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EPPO STANDARD - DIAGNOSTICS

PM 7/ 30 (3) Beet necrotic yellow vein virus

Specific scope: This Standard describes a Diagnostic Protocol for beet necrotic yellow vein virus.

This Standard should be used in conjunction with PM 7/76 *Use of EPPO Diagnostic Protocols*[1].

Specific approval and amendment: This Standard was originally developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and interlaboratory comparison in European countries. Approved as an EPPO Standard in 2003–09. First revision approved in 2006–09. Second revision approved in 2021–08.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Rhizomania disease of sugar beet was first reported in Italy (Canova, 1959) and has since been reported in many EPPO countries (EPPO, 2021). The disease causes economic loss to sugar beet (*Beta vulgaris* var. *saccharifera*) by reducing yield. Rhizomania is caused by beet necrotic yellow vein virus (BNYVV), which is transmitted by the soil protozoan, *Polymyxa betae* (family Plasmodiophoraceae). The virus can survive in *P. betae* resting spores (cystosori) for more than 15 years. The virus is spread by movement of soil, primarily on machinery, sugar beet roots, stecklings, other root crops such as potato, and in composts and soil. Water is important in the spread of the fungal vector; drainage water, ditches and irrigation with water from infected crops can thus favour the spread of the disease. In addition to the high water content of soil, high temperature can stimulate development of *P. betae*. Disease-tolerant sugar beet cultivars are widely used in affected regions. Different pathotypes of BNYVV (A, B, J and P) have been described (Koenig et al., 1994) as well as *Rz1* resistance breaking strains. Tests developed to distinguish pathotypes and resistance breaking strains are not covered in this Diagnostic Protocol, which focusses on tests detecting all strains.

A flow diagram describing the diagnostic procedure for beet necrotic yellow vein virus is presented in Figure 1.

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

2 | IDENTITY

Name: *Beet necrotic yellow vein virus*
Acronym: BNYVV
Taxonomic position: Viruses, Riboviria, Benyviridae, *Benyvirus*
EPPO Code: BNYVV0
Phytosanitary categorization: EPPO A2 list no. 160; EU Protected Zone Quarantine pest (Annex III)

Note. Virus nomenclature in Diagnostic Protocols is based on the latest release of the official classification by the International Committee on Taxonomy of Viruses (ICTV, Release 2019, https://talk.ictvonline.org/taxonomy/). Accepted species names are italicized when used in their taxonomic context, whereas virus names are not, corresponding to ICTV instructions. The integration of the genus name within the name of the species is currently not consistently adopted by ICTV working groups and therefore species names in Diagnostic Protocols do not include the genus name. Names of viruses not included in the official ICTV classification are based on first reports.

3 | DETECTION

Rhizomania affects all subspecies of *Beta vulgaris*[2] and also spinach (*Spinacia oleracea*).

3.1 | Symptoms

3.1.1 | Leaves

Symptoms can often be seen very clearly from aerial photographs, as well on the ground, and consist of distinct yellow patches (Figure 2). On inspection the following may be noted:

- Translucent, pale lettuce-green to lemon-yellow foliage
- Yellow veining following the midrib of the leaf (Figure 3)
- Upright foliage with elongated petioles and narrowed leaf laminae (Figure 4)
- Plants stunted and/or wilted (possibly without leaf symptoms).

[2]The taxonomy of *Beta vulgaris* is mostly unresolved and the text has been simplified compared to the previous version of the Protocol. In the EPPO online Global Database (EPPO, 2021) all cultivated forms are grouped in *B. vulgaris* subsp. *vulgaris*.

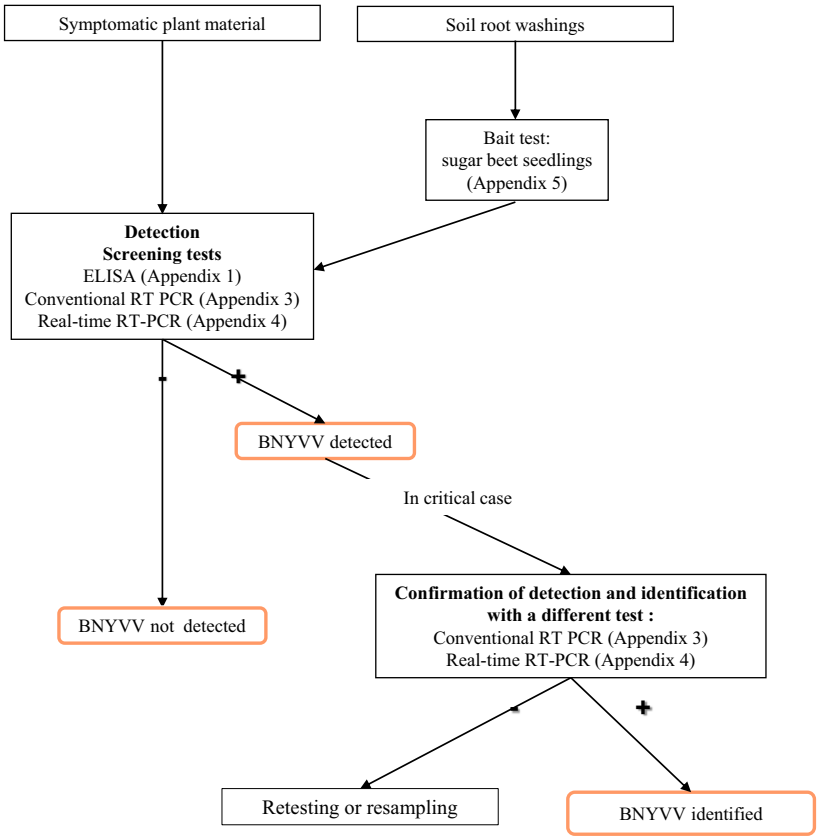

FIGURE 1 Flow diagram describing the diagnostic procedure for beet necrotic yellow vein virus

FIGURE 2 Typical symptoms of rhizomania in the field: a distinct yellow patch of infected sugar beet. Courtesy: Fera Science limited, York (GB). Crown copyright

FIGURE 3 Foliar symptoms of rhizomania: yellow veining following the midrib of the leaf. Courtesy: Fera Science Limited, York (GB). Crown copyright

FIGURE 4 Foliar symptoms of rhizomania: pale green leaves, upright foliage, narrowed leaf laminae. Courtesy: Fera Science Limited, York (GB). Crown copyright

3.1.2 | Roots

Root symptoms include:

- Dark brown bearded roots (this may be slight and/or a single lateral root with bearding near the tip) (Figure 5).
- Root constriction
- Pale yellow to dark brown vascular discoloration in transverse section.
- Nodules (small tumorous growths along the taproot).

The symptoms described above are rarely found together in a single plant. Rhizomania-tolerant cultivars may only show typical symptoms at high virus concentration.

3.2 | Test sample requirement and sample preparation

No sampling recommendation can be made for asymptomatic plant material.

3.2.1 | Symptomatic plant material

Samples should be taken from yellow patches in beet crops. A fork or spade should preferably be used to dig up the roots (especially in dry hard-baked soils). Care should be taken when lifting the beet as the root tip and laterals with 'rat tails' can easily break off and be left behind in the ground. Each sample should consist of the lower third of the taproot of five or six plants showing symptoms.

Sugar beet samples should be thoroughly washed in cold water to remove loose soil from the roots and dried on absorbent paper. Samples should then be placed in plastic bags for processing. A sample of 0.5–1 g of washed lateral or tap roots is used for testing.

Leaves are not used for routine analysis.

FIGURE 5 Typical external root symptoms of rhizomania showing the reduced size of the beet and root proliferation (bearding). Courtesy: Fera Science Limited, York (GB). Crown copyright

3.2.2 | Soil

In fields suspected of being infested by BNYVV, a total of 2.5 L of field soil should be taken by taking multiple samples when walking in a W shape across each of the sampling areas.

3.2.3 | Root washing

Testing of water with soil or decanted water (from root washing) may be required when the water or the decanted water is returned to the fields.

For water with soil, the volume of water with soil should allow for a final volume of 250 mL of solid phase to be obtained after decantation.

For decanted water, the minimum volume should be 3 L.

3.3 | Screening tests

3.3.1 | Plant material

3.3.1.1 | Serological tests

Double antibody sandwich (DAS)-ELISA and triple antibody sandwich (TAS)-ELISA are the most cost-effective screening tests. Further details on DAS-ELISA or TAS-ELISA and instructions to perform an ELISA test are provided in EPPO Standard PM 7/125 *ELISA tests for viruses* (EPPO, 2015a) and further information on antisera is provided in Appendix 1.

3.3.1.2 | Molecular tests

Different RT-PCR tests have been described for the detection of BNYVV. RNA extraction is described in Appendix 2. The following tests are recommended:

- A one-step conventional RT-PCR from Morris et al. (2001), described in Appendix 3
- A real-time RT-PCR from Harju et al. (2005), described in Appendix 4.

The nested RT-PCR recommended in the previous version of this Standard is no longer included due to diagnostic specificity issues. A TaqMan RT-PCR can be used for detection of BNYVV isolates containing additional genomic RNA (RNA 5) (Harju et al., 2002, 2005), but this test is not described in full in this version as it does not allow the detection of all strains of BNYVV.

A real-time RT-PCR has been developed by Anses (FR) but is not yet published and may be considered for inclusion in a future revision of this Standard.

3.3.2 | Soil

Soil samples can be tested by growing susceptible sugar beet seedlings in the soil (bait testing) in a glasshouse or in growing chambers. This bait test is also used for samples from root washing. Details are provided in Appendix 5.

Roots are subsequently tested by ELISA (see Section 3.3.1.1 and Appendix 1) or molecular tests (Appendices 3 and 4). The optimum time for bait testing is 3–4 weeks (see Appendix 5).

3.4 | Other tests

These tests are not used in routine diagnostics.

3.4.1 | Mechanical inoculation of virus to test plants

Test-plant inoculation can be used to increase BNYVV concentrations in plant tissue for subsequent testing with ELISA, molecular tests or electron microscopy. The procedure is described in Appendix 6.

3.4.2 | Electron microscopy

Immunoelectron microscopy (IEM), in combination with symptoms, can give an indication of the presence of BNYVV, when rod-shaped particles (Figure 6) are observed with predominant lengths of about 65–80, 150–160 and 290–310 nm and diameters of 18–20 nm.

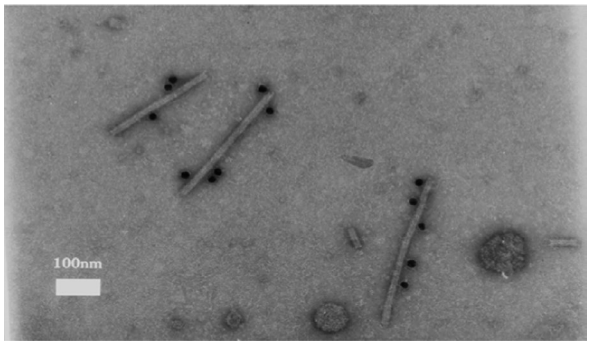

FIGURE 6 Electron micrograph of immunogold-labelling of BNYVV virus particles. Courtesy: Fera Science Limited, York (GB). Crown copyright

Virus particles can be confused with *Beet soil-borne virus*, which is a different rod-shaped beet-infecting virus, also transmitted by *Polymyxa betae*. Instructions to perform EM are provided in EPPO Standard PM 7/126 *Electron microscopy in diagnosis of plant viruses* (EPPO, 2015b).

4 | IDENTIFICATION

In critical cases (see PM 7/76; EPPO, 2018), confirmation of positive results should be performed using a method based on different biological principles or targeting different parts of the genome.

The molecular tests recommended in Section 3.3.1.2 can be used for confirmation.

5 | REFERENCE MATERIAL

Reference material is available from the Leibniz Institute (DSMZ) German Collection of Microorganisms and Cell Cultures GmbH contact@dsmz.de.

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CRITERIA

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from: I Renaudin (Anses, FR) isabelle.renaudin@anses.fr.

9 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

If you have any feedback concerning this Diagnostic Standard, or any of the tests included, or if you can provide additional validation data for tests included in this Standard that you wish to share please contact diagnostics@eppo.int.

10 | STANDARD REVISION

An annual review process is in place to identify the need for revision of Diagnostic Standards. Standards identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

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This Standard was originally developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and interlaboratory comparison in European countries. It was originally drafted by V Harju, Fera (former Central Science Laboratory), York (GB). The French soil bait test was drafted by F. Vey, Anses (former LNPV Fleury les Aubrais). This revision was prepared by a drafting group composed of M Loiseau (Anses, FR lead author), V Harju (Fera, GB), I Renaudin (Anses, FR), A Roenhorst (NVWA-NRC, NL) and R Weekes (Fera, GB).

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APPENDIX 1 - DATA ON ANTISERA FOR DAS-ELISA AND IEM

Instructions to perform DAS- ELISA and IEM are provided in EPPO Standards PM 7/125 *ELISA tests for viruses* (EPPO, 2015a) and PM 7/126 *Electron microscopy in diagnosis of plant viruses* (EPPO, 2015b), respectively.

The source of antibodies is critical. Several BNYVV antisera are available from various suppliers and may differ regarding their analytical sensitivity, analytical specificity and background noise. In general, it is recommended to follow the protocol provided by the supplier of the antiserum.

Performance characteristics for DAS-ELISA as provided by the companies.

1. Agdia (TAS-ELISA)

1.1. Analytical sensitivity (relative) 1:48 600 dilution of infected tissue (pathogen titre unknown)

1.2. Analytical specificity

Inclusivity

This test was designed to detect all strains and isolates of BNYVV. Nineteen distinct samples (different strains or isolates) of BNYVV have been experimentally proven to be detected.

Exclusivity

No cross-reaction observed with *Beet soil-borne mosaic virus* (BSBMS) from Wisler et al. (1999).

1.3. Diagnostic sensitivity 100% (evaluated on 19 samples)

1.4. Diagnostic specificity 100% (evaluated on 18 samples)

1.5. Selectivity

No matrix effect observed with *Beta vulgaris* (leaves, roots or stems), *Solanum tuberosum* (leaves, roots or stems) and *Nicotiana tabacum* (leaves, roots or stems).

2. Bioreba (DAS-ELISA)

2.1. Analytical sensitivity (relative) up to 1: 10 240 dilution

2.2. Analytical specificity

Inclusivity

The antibodies have been validated with a range of isolates (over 30 isolates) from Switzerland, Germany, France, Italy and Austria (F Häni and W Bitterlin, unpublished). All BNYVV isolates tested are recognized. There have been no reports of isolates not being recognized from over nearly 30 years of use from customers from numerous countries.

Exclusivity

Cross-reaction with related beet viruses not evaluated.

2.3. Selectivity

No reaction observed with healthy beet tissue.

3. DSMZ (DAS-ELISA)

3.1. Analytical sensitivity (relative) 1:1 000 dilution (predilution of sample 1:20 w/v)

3.2. Analytical specificity

Inclusivity

Evaluated with five isolates [two from Germany, one from former Yugoslavia (collected before 1997), one from Austria and one from France]. Detected all the different types/resistance breaking strains tested, such as A, B and P type.

Exclusivity

Beet soil-borne mosaic virus (BSBMV) gave a reaction of about 10% of the strength of that of the BNYVV positive control, but BSBMV has not been reported outside the USA.

No cross-reaction with *Beet black scorch virus* (BBSV), *Beet chlorosis virus* (BChV), *Beet mild yellowing virus* (BMYV), *Beet mosaic virus* (BtMV), *Beet oak leaf virus* (BOLV), *Beet ringspot virus* (BRSV), *Beet soil-borne virus* (BSBV), *Beet Virus Q* (BVQ), *Beet yellows virus* (BYV), *Beet western yellows virus* (BWYV)

3.3. Selectivity

No matrix effect noted with *Beta vulgaris*, *Beta macrocarpa*, *Datura stramonium*, *Solanum lycopersicum* and *Spinacia oleracea*.

4. Loewe (DAS-ELISA)

4.1. Analytical sensitivity (relative) 1: 2 000 000 dilution

4.2. Analytical specificity

Exclusivity

No cross-reaction noted with Broad bean stain virus (BBSV), Beet soil-borne virus (BSBV), Beet mosaic virus (BtMV), Beet yellows virus (BYV), Tobacco yellow vein virus (TYV), Turnip mosaic virus (TuMV), Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV).

4.3. Selectivity

Evaluated with *Beta vulgaris*, *Beta vulgaris* subsp. *vulgaris*, *Spinacia oleracea*, *Chenopodium quinoa*, *Brassica napus*.

APPENDIX 2 - RNA EXTRACTION

1. Commercial kit

RNeasy Plant Mini kit from Qiagen provides satisfactory results (I Renaudin and P Gentit, unpublished data). For roots, 1 g of fresh root material is ground with 4.5 mL of RLT buffer (or 0.04 g of freeze-dried sample in 5 mL of RLT buffer). When a PCR test is performed after an ELISA test, 450 µL of ground samples in ELISA extraction buffer are centrifuged for 2 min at 11 000 *g*. The supernatant is removed and the pellet is resuspended in 450 µL of RLT buffer. The supplier's instructions are then followed. ELISA extracts can be stored at approximately 5°C when the molecular test is performed on the same day or frozen at approximately −80°C.

2. CTAB procedure

Modified from Chang et al. (1993). This procedure can be used for ELISA extracts.

2.1. *Solutions*:

CTAB stock buffer

Cetyl tetra ammonium bromide (CTAB), 2%	20 g
Tris-HCl pH 8.0 100 mM	100 mL
EDTA 20 mM	40 mL
Sodium chloride 1.4 M	81.8 g
Water	To make up to 1 L

The stock buffer can be autoclaved and stored at room temperature for at least 1 year.

CTAB grinding buffer

Add 1.0% sodium sulphite and 2% soluble polyvinylpyrrolidone-40 to the stock CTAB solution. The buffer will keep for at least 2 weeks at room temperature.

Chloroform: *isoamyl alcohol* (24: 1 v/v): Add 96 mL chloroform to 4 mL isoamyl alcohol.

4 M lithium chloride: Add 169.56 g of LiCl. Make up to 1 L with water.

5 M NaCl: Add 292.2 g of NaCl Make up to 1 L with water.

Isopropanol: Store at −20°C with water.

TE-SDS buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% (w/v) sodium dodecyl sulphate. Make up to 1 L with water.

2.2. *Protocol*

2.2.1. Root tissue

Grind 100–200 mg of root tissue to a smooth paste in a 10 × 15 cm 500 gauge polythene bag with 1–2 mL of CTAB grinding buffer. Pre-freezing of tissue (at −80°C or in liquid nitrogen may help with the grinding of some tissues). Proceed as explained in Section 2.2.3.

2.2.2. ELISA extracts

Centrifuge 1 mL of ELISA extract at 10 000 *g* for 2 min at room temperature. Discard the supernatant. Add 1 mL of CTAB grinding buffer. Proceed as explained in Section 2.2.3.

2.2.3. Procedure

Transfer 1 mL of the extracts (Sections 2.2.1 and 2.2.2) into a 1.5 mL microcentrifuge tube and incubate at 65°C for 10–15 min. After incubation, centrifuge the tubes in a microcentrifuge at 12 500 *g* for 5 min at room temperature. Remove 700 µL of clarified sap, place it in a fresh microcentrifuge tube and add an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and mix to an emulsion by inverting the tube. Centrifuge at 12 500 *g* in the microcentrifuge tube for 10 min at room temperature. Carefully remove the upper (aqueous) layer and transfer it to a fresh tube. Add an equal volume of chloroform:isoamyl alcohol (24:1 v/v), mix and spin as in previous step. Remove the aqueous layer, taking extra care not to disturb the interphase. Precipitate the RNA by adding an equal volume of 4 M lithium chloride (LiCl), mix well and incubate the sample(s) overnight at 4°C. Pellet the RNA by centrifugation for 25 min at 12 500 *g* at 4°C. Resuspend the resulting pellet in 200 µL of TE-SDS buffer. Precipitate the RNA by adding 100 µL of 5 M NaCl and 300 µL of ice-cold isopropanol, mix well, then incubate sample(s) at −20°C for 20–30 min. Centrifuge the samples for 10 min at 12 500 *g*. Decant off the salt/isopropanol and wash the resulting pellet by adding 400 µL of 70% ethanol. Centrifuge the sample for 4 min at 12 500 *g*. Decant off all the ethanol and leave the tube open to the air until completely dry (at least 45 min). Resuspend the dry pellet in 100 µL of RNase-free water.

APPENDIX 3 - RT-PCR TEST FROM MORRIS ET AL. (2001)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The following RT-PCR protocol is performed for the detection of *Beet necrotic yellow vein virus* (BNYVV).

1.2. The test was optimized by Morris et al. (2001) based on primers of Henry et al. (1995).

1.3. Oligonucleotides

	Primer	Sequence	Amplicon size
Forward primer	BNYVV016	5′-CGA-TTG-GTA-TGA-GTG-ATT-T-3′	500 bp
Reverse primer	BNYVV017	5′-ACT-CGG-CAT-ACT-ATT-CAC-TT-3′	

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. RNA extraction: see Appendix 2.

2.2. One-step RT-PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	To make up to 25 µL	N.A.
PCR buffer (Promega, Southampton, UK)	10×	2.5	1×
dNTPs	10 mM	0.5	0.2 mM
$MgCl_2$	25 mM	1.5	1.5 mM
Forward primer	5 µM	1.0	0.2 µM
Reverse primer	5 µM	1.0	0.2 µM
MMLV	200 U/µL	0.025	5 U
Taq polymerase (Promega, Southampton, UK)	5 U μL^{-1}	0.125	0.625 U
Subtotal		24.0	
RNA		1.0	
Total		25.0	

N.A. : not applicable

2.2.2. RT-PCR conditions: reverse transcription step at 37°C for 30 min, followed by 30 cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Finally, 3 min at 72°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product[3]). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as nad5 (Menzel et al., 2002).

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC (and if relevant IC): a band of 500 bp is visualized.

When these conditions are met

- A test will be considered positive if a band of 500 bp is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that in virology bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting conventional PCR products.

4. Performance characteristics available

Validation data available from a test performance study involving 11 laboratories performed in the framework of DIAGPRO performed in 2002.

The test performance study in different European laboratories was performed with the MMLV reverse transcriptase and a Taq polymerase from Promega. *Note that kits might have changed since the validation data was generated.*

4.1. Analytical sensitivity: not available
4.2. Analytical specificity: not available
4.3. Diagnostic sensitivity: 89%
4.4. Diagnostic specificity: 93%
4.5. Data on repeatability: not available
4.6. Data on reproducibility: not available

[3] *Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products, gBlocks and whole-genome amplicons).*

APPENDIX 4 - REAL-TIME RT-PCR TEST FROM HARJU ET AL. (2005).

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The following RT-PCR protocol is performed for the detection of *Beet necrotic yellow vein virus* (BNYVV).

1.2. The test was developed by Harju et al. (2005).

1.3. Primers and probe were designed within the P21 gene of the RNA 2 capsid protein of BNYVV sequence of Miyanishi et al. (1999) (accession number AB018628).

	Primer	Sequence	Amplicon size
Forward primer	BNYVV-CP 26F:	5′-CAT GGA AGG ATA TGT CTC ATA ATA GGT T-3′	71
Reverse primer	BNYVV-CP 96R	5′-AAC ACT CAC GAC GTC CGA AAC-3′	
Probe	BNYVV-CP 56T	5′-[6-FAM]-TGA CCG ATC GAT GGG CCC G-[BHQ1]-3′	

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. RNA extraction: see Appendix 2.

2.2. Real-time RT-PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	8.75	N.A.
RT-PCR buffer (AgPath ID One-Step RT-PCR, Applied Biosystems)	2×	12.5	1×
Forward Primer	10 mM	0.75	0.3 mM
Reverse Primer	10 mM	0.75	0.3 mM
Probe	10 mM	0.25	0.1 mM
RT-PCR enzyme mix (AgPath-ID One-Step RT-PCR, Applied Biosystems)	25×	1	1×
Subtotal		24	
RNA		1	
Total		25	

N.A. : not applicable

2.2.2. RT-PCR conditions: reverse transcription step at 45°C for 30 min, DNA polymerase activation at 95°C for 10 min, followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product[4]). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as nad5 (Botermans et al., 2013).

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.

[4]Laboratories should take additional care to prevent risks of cross-contamination when using cloned PCR products.

- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential or if it produces an exponential amplification curve with a cycle cut-off value greater than or equal to 32 (see below).
- Tests should be repeated if any contradictory or unclear results are obtained.

Note. The cycle cut-off value of 32 was obtained by Anses (FR) using the equipment and chemicals as described in this appendix. The cycle cut-off value was determined using the ROC curve approach (Delacour et al., 2005). When using the extraction procedure, equipment and chemicals as described in this appendix, cycle cut-off values above 32 could be obtained for non-infected sample. Thus, if the cycle cut-off value is above 32, the test will be considered negative. As a cycle cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance characteristics available

Validation data available from a test performance study organized in 2017 with eight European laboratories.

4.1. Diagnostic sensitivity data

Diagnostic sensitivity without threshold 96%; with the defined threshold 98%.

4.2. Diagnostic specificity data

Diagnostic specificity without threshold 64%; with the defined threshold 98%.

4.3. Data on repeatability

Repeatability without threshold 83%; with the defined threshold 95%.

4.4. Data on reproducibility

Reproducibility without threshold 77%; with the defined threshold 95%.

APPENDIX 5 - SOIL BAIT TESTING

This protocol was developed by INRA (Dijon, FR) and evaluated by Anses (formerly LNPV), and has been used since 1987 for routine analysis (Anses, 2020).

1. Soil samples

Collect a 2.5 L soil sample from the field. If the soil sample is not too wet and sufficiently friable, mix it well and use directly for testing. Air-dry the soil if necessary, pulverize using a hammer, sieve it through a 5 mm sieve and then mix it thoroughly prior to testing.

Six pots should be prepared per soil sample.

a) Fill six 150-mL disposable cups with drainage holes drilled in the base with the sample.
b) Place the six pots together in a tray. Sow about 20 to 30 seeds of a susceptible cultivar in each pot. Cover the seeds with a thin layer of soil sample. Cover each pot with a plant pot saucer, or equivalent, to prevent drying out of the soil before emergence. Plants should be grown in a growing chamber at approximately 25°C under suitable light (15 h day light) and 80% relative humidity. Saucers should be removed as soon as the seedlings emerge. The soil (including the surface) should always be kept moist by regular watering. Do not water from the top but water directly into the tray. A layer of water should be always kept in the bottom of the tray.
c) After 3–4 weeks of growth, roots from individual pots should be washed with clean water. Roots are cut and approximately 1 g is placed in a homogenization bag. A bag should only contain roots from one pot. Only five pots are used. The sixth pot can be used to add to the bags if there are not enough roots in the other five pots. Roots are tested by ELISA (Appendix 1) or molecular tests (Appendices 3 and 4).

2. Root washing: water with soil

The solid phase from water with soil is mixed with sand (ratio ¼ solid phase and ¾ sand) and six pots are filled up with 150 mL of the mix. Continue as in 1b.

3. Root washing: decanted water

450 mL of sterile growing medium should be mixed with 450 mL of sand. Six pots are filled up with 150 mL of the mix. Proceed as described in 1 but use the decanted water only to water the plants.

APPENDIX 6 - MECHANICAL INOCULATION TO TEST PLANTS TO INCREASE VIRUS TITRE

For the preparation of the inoculum, sugarbeet lateral roots are washed and ground in a mortar with a small spatulaful of celite and enough distilled water to make a thin paste. At least two test plants (such as *Chenopodium quinoa*, *Chenopodium amaranticolor*, *Tetragonia expansa*) should be inoculated at the stage of six or more fully expanded leaves by gently covering the

FIGURE 7 Chlorotic lesions of BNYVV in *Chenopodium quinoa*. Courtesy: Fera Science Limited, York (GB). Crown Copyright

leaves in root/celite suspension using a finger covered in a glove. After 5 min, plants are rinsed in tap water to remove debris and left covered overnight to exclude light. On the following day, the cover is removed and the plants are grown for 6–10 days at 18–20°C, with watering daily as required. It should be noted that mechanical transmission of beet isolates of BNYVV can be difficult (Willemen et al., 2002).

Inoculated leaves of test plants develop chlorotic or necrotic lesions after 5–7 days (Figure 7).