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Detection of Beet Necrotic Yellow Vein Virus with Reverse Transcription-Polymerase Chain Reaction

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ABSTRACT

Progressive diagnostic methods such as reverse transcription-polymerase chain reaction (RT-PCR) or nested-PCR have been used to detect plant pathogenic viruses rapidly and precisely during the recent years. Concerning the worldwide economical importance and history of beet necrotic yellow vein Benyvirus (BNYVV), causal agent of rhizomania, particularly in Iran causing high yield losses annually, samples were gathered from rhizomania affected or tentative areas of Darab, Marvdasht and Zarghan (Fars province). Total RNA extraction was carried out using Qiagene kit after the primary ELISA experiments and measurement of infection. Having optimized the procedure, RT-PCR amplified the 500 bp product using specific primers designated based on the triple gene block of BNYVV RNA-2, which showed the presence of viral pathogen in either the infected sugar beet root or test plant leaf tissue precisely. RT-PCR test is very sensitive and specific decreases contaminations risk whilst the work in comparison to ELISA tests. PCR-based diagnostic tests can be optimized as the routine laboratorial methods.

Key Words: Sugar beet; Rhizomania; BNYVV; RT-PCR

INTRODUCTION

Rhizomania disease (root madness) of sugar beet was first reported in Italy in 1952 by Canova. Rhizomania is caused by beet necrotic yellow vein *Benyvirus* (BNYVV); (Tamada & Baba, 1973; Koenig & Tamada, 2000). The virus is transmitted by a soil-inhibiting obligate parasite of sugar beet, *Polymyxa betae,* and is known to survive in the soil for many years protected by resting cystosori of the fungus (Tamada, 1975). Thus, the disease can not be controlled by agronomical practices. Rhizomania has been spread to Far East within less than 20 years and become a serious problem for sugar beet-dependent industries (Hill & Torrance, 1989). Unique nature of pathogenic virus, complex life cycle of fungal vector and wide distribution of three major viral pathotypes and various isolates (Table I) make it very difficult to control rhizomania disease and yield losses exceed 80% occasionally.

Control of rhizomania depends upon the accurate and sensitive detection of BNYVV in plants and soil for two reasons. Firstly, such detection is needed to identify infected fields and adopt appropriate management regimes, e.g. growing a partially resistant cultivar or non-host crop or imposing hygiene restrictions as part of containment policy and secondly, to distinguish truly resistant sugar beet breeding lines from those which are tolerant or partially resistant (Henry *et al*. 1986)

Genetic improvement for rhizomania resistance is the most reliable control way of rhizomania currently. Enzyme linked immuno-sorbent assay (ELISA) is based on direct use of polyclonal and monoclonal antibodies in sap were first applied by Clark & Adam, (1977) and have been used extensively (e.g. *Potyviridae*). Rybicki and Hughes (1990) first used PCR method to detect DNA viruses. Vunsh *et al*. (1990) diagnose RNA plant viruses with RT-PCR method. Wetzel (1991) and Nolasco *et al.* (1993) detected several plant viruses with immunocapture RT-PCR. PCR-based methods benefits the advantages of speed, sensitivity, precision, decrease of infection risk (Spiegel & Martin, 1993). Besides, ELISA methods are not suitable for detecting subviral plant pathogens such as viroids or satellite RNAs.

Beet necrotic yellow vein virus *Benyvirus* has four different particles with distinct RNAs. The virus has three major pathotypes A, B and P. BNYVV monoclonal and polyclonal antibodies were produced and the virus has been detected with ELISA methods (Torrance *et al*. 1988; Koenig *et al*. 1998). Because serological methods are not efficient with the low viral titres, stronger techniques should be used to identify rhizomania resistant sugar beet lines. Torrance *et al.* (1988) also detected BNYVV through TAS-ELISA (triple antibody sandwich; monoclonal antibodies) in infected sap. Specific primers were first designated based on RNA-2 triple gene block motif and BNYVV was detected with RT-PCR technique in infected tissue by Henry (1996). Optimizing Henry (1996) materials and methods, Morris *et al*. (2001) detected the virus in infected tissue with RT-PCR, immunocapture RT-PCR and nested PCR (n-PCR) in infected tissue and compare PCR-based methods to serological methods particularly DAS- (double antibody sandwich) and TAS-ELISA in terms of sensitivity and specificity. Early diagnosis of disease can be easily achieved

via PCR-based techniques is inevitable for following proper regime of crop and disease management. The analysis of nucleotide sequences of the capsid protein epitopes and of different motifs involved in the transmission of the virus to the plant by *Polymyxa betae* reveals that they are well conserved among the different sequences. *In silico* analysis showed that a potential dimerization/dsRNA-binding domain is present on P75. Finally, in order to compare the different BNYVV types, a real-time quantitative (RT)-PCR targeting the readthrough domain of P75 was developed and assessed over a period of 40 days on BNYVV susceptible sugar beet cv. Cadyx (Meunier *et al.*, 2005).

Sequence alignments of Iran Fars CP21 with other isolates showed closed similarities at nucleotide and amino acid levels with BNYVV pathotype A isolates; S from Japan, and YU2 from Yugoslavia. These results suggest that Iran-Fars isolate probably originated from Asia or neighboring European countries rather than from Germany or France (Sohi & Maleki, 2005). The purpose of this study was to demonstrate the sensitivity of existing molecular protocol for BNYVV detection by developing PCR-methods.

MATERIALS AND METHODS

Plant material and virus isolates. Healthy and infected sugar beet plants (*Beta vulgaris*) cv. IC1 and 7233 were gathered from Darab, Zarghan and Marvdasht regions (Fars province; south of Iran). Table II presents the isolates of beet necrotic yellow vein virus gathered from farm province. Samples were incubated at -23ºC in laboratory. Seven days old seedlings of test plants were planted into pots containing either sterilised soil- sand and peat or a 1:1 (v/v) mixture of sand/peat and perlite. The plants were grown for 2 weeks at 25ºC before being tested by ELISA, RT-PCR or n-PCR. The isolates were tested to assure the specificity of the diagnostic tests. The sensitivity of the diagnostic tests was assessed using ten-fold dilution series of extractions from infected *C. quinoa* leaves and sugar beet roots diluted in extractions from healthy *C. quinoa* or sugar beet, respectively. For comparison with the assessments of sensitivity made by Morris *et al*. (2001) and Henry (1996) further dilution series were made in water or extraction buffer for PCR and ELISA, respectively.

Nucleic acid extraction. Total RNA was extracted from 100 mg of root and leave tissue essentially based on QIAGENE® RNeasy plant MiniKit protocol (50 kit, Germany). Root or leave tissue was frozen in liquid nitrogen and ground in 450 µL RLT or RLC kit buffer (4.5 µL ß-mercptoethanol added before use). The homogenate was decanted into a liquid nitrogen frozen 1.5 microfuge vortexed thoroughly and decanted into a Qiagene shredder column (lilac). Qiagene shredder column was entrifuged at 13000 rpm for 2min and the supernatant was decanted into a 1.5 mL microfuge carefully. 0.5 volume of pure ethanol was added to supernatant immediately mixed by pipeting and decanted into a Qiagene RNeasy column (pink). Qiagene RNeasy column was centrifuged at 10000 rpm for 15 sec and discarded the supernatant. 700 µL of RW1 kit buffer was added to the column centrifuged at 10000 rpm for 15 sec and discarded the kit 2 mL microfuge. The column was transferred to a new kit 2 mL microfuge. 500 µL of RPE kit buffer (ethanol added) was added to the column centrifuged at 10000 rpm for 15 sec and discarded the supernatant. The latter stage was repeated for 1min centrifugation and the column was transferred to a 1.5 mL microfuge. 30-50 µL of RNase free water added to the column and centrifuged at 10000 rpm for 1 min. RNA samples were incubated at -23ºC (Fig. 1,2).

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). DAS-ELISA was carried out using polyclonal antibodies raised against BNYVV as described by Koenig *et al*. (1998).

RT-PCR. RT-PCR was carried out using 016 and 017 specific primers (Table III), which amplified a 500 bp fragment of the read-through region of the coat protein of BNYVV (Morris *et al*., 2001). RT-PCR was performed in a one- or two-step format using total RNA purified as described above, or in a one-step immunocapture procedure. In some reactions healthy plant aterial and distilled water controls were used. Amplified products were observed after electrophoresis in ethidium bromide-stained gels.

For two step RT-PCR, production of cDNA was performed using 3 µL of template RNA, 2µL of reverse primer (017, 5 µM) and 100 units of MMLV reverse transcriptase (fermentase,) in a 20µL volume at 42ºC for 50min according to DSMZ instructions (S. Winter, personal communication). PCR amplification was performed in 50 µL volumes containing 5µL of cDNA, 2µL of forward primer (016, 5µM), 5µL of 10X *Taq* reaction buffer (100mM Tris-HCl, 500mM KCl, pH 9.0), 1.5 µL of MgCl2 (50 mM), 1 µL of dNTPs (10 mM) and 2.5 units of *Taq* DNA polymerase (fermentase,). Thermocycling was carried out as follows; 94ºC for 2min, then 30 cycles of 94ºC for 1min, 55ºC for 1min and 72ºC for 1min, followed by 72ºC for 5 min.

One step RT-PCR was undertaken in 50 µL reaction volumes containing 1 µL of template RNA, 2 µL of forward and reverse primers (016 and 017, 5 µM), 5 µL of 10X *Taq* reaction buffer (100 mM Tris-HCl, 500 mM KCl, pH 9.0), 1.5 µL of $MgCl_2$ (50mM), 1 µL of dNTPs (10 mM), 10 units of MMLV reverse transcriptase and 2.5 units of *Taq* polymerase (fermentase,). Thermocycling was carried out as follows; 37ºC for 60min, 94ºC for 2min, then 30 cycles of 94ºC for 2min, 55ºC for 1min and 72ºC for 1min, followed by 72 for 10min.

RESULTS

Specificity of the diagnostic tests. The specificity and reliability of the DAS-ELISA and conventional two-step RT-PCR were reported by Torrance *et al*. (1988) and Morris

et al. (2001). The specificity of conventional RT-PCR was unaffected by replacing the extraction method using by Henry *et al*. (1992) with that of Hughes and Galau (1988) (Morris *et al*., 2001). Similarly, the specificity of one-step RT-PCR using RNA purified by two total RNA extraction methods or immunocapture was the same as the conventional two-step RT-PCR described by Henry (1996) and (Morris *et al*., 2001).

Table I. Isolates of beet necrotic yellow vein virus mechanically transmitted to *Chenopodium quinoa*

Place of origin	Isolate code	first report
West Stow, UK	D4	C.Henry, England
Narford, UK	E11	C.Henry, England
Beachamwell, UK	F10	C.Henry, England
Warren Farm, UK	-	C.Henry, England
France	Fr	C. Putz, France
Pithivier, France	-	M. Richard-Molard, France
Groβ Gerau, Germany	GG	R.Koenig, Germany
Germany	Bav	E.Schlösser, Gernmany
Bologna, Italy	BOL3	L.Giunchedi, Italy
Bologna, Italy	BOL, A2	L.Giunchedi, Italy
Ferrara, Italy	Ferrara C	G. Cianfordini, Italy
Netherlands	Dutch	G. Beemster, Wageningen
Japan	JS2	T. Tamada, Japan
United States of America	US2	K. Richards, France
Switzerland	Swiss	A. Hani, Switzerland

Table II. Isolates of beet necrotic yellow vein virus obtained as infected tap roots gathered from Fars province

Place of origin	fields	Cultivar	Samples
Darab	3	ICI	18
Mardasht	4	7233	32
Zarqan	1	7233	50

Table III. Nucleotide sequences of primers used for RTPCR detection of beet necrotic yellow vein virus

Primer	Nucleotide sequence (5′ to 3′)[a]	Expected amplicon size(bp)
016(forward) [1800]	CGATTGGTATG AGTGATTT (A) [1781]	500bp
017(reverse) [1301]	ACTCGGCATAC TATTCACT (T) [1320]	

Both German and Iranian BNYVV isolates were successfully transmitted mechanically to *C. quinoa* were all detected. All methods produced a product of the expected size (500 bp) and no nonspecific bands were observed (Fig. 3-5). No PCR products were found using total RNA extracted from the root tissue of healthy sugar beet. Neither primer pair produced PCR products using total RNA extracted from the roots of healthy sugar beet.

Evaluation of the diagnostic tests. The diagnostic tests evaluated during this research were assessed on one occasion by testing 20 naturally infected sugar beet using DAS-ELISA, conventional two-step RT-PCR and one-step RT-PCR. All methods detected some infected plants and infection by DAS-ELISA was confirmed by conventional two-step RT-PCR and one-step RT-PCR. Both of these molecular methods detected the same infected plants and

Fig. 1. High quality total RNA bands of a sugar beet root sample extracted using QIAGEN kit: 28S and 16SrRNA bands are well observed top-down route.

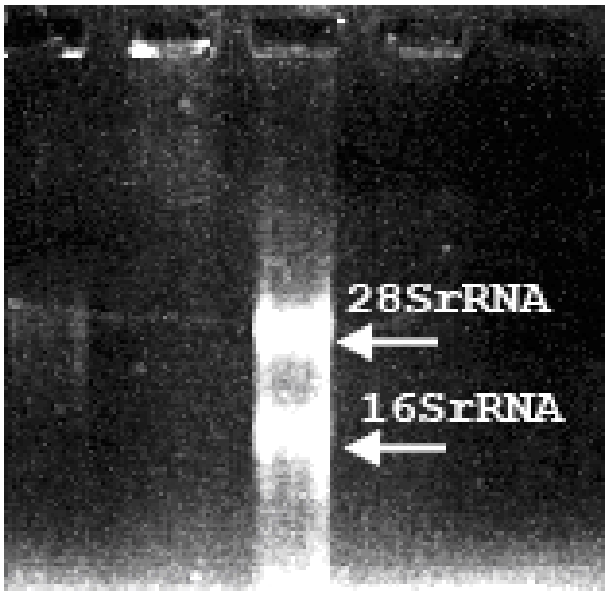
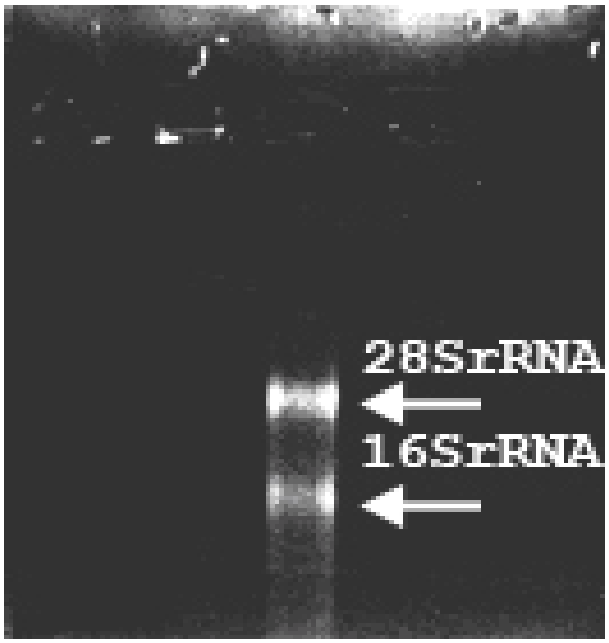


Fig. 2. Moderate quality total RNA bands of a *Chenopodium quinoa* test plant leaf sample extracted using Trizole solution: 28S and 16SrRNA bands are well observed top-down route



were able to detect more infected plants than ELISA.

The sensitivity of DAS-ELISA and conventional two-step RT-PCR were compared by Henry (1996) who showed that the molecular test was 1250 times more sensitive than DAS-ELISA. In this study, both two and one-step RT-PCR using RNA purified by the two total RNA extraction methods were shown to have the same sensitivities; both protocols detected dilutions of total RNA extracted from BNYVV-infected sugar beet and *C. quinoa* in water down to 1×10^{-6} and 1×10^{-7} µL, respectively. When the same RNA was diluted in total RNA extracted from healthy plants, the sensitivity of both two and one-step RT-PCR was reduced to 1×10^{-3} µL in both infected sugar beet and *C. quinoa*. Further comparisons on the sensitivities of the diagnostic methods were made using dilutions in extractions from healthy *C. quinoa* or sugar beet. One-step immunocapture RT-PCR was slightly less sensitive than one-step RT-PCR using purified RNA and detected dilutions of sap extracted from infected sugar beet and *C. quinoa* down to 1×10^{-2} and 1×10^{-3} µL, respectively. Using cDNA amplified from either BNYVV-infected sugar beet or C. *quinoa*, n-PCR increased the sensitivity of detection of amplified cDNA from immunocaptured or purified total RNA to 1×10^{-5} and 1×10^{-6} µL, respectively. Fig. 6 and 7 represent a 1000-fold increase in the sensitivity of BNYVV detection.

DISCUSSION

The RT-PCR assay described by Morris *et al*. (2001) has proven to be a more sensitive method for the detection of Beet necrotic yellow vein *Benyvirus* than DAS-ELISA. This method reduces the time needed to detect rhizomania in soil samples using a seedling bait test from 7 to 4 weeks. The reliability of such tests depends on the sensitivity of the detection method and the distribution of resting spores in the sample, in particular the proximity of spores to the growing roots of the plant. To maximise the likelihood of detecting infection in soils containing little inoculum after a short test period, a n-PCR system was designed which specifically amplified a 326 bp product within the amplicon produced by the primers of Henry (1996). This method improved the sensitivity of BNYVV detection in infected sugar beet roots by 1000-fold compared with the standard RT-PCR.

Further refinements to the RT-PCR method of Henry (1996) have enabled the adoption of a simpler, shorter total RNA extraction method, the replacement of laboratory reagents with standard total RNA extraction kits and the use of a one-step procedure with its innate advantages of convenience and decreased contamination risks. None of these changes affected the specificity or sensitivity of the assay. The one-step RT-PCR also worked well on immunocaptured samples rather than total RNA extracts but with a slight loss of sensitivity. Furthermore, this n-PCR assay can be completed within a few hours and thus results can be produced on the same working day as standard RT-PCR but with increased sensitivity.

Fig. 3. Below arrow show Single 500bp specific bands; (Ch.): BNYVV inoclated leaf of *Chenopodium quinoa* leaf samples; (1, 2, 3, 4): naturally infected sugar beet root samples. No difference was observed between obtained results with test plants and natural host samples.

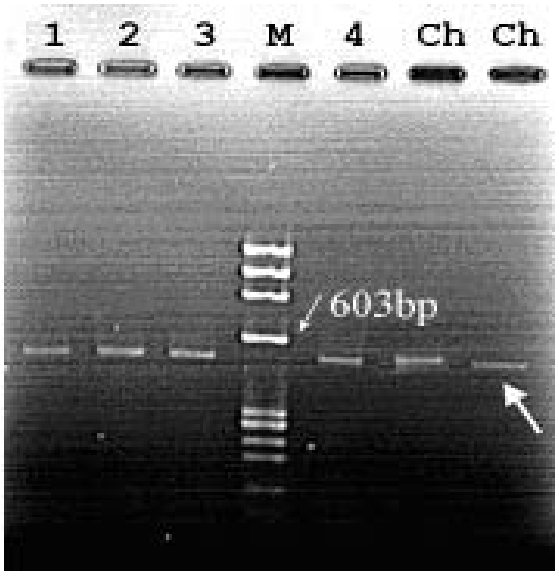
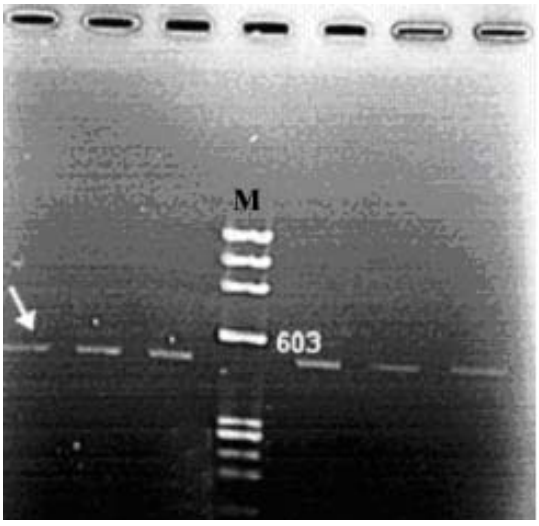


Fig. 4. RT-PCR 500bp bands (arrow) of Iranian sugar beet samples proved the presence of BNYVV virions in plant tissues. All of above six 500bp bands were obtained from naturally infected sugar beet roots. M: ΦX174 DNA size marker. Number on the size marker represent 603 bp.



Apart from its application in early diagnosis of infected plants, the increased sensitivity of RT-PCR lends the system to other applications, for example, the early detection of all BNYVV pathotypes in earlier unaffected

areas or cultivars, including the resistance breaking strain identified in Pithivier, France (Koenig *et al*., 1998). The persistence of the vector, *P. betae*, in soil, makes breeding for resistance a sole method to control rhizomania (Meunier *et al*., 2003). Therefore, a further application of this RT-PCR assay will be to facilitate BNYVV testing for breeding purposes, the increased sensitivity of the assay will help to identify truly resistant sugar beet breeding lines rather than those which are tolerant or partially resistant.

Fig. 5. RT-PCR 500bp bands (below arrow) obtained from German isolate infected *Chenopodium quino* (Ch) leaf samples. M: ΦX174 DNA size marker. Above arrow represent 603 bp of size marker.

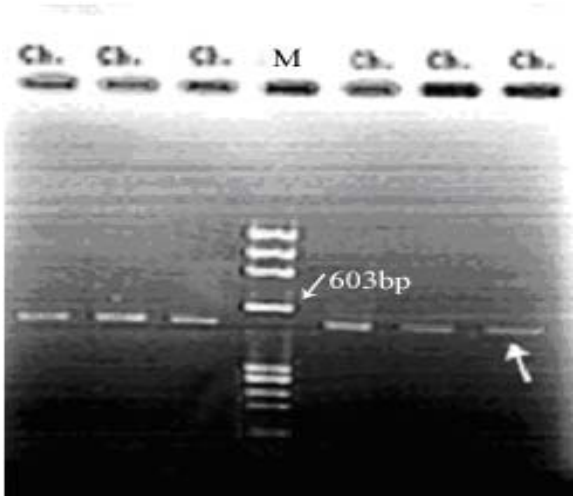


Fig. 6. (Arrow including: 1, 2, 3, 4, 5, 6,7): 10^{-2} dilution RT-PCR 500bp bands obtained from infected *Chenopodium quino* test plant leaf samples. M: ΦX174 DNA size marker

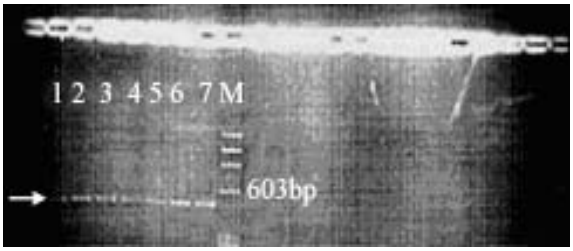


Fig. 7. (Arrow including 1, 2, 3, 4, 5, 6, 7): 10^{-3} dilution RT-PCR 500bp bands obtained from infected sugar beet root samples. M: ΦX174 DNA size marker

Although the specificity of standard RT-PCR was demonstrated by Henry *et al.* (1995), the n-PCR assay is intrinsically more specific since detection is dependent on the binding of four primer designed by Morris *et al.* (2001) rather than two primers. This specificity further decreases the possibilities of false positive results, which is of great importance in countries with quarantine restrictions. To eliminate the possibility of contamination, it is vital to include appropriate negative controls and separate pre- and post-PCR procedures. Recently, a PCR-based method was developed that is able to detect BNYVV A type/B type recombinant RNA molecules up to dilutions of one to a million in pure B type RNA molecules (Koenig & Buttner, 2004). Also, The RT-PCR test system has been developed for detecting the nondescribed mycovirus which is proposed to be called *Helicobasidium purpureum* partitivirus (Mel'nichuk & Spiridonov, 2005).

Sensitivity comparisons showed that for the detection of RNA5, TaqMan was 10,000 times more sensitive than the conventional RT-PCR assay. Further improvements were made to the test procedure by using post-ELISA virus release (VR), as an alternative to RNA extraction. This significantly increased the speed of processing samples and reduced the staff input required, allowing the TaqMan assay to be used routinely as part of an annual survey of UK field samples (Harju *et al*., 2005). Using the Triton X-100 release of RNA and IC-RT-PCR the sensitivity of detection was so high that pg amounts of BYV RNA occurring in dilutions up to 10^{-6} of saps from young tetragonia and sugar beet leaves could be detected (Kunduk & Rysanek, 2004).

It is concluded that RT-PCR assay improves the sensitivity of BNYVV detection compared with existing serological methods and thus reduces the risk of false negative diagnosis. The assay provides a robust, sensitive procedure, which identifies a range of BNYVV isolates obtained from Germany and Iran. The use of the technique to enable early identification of BNYVV from bait test and field grown plants has been demonstrated. The technique could also be used for breeding and quarantine programs as well as for studies on the epidemiology and control of BNYVV.

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