue culture is used, the Test Guidelines could foresee regularly more than one growing cycle which would firstly allow the examiner to detect non-declared micro propagation and secondly to let late effects of tissue culture grow out, thereby reducing the risk of taking a wrong decision on distinctness.

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