Widespread Occurrence of *Wheat spindle streak mosaic virus* in Belgium

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ABSTRACT

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In order to assess the occurrence of *Wheat spindle streak mosaic virus* (WSSMV) in Belgium, a reverse-transcription polymerase chain reaction (RT-PCR) was developed, targeting WSSMV isolates from Canada, France, Germany, Italy, and the United States. The primers also were designed for virus quantification by real-time RT-PCR with SYBR-Green. No cross-reaction with soilborne cereal viruses such as *Barley mild mosaic virus*, *Barley yellow mosaic virus*, *Soil-borne cereal mosaic virus*, and *Soil-borne wheat mosaic virus* was observed. The RT-PCR and real-time quantitative RT-PCR allowed a more sensitive detection of WSSMV than enzyme-linked immunosorbent assay. The incidence of WSSMV in Belgium was evaluated using a bioassay with wheat cvs. Cezanne and Savannah and rye cv. Halo, grown in 104 Belgian soils. The presence of WSSMV was detected from plants grown in 32% of the soils. The RT-PCR methods developed here, combined with large sampling, allowed WSSMV to be detected for the first time in Belgium. The real-time quantitative RT-PCR was developed as a tool for evaluating the resistance to WSSMV by quantifying the virus concentration in wheat cultivars.

Additional keywords: *Polymyxa graminis*, soilborne viruses

Wheat (*Triticum aestivum* L.) is affected by the soilborne bymoviruses *Wheat spindle streak mosaic virus* (WSSMV) and *Wheat yellow mosaic virus* (WYMV), in addition to furovirus infections. WSSMV and WYMV cause mosaic symptoms on wheat, characterized by chlorotic, spindle-shaped streaks on leaves as well as significant yield losses (8,24,30). Both viruses are transmitted by the plasmodiophorid vector *Polymyxa graminis* Ledingham, ensuring their persistence in the soil over a long period (19,25,32).

WSSMV occurs worldwide in wheat-growing areas, whereas WYMV is restricted to China and Japan (15). WSSMV has been detected under temperate and tropical climates in Canada, France, Germany, India, Italy, Japan, the United States, and Zambia (6). It has never been detected in Belgium or in more northern European countries, unlike the bymoviruses affecting barley, such as *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV). These viruses have been reported frequently in Belgium since the

1980s (21,36). This situation raised questions about the possible occurrence of WSSMV in Belgium.

Traditionally, WSSMV has been detected by immunosorbent electron microscopy (ISEM), initially described by Haufler and Fulbright (13,14), and enzyme-linked immunosorbent assay (ELISA), the most commonly used technique (4,6,37). Recently, reverse-transcription polymerase chain reaction (RT-PCR) has started being seen as a sensitive alternative to ELISA, allowing the detection of low viral titer in infected plants. Various RT-PCR methods also have been proposed for the specific detection of WSSMV, WYMV, or both, targeting specific isolates or using a degenerate primers strategy (5,6,10).

In order to evaluate the presence of WSSMV in Belgium, RT-PCR and real-time quantitative RT-PCR were developed for detecting and quantifying WSSMV using a strategy targeting conserved sequences of the coat protein gene of all known isolates (5,6,18,33). Methods were validated on reference isolates of WSSMV. Their specificity was controlled by checking the absence of reaction with other viruses, such as *Soil-borne cereal mosaic virus* (SBCMV) and *Soil-borne wheat mosaic virus* (SBWMV), frequently associated in other countries with WSSMV, as well as BaYMV and BaMMV. The RT-PCR method was used to evaluate the occurrence of WSSMV in wheat plants

showing mosaic symptoms collected in various fields across Belgium as well as in bait plants grown in 104 soil samples collected in the major wheat-growing areas in Belgium. To a limited extent, the presence of WYMV also was evaluated.

MATERIALS AND METHODS

Plant and soil material. During spring 2004, strong yellow mosaic symptoms were observed on wheat plants in fields in various provinces across Belgium: Mechelen (Antwerpen), Corroy-le-Grand and Thines (Brabant wallon), Ath and Comines (Hainaut Occidental), and Flavion and Loncée (Namur). Twelve entire plant samples were collected from each of 12 fields and stored at -20°C until further analysis.

Soil samples ($n = 104$) were collected mainly from fields in the cereal-growing area of southern Belgium (Walloon region) in order to perform bioassays under controlled conditions for assessment of WSSMV distribution. Based on a previous survey of BaYMV and BaMMV distribution in Belgium, the soil sampling was targeted to soilborne viruses and *P. graminis*-prone areas. In each field, composite soil sampling was obtained by taking 50 samples from the upper 20 cm of the soil. The geo-coordinates of each sampled site (latitude and longitude) were recorded using a geographical positioning system (Garmin GPS 12XL; Garmin International Inc., Olathe, KS) and were used to map the location in Belgium using ArcGIS 8.1 (ESRI Inc., Denver, CO).

In addition, soil and plant samples from Belgium, France, Germany, Italy, and the United Kingdom were used as references (Table 1). All soil samples were dried at room temperature, ground to dust, and diluted to 1:5 vol/vol with sterile quartz (1 to 2 mm, one part soil and four parts quartz), to reduce the competition between soil organisms and obtaining soil with a structure conducive to *P. graminis* development (20). The soil-quartz mixture was distributed in hexagonal visotubes pierced at the bottom (12.2 by 2 by 2.2 cm, Cryo Bio System, L'Aigle, France). The wheat cvs. Cezanne (Limagrain Verneuil Holding, Saint Beuzire, France), susceptible to WSSMV and SBWMV, and Savannah (Advanta Seeds UK Ltd., Lincolnshire, UK), susceptible to SBWMV, as well as the rye cv. Halo (Lochow-Petkus GmbH, Bergen, Germany), were used as bait

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plants. The seed surfaces were disinfected by soaking for 5 min in 2.5% sodium hypochlorite solution, followed by rinsing under tap water for 1 h. The seeds were pregerminated on moistened filter paper at 22°C for 2 days before transferring to the visotubes, with one seed by tube. The plants were grown under controlled conditions with a 12-h photoperiod at temperatures often used for baiting soilborne viruses, 17 to 25°C (night to day) and 16 to 18°C (2,3,26,31). The plants were watered with a modified Hoagland nutrient solution at pH 7.2 (16). Eighteen plants grown in reference soils were removed after 10 weeks of growth to check infection. The other plants were removed after 12 weeks of growth. The roots were washed individually and the plant material (roots and leaves) was stored at -20°C until RNA extraction. The root and leaf samples were studied separately and the two youngest leaves were systematically analyzed. Each plant sample grown in Belgian soils was represented by a composite of leaves or roots from 12 plants from the same soil (two wheat cultivars and one of rye, as well as the two temperature conditions mixed). This strategy is used to maximize the chance of detecting the WSSMV.

RNA extraction. Total RNA was extracted from 300 mg of frozen root or leaf samples using FastRNA Pro Green Kit (QBIogene, MP Biomedicals, Illkirch,

France) and the FastPrep system, following the manufacturer's instructions. The homogenization was performed once for leaf samples and twice for root samples during 45 s at the maximum speed of the FastPrep system. RNA extracts were stored at -70°C until further analysis.

Primers design. For the detection of WSSMV, the published sequences available for this virus (accession numbers AJ130982, AJ130981, AJ130980, AJ130979, AB010578, AJ237926, AJ237925, and X73883) initially were aligned to find the homologous region (5,6,18,33). Primers were designed targeting conserved sequences of the coat protein WSSMV gene using the eprimer3 WEMBOSS program (9). Three pairs of primers, WSSMV1-F and WSSMV1-R, WSSMVc1-F and WSSMVc1-R, and WSSMVc2-F and WSSMVc1-R, were developed for amplifying regions of 200, 200, and 199 bp, respectively. The specificity of these primers was assessed using BLAST software (1) and they targeted the sequences of the Canadian, French, German, and U.S. isolates (Fig. 1).

RT-PCR protocol. RNA extract was reverse transcribed and then amplified by PCR using the primers previously described. The RT-PCR protocol was performed as previously described by Meunier et al. (22). PCR was performed using the Bio-Rad iCycler (Bio-Rad), as follows:

after an initial denaturation of 2 min at 94°C, 38 cycles, including denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and elongation for 30 s at 72°C, were carried out. A final elongation was completed at 72°C for 7 min. The annealing temperature was optimized by testing a gradient of temperatures on a set of cDNA samples in a Bio-Rad iCycler. After PCR amplification, 2 µl of loading buffer was added to the PCR products. The samples were loaded on 2% agarose gel in Tris borate-EDTA buffer and the electrophoresis was performed in Sub-Cell GT Agarose Gel Electrophoresis Systems (Bio-Rad). After ethidium bromide staining, the bands of DNA were visualized using the Gel Doc 2000 (Bio-Rad).

RT-PCR was validated using the WSSMV reference isolates from France, Germany, and Italy (Table 1) and then tested on the plants collected in Belgian fields and the bait plants grown in the Belgian soils. The presence of WYMV on these plants also was evaluated using the RT-PCR method described by Clover and Henry (6).

Real-time quantitative RT-PCR. The primers WSSMVc2-F and WSSMVc1-R were used for a real-time RT-PCR assay for quantifying the virus concentration in the samples. The SYBR-Green chemistry was used because of its low cost and flexibility, and the iQ SYBR Green Supermix (Bio-Rad) was selected. Real-time RT-PCR protocol initially was optimized by varying product quantities, times, and temperatures applied in the quantitative PCR. In addition, the influence of the quantity of sampling material and the extraction method used, as well as the amount of RNA used in reverse transcription and the amount of cDNA used in real-time PCR, were analyzed.

RNA extraction and reverse transcription were performed as previously described. To 2.5 µl of cDNA (as in RT-PCR) was added a mixture composed of 12.5 µl of 2x iQ SYBR Green Supermix, 0.5 µl of WSSMVc2-F (20 µM), 0.5 µl of WSSMVc1-R (20 µM), and 9 µl of diethyl pyrocarbonate (DEPC)-treated water. The amplification was conducted on the Bio-Rad iCycler as follows: first denaturation at 95°C for 3 min, and 40 cycles each composed of 95°C for 30 s and 58°C for 1 min. The melting curve was performed for 80 cycles at 55°C for 10 s, with a temperature increase of 0.5°C each cycle.

In each real-time RT-PCR, two repetitions were performed for all tested samples, standards, negative control, and the blank. A DNA standard (production of plasmid DNA) was produced by cloning the PCR product from an RT-PCR obtained using an RNA extract from the German WSSMV isolate (DSMZ, Braunschweig, Germany). Thus, this product was ligated into the pGEM-T vector (Promega Corp., Madison, WI), which was

Table 1. Reference isolates of viruses used in order to validate the reverse-transcription polymerase chain reaction detection method of *Wheat spindle streak mosaic virus* (WSSMV) and to evaluate detection specificity

Origin (country, year)	Material	Virus species ^a	Reference
Belgium, 2003	Barley RNA extract	BaYMV	This article
Belgium, 2003	Barley RNA extract	BaMMV	This article
France	Wheat RNA extract	WSSMV	Gitton et al. (10)
France, 2004	Soil	WSSMV & SBWMV	M. Bonnefoy ^b
Germany	Frozen wheat leaves	SBCMV	DSMZ (PV-0552) ^c
Germany	Frozen wheat leaves	SBWMV	DSMZ (PV-0748)
Germany	Frozen wheat leaves	WSSMV	DSMZ (PV-0541)
Italy, 2001	Soil	WSSMV & SBWMV	V. Vallega ^d
United Kingdom, 2004	Soil	SBWMV	M. Adams & K. Kanyuka ^e

^a BaYMV = *Barley yellow mosaic virus*, BaMMV = *Barley mild mosaic virus*, SBWMV = *Soil-borne wheat mosaic virus*, and SBCMV = *Soil-borne cereal mosaic virus*.

^b ARVALIS, Institut du Végétal, Ouzouer-le-Marché, France.

^c DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany.

^d Istituto Sperimentale per la Cerealicoltura, Rome, Italy.

^e Plant-Pathogen Interactions Division, Rothamsted Research, Harpenden, Hertfordshire, United Kingdom.

Fig. 1. Primer pairs (WSSMV1-F/WSSMV1-R, WSSMVc1-F/WSSMVc1-R and WSSMVc2-F/WSSMVc1-R) used for *Wheat spindle streak mosaic virus* (WSSMV) detection, according to the consensus sequence of a pile up of WSSMV, accession numbers AJ130982, AJ130980, AJ130979, AB010578, AJ237925, and X73883. The sequence of the accession number X73883 is used as reference for nucleotide positions.

used to transform competent cells of *Escherichia coli* (JM109; Promega Corp.) following the manufacturer's instructions. The plasmid DNA from successfully transformed cells was extracted and purified using the QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The concentration in plasmid DNA was measured with a spectrophotometer (28). This purified plasmid DNA was used as a DNA standard in real-time PCR; therefore, four serial dilutions of this sample were performed for obtaining the standard curve, allowing the determination of PCR efficiency and the calculation of the relative quantities of DNA presented in each sample tested. The possibility of using RNA standard also was assessed.

ELISA. ELISA tests for WSSMV detection were performed using LOEWE (Sauerlach, Germany) and DSMZ (Braunschweig, Germany) kits following the manufacturer's instructions unless otherwise stated. The positive and negative controls provided in the LOEWE kit also were used in DSMZ ELISA. Microtiter plates (Nunc maxisorp, 96-well) were coated with WSSMV antibodies (immunoglobulin-G [IgG]) diluted to 1:200 for the LOEWE kit and 1:500 for the DSMZ kit, in a coating buffer at 150 µl/well and incubated for 2 h at 37°C. The wells then were washed three times with Phosphate-Buffered Saline-Tween. Afterward, tested samples were homogenized in an extraction buffer with a dilution of 1:20 (0.1 g of plant material in 1.9 ml of extraction buffer) and 150 µl of this ELISA extract were added per well to the coated plates. The plates were incubated overnight at 4°C and then washed as previously described. The anti-WSSMV conjugate (IgG-AP) was diluted to 1:200 for the LOEWE kit and 1:500 for the DSMZ kit in a conjugate

buffer and 150 µl were added per well. The plates were incubated at 37°C for 2 h and then washed as previously described. Substrate (p-nitrophenyl phosphate, 1 mg/ml) was dissolved in 30 ml of substrate buffer and 150 µl was added per well. Plates then were incubated at room temperature for 3 h. The optical density was measured at 405 nm using the Multiskan Ascent (Labsystems, Helsinki, Finland) from 0 to 180 min, every 15 min, after the addition of the p-nitrophenyl phosphate. Two repetitions were performed on the tested samples for the two kits used.

A sample was considered positive for WSSMV when absorbance at 405 nm was more than three times the mean of the negative control (35).

Comparison between ELISA, RT-PCR, and real-time RT-PCR assays. The sensitivity of ELISA, RT-PCR and real-time RT-PCR assays was evaluated using the German isolate of WSSMV (DSMZ) as the reference sample. ELISA and RNA extracts from freeze-dried leaves of this isolate were diluted 12 times to 1:10 (vol/vol), and these dilutions were tested simultaneously using the three methods. Two repetitions were performed on the sample and its dilution for all tests carried out.

For ELISA, 10 mg of freeze-dried leaves infected by WSSMV were crushed in 1 ml of extraction buffer. From this extract, 12 serial dilutions by a factor of 10 were done in the extraction buffer and 150 µl of each dilution and concentrated extract were added per well to the coated plates.

Simultaneously, freeze-dried leaves of the reference sample were tested by RT-PCR and real-time RT-PCR, using WSSMVc2-F and WSSMVc1-R primers. Total RNA was extracted using FastRNA Pro Green Kit as described previously, with 10 mg of this sample (the same quan-

tity as used for ELISA), and 12 serial dilutions by a factor of 10 were realized in Tris-EDTA solution (1 M Tris, 0.5 M Na₂EDTA, pH 8). A reverse transcription using WSSMVc1-R was performed on concentrated extract and each dilution, as described previously. Complementary DNA produced was amplified by traditional and real-time PCR.

Amplicon sequencing. The amplicon obtained by RT-PCR with WSSMVc2-F and WSSMVc1-R primers on four samples from the wheat cvs. Calisto, Cezanne, N259.3, and Pernel grown in Chambon-sur-Cisse soil was purified using QIAquick 250 Kit (QIAGEN) following the manufacturer's instructions. The purified PCR products were processed for sequencing with the primers WSSMVc2-F and WSSMVc1-R separately, using a Quick Start Kit and CEQTM 2000 x L DNA Analysis System (Beckman Coulter, Fullerton, CA). Sequences obtained were compared and aligned with sequences already known (accession numbers AJ130982, AJ130980, AJ130979, AB010578, AJ237925, and X73883). They were deposited at the European Molecular Biology Laboratory.

RESULTS

Validation of RT-PCR for WSSMV.

Three partially overlapping primer pairs were produced (WSSMV1-F/WSSMV1-R, WSSMVc1-F/WSSMVc1-R, and WSSMVc2-F/WSSMVc1-R), targeting conserved sequences of the coat protein WSSMV gene. Primer pair combinations were tested under optimized conditions on reference isolates from France, Germany, and Italy (Fig. 2A). Whichever primers were used, the RT-PCR method amplified a single PCR product of the expected size for each tested WSSMV isolate, but the signal intensity varied according to the tested sam-

Fig. 2. Profiles of DNA amplifications by **A**, traditional reverse-transcription polymerase chain reaction (RT-PCR) and by **B**, real-time RT-PCR for detection and quantification of *Wheat spindle streak mosaic virus* (WSSMV) (199-bp band) using WSSMVc2-F/WSSMVc1-R primers. Panel B shows the increase in fluorescence with time (Cycle) of accumulated PCR products. Samples in (1) German reference isolate of WSSMV, ordered at DSMZ; (2) French isolate of WSSMV, provided by F. Gitton; (3) leaves of wheat cv. Cezanne grown in Chambon-sur-Cisse soil (France, 17 to 25°C, 12 weeks); (4) leaves of wheat cv. Savannah grown in Rome soil (Italy, 17 to 25°C, 12 weeks); (5) leaves of wheat cv. Cezanne grown in Flavion soil (Belgium, 16 to 18°C, 12 weeks); (6) German reference isolate of SBWMV, ordered at DSMZ; (C-) negative control; (b) blank and (M) size marker: FastRuler DNA Ladder, Low Range (Fermentas, Germany).

Fig. 3. Sensitivity comparison between the enzyme-linked immunosorbent assay (ELISA), reverse-transcription polymerase chain reaction (RT-PCR), and real-time RT-PCR assays for the *Wheat spindle streak mosaic virus* (WSSMV) detection, using dilution series of an extract on German reference isolate. **A**, Histogram shows the evolution of the optical density (O.D.) measured after 75 min at wavelength of 405 nm in ELISA for the eight first dilutions (1→10⁻⁷). The horizontal line represents thrice the mean O.D. of the negative control, it is the threshold above which a sample was considered positive in ELISA. The curve shows the threshold cycle (Ct) determined in the real-time RT-PCR, using WSSMVc2-F and WSSMVc1-R primers, for the eight first dilutions tested and for the negative control (C-). **B**, Gel photography illustrating the detection of WSSMV in eight serial dilutions of the reference sample using traditional RT-PCR. (M) size marker: EZ Load 100-bp Molecular Ruler (Bio-Rad). Data from 10⁻⁸ to 10⁻¹² have not been presented here because WSSMV was not detected by the three methods at these dilutions.

Table 2. Detection by reverse-transcription polymerase chain reaction of *Wheat spindle streak mosaic virus* (WSSMV), using three pairs of primers, on leaf and root samples from different wheat cultivars showing symptoms and collected in Belgian fields

Samples (place, wheat cultivars)	WSSMVc1-F/ WSSMVc1-R ^a	WSSMVc2-F/ WSSMVc1-R ^a
Ath, wheat cv. Belcast	-/-	-/-
Comines, field 1, wheat	+/-	-/-
Comines, field 2, wheat cv. Folio	-/-	-/-
Corroy-le-Grand, field 1, wheat cv. Biscay	-/-	-/-
Corroy-le-Grand, field 2, wheat cv. Biscay	-/-	+/-
Flavion, field 1, wheat cv. Centenaire	+/-	+/-
Flavion, field 2, wheat cv. Maverick	+/-	-/-
Lonzée, field 1, wheat cv. Deben	-/-	+/-
Lonzée, field 2, wheat cv. Koch	-/-	-/-
Lonzée, field 3, wheat cv. Piastre	-/-	-/-
Mechelen, wheat cv. Folio	+/-	+/-
Thines, wheat cv. Koch	-/-	+/-

^a Result of reaction from total RNA extract of leaves/result of reaction from total RNA extract of roots.

ple. No product was obtained with either furoviruses such as SBCMV and SBWMV, frequently associated with WSSMV infection, or with plants infected by BaYMV and BaMMV. In addition, no PCR product was produced using total RNA extracted from the leaf tissue of healthy wheat.

Based on RT-PCR tests conducted on field samples (Table 2), the WSSMVc2-F and WSSMVc1-R primers were chosen for the real-time RT-PCR assay (Fig. 2B).

Detection sensitivity using classical and real-time RT-PCR methods developed here was compared with detection using ELISA (Fig. 3). The LOEWE and DSMZ commercial kits produced similar results. WSSMV was detected using ELISA in the two greatest concentrations, whereas it was detected up to a 10⁻⁵ dilution by RT-PCR and 10⁻⁷ by real-time RT-PCR.

Diagnosis of mosaic symptoms on wheat plants collected in fields. RT-PCR tests performed using the three specific primer pairs designed for WSSMV on the wheat plants showing strong yellow mosaic symptoms and collected in fields in the Belgian provinces of Antwerpen, Brabant wallon, Hainaut Occidental, and Namur confirmed the presence of WSSMV in 7 of the 12 tested leaf samples but not in root samples (Table 2). Depending on the primers used, the results differed. Except for the wheat cv. Centenaire collected from the Flavion field, WSSMV was not detected using ELISA performed by the DSMZ and LOEWE kits on these samples.

Geographical distribution of WSSMV in southern Belgium. The presence of WSSMV tested by RT-PCR using the WSSMV1-F and WSSMV1-R primers was revealed in 32% of the bait plants from the Belgian soils collected across the cereal-growing area of southern Belgium (Fig. 4).

Among the 104 soils tested, WSSMV was detected in 21 leaf samples, 6 root samples, and, for 6 samples, in both leaves and roots of plants grown in those soils. The intensity of the DNA bands observed on electrophoresis gel was generally low.

ELISA tests with DSMZ and LOEWE kits were unable to detect WSSMV in leaves and roots of plants grown in 16 soil samples positive for WSSMV using the RT-PCR test. WYMV was not detected using RT-PCR in tested samples.

The detection tests using RT-PCR confirmed the presence of WSSMV in Belgian soils, but not WYMV. These results show that WSSMV is present in Belgium in a wider area than indicated in the 2004 survey.

Amplicon sequencing. Sequences from isolates Chambon 1 and 2, obtained on the wheat cvs. N259.3 and Cezanne grown in Chambon-sur-Cisse soil, are recorded under the accession numbers AM076767 and AM076768. They match the coat protein WSSMV gene of previously sequenced isolates (accession numbers AJ130982, AJ130980, AJ130979, AB010578,

AJ237925, and X73883). Over the 138 nucleotides, between 1 to 5 nucleotide changes were noted. The sequences obtained for the wheat cvs. Calisto and Pernel were identical with the sequence obtained on the wheat cv. Cezanne.

DISCUSSION

In this study, we show the presence of WSSMV in Belgium, in regions where it has not hitherto been reported. In contrast to previously described RT-PCR methods (6,10), the RT-PCR method used targets a conserved region based on the comparison of existing WSSMV sequences from five countries, enabling European isolates of WSSMV from Belgium, France, Germany, and Italy to be detected. To the best of our knowledge, this is the first time that WSSMV has been reported in Belgium. The WSSMV generally was thought to be confined to areas south of the Loire river in France (12), and the reason why it has now been detected further north is unclear: has it moved northward because of more favorable climatic conditions or does the reason for the findings lie in the sensitivity of the technique applied here?

Sequences of the sources from Chambon-sur-Cisse confirm the correct amplification of a WSSMV amplicon from the coat protein gene, indicating only minor changes in the nucleotide and amino acid sequences when compared with the original French sequence available.

The RT-PCR method, combined with large sampling, enabled WSSMV to be detected from plants grown in 32% of tested Belgian soils, indicating that the

WSSMV is widespread in Belgium. The primer pairs used detected the largest proportion of Belgian isolates, but not all isolates and, therefore, the incidence of the virus may be underestimated. Although mosaic symptoms are indeed spotted, the actual impact on yield in Belgium has still to be evaluated. Slykhuis (30) and Sohn et al. (34) observed yield losses due to WSSMV infection of up to 59%. Cunfer et al. (8) produced a detailed account of reduction in plant development, yield, and grain quality caused by WSSMV infection in Georgia. These yield losses resulted from significant infection levels. In Belgium, the absence of WSSMV detection using ELISA in samples shown by RT-PCR to be infected and the low signal intensity of DNA bands on electrophoresis gel suggest that soil infestation levels are low and build up slowly over time. The high sensitivity of RT-PCR offers a tool for the early detection of WSSMV infections.

Therefore, the RT-PCR method developed allowed a more sensitive detection of WSSMV than ELISA, as already shown by other RT-PCR techniques (6,10,23). This leads to WSSMV detection by RT-PCR in samples appearing negative in ELISA. The primers also were designed directly for use in real-time RT-PCR, allowing a sensitive quantification of the virus concentration in wheat cultivars in order to compare the soil inoculum potential as well as the cultivar resistance to WSSMV.

Belgian WSSMV sources reacted differently, with primers differing by one to three nucleotides. This suggests the pres-

ence of different isolates among the 33 contaminated tested soils.

The molecular method allowed virus detection not only in the leaves but also in the roots of plant samples grown under controlled conditions. This represents a real asset of the RT-PCR method because it offers new prospects for studying the dynamics and epidemiology of viral infection, because the viruses are transmitted by *P. graminis* through the roots. In addition, it is also in the roots that the inoculum potential is regenerated before being released in the soil during root decay (17). The virus detection in the leaves shows that, during the growing period, the virus is able to move from the roots to the leaves. The virus often is detected only in the leaves and less frequently in the roots only or in both leaves and roots, indicating that leaf samples are the best target for WSSMV detection. These results agree with the data on the movement of barley bymoviruses reported by Schenk et al. (29), but they raise the question of the distribution of the virus under natural conditions and over the growing season.

In contrast to WSSMV, WYMV was not detected in Belgium. Hariri et al. (11) reported the presence of WYMV in France, even though Clover and Henry (6) were unable to confirm this report by using primers tested on wheat plants from France.

WSSMV often has been reported in association with the furoviruses responsible for mosaic on wheat, such as SBWMV and SBCMV (7,27,34). The question of the presence of such viruses in Belgium is still open.

Fig. 4. Distribution of *Wheat spindle streak mosaic virus* (WSSMV) in Walloon region (Belgium). Symbols indicate tests on a leaf sample (▲) or root sample (▽). Positive reactions are filled diamonds and negative are open diamonds. The position of the sampled area in Belgium is shown by the insert. These results were obtained by reverse-transcription polymerase chain reaction with WSSMV1-F/WSSMV1-R primers designed.

In conclusion, mosaic represents a new potential threat for Belgian and other European cereal growers. The difficulty of managing viruses responsible for mosaic is linked to the fact that they are transmitted by *P. graminis*. The resting spores of this soilborne organism ensure the persistence of viral particles in soils for several years. The tools proposed here can be used for determining the impact of WSSMV on yield as well as for evaluating wheat cultivar resistance to the virus, with a view to developing an integrated management approach to controlling this noxious and persistent disease.

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LITERATURE CITED

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Cadle-Davidson, L., Schindelbeck, R. R., Van Es, H. M., Gray, S. M., and Bergstrom, G. C. 2003. Using air pressure cells to evaluate the effect of soil environment on the transmission of soilborne viruses of wheat. *Phytopathology* 93:1131-1136.
- Carroll, J. E., Bergstrom, G. C., and Gray, S. M. 1997. Dynamics of *Wheat spindle streak mosaic bymovirus* in winter wheat. *Eur. J. Plant Pathol.* 103:313-321.
- Carroll, J. E., Gray, S. M., and Bergstrom, G. C. 1995. Use of antiserum to a New-York isolate of *Wheat spindle streak mosaic virus* to detect related bymoviruses from North America, Europe, and Asia. *Plant Dis.* 79:346-353.
- Chen, J., Sohn, A., Chen, J.-P., Lei, J., Schulze, S., Steinbiss, H.-H., Antoniow, J. F., and Adams, M. J. 1999. Molecular comparisons amongst wheat bymovirus isolates from Asia, North America and Europe. *Plant Pathol.* 48:642-647.
- Clover, G., and Henry, C. 1999. Detection and discrimination of *Wheat spindle streak mosaic virus* and *Wheat yellow mosaic virus* using multiplex RT-PCR. *Eur. J. Plant Pathol.* 105:891-896.
- Clover, G. R. G., Ratti, C., and Henry, C. M. 2001. Molecular characterization and detection of European isolates of *Soil-borne wheat mosaic virus*. *Plant Pathol.* 50:761-767.
- Cunfer, B. M., Demski, J. W., and Bays, D. C. 1988. Reduction in plant development, yield, and grain quality associated with *Wheat spindle streak mosaic virus*. *Phytopathology* 78:198-204.
- Devereux, J., Haerberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
- Gitton, F., Diao, A., Ducrot, O., Antoniow, J. F., Adams, M. J., and Maraite, H. 1999. A two-step multiplex RT-PCR method for simultaneous detection of *Soil-borne wheat mosaic virus* and *Wheat spindle streak mosaic virus* from France. *Plant Pathol.* 48(5):635-641.
- Hariri, D., Courtillot, M., Zaoui, P., and Lapiere, H. 1987. Multiplication of *Soil-borne wheat mosaic virus* (SBWMV) in wheat roots infected by a soil carrying SBWMV and *Wheat yellow mosaic virus* (WYMV). *Agronomie* 7(10):789-796.
- Hariri, D., Fouchard, M., and Lapiere H. 1998. Mosaïques des céréales à paille, Les virus transmis par le sol. *Phytoma* 509:22-26.
- Haufler, K. Z., and Fulbright, D. W. 1983. Detection of *Wheat spindle streak mosaic virus* by serologically specific electron microscopy. *Plant Dis.* 67:988-990.
- Haufler, K. Z., and Fulbright, D. W. 1986. Identification of winter wheat cultivars and experimental lines resistant to *Wheat spindle streak mosaic virus*. *Plant Dis.* 70:31-33.
- Inouye, T. 1996. *Wheat yellow mosaic bymovirus*. Pages 1388-1390 in: *Viruses of Plants: Descriptions and Lists from the VIDE Database*. A. A. Brunt, K. Crabtree, M. J. Dallwitz, A. J. Gibbs, and L. Watson, eds. Centre for Agricultural and Biosciences International, Wallingford, Oxon, UK.
- Legrève, A., Delfosse, P., Vanpee, B., Goffin, A., and Maraite, H. 1998. Differences in temperature requirements between *Polymyxa* sp. of Indian origin and *Polymyxa graminis* and *Polymyxa betae* from temperate areas. *Eur. J. Plant Pathol.* 104:195-205.
- Legrève, A., Vanpee, B., Delfosse, P., and Maraite, H. 1999. High temperature during storage favours infection potential of resting spores of *Polymyxa graminis* of Indian origin. *Ann. Appl. Biol.* 134:163-169.
- Lu, X., Kashiwasaki, S., Tamura, M., and Namba, S. 1998. The 3'-terminal sequence of RNA1 of *Wheat spindle streak mosaic virus* Canadian isolate (WSSMV-C). *Eur. J. Plant Pathol.* 104:765-768.
- Maraite, H. 1991. Transmission of viruses by soil fungi. Pages 67-82 in: *Developments in Agricultural and Managed-Forest Ecology 23: Biotic Interactions and Soil-borne Diseases*. Proc. First Conf. Eur. Foundation Plant Pathol. A. B. R. Beemster, G. J. Bollen, M. Gerlagh, M. A. Ruissen, B. Schippers, and A. Tempel, eds. Elsevier Science Publishers, Amsterdam.
- Maraite, H., Goffart, J. P., and Bastin, V. 1988. Development of a quantitative method for assessment of *Polymyxa graminis* Led. inoculum potential in soils. Pages 259-266 in: *Integrated Crop Protection in Cereals*. Proc. Meet. EC Experts' Group. R. Cavalloro and K. D. Sunderland, eds. Balkema, Rotterdam, The Netherlands.
- Maroquin, C., Cavelier, M., and Rassel, A. 1982. Premières observations sur le virus de la mosaïque jaune de l'orge en Belgique. *Bull. Rech. Agron. Gembloux* 17:157-176.
- Meunier, A., Schmit, J. F., and Bragard, C. 2005. Comparison of the *Beet necrotic yellow vein virus* P75 nucleotide sequences of Belgian type A and type B sources. *Virus Res.* 108:15-22.
- Meunier, A., Schmit, J. F., Stas, A., Kutluk, N., and Bragard, C., 2003. Multiplex reverse transcription-PCR for simultaneous detection of *Beet necrotic yellow vein virus*, *Beet soil-borne virus*, and *Beet virus Q* and their vector *Polymyxa betae* KESKIN on sugar beet. *Appl. Environ. Microbiol.* 69:2356-2360.
- Miller, N. R., Bergstrom, G. C., and Sorrells, M. E. 1992. Effect of *Wheat spindle streak mosaic virus* on yield of winter wheat in New York. *Phytopathology* 82:852-857.
- Nolt, B. L., Romaine, C. P., Smith, S. H., and Cole, H., Jr. 1981. Further evidence for the association of *Polymyxa graminis* with the transmission of *Wheat spindle streak mosaic virus*. *Phytopathology* 71:1269-1272.
- Ohsato, S., Miyashita, M., and Shirako, Y. 2003. The optimal temperature for RNA replication in cells infected by *Soil-borne wheat mosaic virus* is 17°C. *J. Gen. Virol.* 84:995-1000.
- Rubies-Autonell, C., and Vallega, V. 1987. Observations on a mixed *Soil-borne wheat mosaic virus* and *Wheat spindle streak mosaic virus* infection in durum wheat *Triticum durum* Desf. *J. Phytopathol.* 119:111-121.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning, A Laboratory Manual*, Vol. 3, 2nd ed., Appendix E.5. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schenk, P. M., Antoniow, J. F., Batista, M. D., Jacobi, V., Adams, M. J., and Steinbiss, H.-H. 1995. Movement of barley mild mosaic and barley yellow mosaic viruses in leaves and roots of barley. *Ann. Appl. Biol.* 126(2):291-305.
- Slykhuis, J. T. 1970. Factors determining the development of *Wheat spindle streak mosaic* caused by a soil-borne virus in Ontario. *Phytopathology* 60:319-331.
- Slykhuis, J. T. 1974. Differentiation of transmission and incubation temperatures for *Wheat spindle streak mosaic virus*. *Phytopathology* 64:554-557.
- Slykhuis, J. T., and Barr, D. J. S. 1978. Confirmation of *Polymyxa graminis* as a vector of *Wheat spindle streak mosaic virus*. *Phytopathology* 68:639-643.
- Sohn, A., Schenk, P., Signoret, P. A., Schmitz, G., Schell, J., and Steinbiss, H. H. 1994. Sequence analysis of the 3'-terminal half of RNA1 of *Wheat spindle streak mosaic virus*. *Arch. Virol.* 135(3-4):279-292.
- Sohn, A., Signoret, P. A., Davidson L. E., and Bergstrom, G. C. 2004. *Wheat spindle streak mosaic*. Page 598 in: *Viruses and Virus Diseases of Poaceae (Gramineae)*. H. Lapiere and P.A. Signoret, eds. INRA Editions, Paris.
- Sutula, C. L., Gillett, J. M., Morrissey, S. M., and Ramsdell, D. C. 1986. Interpreting ELISA data and establishing the positive-negative threshold. *Plant Dis.* 70:722-726.
- Vaianopoulos, C., Legrève, A., Barbier, A., Steyer, S., Maraite, H., and Bragard, C. 2003. Detection of *Barley yellow mosaic virus* and *Barley mild mosaic virus* by RT-PCR on resistant barley cultivars. *Parasitica* 59(3-4):67-74.
- Zagula, K. R., Barbara, D. J., Fulbright, D. W., and Lister, R. M. 1990. Evaluation of three ELISA methods as alternatives to ISEM for detection of the *Wheat spindle streak mosaic* strain of *Wheat yellow mosaic virus*. *Plant Dis.* 74:974-978.