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International Union for the Protection of New Varieties of Plants

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Technical Working Party for Vegetables

TWV/55/11

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PARTIAL REVISION OF THE TEST GUIDELINES FOR LETTUCE

Document prepared by an expert from the Netherlands

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1. The purpose of this document is to present a proposal for a partial revision of the Test Guidelines for Lettuce (document TG/13/11 Rev.).

2. The Technical Working Party for Vegetables (TWV), at its fifty-fourth session hosted by Brazil and organized by electronic means, from May 11 to 15, 2020, agreed that the Test Guidelines for Lettuce (*Lactuca sativa* L.) (document TG/13/11 Rev.) be partially revised for Characteristic and Ad. 53 “Resistance to *Lettuce mosaic virus* (LMV) Pathotype II” for the addition of a DNA marker test (see document TWV/54/9 “Report”, Annex III).

3. The following changes are proposed:

(a) Change to point 9.3 “Control varieties” to the current bio-assay method of Ad. 53 “Resistance to *Lettuce mosaic virus* (LMV) Pathotype II”

(b) Addition of a new method for DNA marker test to Ad. 53 “Resistance to *Lettuce mosaic virus* (LMV) Pathotype II”

4. The proposed changes to are presented below in highlight and underline (insertion) and ~~strikethrough~~ (deletion).

Proposed changes to Ad. 53 “Resistance to *Lettuce mosaic virus* (LMV) Pathotype II”

Current wording

Ad. 53: Resistance to *Lettuce mosaic virus* (LMV) Pathotype II

1. Pathogen	*Lettuce mosaic virus*
2. Quarantine status	no
3. Host species	lettuce - *Lactuca sativa* L.
4. Source of inoculum	GEVES[1] (FR) or Naktuinbouw[2] (NL)
5. Isolate	pathotype II (isolates LMV-0 and Ls1 belong to the same pathotype)
6. Establishment isolate identity	resistant and susceptible controls
7. Establishment pathogenicity	susceptible control inoculation
8. Multiplication inoculum	
8.2 Multiplication variety	susceptible control
8.3 Plant stage at inoculation	2-3 leaves
8.4 Inoculation medium	0,05 M PBS, 0,25% (w/v) Na_2SO_3 0,5% $C_5H_{10}NNaS_2.3H_2O$, 4% carborundum and 5% active charcoal
8.5 Inoculation method	rubbing; optionally repeat after 4 d; 1-2 h high humidity after inoculation
8.6 Harvest of inoculum	homogenized fresh leaf in buffer (50% w/v); freeze-dried leaves can be kept less than 1 year in storage, long term storage at -80°C
8.7 Check of harvested inoculum	compare with mock inoculation with LMV buffer + carborundum + charcoal
8.8 Shelf life/viability inoculum	2 h at 4°C or on ice
9. Format of the test	
9.1 number of plants per genotype	at least 20
9.2 number of replicates	1
9.3 Control varieties	susceptible: Bijou (red), Hilde II (green), Sprinter (green), Sucrine (green) resistant: Capitan (green), Corsica (green), Diveria (red)
9.4 Test design	several mock-inoculated plants in the same tray
9.5 Test facility	climate chamber
9.6 Temperature	after inoculation 15-22°C
9.7 Light	12-16 h light ca. 5000 lux
10. Inoculation	
10.1 Preparation inoculum	fresh leaf ground in fresh LMV buffer incl. carborundum and active charcoal
10.3 Plant stage at inoculation	1st leaf well-developed at 1st inoculation, optionally 4 days later 2nd inoculation
10.4 Inoculation method	rubbing, rinse carborundum off
10.7 Final observations	21 days post inoculation
11. Observations	
11.1 Method	visual estimate of mosaic severity; compare with standards, preferably with standards of same growth type.
11.2 Observation scale	resistant = no symptoms susceptible = growth retardation, young leaves with mosaic, leaf curling
11.3 Validation of test	standards should conform to description
12. Interpretation of data in terms of UPOV characteristic states	classify resistant or susceptible per plant, see 11.2.
13. Critical control points	Sprinter is less susceptible than many other susceptible varieties, this variety can be used to detect low inoculation pressure in a specific experiment. anthocyanin coloration in leaves may mask mosaic symptoms and an earlier observation date for green varieties may be possible, depending on the reaction of the standard varieties in the test.

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Proposed new wording

Ad. 53: Resistance to *Lettuce mosaic virus* (LMV) Pathotype II

Resistance to pathotype II to be tested in a bio-assay (method i) and/or in a DNA marker test (method ii).

(i) Bio-assay

1.	Pathogen	*Lettuce mosaic virus*
2.	Quarantine status	no
3.	Host species	lettuce - *Lactuca sativa* L.
4.	Source of inoculum	GEVES[3] (FR) or Naktuinbouw[4] (NL)
5.	Isolate	pathotype II (isolates LMV-0 and Ls1 belong to the same pathotype)
6.	Establishment isolate identity	resistant and susceptible controls
7.	Establishment pathogenicity	susceptible control inoculation
8.	Multiplication inoculum	
8.2	Multiplication variety	susceptible control
8.3	Plant stage at inoculation	2-3 leaves
8.4	Inoculation medium	0,05 M PBS, 0,25% (w/v) Na_2SO_3 0,5% $C_5H_{10}NNaS_2.3H_2O$, 4% carborundum and 5% active charcoal
8.5	Inoculation method	rubbing; optionally repeat after 4 d; 1-2 h high humidity after inoculation
8.6	Harvest of inoculum	homogenized fresh leaf in buffer (50% w/v); freeze-dried leaves can be kept less than 1 year in storage, long term storage at -80°C
8.7	Check of harvested inoculum	compare with mock inoculation with LMV buffer + carborundum + charcoal
8.8	Shelflife/viability inoculum	2 h at 4°C or on ice
9.	Format of the test	
9.1	Number of plants per genotype	at least 20
9.2	Number of replicates	1
9.3	Control varieties	susceptible: Bijou (red), Hilde II (green), Sprinter (green), Sucrine (green) resistant: Capitan (green), Corsica (green), ~~Diveria (red)~~ Multired 80 (red)
9.4	Test design	several mock-inoculated plants in the same tray
9.5	Test facility	climate chamber
9.6	Temperature	after inoculation 15-22°C
9.7	Light	12-16 h light ca. 5000 lux
10.	Inoculation	
10.1	Preparation inoculum	fresh leaf ground in fresh LMV buffer incl. carborundum and active charcoal
10.3	Plant stage at inoculation	1st leaf well-developed at 1st inoculation, optionally 4 days later 2nd inoculation
10.4	Inoculation method	rubbing, rinse carborundum off
10.7	Final observations	21 days post inoculation
11.	Observations	
11.1	Method	visual estimate of mosaic severity; compare with standards, preferably with standards of same growth type.

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11.2	Observation scale	resistant = no symptoms susceptible = growth retardation, young leaves with mosaic, leaf curling
11.3	Validation of test	standards should conform to description
12.	Interpretation of data in terms of UPOV characteristic states	classify resistant or susceptible per plant, see 11.2.
13.	Critical control points	Sprinter is less susceptible than many other susceptible varieties, this variety can be used to detect low inoculation pressure in a specific experiment. anthocyanin coloration in leaves may mask mosaic symptoms and an earlier observation date for green varieties may be possible, depending on the reaction of the standard varieties in the test.

(ii) DNA marker test

Recessive gene *mo1* (with its alleles *mo1*^{1} or *mo1*^{2}) gives resistance to LMV pathotype II. Alleles for resistance *mo1*^{1} and *mo1*^{2} and the presence of the allele for susceptibility *mo1*^{0} can be detected by the co-dominant marker as described by V. Nicaise *et al* (2003). Specific aspects:

1.	Pathogen	*Lettuce mosaic virus* pathotype II
2.	Functional gene	*mo1* (with two alleles for resistance *mo1*^{1} and *mo1*^{2} and one allele for susceptibility *mo1*^{0})
3.	Probes and primers for Taqman PCR	
3.1.	Assay 1	to distinguish *mo1*^{1} genotypes from *mo1*^{0} and *mo1*^{2} genotypes (6 base deletion at nucleotide position 344-349):

Probe	DNA sequence '5-'3	Fluorophore color (optional)
Pr-del-mo1	GGCTCAAGGAGCTGACTTCTATTG	Texas Red (Susceptible)
Pr-del-mo1^1	GGCTCATGACTTCTATTG	6FAM-MGB (Resistant *mo1*^{1})

Primers	DNA sequence '5-'3
Fw-del-mo1	CAACAACATACATCGACCAA
Rev-del-mo1	CTTCCCACTTAGGCTCGAT

Sequence amplicon: '5-'3
The amplicon sequence of the *mo1*^{0} and *mo1*^{2} allele:
TTACAACAACATACATCGACCAAGCAAGTTGGCTCAAGGAGCTGACTTCTATTGTTTCAAGAATAAAATCGAGCCTAAGTGGGAAGACC
The amplicon sequence of the allele for resistance *mo1*^{1}:
TTACAACAACATACATCGACCAAGCAAGTTGGCTCATGACTTCTATTGTTTCAAGAATAAAATCGAGCCTAAGTGGGAAGACC

3.2.	Assay 2	to distinguish *mo1*^{2} genotypes from mo1^0 and mo1^1 genotypes (SNP at nucleotide position 228):

Probe	DNA sequence '5-'3	Fluorophore color (optional)
Pr-SNP228-mo1	CTCCCTCT**G**CTAAGTC	6FAM-MGB (Susceptible)
Pr-SNP228-mo1^2	ACTCCCTCT**C**CTAAGT	VIC-MGB (Resistant *mo1*^{2})

Primers	DNA sequence '5-'3
Fw-SNP228-mo1	GCATCCGCTCGAGCATTC
Rev-SNP228-mo1	CTACCCCAAGCGACTTGCTT

Sequence amplicon: '5-'3

The amplicon sequence of the *mo1⁰* and the *mo1¹* allele:

TCAGCATCCGCTCGAGCATTCTTGGACTTTCTGGTTCGATACTCCCTCT**G**CTAAGTCCAAGCAAGTCG CTTGGGGTAGTTCCATGCGCC

The amplicon sequence of the allele for resistance *mo1²*:

TCAGCATCCGCTCGAGCATTCTTGGACTTTCTGGTTCGATACTCCCTCT**C**CTAAGTCCAAGCAAGTCG CTTGGGGTAGTTCCATGCGCC

4.	Format of the test	
4.1	Number of plants per genotype	at least 20 plants
4.2	Control varieties	Homozygous allele for susceptibility *mo1⁰* present: Sprinter, Sucrine Homozygous allele for resistance *mo1¹* present: Capitan, Kanaryole Homozygous allele for resistance *mo1²* present: Corianas Mix DNA to have a heterozygous control
5.	Preparation	
5.1	Preparation DNA	Harvest per individual plant a part of a young leaf. Isolate total DNA with a standard DNA isolation protocol.
5.2	Preparation PCR	Pipette each DNA sample and a commercial real-time PCR mastermix into individual wells for assay 1 and for assay 2. Analyse the samples in a real-time PCR machine capable of reading the fluorophores of all the probes, with reaction conditions suitable for the mastermix used.
6.	PCR conditions	(detailed test protocol available through Naktuinbouw[5] (NL))

Assay 1:

	Temperature	time	ramping speed
Initial activation of enzyme	95°C	2' 00"	
40 cycles	95°C	0' 15"	5˚C/sec
	65°C	0' 48"	5˚C/sec

Assay 2:

	Temperature	time	ramping speed
	95°C	2' 00"	
40 cycles	95°C	0' 15"	5˚C/sec
	60°C	0' 48"	5˚C/sec

Analysis at end point RFU.

7.	Observations	
7.1	Obervations scale	

Assay 1:

Signal giving Fluorophore		
FAM (*mo1¹*)	Texas Red (*mo1⁰* or *mo1²*)	
-	x	Homozygous *mo1⁰* or *mo1²*, or heterozygous *mo1⁰* and *mo1²*
x	-	Homozygous *mo1¹*
x	x	Heterozygous *mo1⁰* and *mo1¹* or heterozygous *mo1²* and *mo1¹*
-	-	No result, repeat test

[5] Naktuinbouw: resistentie@naktuinbouw.nl

Assay 2:

Signal giving Fluorophore		
FAM (*mo1⁰* or *mo1¹*)	VIC (*mo1²*)	
(x) (FAM RFU << VIC RFU)	x	Homozygous *mo1²*
x	-	Homozygous *mo1⁰* or *mo1¹*, or heterozygous *mo1⁰* and *mo1¹*
x	(x) (FAM RFU >> VIC RFU)	Heterozygous *mo1⁰* and *mo1²* or heterozygous *mo1¹* and *mo1²*
-	-	No result, repeat test

7.2 Validation of the test

Control varieties should give the expected results.
A uniform variety will not show heterozygous plants except variety with (*mo⁰*+*mo1¹ or ²*) alleles combinations.

8. Interpretation of data in terms of UPOV characteristic states

The combination of the two PCR assays leads to the following predicted result in a bio-assay with LMV pathotype II:

		Assay 2 (*mo1²*)		
		absent	**present homozygous**	**heterozygous**
Assay 1 (*mo1¹*)	**absent**	susceptible (*mo1⁰*)	resistant (*mo1²*)	susceptible (*mo1⁰*/*mo1²*)
	present homozygous	resistant (*mo1¹*)	not possible (invalid)	not possible (invalid)
	heterozygous	susceptible (*mo1⁰*/ *mo1¹*)	not possible (invalid)	expected to be resistant, but not yet validated

Heterozygous plants (*mo1⁰*/*mo1¹* or *mo1²*) are susceptible in bio-assay, as *mo1* is a recessive gene.
Heterozygous plants ([*mo1¹*] + [*mo1²*]) need a conclusion from a bio-assay.
Varieties showing a mixture of genotypes (heterozygous plants, homozygous *mo1⁰* plants (susceptible predicted phenotype) and homozygous *mo1¹* or *mo1²* plants (resistant predicted phenotype)) are predicted to be non-uniform in the bio-assay.

In case the DNA marker test result does not confirm the declaration in the TQ, a bio-assay should be performed to observe whether the variety is resistant due to on another mechanism.

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