Diagnostics[1]
Diagnostic

Beet necrotic yellow vein virus (*benyvirus*)

Specific scope

This standard describes a diagnostic protocol for *Beet necrotic yellow vein virus* (*benyvirus*).

Specific approval and amendment

This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) through a partnership of contractor laboratories and intercomparison laboratories in European countries. Approved as an EPPO Standard in 2003-09.
Revision approved in 2006-09.

Introduction

Rhizomania disease of sugar beet was first reported in Italy (Canova, 1959) and has since been reported in more than 25 countries. 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* cystosori for more than 15 years. The symptoms of rhizomania, also known as 'root madness', include root bearding, stunting, chlorosis of leaves, yellow veining and necrosis of leaf veins. 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 favour the disease. In addition to high water content, high temperature can stimulate development of *P. betae*. Control measures include; cleaning soil from agricultural machinery after harvesting beets, avoiding the re-introduction to the farm of factory by-products, careful disposal of waste from seed processing and importing seed potatoes from rhizomania-free areas. Disease-tolerant sugar beet cultivars are widely used in affected regions.

BNYVV is regulated within the European Union in protected zones (EU, 2000), currently Brittany (FR), Finland, Ireland, the Azores (PT), and Northern Ireland (GB).

[1]The figures in this standard marked 'Web Fig.' are published on the EPPO Website www.eppo.org.

Identity

Name: *Beet necrotic yellow vein virus*
Acronym: BNYVV
Taxonomic position: Viruses, *Benyvirus*
EPPO computer code: BNYVV0
Phytosanitary categorization: EPPO A2 list no. 160; EU Annex designation I/B.

Detection

The disease affects all subspecies of *Beta vulgaris*, including sugar beet (*Beta vulgaris* subsp. *maritime*), fodder beet (*Beta vulgaris* subsp. *vulgaris*), red beet (*Beta vulgaris* subsp. *cicla*), mangolds (*Beta vulgaris* subsp. *vulgaris*), sea kale (*Beta vulgaris* subsp. *vulgaris*), Swiss chard (*Beta vulgaris* subsp. *cicla*), and also spinach (*Spinacea oleracea*).

Symptoms of rhizomania

Leaves

Symptoms can often be seen very clearly from aerial photographs, as well on the ground, and consist of distinct yellow patches (Web Fig. 1). On inspection the following may be noted:

- translucent, pale lettuce-green to lemon-yellow foliage
- yellow veining following the midrib of the leaf (Web Fig. 2)
- upright foliage with elongated petioles and narrowed leaf laminae (Web Fig. 3)
- plants stunted and/or wilted (possibly without leaf symptoms).

Roots

- dark brown bearded roots (this may be slight and/or a single lateral root with bearding near the tip) (Web Fig. 4)
- root constriction
- pale yellow to dark brown vascular discoloration in transverse section
- nodules (small tumorous growths along the taproot).

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

Identification

Sampling

Samples should be taken from identified yellow patches in beet crops (identified by aerial photography, etc.). A fork or spade should be used to dig up the roots (especially in dry hard baked soils). Care should be taken to lift the beet whole 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 5 or 6 plants showing symptoms. Each sample should be separately identified and placed in a labelled plastic bag[2].

Sample preparation

For laboratory-based tests, the 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 labelled plastic bags for processing.

Samples for soil-bait testing

Soil samples from the field can be tested for rhizomania by growing susceptible beet in the soil (bait testing) in a glasshouse or in growing chambers. A total of 2.5 kg of field soil should be taken by walking in a W shape across each of the sampling areas. Each sample should be separately identified and placed in a labelled plastic bag.

Sample preparation for lateral flow test kit

See Appendix 6.

Screening tests

ELISA is the best and most cost-effective general screen (Appendix 1). The lateral flow test is appropriate if symptoms are seen in accordance with the pathogen key card provided with the kit (Appendix 6).

[2]In Poland (Jezewska & Piszczek, 2001) sugar beet leaves are routinely sampled for ELISA testing for BNYVV. The suitability of this method has not generally been assessed in the EPPO region and may depend on factors such as virus concentration and local environmental conditions.

Isolation

Mechanical inoculation of virus to test plants

Beta vulgaris (sugar beet): inoculated leaves usually develop chlorotic lesions after 6–8 days. Occasional bright yellow chlorotic lesions can be seen in leaf veins. Infection is rarely systemic. *Chenopodium quinoa*, *Chenopodium amaranticolor*, *Tetragonia expansa*: chlorotic or necrotic lesions develop after 5–7 days. *Nicotiana tabacum*, *Lycopersicon esculentum* and *Phaseolus vulgaris* are non-susceptible and can help to distinguish BNYVV from other rod–shaped viruses, e.g. *Tobacco rattle virus*, *Tobacco mosaic virus*, *Pea early browning virus*. For full description of host range, susceptible and insusceptible plants, see Tamada & Baba (1973). For new hosts, see Horváth (1994).

At least two indicator plants and two non-susceptible hosts should be used. Sugar beet lateral roots are washed and ground in a mortar with a small spatula-full of celite and enough distilled water to make a thin paste. The indicator plant, e.g. *Chenopodium quinoa*, is inoculated at the stage of six or more fully expanded leaves by gently covering the leaves in root/celite suspension, using a finger covered in a disposable 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. Test plants infected with BNYVV produce characteristic chlorotic lesions (Web Fig. 5). There should be no symptoms on non-susceptible hosts. As mechanical inoculation is not always successful, false negative results may occur.

Polymyxa betae

The presence in roots of *Polymyxa beta*, the protozoan vector of BNYVV, can aid presumptive diagnosis of BNYVV, but its absence does not indicate that the roots are not infected. Suspect rootlets are washed in cold water and dried on absorbent paper. A selected sample, mounted gently flattened in water on a simple microscope slide with cover slip, is examined at × 10 under a light microscope for characteristic cystosori in the root cells. A magnification of × 40 may be used for closer examination (Web Fig. 6). Other stages in the life cycle of *P. betae* may also be seen, such as sporangia and plasmodia.

Confirmation test(s)

ELISA test

A sample of 0.5–1 g of washed lateral or tap roots is processed following the procedure of Appendix 1. The ELISA value of the sample should be more than 2–3 times greater than the negative control. See specific instructions enclosed with the antisera.

RT-PCR test

A sample of washed lateral or tap roots (which may have been

stored frozen) is processed following the procedure of Appendix 2[3].

Immunocapture PCR

A sample of washed lateral or tap roots (which may have been stored frozen) is processed following the procedure of Appendix 3.

TaqMan® RT-PCR

A sample of washed lateral or tap roots (which may have been stored frozen or freeze dried) is processed following the procedure of Appendix 4.

Electron microscopy tests

A washed sample of lateral or tap roots is examined following EM, IEM or gold labelling procedures (Appendix 5).

Soil tests

Soil from fields suspected of being infested by rhizomania can be tested by baiting with seedling sugar beet (Appendix 7), which are then tested by ELISA (Appendix 1). The optimum time for bait testing is 6 weeks for the English test (Henry *et al*., 1992; Tuitert & Bochen, 1993) or 3–4 weeks for French test (LNPV Fleury method). If a more rapid method is needed, RT-PCR (Appendix 2) or TaqMan® RT-PCR (Appendix 4) can be done after 3 weeks using the English test (Henry *et al*., 1995).

Beet soil-borne virus (*pomovirus*) is a different rod-shaped beet-infecting virus, also transmitted by *Polymyxa betae*. *Beet soil-borne mosaic virus* (*benyvirus*) is also closely related but serologically distinct. The ring-tested diagnostic tests recommended in this protocol are specific for BNYVV, and will not detect any other viruses.

The procedures for detection and identification described in this protocol, and the decision scheme in Fig. 1 or 2, should have been followed. Positive identification of BNYVV (in the original plant or in an indicator plant) should be made using ELISA and/or PCR methods (see Appendices). A confirmation of the presence of the virus may be required, using a method distinct from that originally used (e.g. if a serological method was used first, a molecular method is used for confirmation). See Figs 1 and 2 and Appendices. Any first finding should be confirmed by other tests.

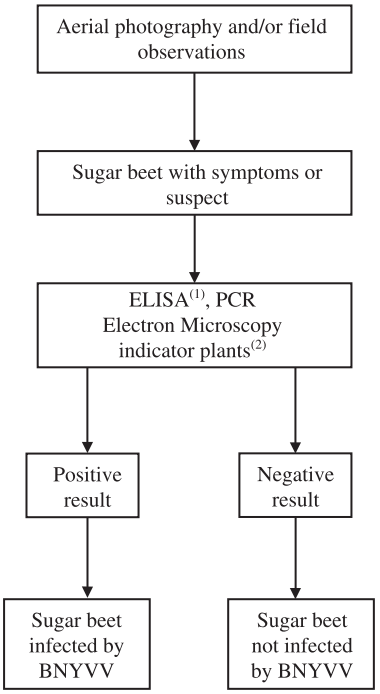
Other tests used for BNYVV mainly for research purposes

Protein profiling

Whole cell extracts can be denatured and separated by SDS–PAGE. The coat protein of BNYVV is Mr 21 kDa. Western blotting can also be carried out (Torrance *et al*., 1988).

[3]A multiplex PCR that can detect BNYVV, BSBV, Beet virus Q and *Polymyxa betae* has been developed (Meunier *et al*., 2003). This has been tested by the authors (Université Catholique de Louvain-la Neuve) (UCL) in comparison with the DIAGPRO PCR protocol.

Fig. 1 Flow-diagram for detection and identification of *Beet necrotic yellow vein benyvirus* in sugar beet.



(1) The most appropriate and rapid screening test would be ELISA (Appendix 1), which should be repeated if the result is unclear. Lateral flow devices (Appendix 6) can be used in the field and for small numbers of lab samples.

(2) ELISA testing is normally sufficient for diagnosis but the following alternative tests can be performed as required: PCR (Appendix 2) and TaqMan® PCR (Appendix 4) Additional tests include mechanical inoculation of indicator plants and electron microscopy (Appendix 5).

Quantification of *Polymyxa betae* in rhizomania soil samples

It is possible to estimate the number of infectious units of viruliferous *P. betae* in an infested soil by a series of soil dilutions, by the most probable number (MPN method) and bait testing (Ciafardini, 1991).

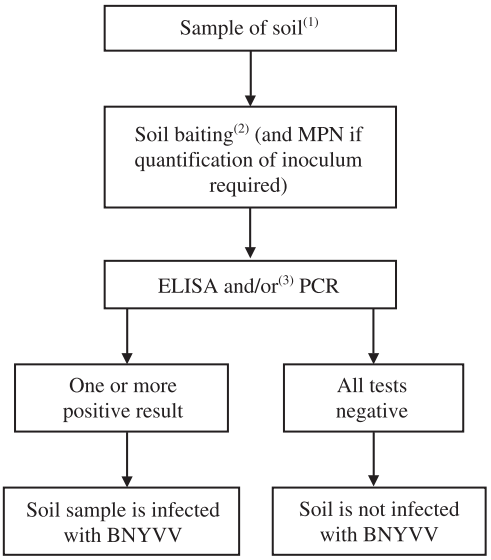
Tissue print-immunoblotting of roots

It is possible to show, by immunoblotting of a longitudinal section of a sugar beet root, where the virus is concentrated in a given plant. This has been used more as a research and development technique than in general diagnosis (Kaufmann *et al*., 1992).

A, B & P pathotypes

Different pathotypes of BNYVV, designated A and B, were first identified by Koenig *et al*. (1994). They have been classified into groups based on a number of molecular characteristics. The

Fig. 2 Flow diagram for detection and identification of *Beet necrotic yellow vein benyvirus* in soil samples.



(1) The standard size of soil sample is 0.5–2.5 kg. However, the procedure can be used for smaller samples.

(2) Soil bait tests: (Appendix 7).

(3) ELISA testing (Appendix 1) is the normal way of screening many samples for BNYVV and can be used as the sole screening test provided the antisera is of high specificity. The test can detect virus concentrations down to 10^{-3}) and is cheap and fast. If an additional confirmatory test is required it should be preferably based on different biological principles.

PCR (Appendix 2) being approximately 800 times more sensitive than the standard TAS ELISA, the detection limit is 10^{-6} for root extracts. TaqMan® PCR (Appendix 4) is approximately 10 000 times more sensitive than conventional PCR. Skilled operators are needed however, as extreme care is required during preparation to prevent contamination and false positives.

sequence differences between A & B types are subtle, a high percentage of the sequence being the same. Sequencing is now reliably used to detect strain differences. Koenig & Lennefors (2000) have used sequencing to provide a more reliable method of differentiating the European A, B and P types of BNYVV than using RFLP and SSCP analyses (Koenig *et al*., 1995), the genomes of the BNYVV isolates having been found to be very stable.

The common isolates of BNYVV contain RNAs 1–4. The A type is widespread in most European countries (Kruse *et al*., 1994), the USA, China and Japan. The B type is more restricted, generally to Germany, France and the UK. Mixtures of these strains can occur. A and B isolates can be also differentiated using PCR (Ratti *et al*., 2005). BNYVV isolates containing additional genomic RNA (RNA 5) are found in Japan and China (Tamada *et al*., 1989; Miyanishi *et al*., 1999). Such isolates, which have been described as P pathotype have also been reported in Europe, near Pithiviers (FR) (Koenig *et al*., 1997), and near Norwich (GB) (Harju *et al*., 2002). Similar BNYVV strains with RNA 5 have been found in Kazakhstan (Koenig & Lennefors, 2000). There is some evidence to suggest that isolates containing RNA 5 are more virulent than those of the other pathotypes (Tamada *et al*., 1996), and that sugar beet cultivars with different degrees of resistance vary in their response to various pathotypes of BNYVV. B types appeared to be less damaging than A or P types. P types appear to give a higher virus content than A or B types (Heijbroek *et al*., 1999). A Japanese PCR test for RNA 5 was published by Kiguchi *et al*. (1996) and TaqMan® PCR has been used for detection of RNA 5 in the UK (Harju *et al*., 2002) and (Harju *et al*., 2005) (see Appendix 4).

Future diagnostic developments

Recently published papers described the use of a new type of antibody production from BNYVV-specific single-chain antibody variable fragments (scFvs) (Griep *et al*., 1999; Uhde *et al*., 2000). The latter authors achieved good results when testing stored sugar-beet roots with antibodies produced from scFvs. The specificity of these new antibodies in ELISA may in future have potential for their use as reagents in sensitive diagnostic assays for testing. A recombinant antibody ELISA test has been developed for *Polymyxa betae* (Kingsnorth *et al*. 2003). A close correlation was found between the numbers of *P. betae* zoospores in serially diluted suspensions and absorbance values in the ELISA test.

In the United States remote detection using hyper-spectral leaf reflectance and multi spectral canopy reflectance to study rhizomania has been tested (Steddom *et al*., 2003). They found the total leaf nitrogen was significantly lower in symptomatic beets than in healthy beets. Chlorophyll and carotenoid levels were also reduced in symptomatic beets. Classification was best in August gradually decreasing in accuracy until harvest. These results indicate that remote sensing technologies can be used to facilitate detection of rhizomania.

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) Documentation and reporting on a diagnosis.

Further information

Further information on *Beet necrotic yellow vein virus* can be obtained from the Pest and Disease Identification Team (PLHB) and Immunological and Molecular Methods Team (PLHC), Central Science Laboratory, Sand Hutton, York YO41 1LZ, United Kingdom, e-mail: v.harju@csl.gov.uk.

Information can also be obtained from other institutes including the Institute for Plant Virology, Microbiology and Biosafety, Messeweg 11/12, D-38104, Braunschweig, Germany, e-mail: Biosearch@bba.de and Institut International de Recherches Betteravièrs 195, Avenue de Tervuren B-1150 Bruxelles, Belgium, e-mail: mail@iirb.org.

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The French soil bait test (LNPV Fleury method) was drafted by F. Vey, LNPV Fleury les Aubrais.

The main diagnostic tests recommended in this protocol were ring-tested in different European laboratories[4]. Individual samples were tested in all cases. Bulked samples sometimes used in large surveys were not tested.

References

Canova A (1959) [On the pathology of sugar beet]. *Informatore Fitopatologico* **9**, 390–396 (in Italian).

Chang S, Puryear J & Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Bioliology Reporter* **11**, 113–116.

Ciafardini G (1991) Evaluation of *Polymyxa betae* Keskin contaminated by beet necrotic yellow vein virus in soil. *Applied and Environmental Microbiology* **57**, 1817–1821.

EU (2000) Council Directive 2000/29 of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* L169, 1–112.

Grassi G, Cerato C, Benso P & Borgatti S (1988) Monoclonal and conventional antibodies for the detection of beet necrotic yellow vein virus (BNYVV) in sugar beet. *Phytopathologia Mediterranea* **27**, 125–132.

Griep RA, Twisk C & van Schots A (1999) Selection of beet necrotic yellow vein virus specific single-chain Fv antibodies from a semi-synthetic combinatorial antibody library. *European Journal of Plant Pathology* **105**, 147–156.

Harju VA, Mumford RA, Blockley A, Boonham N, Clover GRG, Weekes R & Henry CM (2002) The identification of isolates of *Beet necrotic yellow vein virus* from the United Kingdom which contain RNA 5. *Plant Pathology* **51**, 811.

Harju VA, Skelton A, Clover GRG, Ratti C, Boonham N, Henry CM & Mumford RA (2005) The use of real time RT- PCR (TaqMan®) and post ELISA virus release for the detection of Beet necrotic yellow vein virus types containing RNA 5 and its comparison with conventional PCR. *Journal of Virological Methods* **123**, 73–80.

Harness AM, Johnson JC, Kulemeka BP, Sutula CL & Badla MD (2003) Immunocapture RT-PCR as a confirmation tool for ELISA. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* **110**, 101. (Abstract) [http://www.agdia.com/references/immunocaptureposter.pdf].

Heijbroek W, Musters PMS & Schoone AHL (1999) Variation in pathenogenicity and multiplication of *Beet necrotic yellow vein virus* (BNYVV) in relation to the resistance of sugar beet cultivars. *European Journal of Plant Pathology* **105**, 397–405.

Henry CM, Barker I, Morris J & Hugo SA (1995) Detection of beet necrotic yellow vein virus using reverse transcription and polymerase chain reaction. *Journal of Virological Methods* **54**, 15–28.

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Henry CM, Harju V, Brewer G & Barker I (1992) Methods for the detection of Rhizomania in soil. *Aspects of Applied Biology* **32**, 129–133.

Horváth J (1994) Beet necrotic yellow vein furovirus 1 new host. *Acta Phytopathologica et Entomologica Hungarica* **29**, 109–118.

Hughes DW & Galau G (1988) Preparation of RNA from cotton leaves and pollen. *Plant Molecular Biology Reporter* **6**, 253–257.

Jezewska M & Piszczek J (2001) Surprisingly high frequency of detection of *Beet necrotic yellow vein virus* in sugar beet leaves by ELISA. *Phytopathologic Polonica* **21**, 165–170.

Kaufmann A, Koenig R & Lesemann DE (1992) Tissue print-immunoblotting reveals an uneven distribution of beet necrotic yellow vein and beet soil-borne viruses in sugar beets. *Archives of Virology* **126**, 329–335.

Kiguchi T, Saito M & Tamada T (1996) Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. *Journal of General Virology* **77**, 575–580.

Kingsnorth CS, Asher MJC, Keane GJP, Chwarszczynska DM, Luterbacher MC & Mutasa-Göttgens ES (2003) Development of a recombinant antibody ELISA test for the detection of Polymyxa betae and its use in resistance screening. *Plant Pathology* **52**, 673–680.

Koenig R, Haeberle AM & Commandeur U (1997) Detection and characterization of a distinct type of beet necrotic yellow vein virus RNA 5 in a sugar beet growing area in Europe. *Archives of Virology* **142**, 1499–1504.

Koenig R, Kruse M, Hoffmann H, Heijbroek W, Buttner G, Lindsten K & Paul (1994) The existence of possible pathotypes of *Beet necrotic yellow vein virus* (BNYVV) and their possible impact on partially resistant sugar beet varieties. In *Proceedings of the 57th IIRB Winter Congress*. IIRB, Bruxelles (BE).

Koenig R & Lennefors BL (2000) Molecular analyses of European A, B and P type sources of beet necrotic yellow vein virus and detection of the rare P type in Kazakhstan. *Archives of Virology* **145**, 1561–1570.

Koenig R, Lesemann DE & Burgermeister W (1984) Beet necrotic yellow vein virus: purification, preparation of antisera and detection by means of ELISA, immunosorbent electron microscopy and electro-blot immunoassay. *Phytopathologische Zeitschrift* **111**, 244–252.

Koenig R, Luddecke P & Haeberle AM (1995) Detection of beet necrotic yellow vein virus strain, varieties and mixed infections by examining single-strand confirmation polymorphism of immunocapture RT-PCR products. *Journal of General Virology* **76**, 2051–2055.

Kruse M, Koenig R, Hoffmann A, Kaufmann A, Commandeur U, Solovyev AG, Savenkov I & Burgmeister W (1994) Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. *Journal of General Virology* **75**, 1835–1842.

Meunier A, Schmit J-F, Stas A, Kutluk N & Bragard C (2003) Multiplex RTPCR for the simultaneous detection of *Beet necrotic yellow vein virus*, *Beet soil borne virus*, *Beet virus Q* and their vector *Polymyxa betae* Keskin on sugar beet. *Applied and Environmental Microbiology* **69**, 2356–2360.

Miyanishi M, Kusume T & Tamada T (1999) Evidence for three groups of sequence variants of *Beet necrotic yellow vein virus* RNA 5. *Archives of Virology* **144**, 879–892.

Morris J, Clover GRG, Harju VA, Hugo SA & Henry CM (2001) Development of a highly sensitive nested RT-PCR method for Beet necrotic yellow vein virus detection. *Journal of Virological Methods* **95**, 163–169.

Mumford RA, Walsh K, Barker I & Boonham N (2000) Detection of Potato mop-top and Tobacco rattle virus using a multiplex real-time fluorescent reverse transcription polymerase chain reaction assay. *Phytopathology* **90**, 448–453.

Ratti C, Clover GRG, Rubies-Autonell C, Harju VA & Henry CM (2005) A Multiplex RT- PCR assay capable of distinguishing beet necrotic yellow vein virus types A and B. *Journal of Virological Methods* **124**, 41–47.

Spiegel S & Martin RR (1993) Improved detection of potato leafroll virus in dormant potato tubers and microtubers by the polymerase chain reaction and ELISA. *Annals of Applied Biology* **122**, 493–500.

Steddom K, Heidel G, Jones D & Rush CM (2003) Remote detection of rhizomania in sugar beets. *Phytopathology* **93**, 720–726.

Tamada T & Baba T (1973) Beet necrotic yellow vein virus from rhizomania-affected sugar beet in Japan. *Annals of Phytopathological Society of Japan* **39**, 325–331.

Tamada T, Kusume T, Uchino H, Kiguchi T & Saito M (1996) Evidence that Beet necrotic yellow vein virus RNA 5 is involved in symptom development of sugar beet roots. In *Proceedings of the Third Symposium of the International Working Group on Plant Viruses with Fungal Vectors* (Ed. Sherwood, JL & Rush, CM), pp. 49–52. American Society of Sugar beet Technologists, Denver (US).

Tamada T, Shirako Y, Abe H, Saito M & Kiguchi T (1989) Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. *Journal of General Virology* **70**, 399–409.

Torrance L, Pead MT & Buxton G (1988) Production and some characteristics of monoclonal antibodies against beet necrotic yellow vein virus. *Annals of Applied Biology* **113**, 519–530.

Tuitert G (1990) Assessment of the inoculum potential of *Polymyxa betae* and beet necrotic yellow vein virus (BNYVV) in soil using the most probable number method. *Netherlands Journal of Plant Pathology* **96**, 331–341.

Tuitert G & Bochen GJ (1993) Recovery of resting spores of *Polymyxa betae* from soil and the influence of duration of the bioassay on the detection level of beet necrotic yellow vein virus in soil. *Netherlands Journal of Plant Pathology* **99** (Suppl. 3), 219–230.

Uhde K, Kerschbaumer RJ, Koenig R, Hirschl S, Lemaire O, Boonham N, Roake W & Himmler G (2000) Improved detection of *Beet necrotic yellow vein virus* in a DAS ELISA by means of antibody single chain fragments (scFv) which were selected to protease-stable epitopes from phage display libraries. *Archives of Virology* **145**, 179–185.

Appendix 1. ELISA test

Materials recommended for the ELISA test

Homogenization buffer
This buffer is used for tissue maceration: polyvinylpyrrolidone (PVP) 20.0 g; phosphate-buffered saline (PBST) 1 L (see below). Add 500 mL of PBST to 20 g of PVP. Dissolve by machine stirring rapidly. Make up to 1 L, stirring thoroughly. This buffer should be made up freshly as required.

Carbonate coating buffer
pH 9.6: Na_2CO_3 1.59 g; $NaHCO_3$ 2.93 g; distilled water 1 L. Dissolve the ingredients and check pH. Store at 5°C.

10 × Phosphate Buffered Saline (PBS), 1× = pH 7.2: NaCl 80 g; KH_2PO_4 2 g; $Na_2HPO_4 \cdot 12H_2O$ 29 g; KCl 2 g; distilled water 1 L. Dissolve all ingredients and check pH. Dilute to 1 × for use.

Phosphate-Buffered Saline-Tween (PBST)
10 × PBS 100 mL; 10% Tween-20 5 mL; distilled water 895 mL. Stir ingredients briefly.

Antibody buffer (prepare fresh)
PBST 100 mL; 5% dried milk powder 5 g or 0.2%; bovine serum albumin 0.2 g. Stir ingredients briefly.

Alkaline phosphatase substrate solution pH 9.8:
Diethanolamine 97 mL; distilled water 800 mL. Mix and adjust to pH 9.8 with concentrated HCl. Make up to 1 L with distilled water. Add 0.203 g of $MgCl_2$ and store at 5°C. Dissolve two phosphatase substrate 5 mg tablets (Sigma) per 15 mL of substrate solution.

Antibodies
Suitable antibodies (including Anti-species AP-conjugated antibody) for use in the ELISA test for BNYVV are those of Koenig *et al*. (1984), Grassi *et al*. (1988) and Torrance *et al*. (1988).

Commercial detection kits
- Bio-Rad, Phyto-Diagnostics, 3 bd. Raymond Poincaré, 92430 Marnes-la-Coquette (FR) [previously Sanofi Cie (Libourne, FR] . Tel. +33 (0)1 47 60 00, Fax +33 (0)1 47 41 91 33, www.bio-rad.com;
- Bioreba AG (Switzerland), Chr. Merian-Ring 7, CH 4153 Reinach BL1 (CH) www.bioreba.ch;
- Adgen Ltd, Nellies Gate, Auchincruive, Ayr KA6 5HW, Scotland (GB). Tel. +44 (0)1292 525275, Fax +44 (0)1292 525477, www.adgen.co.uk;

ELISA test – Triple Antibody Sandwich (TAS)

This test, based on Henry *et al*. (1992), has been ring–tested. The summary of the ring testing data analysis of the results of BNYVV diagnostic tests are presented below:

TP (True positives) 76	TN (True negatives) 54
FP (False positives) 0	FN (False negatives) 2
Total samples	132
Sensitivity = 0.97 (97%)	Specificity = 1.00 (100%)
Positive predictive value = 1.00 (100%)	Negative predictive value = 0.96 (96%)
Accuracy = 0.98 (98%)	

Ring testing conclusion for ELISA: recommended.

Data analysis has been made following the method used by the Medical University of South Carolina as follows:
Sensitivity = true positives/(true positives + false negatives)
Specificity = true negatives/(true negatives + false positives)
Positive predictive value = true positives/(true positives + false positives)
Negative predictive value = true negatives/(true negatives + false negatives)
Hit rate (accuracy) = (true positives + true negatives)/Total samples.

The use of Nunc-Maxisorp microtitre plates or those of a similar quality is recommended. Include negative sugar beet controls, homogenizer buffer control and positive control (either known positive sugar beet material or positive leaf material from BNYVV inoculated onto *Chenopodium quinoa*). Add BNYVV antibody at the recommended dilution to the coating buffer. Pipette the solution onto the microtitre plates, 100 µL per well. Incubate for 3 h at 33°C. Flick out the contents of the wells, and wash wells three times with PBS-Tween with 3-min soaks between washes. Blot dry on absorbent paper.

Add sample homogenate at 100 µL per well, using two wells per test sample. Incubate at 4°C overnight. Flick out the contents of the wells as before but wash 4 times. Add specific BNYVV monoclonal antibody diluted as appropriate in antibody buffer at 100 µL per well. Incubate for 2 h at 33°C. Flick out contents of

wells and wash 4 times. Prepare antispecies alkaline phosphatase conjugate at appropriate dilution in antibody buffer. Add 100 µL to each well. Incubate for 2 h at 33°C. Flick out the contents of the wells and wash 4 times. Prepare alkaline phosphatase substrate solution. Add 100 µL to each well. Incubate at ambient temperature for 1 h. Read absorbance at 405 nm.

Some commercial kits for BNYVV detection use the double antibody sandwich (DAS) ELISA. If this method is being used add antibody conjugate instead of monoclonal, and omit the following step of adding conjugate.

The ELISA test is negative if the absorbance of the sample is less than 3 times the absorbance of the healthy control, or positive if equal or greater than 3 times that value (Web Fig. 7).

This protocol is recommended but it can be modified according to the instructions supplied with the antibody, the experience of the laboratory or as the result of specific study.

Appendix 2. PCR test

Materials for the PCR test

Oligonucleotide primer sequence
Downstream primer:
(BNYVV 017 (R) 5′-ACT-CGG-CAT-ACT-ATT-CAC-T(T)-3′
Upstream primer:
(BNYVV 016 (F) 5′-CGA-TTG-GTA-TGA-GTG-ATT-T (A)-3′
Expected size of amplicon 500 bp.

Nested PCR primers
Downstream primer:
(Rhzn 17 (R) 5′-GAC-GAA-AGA-GCA-GCC-ATA-GC)-3′
Upstream primer:
(Rhzn 15 (F) 5′-ATA-GAG-CTG–TTA-GAG-TCA-CC)-3′
Expected size of amplicon 326 bp.

RNA extraction method
RNA is extracted from sugar beet roots by a method adapted from Hughes & Galau (1988) with modifications from Spiegel & Martin (1993).

Extraction buffer
Tris base-HCl 200 mM pH 8.5; 1.5% lithium dodecyl sulphate; EDTA 10 mM; NaCl 300 mM; 1% Sodium deoxycholate. Autoclave (121°C at 1.2 bar for 30 min) and store. Prepare fresh extraction buffer in required amount for the number of samples extracted (3 mL per sample) by adding 1% Ipegal CA-630 (Sigma-Aldrich); DTT (DL-Dithiothreitol) 10 mM; Thiourea (CH_4N_2S) 5 mM. The resulting buffer will keep for 2 weeks at room temperature.

Potassium acetate
Potassium acetate 6 M; molecular grade water. Adjust pH to 6.5 using acetic acid and make up to 1 L. Autoclave prior to use.

Lithium chloride
LiCl 4 M; molecular grade water. Make up to 1 L.

TE Buffer: Tris-HCl 10 mM; EDTA 1 mM; molecular grade water. Adjust pH to 8.0 and make up to 1 L.

Sodium chloride
NaCl 5 M; molecular grade water. Make up to 1 L. Autoclave prior to use.

Ethidium bromide
TBE 600 mL; ethidium bromide 60 µL (0.5 µg/mL). Keep in covered plastic box covered to prevent photo degradation.

1× Tris borate EDTA buffer (for gel electrophoresis)
Tris BASE 107.8 g; EDTA 7.4 g; H_3BO_3 55.0 g; de-ionized water to 10 L. Adjust to pH 8.2.

RNase-free DEPC water
Add 0.05% diethyl pyrocarbonate (DEPC) to water that needs treating. Decant into small bottles, or as required (coat all internal surfaces). Leave bottle tops loose. Leave overnight in fume hood. Autoclave at 121°C at 1.2 bar for 30 min. This treated water cannot be used for Tris buffers.

6× loading buffer (for gel electrophoresis)
0.25% bromophenol blue; 0.25% xylene cyanol FF; 30% glycerol in water. Store at 4°C.

PCR test

This test, based on Morris *et al*. (2001), has been ring-tested. The summary of the ring testing data analysis of the results of BNYVV diagnostic tests are presented below.

One-step RT-PCR

TP (True positives) 82	TN (True negatives) 31
FP (False positives) 2	FN (False negatives) 10
Total samples	125
Sensitivity = 0.89 (89%)	Specificity = 0.93 (93%)
Positive predictive value = 0.97 (97%)	Negative predictive value = 0.75 (75%)
Accuracy = 0.90 (90%)	

Ring testing conclusion for PCR protocols: normal PCR works well as a confirmation test (if required). Other methods of RNA extraction could also be used if necessary.

Nested One-step RT-PCR

TP (True positives) 44	TN (True negatives) 12
FP (False positives) 23	FN (False negatives) 2
Total samples	81
Sensitivity = 0.91 (91%)	Specificity = 0.34 (34%)
Positive predictive value = 0.65 (65%)	Negative predictive value = 0.85 (85%)
Accuracy = 0.69 (69%)	

Ring testing conclusion for Nested PCR: as a routine diagnostic method, needs care as it is only reliable with all controls working well.

Data analysis of ring-tests has been made following the method used by the Medical University of South Carolina (see Appendix 1).

Sterile filter-plugged pipette tips, Eppendorfs, etc. should be used, and gloves worn during all stages of sample preparation and other manipulations involving PCR.

Nucleic acid extraction (Hughes & Galau, 1988)

Weigh 200 mg of fresh or frozen root material (or 100 mg dried sample). Place in suitable labelled, small, strong, polythene bags (such as Stomacher) and immerse in a flask of liquid nitrogen. Add 0.5–1 mL of RNA extraction buffer with DTT and thiourea freshly added. Homogenize. Decant 600 µL of the homogenate into a labelled 1.5 mL microfuge tube. Add an equal volume of 6 M potassium acetate to each tube and incubate on ice for 15 min. Spin the samples in a microfuge at 12 000 × g for 10 min. Transfer 600 µL of the supernatant to a fresh, labelled microfuge tube. Add an equal volume of 4 M LiCl. Incubate the samples at 4°C overnight. Centrifuge samples at 12 000 × g at 4°C for 30 min. Resuspend the pellet in 200 µL TE buffer containing 1% SDS. Add 100 µL of 5 M NaCl and 300 µL ice-cold isopropanol, vortex and incubate samples at −20°C for 30 min. Centrifuge the samples at 12 000 × g at 4°C for 10 min. Discard the supernatant and wash the pellet with 500 µL of 70% ethanol. Centrifuge at 13 000 rev min^{-1} at 4°C for 5 min. Carefully pour off the ethanol and dry the pellet in a vacuum drying centrifuge for about 15 min. Resuspend the pellet in 50 µL of RNase-free water. Use for PCR or freeze at −20°C until needed.

One-step RT-PCR

Keep defrosted extractions, chemicals and buffers on ice prior to use. For each sample: pipette 1 µL of RNA template into a labelled 0.5 mL Eppendorf tube. Include positive and negative controls and RNAse–free water control.

Make up the following RT master mix in a 1.5-mL Eppendorf tube (amounts per sample, but adding 2 extra multiples to allow for pipetting errors): 10 × PCR buffer 5 µL (Promega) 100 mM Tris-HCl, 500 mM KCl, pH 9.0; dNTPs (10 mM) 1 µL: forward primer 016F (5 µM) 2 µL; reverse primer 017R (5 µM) 2 µL; $MgCl_2$ (25 mM) 3 µL; DEPC water (RNAse free) 35.45 µL; MMLV 0.05 µL (10 units, Promega, Southampton, GB); Taq polymerase (add last) 0.5 µL (2.5 units, Promega). Add this 49 µL of the master mix to each Eppendorf already containing 1 µL of template RNA.

Run the following programme in the thermal cycler: 30 min at 37°C, 2 min at 94°C; followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Finally 3 min at 72°C. Analyse by running samples on a gel or store vials at 5°C (short-term) or −20°C until analysis is performed. When normal PCR is complete, use 0.5 µL of PCR product as the template for the nested PCR as below or run products on a gel.

Nested PCR

PCR nested primers are used as an additional amplification step, if required. The method is 1000 times more sensitive than conventional PCR. Special care is needed to ensure the product does not become contaminated. An additional Eppendorf tube containing 0.5 µL of DEPC water that has not been through the initial PCR should be used as an additional control with the nested PCR.

Master mix (per sample)

10 × PCR Buffer (Promega, 100 mM Tris-HCl, 500 mM KCl, pH 9.0) 5 µL; dNTPs (10 mM) 1 µL; Primer rhzn15 (5 µM) 2 µL; Primer rhzn17 (5 µM) 2 µL; $MgCl_2$ (25 mM) 3 µL; Taq polymerase (Promega 2.5 units) 0.5 µL; DEPC water (RNAse free) 36 µL; amplified product from normal BNYVV PCR 0.5 µL. Add the 49.5 µL of Master mix to each tube containing 0.5 µL of PCR product.

Run the following programme in the thermal cycler: 94°C for 2 min; 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min; finally, 72°C for 3 min. Analyse PCR products or store vials at −20°C until analysis is performed.

RT-PCR for RNA 5 (P-type) (Harju *et al.*, 2005)

(Not ring tested.) Specific assays for RNA 5 detection of BNYVV have been included as this type is considered to possibly be more aggressive therefore laboratories may wish to use the RT-PCR or TaqMan® RT-PCR (Appendix 4) to screen BNYVV positive samples for its presence.

Specificity

Using the RNA 5-specific primer set, PCR products measuring 530 French (Pithiviers) (English and Japanese isolates) and 527 (Chinese isolate) base pairs (bp) were obtained from beet samples containing RNA 5. RNA extracts made from beet infected with the A or B isolates of BNYVV and uninfected beet did not produce PCR products.

Sensitivity

The BNYVV RNA 5 assay detects to a dilution of 1 : 10.

Materials for the RT-PCR for RNA 5 (P-type)

Oligonucleotide primer sequence

Upstream primer: BNYVV 5F1 Forward Primer GATATGGCATATAGCGACG.

Oligonucleotide primer sequence

Downstream primer: BNYVV 5R1 Reverse Primer GGTCGTTGCCAAAATCTC.

Expected size of amplicon 530(bp) French (Pithiviers), English and Japanese isolates), 527(bp) (Chinese isolate).

(Primer design based on BNYVV RNA 5 sequence (Koenig *et al.*, 1997)).

Reverse transcription of the sample RNA is performed essentially according to manufacturer's instructions, using 1 µL of template RNA, 1 µL (10 pmol) of BNYVV 5RI and 100 units of M-MLV reverse transcriptase (Promega, Southampton, UK or similar) in a final volume of 10 µL. Incubate sample(s) in a heating block or water bath for 1 h at 37°C.

For the PCR reaction add the following constituents to the 10 µL of the cDNA reaction; 10pmols of primer BNYVV 5F1, 1.5 mM $MgCl_2$, 1 × *Taq* reaction buffer (Promega; 10 mM Tris-HCl, 50 mM KCl, pH 9.0), 0.2 mM dNTPs (Promega) and 2.5 units of Taq DNA polymerase (Promega) and make up to the final reaction volume of 50 µL with sterile, RNAse-free water.

Thermo-cycling is performed as follows: 94°C for 1 min, then 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by 72°C for 3 mins. The PCR products (10 µL per well) are analysed by gel electrophoresis (see Analysis of PCR product).

Analysis of PCR product

The PCR products are detected by agarose gel electrophoresis and staining with ethidium bromide. Prepare a 1.2% agarose gel by gently bringing to the boil (molecular grade, general purpose) agarose in Tris borate EDTA buffer. Cool the molten agarose to 50–60°C, pour into the gel tray and insert the comb. Allow the gel to set. Remove the comb, submerge the gel in Tris Borate EDTA buffer so that it is covered with 2–3 mm of liquid.

On parafilm or in new Eppendorf tubes, take 2 µL of 6 × loading buffer and mix with 10 µL of PCR product. Load 10 µL of loading buffer/PCR product mix into the wells carefully. Include appropriate 1 Kb marker(s) used at 5 µL per well or 100 base-pair ladder and positive control amplified DNA.

Run gel at 100 V for 1–1.5 h (gel dye front about 15 cm). Remove and soak in ethidium bromide solution (0.5 µg/mL) for 30−45 min. Destain by rinsing in distilled water. Visualize the amplified DNA products with a UV transilluminator. The PCR product of BNYVV with primer set complementary to the nucleotides 1301–1320 and 1781–1800 on RNA 2 is 500 bp in length. The nested PCR product is 326 bp. Check against DNA marker and against positive control. The water control should be negative in every case. If positive contamination has occurred, the test should be repeated. Photograph the gel to provide a permanent record.

The PCR test is negative if the characterized 500 bp (PCR) fragment (or 326 bp for nested PCR) is not detected and the fragment for the positive control isolate of BNYVV is detected, and positive if the 500 bp (PCR) fragment (or 326 bp for nested PCR) is detected and it is identical in size with the fragment for the positive control isolate of BNYVV. The RNA 5 PCR test is negative if the characterized 527 or 530 bp (PCR) fragment is not detected and the fragment for the positive control isolate of BNYVV RNA 5 is detected, and positive if the 527 or 530 bp (PCR) fragment is detected and it is identical in size with the fragment for the positive control isolate of BNYVV RNA 5.

Appendix 3. One-step Immunocapture RT-PCR

Materials for Immunocapture PCR

For ELISA grinding buffer and PBST, see Appendix 1. For PCR primers, etc., see Appendix 2. The following one-step Immunocapture RT-PCR has been found to be slightly less sensitive than a one-step RT-PCR (Morris *et al*., 2001) using purified RNA and detects dilutions of sap extracted from infected sugar beet and *C. quinoa* down to 1×10^{-2} and 1×10^{-3}, respectively.

Ring testing conclusion for Immmunocapture PCR: Due to only few participants wishing to test the method, no firm conclusions can be drawn, but the method appears to work well with experienced operators.

Coat 500-µL microfuge tubes with polyclonal anti-BNYVV antiserum (e.g. Adgen) 3 h at 33°C. Wash tube three times with PBST (Appendix 1). Grind sample roots in ELISA extraction buffer 1 : 9 w/v (as in ELISA Appendix 1). Add 100 µL of root homogenate to the coated microfuge tube and incubate overnight at 4°C. Wash three times with PBST and twice with sterile distilled water.

Add 50 µL of the following RT Master mix to each washed sample tube: Forward primer (016F, 5 µM) 2 µL; Reverse primer (017R, 5 µM) 2 µL; 10 × Taq reaction buffer (100 mM Tris-HCl, 500 mM KCl, pH 9.0) 5 µL; $MgCl_2$ (25 mM) 3 µL; dNTPs (10 mM) 1 µL; MMLV reverse transcriptase (Promega, Southampton, GB) 10 units 0.05 µL; RNAse inhibitor (Promega, Southampton, GB) 20 units 0.5 µL; *Taq* DNA polymerase (Promega, Southampton, GB) 2.5 units 0.5 µL; molecular grade/DEPC water 35.95 µL.

Place tubes in a thermocycler set to the following programme: 30 min at 37°C, 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, followed by 3 min at 72°C. Analyse PCR product (see previous section) or store vials at −20°C until analysis is performed.

Appendix 4. TaqMan® PCR test (not ring-tested)

Gain in sensitivity, time and labour-savings, made by avoiding the needs for post-PCR gel electrophoresis are some of the main advantages of real-time over conventional PCR, however, machinery and consumables are relatively expensive.

Specificity

12 BNYVV isolates from around the world were tested using these assays. These included three isolates known to contain RNA 5, one isolate from Pithiviers, France (P) and two Far Eastern isolates, from China and Japan, in addition to nine European isolates that were identified as A or B types.

With the RNA 5 assay, only the three isolates known to contain RNA 5 (Pithiviers, France (P), Japanese and Chinese) tested positive, while the remaining A or B isolates all tested negative. In contrast, all 12 isolates tested positive when using the RNA 2 specific assay. RNA extracts made from uninfected sugar beet tested negative using both assays.

The results were validated using UK field samples over three growing seasons. When tested using the RNA 2-specific primers described by Morris *et al*. (2001), products of the correct predicted size were obtained for all 12 isolates, with both the standard (500 bp) and (326 bp) nested primer pairs.

Sensitivity
A dilution series of the infected sample in uninfected beet root ground in CTAB RNA buffer was performed. The sensitivity of both the real-time (TaqMan® RNA 2 and RNA 5) and conventional RNA 5 RT-PCR assays was directly compared. Results showed that the two TaqMan® assays were 10 000 times more sensitive than the conventional PCR assay, detecting down to a dilution of 1 : 100 000, in contrast to the conventional PCR BNYVV RNA 5 assay which only detected to a dilution of 1 : 10.

Post-ELISA virus release (VR) extraction for TaqMan® RT- PCR

Sensitivity
In addition to the CTAB method, a more rapid method, based on post-ELISA virus release (VR) (Harness *et al*., 2003) was also tested. The results show that this method gave reliable detection when using the BNYVV TaqMan® RT-PCR assays, giving a typical range of positive C_T values of between 19 and 31 for RNA 2. It offers a reliable means to both confirm and type ELISA positive samples, without the need to resample and perform complex RNA extraction procedures. Overall, while the results do indicate that there is a reduction in sensitivity using VR as an alternative to a traditional total RNA extraction method, they do show that VR TaqMan® detection is at least as sensitive as ELISA detection alone. The overall savings in time and labour offered by VR TaqMan® make it an extremely attractive option when large numbers of samples are involved. A direct sensitivity comparison of ELISA detection alone with VR TaqMan® detection following ELISA showed that both ELISA and VR TaqMan® (using the BNYVV RNA 2 assay) could detect down to the same dilution of 1 : 100.

Materials for the TaqMan® PCR test

Primer sequence
Sequences of BNYVV-specific primers and probes used for TaqMan® for RNA 2 and RNA 5 (P -type).

TaqMan® RNA 2
Sequence (5′-3′)
BNYVV-CP 26F (Forward Primer)
CATGGAAGGATATGTCTCATAATAGGTT
BNYVV-CP 96R (Reverse Primer)
AACACTCACGACGTCCGAAAC
BNYVV-CP 56T (Probe, FAM-labelled)
TGACCGATCGATGGGCCCG

TaqMan® RNA 5 (P-type)
BNYVV-R5 96F (Forward Primer)
CAATTTGAAAACGAGTGTAAAGTAAAAGG
BNYVV-R5203R (Reverse Primer)
CTG CTT CTG AGT GAC ACC AAG TG
BNYVV-R5123T(Probe,VIC-labelled)
AGGTTACTAAACAAAATAGCCCTCCATACGGTACGA

CTAB stock buffer
The stock buffer can be autoclaved and stored at room temperature for at least 1 year: 2% cetyl tetra ammonium bromide (CTAB), 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M sodium chloride.

CTAB grinding buffer
1.0% sodium sulphite, 2.0% soluble polyvinylpyrrolidone-40, should be added fresh to the CTAB stock buffer (containing the first four reagents) prior to extraction. The buffer will then 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.
5 M NaCl:
Add 292.2 g of NaCl Make up to 1 L.
Isopropanol:
Store ice-cold at −20°C.
TE-SDS buffer:
10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% (w/v) sodium dodecyl sulphate. Make up to 1 L.
Post-ELISA virus release (VR) buffer:
10 mM Tris-HCl, pH 8.0, 1.0% (v/v) Triton X-100. Make up to 1 L.
PBST buffer:
Phosphate-buffered saline containing 0.05% Tween 20 (v/v) (PBST).

TaqMan® PCR test

Sterile filter-plugged pipette tips, Eppendorfs, etc. should be used, and disposable gloves worn during all stages of sample preparation and other manipulations involving TaqMan® PCR.

CTAB Total RNA extraction

Modified from Chang *et al*. (1993).

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 N_2 may help with the grinding of some tissues). Transfer ground sap into a 1.5 mL microfuge tube and incubate at 65°C for 10–15 min. After incubation, centrifuge the tubes in a microfuge at 12 500 g for 5 min at room temperature. Remove 700 µL of clarified sap, place in a fresh microfuge 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 microfuge for 10 min at room temperature. Carefully remove upper (aqueous) layer and transfer to a fresh tube. Add an equal volume of chloroform: isoamyl alcohol (24 : 1 v/v), mix and spin as in previous step. Remove aqueous layer, taking extra care not to disturb 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. Microfuge the samples for 10 min at 12 500 g. Decant off the salt/isopropanol and wash the resulting pellet by adding 400 µL 70% ethanol. Microfuge 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.

Post-ELISA virus release (VR) for TaqMan®. RT-PCR

Following ELISA, the trapped virus can be released using the method of Harness *et al*. (2003).

Wash positive or potentially positive ELISA plate wells with phosphate-buffered saline 0.005% Tween (PBST) and tap dry on absorbent paper. Fill these wells with 50 µL of virus release buffer (VRB). Cover with cling wrap or similar, to prevent evaporation, and put on a shaker for 5 min at 65°C. After incubation, decant the extracts into labelled Eppendorf tubes and store at 5°C. Test the same day or freeze at -80°C to store prior to testing. Use 5 µL of extract for testing by TaqMan®.

TaqMan® RT-PCR assay

Set up single-tube TaqMan® RT-PCR reactions (25 µL) in 96-well or 384-well reaction plates using a TaqMan® core reagent kit (Applied Biosystems). Make up primers to a final concentration of 200 µM. Add an additional 10 units of M-MuLV reverse transcriptase enzyme (Promega, Southampton, UK or similar) and 1 µL of sample RNA per reaction. Real time PCR can be performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) or similar, using generic cycling conditions (Mumford *et al*., 2000). The threshold cycle (C_T) is the cycle at which a significant increase in fluorescence occurs, hence a C_T value below 40 indicates a positive result and a C_T value of 40 indicates a negative result. (Typical C_T values obtained from BNYVV-infected beet are between C_T 16 and C_T 31).

Appendix 5. Electron microscopy tests (not ring tested)

Materials for electron microscopy tests

2% Uranyl acetate (U/A) stain
Uranyl acetate 2 g; distilled water to 100 mL. Dissolve and store in a brown glass dropper bottle at 4°C.

Phosphate buffer pH 6.5 (Sorenson's) (SPB)
Mix 3 mL of Na_2HPO_4 (9.469 g/L) with 7 mL KH_2PO_4 (9.079 g/L). PBS – see Appendix 1.

Transmission Electron Microscopy (TEM)

Wash roots free from soil and blot dry on absorbent paper. Grind 1 g of root using a mortar and pestle in about 1 mL of SPB pH 6.5 (choose lateral roots or thin slivers from lateral root). Take up the liquid with a pipette and place two 20-µL drops in a Petri dish lined with plastic laboratory film. Place two TEM carbon-coated grids, one on each of the drops, coated side down, using fine forceps. Incubate with lid on Petri dish for 10 min. Remove grids using forceps and wash off excess sap with 20 drops of distilled water from a Pasteur pipette. Stain sample with 3 drops of 2% U/A using a dropper bottle. Gently dry the grids by touching their edge on filter paper, and store. Examine in the TEM at × 46 000 for the following particle morphology: rod-shaped virions, usually straight, not enveloped (Web Fig. 9); four clear modal lengths (70, 100, 265, 390 nm), width 20 nm; axial canal obscure, obvious basic helix-pitch 2.6 nm.

Immune Electron Microscopy (IEM)

Prepare and grind roots as above. Decant homogenate into an Eppendorf tube and centrifuge at 1000 g for 1 minute. To trap the virus particles, incubate carbon-coated TEM grids on two 20 µL drops of Adgen BNYVV monoclonal or similar, diluted 500 times in SPB pH 6.5 for 15 min at room temperature. Wash grids to remove excess antibody with 20 drops of SPB pH 6.5 using a Pasteur pipette, and gently dry on filter paper. Incubate grids on two 20 µL drops of sample homogenate for 1 h. Wash grids with 20 drops of SPB pH 6.5, and dry as before. To label the virus particles, place grids on 20 µL drops of Adgen polyclonal coat or similar 1/50–1/100 (to decorate) for 15 min. Wash grids with 20 drops of distilled water, and dry as before. Stain grids with 3 drops of 2% U/A, dry as before, and store. Examine in the TEM at × 46 000 for labelled particles.

Protein-A Immunogold with trapping

Dilute specific antiserum 1 : 500 in SPB pH 6.5. Float a carbon-coated EM grid on the liquid, carbon side down. Incubate at room temperature for 10 min. Wash with 20 drops of PBS buffer and drain on filter paper. Grind root material and dilute to 1 : 10 in SPB pH 6.5. Place 20 µL of root solution on laboratory film. Place the grid on the liquid, carbon side down. Incubate at room temperature for 10 min and drain. Dilute antiserum, e.g. Adgen detection antibody (MAFF 9) or equivalent in SPB pH 6.5 1 : 500. Incubate grid on a 20 µL drop at room temperature for 10 min and drain. Wash grid with 20 drops of PBS buffer and place on a 20 µL drop of Protein A gold (20 nm) (British Biocell International) or similar, diluted 1 : 50 in SPB pH 6.5. Incubate at room temperature for 10 min and drain. Wash with 10 drops of PBS buffer, 5 drops of distilled water and stain with 3 drops of 2% U/A, drain. Examine under TEM at × 46 000. (Web Fig. 10).

Appendix 6. Lateral Flow Tests

The Rhizomania Pocket Diagnostic lateral flow test kit (produced

and marketed at Central Science Laboratory, York, GB) has been ring-tested. The summary of the ring testing data analysis of the results of BNYVV diagnostic tests are presented below.

TP (True positives) 35	TN (True negatives) 37
FP (False positives) 0	FN (False negatives) 3
Total samples	75
Sensitivity = 0.92 (92%)	Specificity = 1.0. (100%)
Positive predictive value = 1.0 (100%)	Negative predictive value = 0.92 (92%)
Accuracy = 0.96 (96%)	

Ring testing conclusion for Lateral flow field kits: work well (use another lab test to confirm result).

Data analysis of ring-tests has been made following the method used by the Medical University of South Carolina (see Appendix 1).

Consult instructions before use. For beet roots with symptoms of rhizomania, take tap-root and fine hairs, shake off excess soil, break into small pieces and add to bottle (the kit bottle contains buffer and sodium azide). Replace lid tightly, ensuring dropper cap is on. Shake for 30 s. A brown extract should become visible.

Remove the test device from its foil pack, avoiding touching the viewing window. Remove cap from bottle, and discard 2–3 droplets by inverting and gently squeezing bottle. Hold device horizontally and gently squeeze 2 drops onto the sample well of the device. Keep device horizontal until extract is absorbed (about 30 s) and a blue dye appears in the viewing window. The control line should become clearly visible in the viewing window after 3 min, and the test (labelled T on the device) result visible in 1–3 min. Two blue lines (C & T) indicates a positive result, test correctly performed (Web Fig. 8b). One blue line (C only) indicates a negative result, test correctly performed (Web Fig. 8a). Faint blue T line, strong C line indicates a possible positive, test correctly performed. A faint or absent line may indicate a low concentration of the pathogen, uneven distribution within the plant or recent infection. This test should primarily be used for screening purposes. Any sample giving a positive result should be sent to the laboratory for confirmation.

Appendix 7. Soil bait testing

English test

This test, which has not been ring-tested, is based on a modified version of Tuitert (1990) and D. Wright (pers. comm., 1998). 500 g of rhizomania-infested soil collected from the top 15 cm of the field should be air-dried, mixed thoroughly in an inflated plastic bag (pulverized if necessary using a hammer) and sieved through a 2 mm sieve. Six replicate tests should be done for each soil sample.

Mix soil-less seed and potting compost 3 : 1 with grit and use as the potting mix. Thoroughly mix the potting mix 1 : 1 with test soil (600 mL of each) in an inflated plastic bag. Fill six 250-mL disposable cups with drainage holes drilled in the base with the sample/potting mix. Stand the six pots together in a plastic tray. Water them gently from the top and in the tray.

Pre-germinate sugar beet seeds, of a susceptible cultivar, by soaking in tepid water for 4 h. Spread seeds on damp kitchen paper in a sealed plastic container, and hold for about 24 h, until radicles emerge. Sow two pre-germinated seeds per pot. Use disposable sticks or forceps for planting. Sterilize forceps between different soil samples (e.g. with 70% ethanol). Cover the seeds with a little sterile sand. Grow plants for 6 weeks in a glasshouse with a day temperature of 23°C and night temperature of 15°C. Do not over-water or allow pots to stand in water for prolonged periods as this will inhibit root growth. Water lightly from the top to direct water into individual pots. Avoid cross contamination between samples. Allow soil to dry out slightly between watering. Feed once a week with a suitable liquid feed.

Wash out the roots one pot at a time into a bucket or tray. Rinse in clean water to remove grit. Change water between different test soils. Cut off roots and lateral roots, and place about 1 g in a labelled homogenization bag. Test by ELISA (Appendix 1), reaction volumes being added at 100 µL per well.

French test (LNPV Fleury method)

This protocol has been developed by INRA of Dijon, tested by the LNPV and used since 1987 for serial analysis. It was published in the French Official Journal on the 2nd of June 2005 under the reference 'VS/04/07 version b'. It is currently being ring-tested.

Collect a 2.5 kg 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 replicate tests should be done for each soil sample. Fill six 150-mL disposable cups with drainage holes drilled in the base with the sample. Stand the six pots together in a tray. Sow about 20 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 similar, to prevent drying out of the soil before emergence. Water lightly in the bottom of the tray. The soil surface must be wet. Grow plants for 3–4 weeks in a growing chamber regulated around 25°C under suitable light (15 h day light). Remove the saucers as soon as the seedlings emerge. Always keep the soil wet by regular watering. Do not water from the top but water directly into the tray. A layer of water must be always kept in the bottom of the tray. After 3–4 weeks of growth, wash the roots one pot at a time with clean water. Cut off the roots, and place about 0.5 g in a labelled homogenization bag. Each bag should contain roots from one pot. Only five pots are used. The sixth pot is used to complete the bags if there are not enough roots in the other five pots. Test by ELISA (Appendix 1).

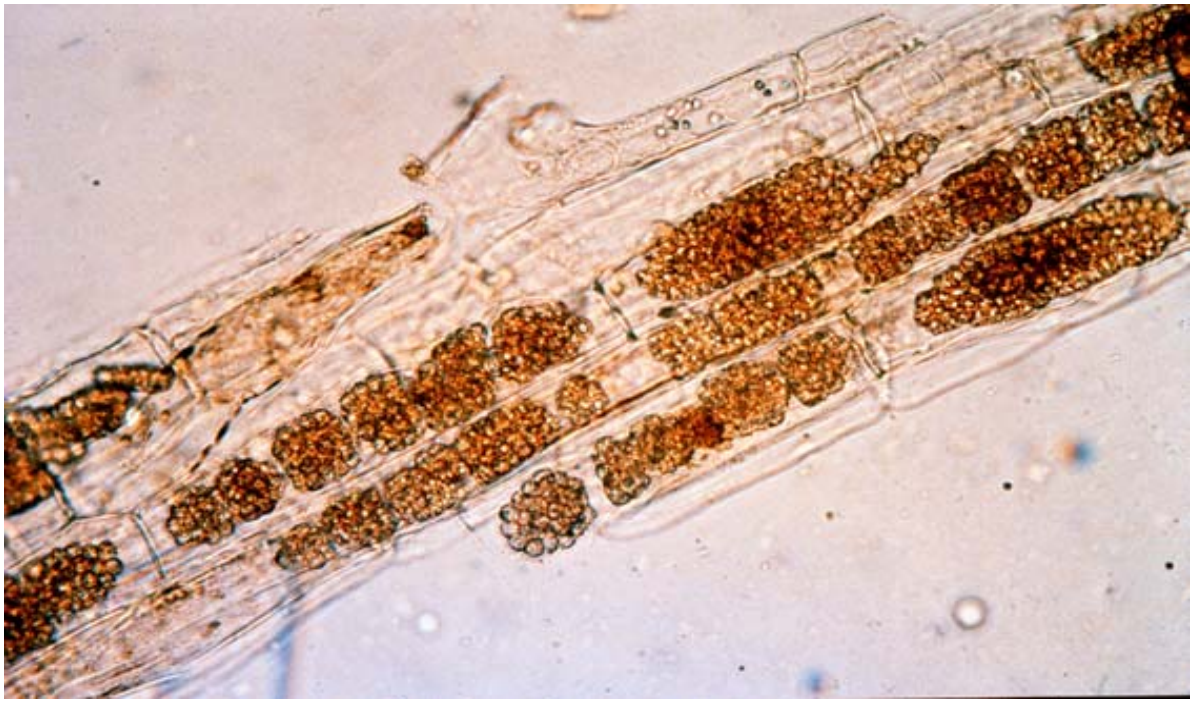
Web Fig. 1 Typical symptoms of rhizomania in the field: a distinct yellow patch of infected sugar beet.

Web Fig. 2 Foliar symptoms of rhizomania: yellow veining following the midrib of the leaf (very rarely seen in the UK).

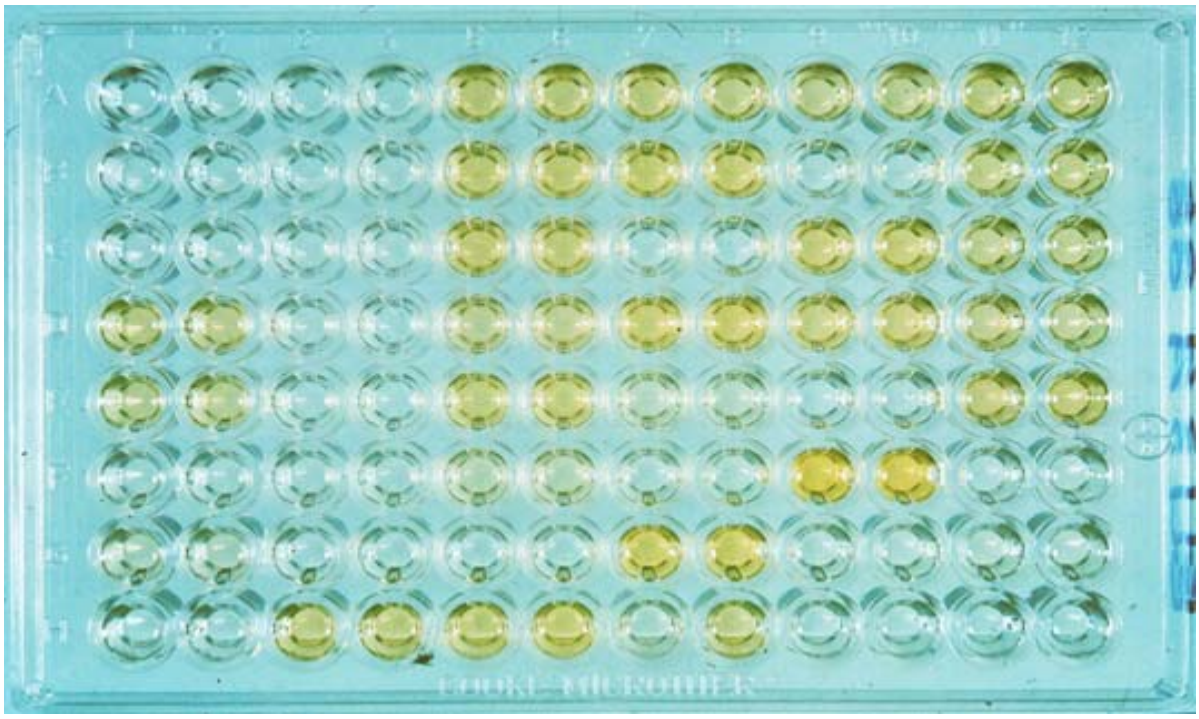
Web Fig. 3 Foliar symptoms of rhizomania: pale green leaves, upright foliage, narrowed leaf laminae.

Web Fig. 4 Typical external root symptoms of rhizomania showing the reduced size of the beet and root proliferation (bearding).

Web Fig. 5 Chlorotic lesions of BNYVV in *Chenopodium quinoa.*



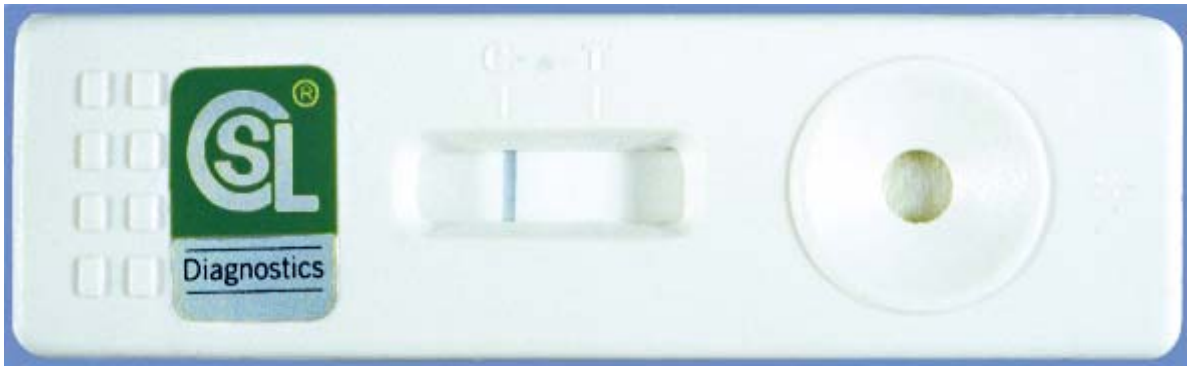
Web Fig. 6 Microscope slide of *Polymyxa betae* cystosori.



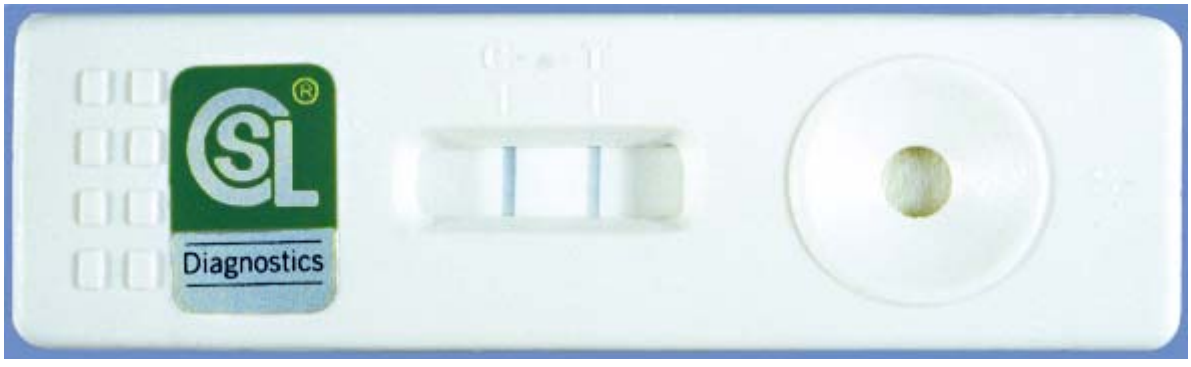
Web Fig. 7 ELISA diagnostic test: yellow wells, when using alkaline phosphatase substrate, indicate a positive result - BNYVV is present.

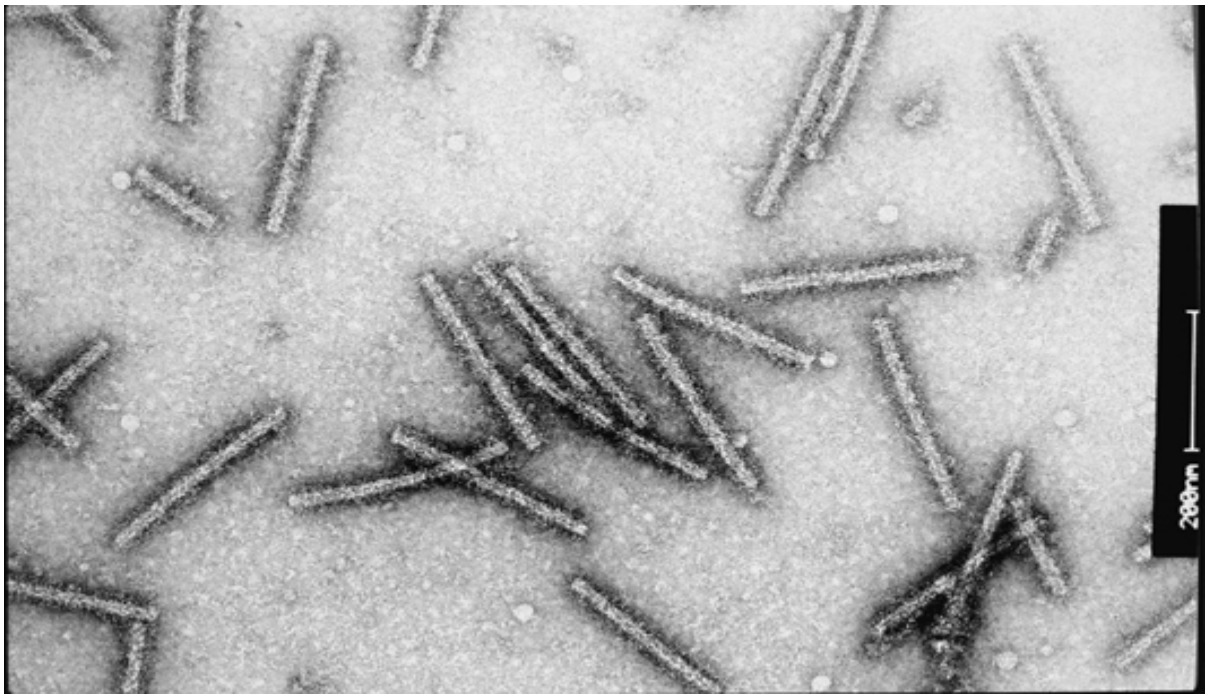
Web Fig. 8 Rhizomania Pocket Diagnostic lateral flow test kit
a. Lateral Flow device- negative
b. Lateral Flow device- positive

a.

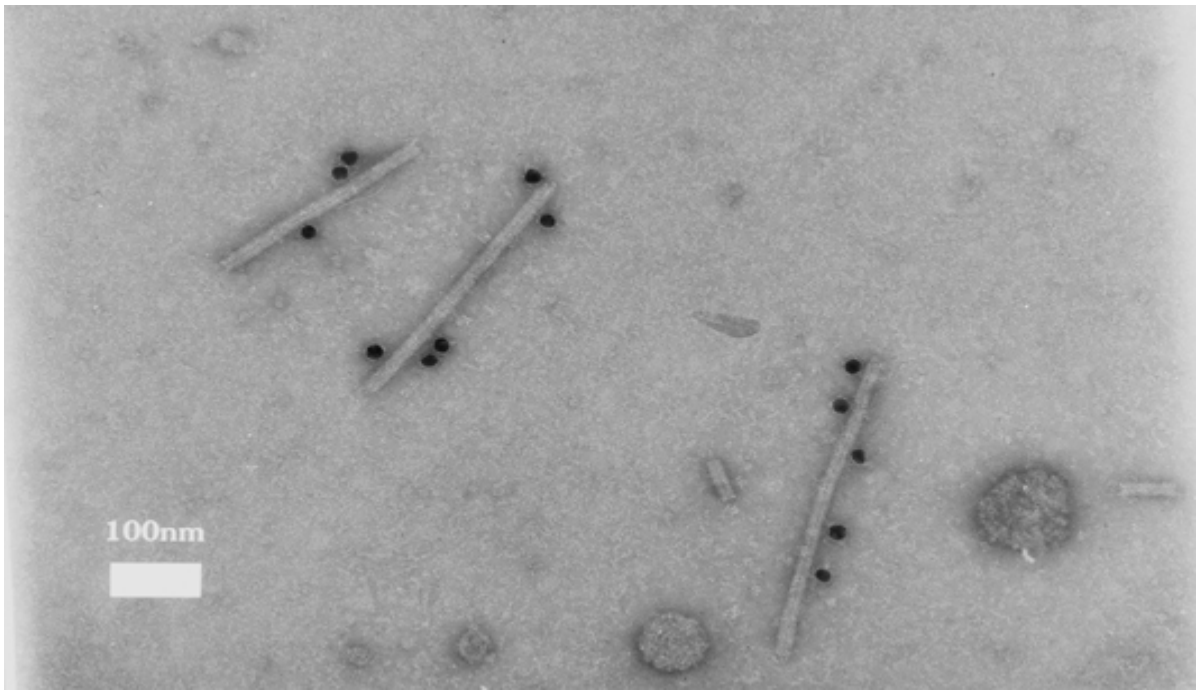


b.





Web Fig. 9 Electron micrograph of rod-shaped virions of BNYVV, using the IEM method.



Web Fig. 10 An Electron micrograph of immunogold - labelling of BNYVV virus particles.