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| International Union for the Protection of New Varieties of Plants |  |

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| Technical Working Party for Vegetables  Fifty-Fifth Session Antalya, Turkey, May 3 to 7, 2021 | TWV/55/11  Original: English  Date: April 1, 2021 |

Partial revision of the Test Guidelines for Lettuce

Document prepared by an expert from the Netherlands

Disclaimer: this document does not represent UPOV policies or guidance

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.).

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).

The following changes are proposed:

1. Change to point 9.3 “Control varieties” to the current bio-assay method of Ad. 53 “Resistance to *Lettuce mosaic virus* (LMV) Pathotype II”
2. Addition of a new method for DNA marker test to Ad. 53 “Resistance to *Lettuce mosaic virus* (LMV) Pathotype II”

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]](#footnote-2) (FR) or Naktuinbouw[[2]](#footnote-3) (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) Na2SO3 0,5% C5H10NNaS2.3H2O, 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. |

*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).

1. Bio-assay

|  |  |  |
| --- | --- | --- |
| 1. | Pathogen | *Lettuce mosaic virus* |
| 2. | Quarantine status | no |
| 3. | Host species | lettuce - *Lactuca sativa* L. |
| 4. | Source of inoculum | GEVES[[3]](#footnote-4) (FR) or Naktuinbouw[[4]](#footnote-5) (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) Na2SO3 0,5% C5H10NNaS2.3H2O, 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. |
| 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 *mo11* or *mo12*) gives resistance to LMV pathotype II. Alleles for resistance *mo11* and *mo12* and the presence of the allele for susceptibility *mo10* 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 *mo11*and *mo12*and one allele for susceptibility *mo10*) |
| 3. | | Probes and primers for Taqman PCR | | |  |
| 3.1. | | Assay 1 | | | to distinguish *mo11* genotypes from *mo10* and *mo12* 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-mo11 | GGCTCATGACTTCTATTG | 6FAM-MGB (Resistant *mo11*) |  |  |  | | --- | --- | | Primers | DNA sequence ‘5-‘3 | | Fw-del-mo1 | CAACAACATACATCGACCAA | | Rev-del-mo1 | CTTCCCACTTAGGCTCGAT |   Sequence amplicon: ‘5-‘3  The amplicon sequence of the *mo10*and*mo12* allele:  TTACAACAACATACATCGACCAAGCAAGTTGGCTCAAGGAGCTGACTTCTATTGTTTCAAGAATAAAAT  CGAGCCTAAGTGGGAAGACC  The amplicon sequence of the allele for resistance *mo11*:  TTACAACAACATACATCGACCAAGCAAGTTGGCTCATGACTTCTATTGTTTCAAGAATAAAATCGAGCC  TAAGTGGGAAGACC | | | | | |
| 3.2. | Assay 2 | | | to distinguish *mo12* genotypes from mo10 and mo11 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-mo12 | ACTCCCTCT**C**CTAAGT | VIC-MGB (Resistant *mo12*) |  |  |  | | --- | --- | | 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]](#footnote-6) (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 | | | | | | |
| 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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