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Protocols

Comparative analysis of beet western yellows virus detection methods: Development and validation of a novel real-time RT-PCR assay also targeting beet leaf yellowing virus

Hellin Pierre [a,*], Everaert Ellen [b], Demonty Elisabeth [a], Richet Pauline [a], Muhovski Yordan [c], De Jonghe Kris [b], Hautier Louis [a], Steyer Stéphan [a]

[a] Walloon Agricultural Research Center – Plant and Forest Health Unit, Gembloux, Belgium
[b] Flanders Research Institute for Agriculture, Fisheries and Food - Plant Sciences Unit, Merelbeke-Melle, Belgium
[c] Walloon Agricultural Research Center – Biological Engineering Unit, Gembloux, Belgium

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ABSTRACT

Beet western yellows virus (BWYV – *Polerovirus BWYV*) is a polerovirus associated with yellowing and stunting symptoms in sugar beet and several other crops. Its close relationship with other beet-infecting poleroviruses, has long complicated accurate diagnosis due to serological cross-reactivity and limited molecular data. To overcome these issues, we developed a novel real-time RT-PCR assay enabling the specific and sensitive detection of BWYV and beet leaf yellowing virus (BLYV – *Polerovirus BLYV*). The assay was designed from a curated genomic dataset to ensure inclusivity and tested in parallel with validated reference methods, including a semi-generic ELISA and a generic polerovirus RT-PCR. Comprehensive validation following the EPPO PM 7/98(5) standard confirmed the method's high analytical sensitivity, specificity, repeatability, and reproducibility. The assay proved robust to methodological variations and compatible with multiplex diagnostic workflows. Compared with existing approaches, it offers faster, more reliable detection and eliminates cross-reactivity with non-target poleroviruses. This method provides a valuable tool for accurate BWYV surveillance and supports improved management of beet virus yellows in agricultural and phytosanitary contexts.

1. Introduction

Beet western yellows virus (BWYV; *Polerovirus BWYV*) is a polerovirus that induces yellowing and stunting symptoms in sugar and table beet as well as on a broad variety of host including other economically important crops such as spinach and peppers (Beuve et al., 2008; Yoshida and Tamada, 2019). It has been reported in various countries including the USA, France, Greece, Ecuador, South Korea, Japan and China (Beuve et al., 2008; Lotos et al., 2014; Miguel et al., 2016; Yan et al. 2019; Yoshida and Tamada, 2019; Kwak et al. 2023; Alvarez-Quinto et al., 2024). On sugar beet, BWYV is part of a complex of viruses responsible for the "virus yellows" disease, which can cause significant yield losses in affected crops. Although commonly affecting sugar beet in the USA (Wintermantel, 2005), BWYV has only been officially reported once on beet in the EU (Greece; Lotos et al., 2014), where the beet yellowing viral complex is commonly composed of beet yellows virus (BYV; *Closterovirus favibetae*), beet mosaic virus (BtMV; *Potyvirus betaceum*) and two poleroviruses namely beet mild yellowing virus (BMYV; *Polerovirus BMYV*) and beet chlorosis virus (BChV; *Polerovirus BCHV*) (Hossain et al., 2021; Mahillon et al., 2022). Viruses responsible for beet yellows are transmitted by aphids, mainly the green peach aphid (*Myzus persicae*). As no tolerant beet varieties are currently commercialized, the most effective control measure has been insecticide spraying and seed dressing. In recent years, neonicotinoid-based insecticides have been banned in Europe, leaving farmers with limited tools to manage virus diseases during growing seasons that are especially favorable for aphid outbreaks and virus transmission.

Due to its close relationship with turnip yellows virus (TuYV; *Polerovirus TUYV*) and BMYV, BWYV has been frequently misidentified in the past (Stevens et al., 2005; Beuve et al., 2008). Their high genetic and immunological similarity often led to misdiagnosis because commercially available serological tests, based on polyclonal antibodies, cross-react with the aforementioned viruses and with BChV (Hossain et al., 2021). This cross-reaction has complicated efforts to accurately

* Correspondence to: Walloon agricultural reasearch center, Rue de Liroux 4, 5030 Gembloux, Belgium.
E-mail address: p.hellin@cra.wallonie.be (H. Pierre).

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detect and study the virus. Other closely related Polerovirus species have also been described, such as brassica yellows virus (BrYV; *Polerovirus TUYV*), beet leaf yellowing virus (also infecting sugar beet; BLYV; *Polerovirus BLYV*), cucurbit aphid-borne yellowing virus (CABYV; *Polerovirus CABYV*), and melon aphid-borne yellowing virus (MABYV; *Polerovirus MABYV*), which form a well-supported phylogenetic clade but may show varying levels of similarity depending on the genomic region analyzed (Yoshida and Tamada, 2019; Latourrette et al., 2021).

PCR-based methods, both generic for polerovirus and BWYV-specific (Robertson et al., 1991; Abraham et al., 2007; Beuve et al., 2008; Knierim et al., 2010, 2013; Lotos et al., 2014; Eicholtz et al., 2018; Yan et al., 2019; Kwak et al., 2023; Alvarez-Quinto et al., 2024), have been developed as alternatives, each with their own advantages and limitations. Although post amplification sequencing is required to effectively identify the virus present, generic methods are often preferred by plant diagnostic laboratories because they allow for detection of multiple viruses with a single test, thus limiting the burden of validating a plethora of species-specific tests. For broader polerovirus detection, the use of generic Gen1–2 primers (Knierim et al., 2013) has been suggested by the EPPO Q-bank consortium in virology (https://qbank.eppo.int/). Nevertheless, no validation data is currently available for any PCR-based methods targeting BWYV or poleroviruses.

Table 1
Analytical specificity of the different BWYV detection methods (ELISA, RT-PCR and RT-qPCR) on target and non-target viruses.

	Species	Host matrix	Isolate	ELISA[a]	RT-PCR	RT-qPCR	
				CRA-W[b]	CRA-W	CRA-W	ILVO
Target Virus	BWYV	*Capsella bursa-pastoris*	P6-6D-W11_BWYV	+	+	22.6	24.2
	BWYV	*Beta vulgaris*	USA	+	+	19.8	22.6
	BWYV	*Capsella bursa-pastoris*	USDA	+	+	21.2	20
	BWYV	Synthetic control[c]	NC_004756.1	nt	nt	22.9	nt
	BWYV	Synthetic control	MW349137.1	nt	nt	23.9	nt
	BWYV	Synthetic control	HM804471.1	nt	nt	22.4	nt
	BWYV	Synthetic control	KU521324.1	nt	nt	21.9	nt
	BWYV	Synthetic control	MW674791.1	nt	nt	22.9	nt
	BWYV	Synthetic control	OL449448.1	nt	nt	23.5	nt
	BLYV	*Beta vulgaris*	BLYV-NN[d]	nt	+	17	15.2
	BLYV	Synthetic control	LC428352.1	nt	nt	21.8	21
Non-target virus							
Polerovirus	TuYV	*Raphanus sativus*	DSMZ PC-1209	+	+	-	-
	TuYV	*Brassica napus*	DSMZ PV-1284	nt	+	-	-
	TuYV	*Brassica napus*	20/0106/VI.16	+	+	-	nt
	TuYV	*Brassica oleracea*	20/0013/RVI.1	nt	+	-	nt
	TuYV	*Crepis* sp.	22/0136/VI.170	+	nt	-	nt
	TuYV	*Viola arvensis*	22/0136/VI.172	+	nt	-	nt
	TuYV	*Artemisia vulgaris*	22/0136/VI.196	+	nt	-	nt
	BChV	*Beta vulgaris*	DSMZ PV-1211	nt	+	-	-
	BChV	*Beta vulgaris*	USDA	+	+	-	nt
	BChV	*Beta vulgaris*	BChV-2A	+	+	-	nt
	BChV	*Beta vulgaris*	BChV-Irbab	nt	nt	-	nt
	BChV	*Beta vulgaris*	BChV PC ILVO	nt	nt	nt	-
	BMYV	*Beta vulgaris*	IfZ	+	+	-	nt
	BMYV	*Beta vulgaris*	SES	nt	nt	-	nt
	BMYV	*Beta vulgaris*	DSMZ PV-1210	nt	nt	nt	-
	PLRV	*Solanum tuberosum*	23/0015/VI	-	+	-	nt
	CABYV	*Cucumis sativus*	CABYV DCP[c]	nt	+	-	-
	CABYV	Synthetic control	MN688220.1	nt	nt	-	nt
	CABYV	Synthetic control	NC_003688.1	nt	nt	-	nt
	MABYV	Synthetic control	MW023665.1	nt	nt	-	-
	MABYV	Synthetic control	MW528313.1	nt	nt	-	nt
	MABYV	Synthetic control	NC_010809.1	nt	nt	-	nt
	MABYV	Synthetic control	OK120863.1	nt	nt	-	nt
	CYDV-RPV	*Avena sativa*	24/0122/VI.05	-	+	-	nt
	CYDV-RPV	*Avena sativa*	24/0122/VI.06	-	+	-	nt
	UPolV	*Ullucus tuberosus*	UL20004B	nt	+	-	-
	PeVYV	*Capsicum annuum*	DSMZ PV-0554	-	+	-	-
Non-polerovirus	BYV	*Beta vulgaris*	DSMZ PV-1237	-	-	-	-
	BYV	*Beta vulgaris*	BBRO	-	nt	-	nt
	BYV	*Chenopodium foliosum*	DSMZ PV-0981	nt	nt	nt	-
	BtMV	*Beta vulgaris*	DSMZ PV-1218	nt	-	-	-
	BtMV	*Beta vulgaris*	DSMZ PV-1228	-	nt	-	-
	BtMV	*Beta vulgaris*	DSMZ PV-1169	nt	nt	nt	-
	BNYVV	*Chenopodium quinoa*	DSMZ PV-0990	nt	nt	nt	-
	BNYVV	*Beta vulgaris*	DSMZ PC-0467	nt	nt	nt	-
	CMV	Unknown		nt	nt	nt	-
	AMV	*Nicotiana tabacum*	DSMZ PC-0779	nt	nt	nt	-
	BSBMV	*Chenopodium quinoa*	DSMZ PC-1035	nt	nt	nt	-
	BSBV	*Beta vulgaris*	DSMZ PC-0576	nt	nt	nt	-
	BVQ	*Beta vulgaris*	DSMZ PV-0961	nt	nt	nt	-

Note: [a] Detection method used. Result of each test are given as follows: "+" = detected, "-" = not detected, "nt"= not tested. Numerical values indicated as the RT-qPCR results correspond to Ct values obtained in the case of a BWYV or BLYV detection.
[b] Operators who performed the analysis.
[c] Synthetic controls are single stranded DNA molecules corresponding to the target sequence of the RT-qPCR test from a particular isolate for which the GenBank accession number is given as isolate name.
[d] RNA extracted at ILVO.

In this study, we report on the development of a novel RT-qPCR method for the detection of both BWYV and BLYV, collectively referred to as BWYV-like, owing to their close genetic relatedness and shared ability to infect sugar beet. The method was compared to other reference detection methods, namely ELISA and conventional RT-PCR. Based on thorough validation following international standards (EPPO, 2021), our research aims to provide a more specific and sensitive tool for BWYV detection as well as validation data for generic polerovirus detection, addressing the limitations of existing detection methods.

2. Materials and methods

2.1. Plant material and positive controls

Viral isolates were mostly obtained through DSMZ's collection (Table 1). Other isolates were sourced from CRA-W and ILVO's own viral collection or generously provided by other research institutes. To evaluate the performance of the diagnostic tests, some positive samples were diluted into healthy plant matrices. Healthy plants were grown from seeds (*Beta vulgaris* cv. Lysana, *Nicotiana tabacum* cv. Xanthi, *Solanum lycopersicum* cv. Marmande, *Cucurbita pepo* cv. Black Beauty, *Capsicum annuum* cv. Yolo wonder, *Brassica juncea*, *Cyanus segetum*, *Fagopyrum esculentum*, *Matricaria chamomilla*, *Pisum sativum*, *Raphanus sativus*, *Trifolium repens*, *Vicia sativa* and *Gypsophila* sp.) in controlled growth chambers (16/8 h light/dark, photoperiod at 22°C), except for spinach for which freshly bought leaves were used. To assess the specificity of the RT-qPCR assay against multiple viral isolates of BWYV, BLYV, CABYV and MABYV, synthetic DNA oligonucleotides matching the target amplicon of each isolate (with accession numbers shown in Table 1) were ordered from Eurofins and used as defined template controls.

2.2. Detection of BWYV by ELISA

The BWYV TAS-ELISA (RT-0049) kit from DSMZ was used as the reference method for ELISA detection of BWYV. All ELISA buffers were prepared according to the manufacturer's instructions (DSMZ), except that sodium azide was omitted from all buffers, and ovalbumin (2 g/L) was added to the sample extraction buffer formulation. 100 mg of fresh, frozen or rehydrated leaf material were grinded in a 2 mL Eppendorf tube with a 5 mm steel ball using a Retsch MM40 for 1.5 min at 30 Hz. 1 mL of Sample extraction buffer was then added to each tube which is then shaken vigorously and briefly centrifuged to pellet the remaining leaf debris. Serological virus detection was then performed on the supernatant following the manufacturer's recommendations. The absorbance was measured after an hour at 450 nm using a MultiSkan FC (Thermo Fisher Scientific). The antiserum used in the kit is known to cross-react with other members of the *Polerovirus* such as TuYV, BChV and BMYV.

2.3. RNA extraction

At CRA-W, RNA extraction was performed on 100 mg of leaf samples, homogenized as previously described. Nucleopsin Plant and Fungi RNA extraction kit (Macherey-Nagel) was done with a final elution in 50 µL of the provided Elution Buffer. At ILVO, leaf samples were either homogenized in GH+ buffer (Botermans et al., 2013) at a 1:5 w/v ratio or bead-beaten in TissueLyser II with 5 mm steel beads (Simply Bearings) for 1 min at 30 Hz. RNA was then extracted using MagMax RNA isolation kit (Thermo Fisher Scientific) on the KingFisher Flex platform and eluted in 100 µL nuclease free water. Each method was performed according to the manufacturer's instructions.

2.4. Generic Polerovirus detection by RT-PCR

RT-PCR using the generic primers Gen1/2 (Knierim et al., 2013) was performed using the Titan One Tube RT-PCR System (Roche) in 20 µL reaction containing Titan reaction buffer 5X, 500 nM of each primers, dNTPs at 10 nM each, and 0.4 µL of enzyme mix. Thermocycling conditions consisted of a first cycle at 50°C for 30 min for the RT followed by 2 min at 94°C and then 40 cycles of 94°C for 15 s, 54°C for 30 s and 72°C for 45 s. A final elongation step of 5 min at 72°C ended the run. Amplification was visualized using TAE gel electrophoresis stained with GelRed (Biotium). Viral identification was carried out through Sanger sequencing of the amplicons at the Eurofins facility, followed by comparison of the obtained sequence to the "core_nt" database of GenBank® using megablast.

2.5. Design of the RT-qPCR assay for the detection of BWYV

Genomic sequences of BWYV were retrieved from GenBank, aligned and visually filtered by comparison against the genome of the BWYV-USA isolate (Acc. No. AF473561 syn. NC_004756) to exclude misannotated entries. BLYV isolates were also included, as the virus shares highly similar genomic sequences with BWYV and is also a beet-pathogenic polerovirus. The final subset of 38 sequences (Supplementary Table1) obtained was aligned and merged into a single consensus sequence which was then used to design a set of primer and probes with the PrimerQuest webtool (IDT DNA technologies). Based on the selected BWYV sequences, the primers BWYV-F1 (5'-CTACAAGCCGCCAGGTG-3'; nt positions 2069–2085) and BWYV-R1 (5'-GTAGGCCTCGCAGCATTT-3'; nt positions 2147–2164) and the probe BWYV-Pr1 (5'-TTTAACTTGCTGGGCGCGTTCC-3'; nt positions 2089–2110) were designed on the RNA-dependent RNA polymerase (RdRp) coding region (nucleotide positions refer to NC_004756).

2.6. Execution of the RT-qPCR assay

Two different methodologies were followed to conduct the RT-qPCR by CRA-W and ILVO.

At CRA-W, reactions were performed in 25 µL of final volume using One-Step Takyon Ultra Probe 4X MasterMix (Eurogentec, Belgium) and primers and probe at 250 nM and 80 nM, respectively. The primers and probe set (CyOXID-F, R and -TAQ) designed by Papayiannis et al. (2011) to quantify the level of the mitochondrion cytochrome oxidase subunit I (mtCOXI) from plants were also added in multiplex (at 300 and 100 nM, respectively) to serve as an internal control. Samples were run in duplicates with 3 µL of sample RNA per reaction. The cycling program consisted of a 15 min step at 50°C to enable reverse transcription followed by an activation step at 95°C for 3 min, then by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 1 min, with the intensity of fluorescence captured at the end of every cycle. Reactions were carried out on a CFX96 optical reaction module with C1000 Touch thermal cycler (Bio-Rad). The sequence of the target amplicon (96 bp), extrapolated from the BWYV-USA genomic sequence, was synthetized by Eurofins and subsequently cloned in pJet 1.2 vector (Thermo Fisher Scientific) to serve as positive amplification control. The performance of the assay was tested using serial dilutions of the cloned amplicon. The correspondence between cycle threshold values (Ct values; determined at the threshold automatically estimated by the CFX manger software) and the potential number of viral plasmid copies was evaluated using a standard curve made from serial dilutions of the plasmid control.

At ILVO, reactions were carried out in 20 µL final volume with the TaqPath No Rox Kit (Applied Biosystem) in multiplex with primers and probes targeting BtMV (confidential), TuYV (Buxton-Kirk et al., 2021) and COX as internal control (Cullen et al., 2005). All primers and probes were used at 300 and 150 nM, respectively, except for COX, which were used at 150 nM and 250 nM. Samples were run in duplicates with 2 µL of

sample RNA per reaction. The cycling program consisted of a 2 min step at 25°C and a 10 min step at 53°C to enable reverse transcription followed by an activation step at 95°C for 2 min, then by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min, with the intensity of fluorescence captured at the end of every cycle.

A cut-off value for BWYV detection was set at 35 cycles at both laboratories, corresponding to the validated threshold ensuring reliable detection of the virus while minimizing the risk of false-positive results.

2.7. Diagnostic validation

Validation of the ELISA and the two PCR-based methods were performed at CRA-W, following the EPPO guideline on diagnostic validation (EPPO, 2021). Briefly, sensitivity was assessed by testing serial dilutions of three BWYV isolates spiked into healthy plant matrices to determine the limit of detection. Repeatability was evaluated by analyzing dilutions of isolate P6–6D-W11_BWYV using 3 biological replicates. Reproducibility was assessed on a different day by a second operator, who tested an independent set of 3 biological replicates of the same isolate, serially diluted to concentrations approaching the detection threshold of each assay. Analytical specificity was evaluated for each method using a broad panel of viruses and isolates (Table 1), to assess the ability to detect the target virus without cross-reactivity with non-target viruses. Selectivity was evaluated by testing viruses in different host matrices to evaluate any matrix effects that could interfere with detection. Robustness was assessed by parallel testing at the ILVO laboratory, where the same assays were performed following an entirely independent procedure, as previously described. Validation data of each method were deposited on the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validation list).

3. Results

3.1. Analytical sensitivity

Comparison of the sensitivity between ELISA, RT-PCR and RT-qPCR was performed on 3 BWYV isolates (Table 2). Overall detection using the 3 methods showed a variable viral titer across the starting infected material, with the BWYV-USA infected plant containing the lowest amount of virus. The overall limit of detection (LOD) of each method was therefore based on the lowest dilution which gave a positive result for all isolates. The ELISA test showed a much lower sensitivity compared to the PCR-based methods with only the undiluted samples being reliably detected across all isolates and biological replicates. The generic RT-PCR had a higher sensitivity, reliably detecting BWYV up to a 10-fold dilution, although the detection limit was much lower in P6–6D-W11_BWYV and BWYV-USA than for BWYV-USDA. Lastly the RT-qPCR assay showed to be even more sensitive, allowing to reliably detect all 3 BWYV isolates at least at a 10^4-fold dilution. The standard curve obtained with serial dilutions of plasmid containing the target amplicon showed a detection for as low as 10 plasmid copies/µL (Fig. 1). Although using DNA does not take into account reverse transcription, applying the Ct cut-off value implemented for plant sample analysis (i.e. cut-off = 35 cycles) brings the number of copies potentially detected by the RT-qPCR test to less than 800 copies. Sensitivity of the ELISA and the generic RT-PCR were tested as well against dilutions of BMYV, BChV, and TuYV in beet matrix (Table 3) with a single replicate. The results showed a better detection limit of the ELISA compared to BWYV, especially for BChV and BMYV (around 10^2-fold dilution). Nevertheless, as for the BWYV isolates, the LOD of the RT-PCR was more sensitive than the ELISA, detecting BMYV, BChV and TuYV at a 10^3-fold dilution. PLRV was also detected by the generic RT-PCR test up to a 10^4-fold dilution of the starting infected material in potato leaf matrix.

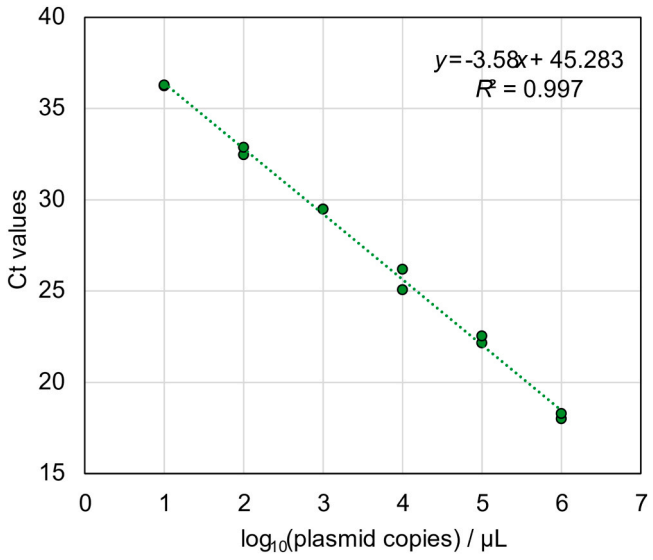

Fig. 1. Standard curve obtained for serial dilution of plasmid (from 10^6 to 10 copies) containing the sequence region of isolate BWYV-USA (NC_004756.1) targeted by the RT-qPCR. Results from two biological replicates are shown with each point being the average of two technical replicates.

Table 2
Comparison of the analytical sensitivity, repeatability and reproducibility of the BWYV detection methods.

Dilution factor	P6-6D-W11_BWYV[a]						BWYV-USA				BWYV-USDA		
	ELISA[b]	RT-PCR		RT-qPCR			ELISA	RT-PCR	RT-qPCR		ELISA	RT-PCR	RT-qPCR
	CRA-W	CRA-W1	CRA-W2	CRA-W1	CRA-W2	ILVO	CRA-W	CRA-W	CRA-W	ILVO	CRA-W	CRA-W	CRA-W
undiluted	+,+,+	+,+,+	nt	15.9, 16.8, 13.6	nt	nt	+	+	13	nt	+	+	20.1
101	+,-,-	+,+,+	nt	19.2, 20.1, 16.8	nt	nt	-	+	17.4	nt	-	+	24.2
102	-,-,-	+,+,+	+,+,+	21.9, 23.4, 20.4	23, 23.2, 23.6	24.2	-	+	19.8	22.58	-	-	25
103	-,-,-	+,+,+	+,+,+	25.9, 26.5, 24.2	26.9, 26.8, 26.3	nt	-	+	23.3	nt	-	-	28.7
104	-,-,-	+,-,-	-,-,-	28.4, 30.8, 28	28, 29.1, 28.4	29.1	-	+	26.8	26.1	-	-	31.9
105	-,-,-	-,-,-	-,-,-	32.5, 31.7, 30.1	32.3, 32.2, 31.9	nt	-	+	30.4	29	-	-	38.6
106	-,-,-	-,-,-	-,-,-	nd, 37.9, 34.9	35.6, 38, 35.5	nt	-	-	34.8	31.8	nt	nt	nt
107	-,-,-	-,-,-	-,-,-	nd	nd	nt	-	-	35.7	33.3	nt	nt	nt
108	nt	nt	nt	nt	nt	nt	-	-	-	35.7	nt	nt	nt

Notes: [a] Name of the BWYV isolate tested. Diagnostic tests for isolate P6-6D-W11_BWYV at CRA-W were performed in biological triplicates to evaluate the repeatability as well as by two operators (CRA-W1 and CRA-W2) to evaluate the reproducibility of the method. The result of each test is given in the corresponding column. Reproducibility was not evaluated for ELISA.
[b] Detection methods evaluated on the coresponding isolate. Result of each test are given as follows: "+" = detected, "-" = not detected, "nt"= not tested. Numerical values indicated as the RT-qPCR results correspond to Ct values obtained in the case of a BWYV-like detection.
[c] Operator who performed the detection assay.

Table 3
Comparison of the analytical sensitivity of the (semi)-generic polerovirus detection methods (ELISA and RT-PCR) on different polerovirus.

Dilution factor	BMYV (isolate IfZ)		BChV (BChV-2A)		TuYV (DSMZ PC-1209)		PLRV (23/0015/VI)	
	ELISA[a]	RT-PCR	ELISA	RT-PCR	ELISA	RT-PCR	ELISA	RT-PCR
undiluted	+	+	+	+	+	+	-	+
10^1	+	+	+	+	(+)	+	nt	+
10^2	+	+	+	+	-	+	nt	+
10^3	-	+	-	+	-	+	nt	+
10^4	-	-	-	-	-	-	nt	+
10^5	-	-	-	-	-	-	nt	-
10^6	-	-	-	-	nt	nt	nt	nt

Note: [a] Detection method used. Result of each test is given as follows: "+ " = detected, "(+)" = doubtful, "-" = not detected, "nt"= not tested

3.2. Repeatability and reproducibility

Repeatability and reproducibility of each test were evaluated on plant tissues infected with P6–6D-W11_BWYV (Table 2). The dilution series were biologically replicated 3 times by 2 operators (CRA-W1 and CRA-W2) with the second operator only investigating the dilutions around the LOD as identified by the first operator. Repeatability and reproducibility were both at 100 % at the isolate-specific LOD of each test, corresponding to the undiluted sample for ELISA, a 10^3-fold dilution for the RT-PCR and a 10^5-fold dilution for the RT-qPCR (mean=31.8 and sd=0.87). At the LOD, all RT-PCR products allowed for the correct identification of BWYV using Sanger sequencing (data not shown).

3.3. Specificity

In silico analysis of the novel primers and probes showed a low level of mismatches when compared to BWYV sequences with a maximum of one mismatch per accession for all 3 oligos (Supplementary Figure 1). Primer-BLAST searches using primers BWYV-F1 and-R1 also showed complementarity with some CABYV and MABYV accessions, while searches with BWYV-Pr1 showed a minimum of 4 mismatches with the probe sequence for both CABYV and MABYV. These findings were confirmed in the lab with no amplification signals detectable for any of the RNA extracts from non-target virus and various plant matrices, while all BWYV isolates could be detected. Specificity was therefore 100 % according to the EPPO Standard PM7/98 (5). In comparison, other BWYV specific RT-PCR detection methods (Beuve et al., 2008; Lotos et al., 2014; Eicholtz et al., 2018; Yan et al., 2019; Kwak et al., 2023; Alvarez-Quinto et al., 2024) generally showed a lower inclusivity for BWYV isolates as the in silico analysis revealed multiple nucleotide mismatches between the primers and the corresponding target regions in several isolates (Supplementary Figure 2). As expected, the ELISA assay cross-reacted with all tested strains of BMYV, BChV and TuYV while other tested poleroviruses - potato leafroll virus (PLRV; *Polerovirus PLRV*), cereal yellow dwarf virus-RPV (CYDV-RPV; *Polerovirus CYDVRPV*) and pepper vein yellows virus (PeYVV; *Polerovirus PEVYV*) - were not detected. This semi-generic nature of the test was not considered as a flaw in specificity as it is a known and advertised behavior. On the other hand, the generic RT-PCR detection assay showed a 100 % inclusivity for all tested poleroviruses and no cross-reaction with any of the other viruses or matrix tested (Table 2). Moreover, Sanger sequencing confirmed the virus identity of all tested poleroviruses (data not shown). As for the RT-qPCR, the ELISA and RT-PCR test did not cross react with other plant matrices.

3.4. Selectivity

The possibility of reliably detecting BWYV in other plant matrices was compared for the P6–6D-W11_BWYV isolates by diluting (10^2-fold) infected material in tobacco (Ct = 23.3 [CRA-W]), tomato (Ct = 23.9 [CRA-W] and 24.7 [ILVO]), zucchini (Ct = 24.6 [CRA-W]) and bell pepper (Ct = 22 [CRA-W] and 23.6 [ILVO]). The overall values obtained were overly similar with average Ct value obtained for P6–6D-W11_BWYV in beet (mean Ct = 22.6 [CRA-W] and 24.2 [ILVO]). Moreover, the BWYV USA isolate diluted in spinach was detected at high dilution rates with both the RT-PCR (up to 10^5-fold) and the RT-qPCR (up to 10^7-fold) assays (Table 1). Virus isolates used for specificity testing infected different hosts such as potato (PLRV), barley (CYDV-RSV) or oilseed rape (TuYV), which did not appear to hinder polerovirus detection. On the other hand, in RT-qPCR, all plant RNA extracts produced a clear amplification signal for internal control (data not shown).

3.5. Robustness

To evaluate the robustness of the test as its capacity for providing reliable results when being implemented in other laboratories, samples were sent to ILVO for analysis using their own protocol. Despite having a different RNA extraction procedure, a different RT-qPCR reaction mixture, a different thermocycler and a different analysis pipeline, the results from ILVO were comparable to that of CRA-W (Tables 1 and 2).

4. Discussion

The results presented in this study show that the newly developed RT-qPCR test allows specific and sensitive detection of BWYV and BLYV. The assay consists of a one-step RT-qPCR assay using specific primers and probe and that is multiplexed with an internal RNA isolation control. It was designed using all currently available relevant full BWYV-like sequences selected from GenBank. Performance criteria, evaluated following EPPO PM 7/98 (5), showed clear advantage of the developed RT-qPCR compared with other available methods. The test is readily applicable to any laboratory equipped for RT-qPCR analysis and is likely sufficiently robust to withstand modifications of any step of the protocol, as shown in this study, provided that sufficient verification is undertaken to support the validity of the results.

The specific BWYV RT-qPCR is routinely applied in multiplex assays at CRA-W (with BYV, BtMV, BMYV, and BChV; Ruh et al., 2023) and at ILVO (with BtMV and TuYV). Both tests are used for the high-throughput screening of thousands on plant and aphid samples in the framework of the VIROBETT (PRW) and VIRBICON (VLAIO-LA) projects aiming at developing knowledge and strategies around beet virus yellows. Performance of the BWYV assay was compared with different multiplexing scenarios (single assay, multiplexed with internal control and multiplexed with the method from GEVES), without apparent impact on its sensitivity (data not shown). The BWYV RT-qPCR could therefore likely be multiplexed with other assay depending on the goal.

The novel RT-qPCR assay showed clear advantages over ELISA and conventional RT-PCR in sensitivity. It reliably detected BWYV at higher dilutions than the other methods, demonstrating a limit of detection as low as 10 copies/µL for the target sequence. Depending on the BWYV sample, the sensitivity of the RT-qPCR assay was up to 10^6-fold higher than the ELISA and up to 10^3-fold higher than the RT-PCR. Nevertheless, the RT-PCR showed a very good sensitivity, detecting BWYV up to a 10^5-fold dilution of the starting infected material. Many BWYV specific RT-PCR tests have been developed over the years but unfortunately none of the studies performed validation which could help comparing the tests

based on performance criteria such as sensitivity. As a reference point, a multiplex RT-qPCR method for the detection of the beet-infecting poleroviruses BChV and BMYV was recently developed by Borgolte et al. (2025). The authors evaluated, based on dilution of cDNA copies of viral genome, that both assays could detect as low as about 9.000 copies. Considering that our RT-qPCR test could detect less than 800 copies, it therefore appears to be highly sensitive for BWYV detection.

The low sensitivity of the ELISA might not be such an issue for BMYV and BChV since their viral titer in beet is very high. Nevertheless, the test semi-generic nature makes it a bit impractical when monitoring beet viruses, as BChV, BMYV, BWYV and TuYV can infect beet requiring a second test for species confirmation. On the other hand, the primers developed by Knierim et al. (2013) appeared to be highly generic, effectively detecting all polerovirus tested. These primers were initially designed to be able to detect poleroviruses in samples for which the quality and quantity of RNA were not sufficient to use generic polerovirus primers yielding longer amplicons (>1 kb) such as Pol-G-F/R or Pocon-F/-R (Knierim et al., 2010). Nevertheless, another generic test developed by Abraham et al. (2007) might outcompete the one from Knierim et al. (2013) in terms of inclusivity based on *in silico* analysis with MFEprimer (Wang et al., 2019) on the "NCBI virus" database (data not shown). Other generic primers producing short amplicons have been designed (Robertson et al., 1991; Lotos et al., 2014) but were dismissed because of low inclusivity. The validation data on the Gen1/2 primer set therefore is valuable information for diagnostic laboratories looking to adopt a generic polerovirus detection assay.

As some poleroviruses are closely related and in the past, they were often misidentified as one another, which was the case for TuYV, BMYV and BWYV (Beuve et al., 2008). Databases such as GenBank therefore contain genome sequences that are incorrectly assigned to a species. Working on a curated set of sequences, selected by careful comparison with the sequence of the reference isolate BWYV-USA is therefore mandatory in order to design an effective test. As BLYV genomic sequence is highly similar to that of BWYV and that it is also infecting beet (Yoshida and Tamada, 2019), this virus was also included in the genomic sequence set. The RT-qPCR assay was highly specific for BWYV and BLYV as demonstrated in the laboratory and *in silico* and did not generate false positive with the closely related CABYV. The differences in Ct values observed among the BWYV isolates reflect variations in virus titer within the samples, as indicated by the analytical sensitivity results, rather than differences in assay specificity. Alternatively, the RT-PCR assay developed by Lotos et al. (2014) appeared to be also specific for BWYV and BLYV. The short amplicon generated by this test might also give a good sensitivity (especially if used in a SYBR-based RT-qPCR assay). Nevertheless, the actual performance of the assay has not been tested and the presence of degenerate nucleotides in the oligo as well as the primers characteristics (especially GC% and dimer formation) might not be favorable.

Based on literature and GenBank records, BWYV appears to be geographically limited to the American and Asian continents, and is listed as a quarantine pathogen in countries such as Mexico and Jordan. In Europe, its presence has only been documented in *Nepenthes mirabilis* from a French botanical garden (Miguel et al., 2016) and on multiple hosts, including sugar beet, in Greece (Lotos et al., 2014). Notably, BWYV has never been officially reported in the major beet-producing regions of Europe, despite the widespread cultivation of susceptible crops. Although BWYV caused significant yield losses in California in the past (Wintermantel, 2005; Beuve et al., 2008), the reduction of sugar beet production in that region, together with its absence in a recent limited survey from major U.S. beet-growing areas (Chinnadurai et al., 2024), suggest that its current economic impact in the United States might be minimal. Nevertheless, its potential establishment in Europe could exacerbate the already complex viral landscape affecting this crop. The development and availability of a sensitive, specific, and reliable diagnostic tool are therefore essential to monitor, prevent, and mitigate the potential spread of this virus across vulnerable agricultural regions.

CRediT authorship contribution statement

Richet Pauline: Validation, Formal analysis. **Muhovski Yordan:** Writing – review & editing, Formal analysis. **De Jonghe Kris:** Writing – review & editing, Supervision. **Hautier Louis:** Writing – review & editing, Project administration. **Steyer Stéphan:** Writing – review & editing, Supervision. **Hellin Pierre:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Everaert Ellen:** Writing – review & editing, Validation, Investigation. **Demonty Elisabeth:** Validation, Formal analysis.

Declaration of Competing Interest

The authors have no conflict of interest to disclose

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2026.115352.

Data Availability

Data will be made available on request.

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