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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

Geneva

TECHNICAL WORKING PARTY FOR VEGETABLES

Fiftieth Session
Brno, Czech Republic, June 27 to July 1, 2016

ADDENDUM TO

NEW ISSUES ARISING FOR DUS EXAMINATION

Document prepared by the Office of the Union

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This document contains presentations to be made at the fiftieth session of the Technical Working Party for Vegetables (TWV), as follows:

- Annex I: "Vegetatively propagated varieties in a normally seed propagated species", by an expert from the Netherlands;

- Annex II: "Resistance-specific molecular markers", by an expert from the Netherlands;

- Annex III: "Effect of seed Priming on vegetable DUS tests" by an expert from the Community Plant Variety Office of the European Union (CPVO)

[Annexes follow]

ANNEX I

VEGETATIVELY PROPAGATED VARIETIES IN A NORMALLY SEED PROPAGATED SPECIES
BY AN EXPERT FROM THE NETHERLANDS

Background

- The method of propagation should not influence the expression and observation of characteristics
- In some vegetable crops such as tomato, pepper, etc., techniques and methods for breeding and propagation nowadays are used different from the classical ones: instead of propagation by seed, vegetative propagation.
- The identity material consists of rooted cuttings.
- Nb.: Grafted cuttings are not accepted as identity material.

Pepper and tomato: Vegetatively propagated plant material to submit

- The DUS test is performed with young rooted cuttings (basic material) provided by the applicant;
- If a second cycle is needed, new rooted cuttings are required;
- The quality of the material is very dependent on the source of the material; i.e. the propagation by the applicant;
- It is very difficult to compare the vegetatively propagated material with seed propagated material, because the growing cycles are not simultaneous

Pepper and tomato: Consequences for characteristics

- Seedling characteristics cannot be observed;
- Characteristics like plant height, time of beginning of flowering and time of maturity are influenced by the development stage of the plant material;
- Resistance characteristics: additional (p.e. inoculation/type of medium, reading etc.) or new methods necessary especially for soil borne diseases.

Pepper and tomato
Approach to perform a satisfactory examination

- In case of doubts about a vegetatively propagated variety possibly directly derived from an existing seed propagated variety, DNA analysis may be used;
- Another option would be cuttings of similar seed propagated varieties;
- The plant material needs to meet clearly defined conditions on quality, health, size, root system;
- Vegetatively propagated material of known seed propagated example varieties to be included in trials to calibrate the expression for these characteristics;

Pepper and tomato
Assessment of consequences for DUS testing

- Are there morphological differences when one variety is both vegetatively propagated and seed propagated?
- Are there morphological differences when cuttings are taken from young plants or older plants?
- In vegetatively propagated varieties: is it possible to observe the characteristics Plant height, Time of flowering and Time of harvest maturity?
- What consequences for DUS-testing are there when examining a vegetatively propagated tomato or pepper variety?

Assessment DUS testing of vegetative varieties compared to seed varieties

- **Method**: Varieties where chosen to represent the whole range of expressions for the characteristics Plant height, Time of flowering and Time of harvest maturity.

- **Comparison of**
  - Plants from seedlings vs. plants from cuttings
  - Cuttings from young plants vs. cuttings from older plants
  - From cuttings only

Pepper and tomato

Results

Pepper:

- Plants from seedlings vs. plants from cuttings: no differences found.
- Cuttings from young plants vs. cuttings from older plants: no differences found.
- Observation of characteristics Plant height, Time of flowering and Time of harvest maturity was not possible due to an unequal development of plants from seedlings and plants from cuttings. Especially the cuttings had a different speed of root formation.

Pepper and tomato

Results

- Tomato:
  - Plants from seedlings vs. plants from cuttings: slightly lighter green leaves in all three varieties.
  - Cuttings from young plants vs. cuttings from older plants: no differences found.
  - Observation of characteristics Plant height, Time of flowering and Time of harvest maturity was not possible due to an unequal development of plants from seedlings and plants from cuttings.

Pepper and tomato
Results

- Pepper:
  - It is difficult to have all cuttings to form roots at the same time;
  - When both plants from seeds and plants from cuttings are in the same plant stage at the time of planting, no differences are found. To observe distinctness is therefore possible in the same trial. But, for good comparison of time-influenced characteristics like Plant height, Time of flowering and Time of harvest maturity, it is very important to have synchronized plants from seeds and plants from cuttings, planted in the same time in the trial. A growing instruction needs to be developed.

Pepper and tomato
Results

For Pepper resistance tests some slight adaptations are needed to the regular protocol:

Tobacco Mosaic Virus (TMV) – race 0, race 1-2 and race 1-2-3

- Use the youngest, fully developed leaf (length and age of the plant is not critical) instead of young plants at the stage of developed cotyledons - first pointing leaf. Inoculate the detached leaves according to the regular protocol. After 7-14 days, make observations according to regular protocol. Detached leaves of tobacco (N. xanthi) should be added to check the inoculum quality.

Potato Virus Y (PVY) – race 0

- After transplanting the cuttings, inoculate them according to the regular protocol.

Pepper and tomato
Results

- Tomato
  - By taking cuttings, the intensity of leaf colour becomes slightly darker green than in plants from seeds. To be able to observe distinctness between a vegetatively propagated candidate and seed propagated comparisons, the comparisons should be grown from cuttings as well. This will make the trial more expensive.
  - It takes secure planning to have plants from seeds and plants from cuttings in the same plant stage when planting.

Pepper and tomato
Results

For Tomato resistance tests adaptations are needed to the regular protocols:

Meloidogyne incognita

- When cuttings are ready for transplanting (ca. 13 days after cutting), transplant them into infected soil according to regular test protocol.
- Test may take approximately one week longer as cuttings may be stronger against Nematodes.

Verticillium sp. (Va and Vd) – race 0 and Fusarium oxysporum f. sp. lycopersici race 0 (ex 1) and race 1 (ex 2)

- When cuttings are ready for transplanting (ca. 15 days after cutting), immerse the roots in a spore suspension with a spore concentration 5 times higher than regular. This high spore concentration is necessary to break through the mature-plant-resistance.

Tomato Mosaic Virus (ToMV) – strain 0

- After transplanting the cuttings, grow them for ca. 7 days more until the plants are developing well. Then, inoculate them according to the regular protocol.

Potato Virus Y (PVY) – race 0

- After transplanting the cuttings, inoculate according to the regular protocol

Conclusions and discussion

- DUS testing of vegetatively propagated varieties needs more labour and planning to synchronise with seed varieties;
- Protocols are needed for taking and growing cuttings;
- Protocols are needed for resistance tests;
- The testing of vegetatively propagated varieties is more expensive.

- Is it acceptable that the DUS examiner propagates vegetative applications/comparison varieties for use in DUS tests?

[Annex II follows]

ANNEX II

RESISTANCE-SPECIFIC MOLECULAR MARKERS
BY AN EXPERT FROM THE NETHERLANDS

Resistance-specific molecular markers

Amanda van Dijk, Naktuinbouw Variety Testing
Hedwich Teunissen, Naktuinbouw R&D

TGP/15/1

Model: Characteristic-Specific Molecular Markers

Molecular markers can be used as a method of examining DUS characteristics on the following basis:

- same number of individual plants as for the examination by a bioassay;
- verification of the reliability of the link between the marker and the characteristic;

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- different markers for the same characteristic are different methods for examining the same characteristic;
- markers linked to different genes conferring expression of the same characteristic are different methods for examining the same characteristic;
- markers linked to different regulatory elements for the same gene conferring expression of the same characteristic are different methods for examining the same characteristic.

- Example: “Gene Specific Marker for Herbicide Tolerance”, CMS in Brassica.

Current situation

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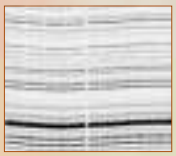
DUS testing tomato

- Resistance / Susceptibility for the obligatory diseases (information on the TQ) is used to select relevant reference varieties (grouping characteristics)
- Information on TQ for a candidate variety must be confirmed
- Confirmation is done by bioassay
- PCR test can be performed when problems in bioassay arise, as extra confirmation
- Some cases bioassay is not available, not possible (quarantine status as for Rs, TYLCV, TSWV), difficult to perform and/or to reproduce (false positives and false negatives or mild symptoms as in Fol)

Proposed strategy



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1. PCR result	Resistance marker present (dominant marker)	Resistance marker absent (dominant marker)	Homozygous resistant or heterozygous (co-dominant marker)	Homozygous susceptible (co-dominant marker)
2. Conclusion DNA	Resistant	Susceptible, or a mistake in the test, or Resistant (based on a different gene)	Resistant	Susceptible, or Resistant (based on a different gene)
3a. TQ info **RES**	Okay: conclusion resistant	Not okay: bioassay	Okay: conclusion resistant	Not okay: bioassay
3b. TQ info **SUSC**	Not okay: bioassay	Confirmation by bioassay (# plants)	Not okay: bioassay	Okay: conclusion susceptible

Benefits of PCR tests

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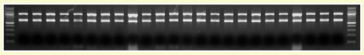
1. PCR results are complementary to bio-assay results. Increased reliability, especially in cases of difficult bioassays.
2. PCR tests are faster and often cheaper than bio-assays.

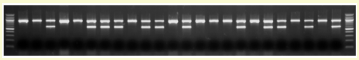
What is needed

- Robust (validated) marker system: repeatable results
- Validated marker: reliable link between marker and resistance characteristic
- Test on sufficient number of plants to fulfil the requirements
- Knowledge of gene(s) in use
- Preferably co-dominant marker

Lower band: 24 homozygous resistant plants



2 bands: 24 heterozygous resistant bands



12 plants (heterozygous) resistant and 12 plants (homozygous) susceptible

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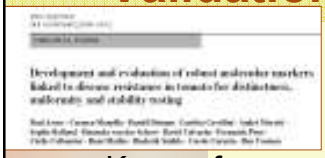
Validation of I-2 marker for FoI: 1



- Known from many literature references that resistance to *Fusarium oxysporum f. sp. lycopersici* race 1 is based on gene I-2, no other genes described (yet).
- Dominant marker described by Arens et al (2010).
- Validated in an international project, funded by CPVO.
- Dominant marker described by El Mohtar et al (2007) is preferred, due to easier performance in lab.
- A new combination of the susceptible allele from Arens and the resistant allele from El Mohtar resulted in a co-dominant marker which shows both alleles, ánd performs well in the lab.

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Validation of I-2 marker for Fol: 1

- 64 applications for DUS (2015) tested for Fol: 1 in a bioassay.
- 32 samples from the reference collection from other sources (breeding companies from outside The Netherlands)
- All (-1) samples show the same result in
  - Bioassay
  - Arens marker for I-2
  - El Mohtar marker for I-2

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Validation of I-2 marker for Fol: 1

- Sample X in bioassay 2015:

  17 plants resistant, 5 plants mild symptoms.
  Resistant control also 5 plants with mild symptoms.

Resistant control is known to be genetically uniform resistant.

- Sample X with Arens marker 2016:

  12 plants resistant, 10 plants susceptible

- Sample X with El Mohtar marker 2016:

  12 plants resistant, 10 plants susceptible

- Sample X in bioassay 2016:

  12 plants resistant, 10 plants severe symptoms

Conclusions: both markers validated, sample X not uniform, in case of doubt (mild symptoms) use the DNA marker test.

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Known markers for tomato resistances I

- ***Meloidogyne incognita***
  - MI1.2 (traditional PCR)
- ***Tomato Mosaic Virus (ToMV)***
  - Tm1 (traditional PCR)
  - Tm2 and $Tm2^2$ (tetra ARMS)
- ***Verticillium dahliae***
  - Ve1 and Ve2 (same locus) (tetra ARMS)
- ***Fusarium oxysporum f. sp. lycopersici***
  - I-2 gene (traditional PCR)

No significant problem with bioassays

1. Complementary results – more reliability
2. Faster and cheaper – cost efficienty
3. Management of reference collections:
   - To screen (old) reference varieties
   - Gain new/additional data for old varieties

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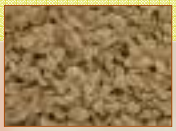
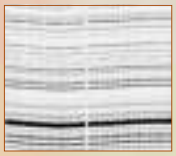
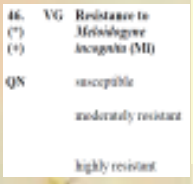
Correlation







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Verticillium dahliae Ve1 and Ve2 genes

Total # varieties	Correlation PCR vs TQ and Bioassay
94	98%
2	Ve1-ve2 new haplotype = intermediate resistance

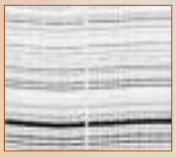
Discovery of combination of new alleles (=haplotype) that might explain newly observed intermediate resistance levels for Verticillium.

Meloidogyne incognita MI1.2 gene

Total # varieties	Correlation PCR vs Bioassay
130	99%, but uncertainty about differentiation between HR and IR
1 resistant fragment	Susceptible in bioassay

This candidate variety also had intermediate resistance levels for Ve.
This application was not registrated. Not DUS.

Correlation



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Tomato Mosaic Virus (ToMV) Tm2 and $Tm2^2$

Total # varieties	Correlation PCR vs TQ and Bioassay
100	100%

Fusarium oxysporum f. sp. lycopersici I-2 gene

Total # varieties	Correlation PCR vs TQ and Bioassay
196	100%

Known markers for tomato resistances II

TSWV reference:
Dianese E.C., Fonseca M.E.N., Goldbach R., Kormelink R., Inoue-Nagata A.K., Resende R.O., Boiteux L.S.(2009) Development of a locus-specific, co-dominant SCAR marker for assisted-selection of the *SW-5* (*Tospovirus* resistance) gene cluster in a wide range of tomato accessions. Mol Breeding (2010) 25:133-142.

TyLCV reference:

Verlaan M.G.: The Tomato Yellow Leaf Curl Virus Resistance Gene Ty-1 and TY-3 are allelic and Code for DFGD-Class RNA Dependent RNA Polymerases. PLOS Genetics March 2013 Volume 9 issue 3.

Patent:
http://www.google.com/patents/WO2012125025A1?cl=en

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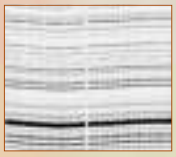
- ***Tomato Spotted Wilt Virus (TSWV)***
  - Sw-5 (TaqMan PCR)
- ***Tomato Yellow Leaf Curl Virus (TyLCV)***
  - Ty-1 / Ty-3 (Melt Curve analysis)

Problems:

TSWV:
- Quarantine pathogen in EU
- Difficult bioassay in a tent
- Trips
- Very instable virus
- Many false negatives sometimes false positives

TYLCV:
- Quarantine pathogen in EU
- White Fly
- Difficult bioassay based on Agrobacterium inoculation with transgen

Correlation



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TSWV Sw-5

Total # varieties	Correlation PCR vs TQ
118	100%

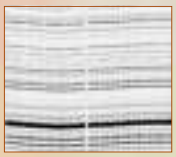
Total # varieties	Correlation PCR vs TQ and Bioassays
37	100%

TyLCV Ty-1/Ty-3

Total # varieties	Correlation PCR vs TQ
15	100%

Bioassay is not (yet) possible in NL.

Future perspective



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- ❑ PCR is complementary to the bioassay
- ❑ Faster and cheaper
- ❑ Good help to screen and manage the reference collection

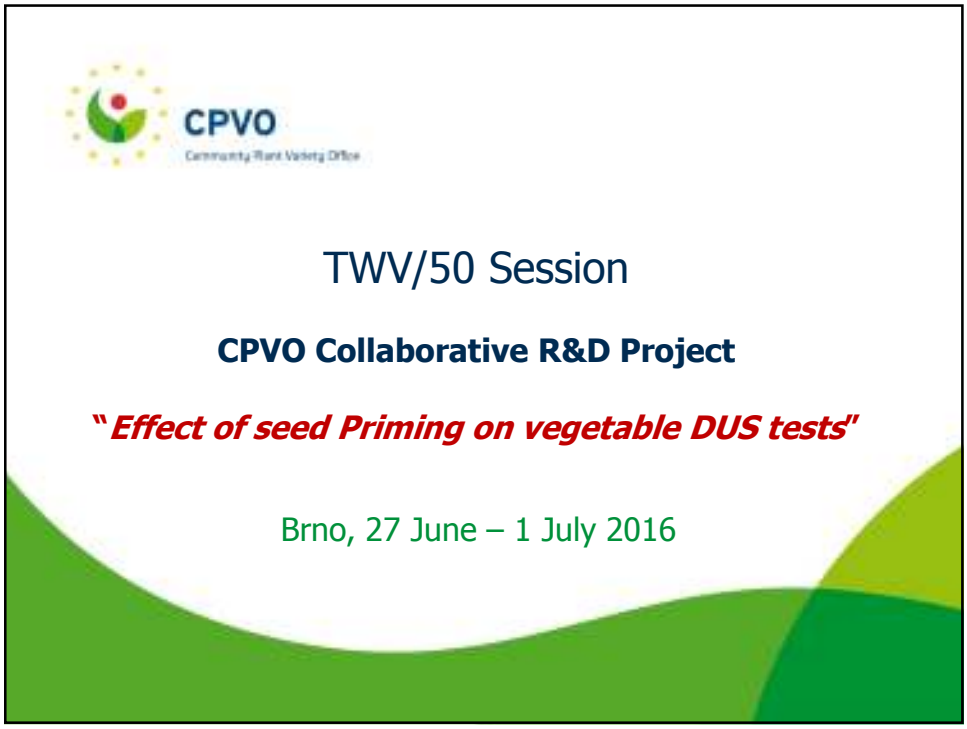
Proposal:
include DNA marker tests in the UPOV guideline tomato (TG/44/11) as an alternative method for the traditional bioassay

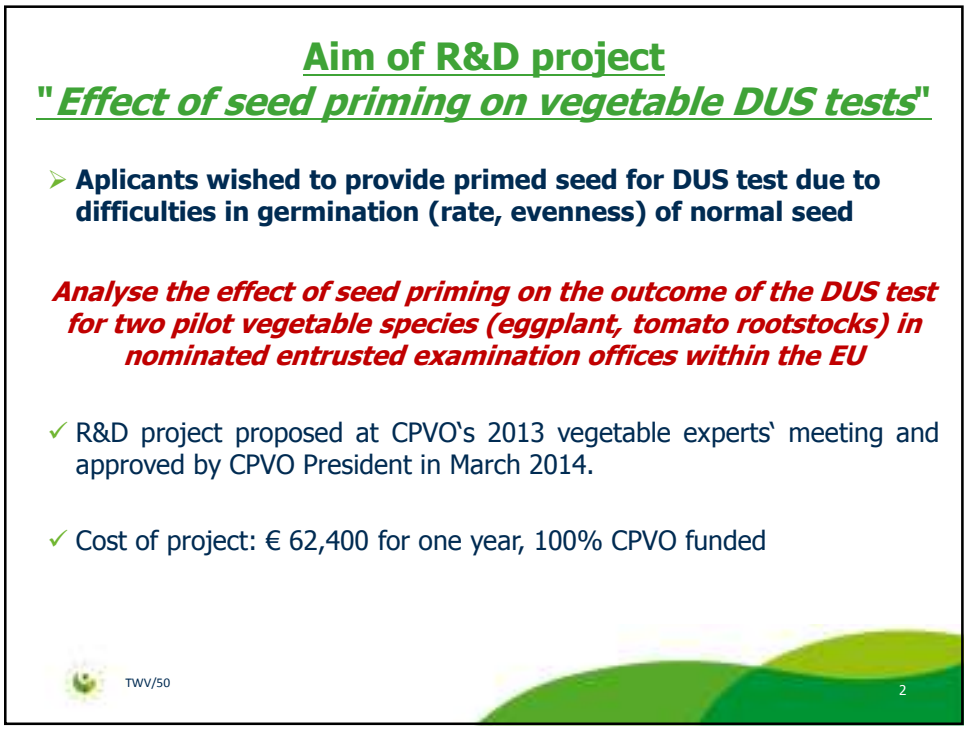
- Characteristic 48.2 Resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Fol) race 1
- Characteristic 51 Resistance to Tomato mosaic virus (ToMV)
- Characteristic 58 Resistance to Tomato spotted wilt virus (TSWV)

[Annex III follows]

ANNEX III

EFFECT OD SEED PRIMING ON VEGETABLE DUS TESTS
BY AN EXPERT FROM THE COMMUNITY PLANT VARIETY OFFICE OF THE EUROPEAN UNION (CPVO)





Partners involved in R&D project

- CPVO (coordinator)
- Natktuinbouw (NL): eggplant + tomato rootstocks
- GEVES (FR): eggplant
- OEVV/INIA (ES): tomato rootstock
- ESA

Technical details of R&D project

- 3 Varieties per species, delivered as primed and unprimed seed lots:
  - Eggplant: 'Adele', 'Brigitte', 'Dalia'
  - Tomato rootstock: 'He-Man', 'Protector', 'Unifort,
- Duplicate DUS tests for one year using CPVO protocols:
  - TP-117/1: Eggplant
  - TP-44/4: Tomato
  - TP-294/1: Tomato rootstock

Technical details of R&D project

- Special attention to be given to characteristics which may be influenced by the germination rate of seed sample (e.g. vigour, time of harvest maturity, etc.)
- Related study on gemination rates of primed/unprimed seed samples for reference collection purposes
  - 2nd germination test after six months
- CPVO to compile results from each partner examination office and conclude on study in 2015

..... *will primed seed samples be allowed for DUS tests ???*

Partner results

Naktuinbouw (NL) (*tomato rootstock + eggplant*)

- Trials carried out according to plan on both sets of species based upon prescribed methodology
- Better start for seedlings primed seed lots, but seedlings for non-primed seed lots then caught up
- Morphological characteristics in CPVO protocol for both species were unaffected by priming process
- Slight variations in symptoms for some disease resistance charcteristics in one tomato rootstock variety
- No germination loss for any sample after 6 months, but faster germination in some primed samples of tomato rootstocks

Partner results

OEVV/INIA (ES) (*tomato rootstock*)

- Trials carried out according to plan on both sets of species based upon prescribed methodology
- Better start for seedlings in 2 out of 3 primed seed lots, but seedlings for non-primed seed lots then caught up
- Expression of morphological characteristics in CPVO tomato & tomato rootstocks protocol were unaffected by priming process
- Slight difference in uniformity for Fusarium oxysporum race 2 (ex 3) in one tomato rootstock variety
- No germination loss for any sample after 6 months

Partner results

GEVES (FR) (*eggplant*)

- Trials carried out according to plan on both sets of species based upon prescribed methodology
- Faster seedling emergence in all 3 primed seed lots, but seedlings for non-primed seed lots then caught up
- Expression of morphological characteristics in CPVO eggplant protocol were unaffected by priming process
- No disease ressitance characteritics in testing protocol
- After 6 months, faster seedling emergence in all 3 primed seed lots, and decrease in germination for one non-primed seed lot
- GEVES expresed reservations in using primed seed samples for DUS test

Conclusions based upon the results from NL, ES, FR

- Results from all three R&D test sites correlate with each other
- Under the 2014 growth conditions, seed priming does not affect expression of characteristics of the two pilot crops
  - thus primed or non-primed seed sample would appear to give same outcome to a DUS test
- Primed seed samples germinated earlier and more evenly. No loss in germination for primed seed sample over a relatively short time period
- Based upon above assumptions of R&D project, primed seed samples could be acceptable for DUS test

Discussion issues emanating from R&D project

- If allowed, should samples be indicated as primed or not?
- Effects on DUS test between 1st and 2nd growing periods?
- Can results be extrapolated to other vegetable species?
- How long can a primed sample be stored (germination)?
  - *Indications that optimal lifespan of primed seed is 3 years*
- How to ensure upkeep/renewal of sample for variety collection?
- Are benefits mostly for applicants, or are there also gains to be made by examination authorities?

Implementation of R&D project results

- CPVO **agrees with possibility** to accept primed seed for tomato rootstocks and eggplant
- NL, ES and FR to analyse separtely whether to allow the submission of primed seed for DUS tests conducted by them for the two aforesaid crops
- Examination authorities offering capability publish detailed seed submission requirements in CPVO's S2 Gazette (2016)
- First seed priming samples to be sown in DUS tests for candidate varieties starting early 2017
- Ongoing germination tests to establish longevity of primed seed samples under optimal storage conditions for DUS/variety collection purposes

[End of Annex III and of document]