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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

REVISED ADDENDUM TO

USE OF DISEASE AND INSECT RESISTANCE CHARACTERISTICS IN DUS EXAMINATION

Document prepared by the Office of the Union

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

- Annex I: "MatRef: a national network managing seeds and strains for disease resistance tests", by an expert from France;
- Annex II: "Harmonization of resistance tests to diseases for DUS testing: Harmores 2", by an expert from the Community Plant Variety Office of the European Union (CPVO);
- Annex III: "Resistance-specific molecular markers", by an expert from the Netherlands

[Annexes follow]

ANNEX I

MATREF - A NATIONAL NETWORK MANAGING SEEDS AND STRAINS FOR DISEASE RESISTANCE TESTS BY AN EXPERT FROM FRANCE

MatRef

a national network managing seeds and strains for disease resistance tests

Presented by France - 50th TWV, Brno 2016

GEVES
Expertise & Performance

GEVES missions

- Conduct studies for
  - Registration
  - Plant breeder's rights
  - Seed certification
- Training
- Seed companies monitoring
- Methodological research
- Expertise

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Use of disease resistance tests

- For registration or protection :
  - For DUS (mainly vegetables)
    - Majority of lab tests, carried out by SNES
    - Some tests carried out by INRA
    - Some tests carried out by seed companies (blind test)
  - For VCUS (other species)
    - Majority of field tests, carried out by SEV (network)
    - Lab tests carried out by SNES
    - Some tests carried out by INRA
- For seed companies, phytosanitary companies…

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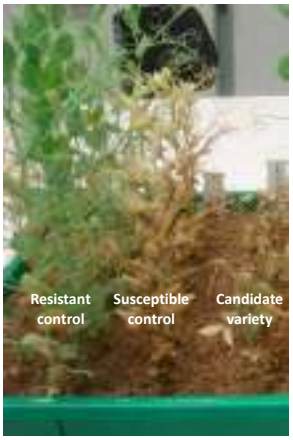
…but sometime we are facing discrepencies

Applicant's claim

Official observation



Requirements for resistance tests

We respect these 3 requirements for resistance tests

- Controls (S, IR, R) ➔ validated and available
- Differentials ➔validated and available
- Reference strains ➔ validated, available, stable and representative of natural conditions

2000 isolates in GEVES collection

250 reference isolates in GEVES

70 reference isolates in MATREF

We guarantee stable and representative strains.
We provide seeds of controls and differentials.

Seeds are checked for :
- germination
- health analysis
- expected behavior to the pathogen

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What is MatRef ?

- A network created in 2002
  - Public partners:
    - National regulatory authority : GEVES, coordinator
    - Research institute : INRA Avignon
  - Private partners: seed companies

- Managing MATERIAL of REFERENCE
  - Controls: susceptible, resistant cultivars
  - Differential hosts: for identification of races
  - Pests and pathogen strains

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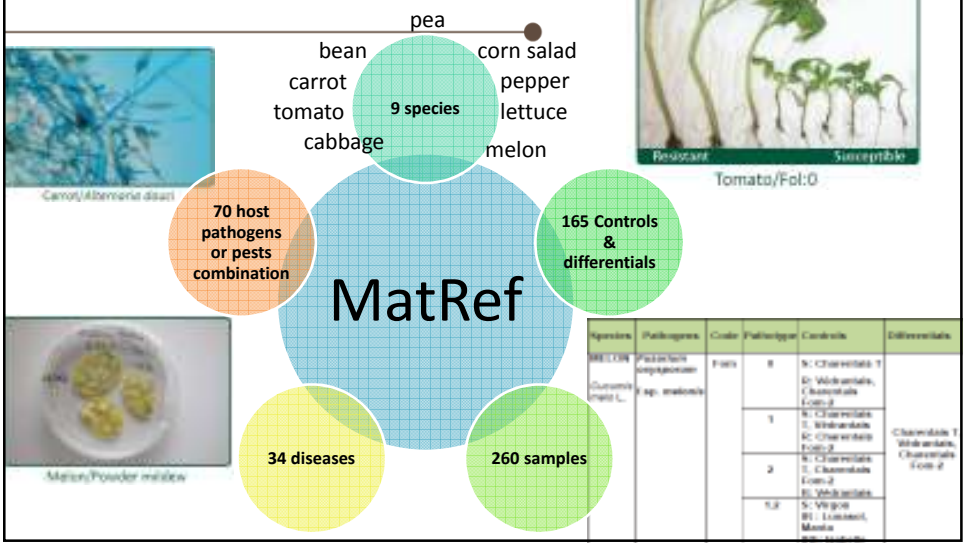
MatRef : overview



Aim of MatRef

- **To make available a validated reference collection** used in disease resistance tests for official labs in charge of registration or protection
- **To harmonize the controls and the isolate** between official labs (SNES & INRA) and breeders
- **To optimize the consistency** of results for disease resistance tests
- **To exchange information** about developments in new disease resistance tests, new resistance genes or new races, harmonization of terminology of pathogens, in collaboration with ISF working group and other EU country authorities
- **To enhance / improve** internal work
- To have a **global harmonization** of protocols
- To have **robust, common and unanimous results**

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Activities of MatRef

- **Task-sharing between network-members :**
  - *database management,*
  - *seeds multiplication and storage,*
  - *control of germination, of seed health and behaviour in relation to the pathogen*
  - *strains maintenance*
  - *distribution of seeds and strains*

Activities of MatRef

- **Providing of a collection of reference material, managed by database**
  - **Seed material**
    - GEVES or seed companies multiply
    - GEVES validates resistance / susceptibility behavior , but also germination and sanitary aspect
    - GEVES stores in an active bank at 4°C and back up at -18 °C
  - **Isolates**
    - GEVES validates aggressiveness and virulence on controls and differentials
    - GEVES and INRA (official laboratories) maintain as an active bank and store as long term back-up in 2 secure locations

Activities of MatRef

Immunofluorescence carried out to detect *Clavibacter michiganensis subsp michiganensis*

Seed health tests : quarantine & quality

To be able to distribute these differentials and controls to breeders and official labs, seed must be guaranteed free of disease, with at least , the list of quarantine disease

Species	16 pathogens
Carrot	*Xanthomonas hortorum* pv. *carotae*
Bean	*Pseudomonas savastanoi* pv. *phaseolica* *Pseudomonas syringae* pv. *syringae* *Xanthomonas axonopodis* pv. *phaseoli* (quarantine)
Lettuce	LMV (except for Bremia)
Melon*	*Acidovorax avenae* subsp *citrulli* Squash mosaic virus
Pepper	TMV PMMoV *Xanthomonas vesicatoria* & *X. axonopodis* pv. *vesicatoria* (quarantine)
Pea	*Pseudomonas syringae* pv. *pisi* *Pea seed-borne mosaic virus* **
Tomato	*Clavibacter michiganensis* subp *michiganensis* (quarantine) Pepino Mosaic Virus (quarantine) ToMV *Xanthomonas vesicatoria* & *X. axonopodis* pv *vesicatoria* (quarantine)

MATREF storage and distribution

Elisa carried out to detect LMV

* if regenerated out of France ** not compulsory

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Activities of MatRef

Distribution of reference material :

- **Free of charge** for MatRef members
- **Available for non-members** , with a financial contribution and a restricted seed quantity

*List of MatRef material and prices : **www.geves.fr***
Products & services / MATREF Network

*Contact : **matref@geves.fr***

Seeds

Species (200 seeds/genotype)	Price
Carot, Cornsalad	€ 31.50
Bean, Lettuce, Pea	€ 42.00
Melon	€ 53.00
Pepper, Tomato	€ 63.00
Complete pack of Bremia differentials	€ 242.00

Strain

Pathotype	Price
1 *Bremia* race	€ 56.00
Nr:0	€ 74.00
Podosphaera xanthii	€ 74.00

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Activities of MatRef

These collections (strains and seeds) are now being used at

➔ **a national level by official French laboratories** (GEVES and INRA), **breeders** (Matref members and non-members), **research laboratories, schools and universities, …**

➔ **an international level by Matref members-breeders and Matref non-members-organization: GEVES counterparts** (Naktuinbouw-NL, CeRSAA-IT,…) **, research laboratories** (CPPSI-USA, Research Institute of Plant Production -SK,….) **seed companies, schools and universities, …**

Involvement of MatRef in research projects

CPVO · OCVV **R&D Project**

Harmores : **Harmo**nization of **res**istance tests to diseases for DUS testing
European projects set up and managed by GEVES in collaboration with partners

Harmores (2004-2006)

Bean
BCMNV/BCMV
Colletotrichum lindemuthianum
Pseudomonas savastanoi pv *phaseolica*

Tomato
Fol race 0 et race 1
Verticilium dahliae
ToMV 0

Harmores 2 (2012-2014)

Lettuce
Bremia lactucae

Pea
Fop race 1
Ascochyta pisi race C

Pepper
TMV:0
PMMoV:1.2
PMMov:1.2.3
PVY:0

GEVES organized 2 WS
- **Lettuce: 04/11/2014 , 23 part.**
- **Pea: 05/11/2014, 6 part.**

Harmores 3 (2016-2019)

Tomato
Fusarium oxysporun f.sp. *lycopersici* Race 0 (ex 1) and Race 1 (ex 2)
Meloidogyne incognita

Pea
Erysiphe pisi

Melon
Fusarium oxysporun f.sp. *melonis* race 1.2
Fusarium oxysporun f.sp. *melonis* race 2 (+ validation with also Fom:0 and 1)
Podosphaera xanthii

6 WS planned during the 3 years (organization : GEVES)

CASDAR - CTPS Caracterization of virulence of ***Podosphaera xanthii***, causing powdery mildew of melon, and development of a race codification system

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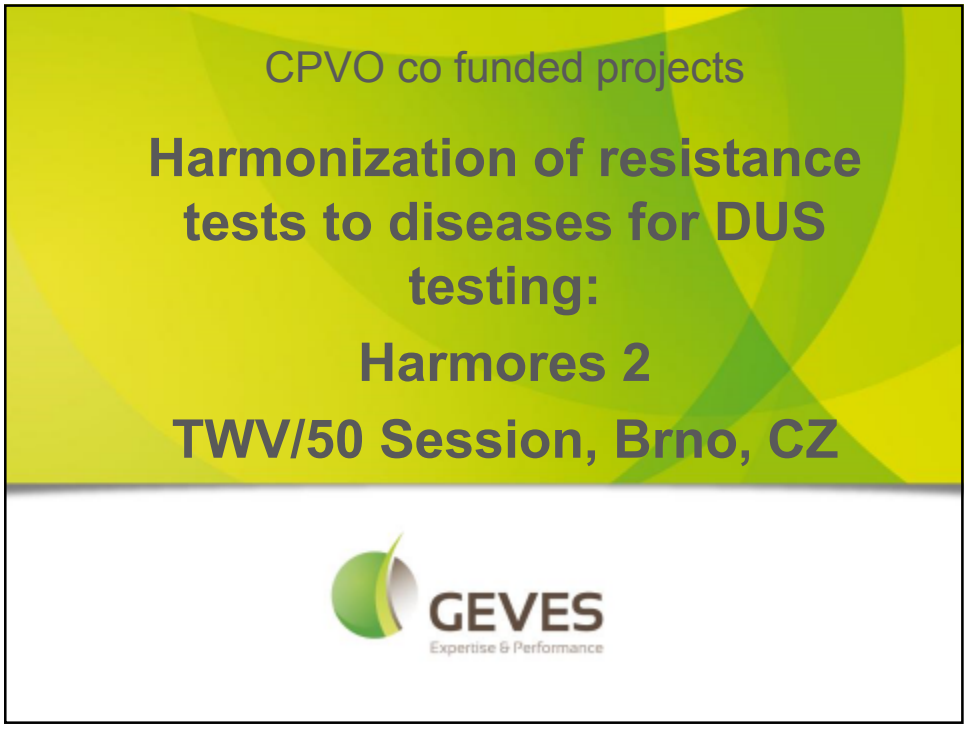
[Annex II follows]

ANNEX II

HARMONIZATION OF RESISTANCE TESTS TO DISEASES FOR DUS TESTING: HARMORES 2
BY AN EXPERT FROM THE COMMUNITY PLANT VARIETY OFFICE OF THE EUROPEAN UNION



Harmores 2 | Goal/deliverable | Results | Conclusion

Harmonize DUS resistance protocols:

- better coherence of results between countries
- between declarations of breeders and official tests,
- better definition and exchange of reference material
  - ✓ updated bibliography on the selected host/pathogen combinations
  - ✓ available reference isolates with maintainers laboratories
  - ✓ available reference resistant and susceptible controls
  - ✓ optimised culture conditions for all the studied pathogens
  - ✓ optimised test conditions
  - ✓ harmonised protocols to be proposed to CPVO

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Harmores 2 | Goal/deliverable | Results | Conclusion

- Phase 1 • *Description and comparison of the existing tests*
- Phase 2 • *Selection of common reference material*
- Phase 3 • *Harmonisation of protocols*
- Phase 4 • *Validation of harmonized protocols*

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Harmores 2 | Goal/deliverable | Results | Conclusion

• Pepper

		Tested	Validated	Selected
Nb of controls	**TMV: 0**	10	6	**5**
	PMMoV: 1.2	12	8	**5**
	PMMoV: 1.2.3	10	8	**4**
	PVY: 0	10	7	**6**
		Tested	**Validated**	**Selected**
Nb of strains	**TMV: 0**	5	2	**1**
	PMMoV: 1.2	5	2	**1**
	PMMoV: 1.2.3	4	1	**1**
	PVY: 0	4	1	**1**
		Tested	**Validated**	**Selected**
Nb of stage of inoculation	**TMV: 0**	2	2	**2**
	PMMoV: 1.2	2	1	**1**
	PMMoV: 1.2.3	2	1	**1**
	PVY: 0	2	1	**1**

TMV➔ 1 strain not validated as TMV: 0

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TMV **: comparison of strains**

Harmores 2 | Goal/deliverable | Results | Conclusion

• Lettuce

		Tested	Validated	Selected
Nb of substrate	**Bl: 24**	4	3	**3**
	Bl: 26	4	3	**3**
		Tested	**Validated**	**Selected**
Nb of notation scales	**Bl: 24**	3	1	**1**
	Bl: 26	3	1	**1**

Bremia **: comparison of substrates**

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Harmores 2 | Goal/deliverable | Results | Conclusion

- Lettuce
  - A workshop was planned on the 4th November 2014 to harmonize the different official notation scales: CPVO, UPOV and IBEB.
  - Objectif:
    - ✓ to draw the line between resistance and susceptibility around light or sparse sporulation,
    - ✓ to harmonize a common notation scale and interpretation of observed symptoms.
  - 25 people from national examination offices and ESA representatives of 7 European countries participated.
  - 21 "difficult to judge" varieties, on 3 substrates with 2 races

Common notation scale and interpretation rule

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Harmores 2 | Goal/deliverable | Results | Conclusion

RESISTANT	 No sporulation + no necrosis	 No sporulation + necrosis	 Weak sporulation (much less than susceptible control) + necrosis	 Weak sporulation less than susceptible control not evolving between second and third notation + necrosis	 In some cases very sparse sporulation can occur (without necrosis) and does not evolve between 2nd and 3rd notation
SUSCEPTIBLE	 Reduced sporulation (compared to susceptible control) without necrosis	 Normal sporulation without necrosis			
OTHER CASE			Normal sporulation (same level as susceptible control) with necrosis ➔ **in this case another test on bigger plants or other substrate must be undertaken**		

Harmores 2 | Goal/deliverable | Results | Conclusion

- Pea

		Tested	Validated	Selected
Number of controls	*Ascochyta pisi*	5	5	**5**
	Fusarium oxysporum f. sp. *pisi*	10	3	**3**
		Tested	**Validated**	**Selected**
Number of strains	*Ascochyta pisi*	2	2	**2**
	Fusarium oxysporum f. sp. *pisi*	3	1	**1**
		Tested	**Validated**	**Selected**
Number of stage of inoculation	*Ascochyta pisi*	2	1	**1**
	Fusarium oxysporum f. sp. *pisi*	2	2	**2**
		Tested	**Validated**	**Selected**
Number of notation scales	*Ascochyta pisi*	2	1	**1**
	Fusarium oxysporum f. sp. *pisi*	2	1	**1**

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Harmores 2 | Goal/deliverable | Results | Conclusion

- Pea
  - A workshop was planned 5th November 2014 to describe a harmonized notation scale of *Fusarium oxysporum* f. sp. *pisi*
  - Objectif:
    - ✓ Harmonization of a common notation scale
    - ✓ Definition of an interpretation rule
  - 5 people from national examination offices from 4 European countries participated.
  - Comparison on a panel of two methods of inoculation and two notation scale

↳Common notation scale and interpretation rule

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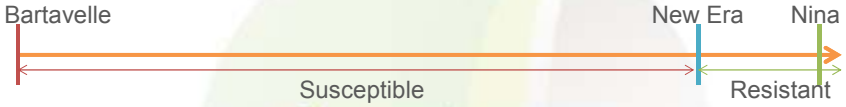
Harmores 2 | Goal/deliverable | Results | Conclusion

- Harmonized rules of decision

  ↳ Proposal for *Ascochyta pisi:* Interpretation of varieties with same level of resistance than Nina and/or Rondo and Madonna as resistant

  ↳ Proposal for *Fusarium oxysporum* f. sp. *pisi:* Interpretation of varieties with same level of resistance than New Era and/or Nina as resistant



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Harmores 2 | Goal/deliverable | Results | Conclusion

- Exemple of results for *Fusarium oxysporum* f. sp. *pisi*

Seed sample code	number of plants			Interpretation	Nb plants R	Nb plants S
	Note 0	Note 1	Note 2			
Sample N° 4			26	S		26
Sample N° 21		26		R	26	
Sample N° 41	26			R	26	
Sample N° 49		26		R	26	
Sample N° 57		26		R	26	
Sample N° 79			26	S		26
Sample N° 87			26	S		26
Bartavelle (S control)			26	S		26
New Era (IR control)		26		R	26	
Nina (R control)	26			R	26	

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Harmores 2 | Goal/deliverable | Results | Conclusion

- **Reference material**
  - Should be available:
    - ✓ Maintainers of isolates defined and indicated in protocols
  - Mention in each protocol “this protocol has been validated on…”
  - MTA shared, to be harmonized
  - A general rule for maintenance of isolates and controls established:
    - ✓ Availability in the network of maintainers
    - ✓ If outside of maintainers network, forward the request to original provider of the isolate
    - ✓ Control by maintainers of isolates on differentials and control varieties twice every 5 years

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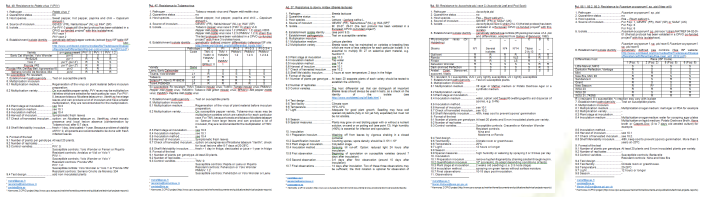
Harmores 2 | Goal/deliverable | Results | Conclusion

- Dissemination
  - 5 updated robust protocols proposed to CPVO



  - Presentation to the new notation scale to IBEB
  - Presentation of a poster on harmonized lettuce notation scale during the Eucarpia Leafy Vegetables congress in April 2015
  - Proposition of revision of the Lettuce UPOV guideline
  - Planned communication at general Eucarpia congress in 2016.

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[Annex III follows]

ANNEX III

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;

and….

nak / tuinbouw

and….

- 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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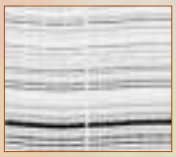
DUS testing tomato

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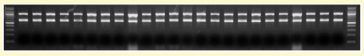
1. PCR results are complementary to bio-assay results. Increased reliability, especially in cases of difficult bioassays.
2. PCR tests are faster and often cheaper than bio-assays.

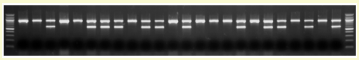
What is needed

- 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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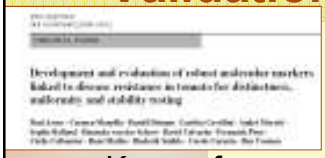
Validation of I-2 marker for FoI: 1



- 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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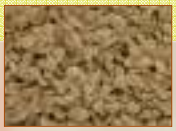
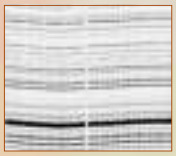
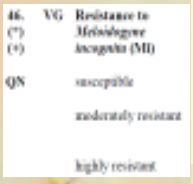
Correlation







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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

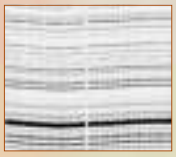
Discovery of combination of new alleles (=haplotype) that might explain newly observed intermediate resistance levels for Verticillium.

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 Tm2²

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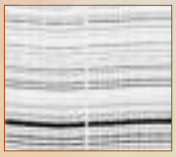
- ***Tomato Spotted Wilt Virus (TSWV)***
  - Sw-5 (TaqMan PCR)
- ***Tomato Yellow Leaf Curl Virus (TyLCV)***
  - Ty-1 / Ty-3 (Melt Curve analysis)

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%

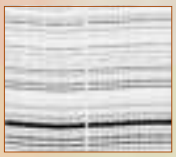
Total # varieties	Correlation PCR vs TQ and Bioassays
37	100%

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)

[End of Annex III and of document]