Identification and Quantification of *Polymyxa graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* on Barley and Wheat

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ABSTRACT

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Polymyxa graminis f. sp. *temperata* and *P. graminis* f. sp. *tepida* are distinguished on the basis of their specific ribosomal DNA sequences. In order to evaluate whether or not host specialization is associated with the special form, the occurrence of infection of both forms on barley and wheat was studied. *P. graminis* inocula were obtained from soils collected in Belgium and France. Their ribotypes were characterized using molecular tools specific to *P. graminis* f. sp. *temperata* or *P. graminis* f. sp. *tepida* such as restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified rDNA, nested and multiplex PCR. Both special forms were found in each country and coexisted in some soils. The host specificity of *P. graminis* special forms for barley and wheat was studied from two soils collected at Gembloux (Belgium) and Chambon-sur-Cisse (France), each infested by bymo- and furoviruses. *P. graminis* f. sp. *temperata* is more frequent on barley and *P. graminis* f. sp. *tepida* on wheat. Furthermore, the quantification of each form on barley and wheat by two separated real-time quantitative PCR assays confirms the observations on the vector specialization. These results suggest a certain but not exclusive host specificity of *P. graminis* special forms.

Additional keyword: plasmodiophorid

The plasmodiophorid *Polymyxa graminis* Ledingham is a ubiquitous obligate root endoparasite. *P. graminis* transmits at least 14 virus species from the genera *Beny*-, *Bymo*-, *Furo*-, and *Pecluvirus*, causing major diseases and significant yield losses in cereals in both temperate and tropical regions (1,10,17).

Polymyxa belongs to the kingdom Protista and the order Plasmodiophorales. Its taxonomic position is a matter of debate and was reviewed recently (4,6). Molecular and ecological characterization (11,20, 27,28) have shown a high level of diversity of *P. graminis* isolates, leading to the distinction of five special forms of *P. graminis* (11), with specific ribotypes and ecological features. Two special forms, *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*, occur in temperate areas and are characterized by strongly differing ribotypes (11,29).

P. graminis is found mainly on a wide range of Gramineae, including barley, oats, pearl millet, rice, rye, sorghum, and wheat (2,15,19). Host range studies involving

barley, oats, rye, and wheat have revealed different levels of susceptibility to *P. graminis* isolates from various countries (Belgium, Canada, France, Germany, India, Pakistan, Senegal, and the United Kingdom) depending on the cultivars/species tested (2,5,15).

Differences in the host range and the ability to parasitize cereal species raise the possibility of a host specialization in *Polymyxa* linked to the ribotype previously described. This possibility is of major importance in improving the understanding of the specific interactions between *P. graminis* and viruses. Therefore, specific and sensitive tools such as restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified rDNA, and nested, multiplex, and real-time quantitative PCR assays were developed to discriminate between *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*, and to study the host specialization of *P. graminis* special forms. These methods were targeted on two cereal species cultivated mainly in temperate areas – barley and wheat – and grown on soils infested by bymo- and furoviruses.

MATERIALS AND METHODS

Origin of *P. graminis* inocula. *P. graminis* inocula were obtained from plants grown under controlled conditions on infested soils or collected in fields. Seven Belgian soil samples were collected

in fields at Baisy-Thy, Flavion, Gembloux, Malines, Marbais, Marneffe, and Ramillies. Their sampling was based on a previous survey of the distribution of *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) in Belgium and was targeted at soilborne viruses and *P. graminis*-prone areas. In addition, one French soil (Chambon-sur-Cisse) was studied. Three infested soils were used for the precise characterization of *P. graminis* inocula. The soil sample collected in France at Chambon-sur-Cisse is from an experimental field of ARVALIS (Technical Institute for Cereals and Forage, France) with a wheat monoculture since the 1990s and known to be infested by mosaic viruses of wheat (25,26). The Gembloux soil is from an experimental field of CRA-W (Centre Wallon de Recherches Agronomiques, Gembloux, Belgium) infested by BaMMV and BaYMV (24) and in which barley cultivars have been grown since the 1980s with wheat and rye cultivars since 2004. In the soil sample from a farmer field at Flavion (Belgium), mosaic symptoms were observed for the first time in Belgium in 2004, and furoviruses and the *Wheat spindle streak mosaic virus* (WSSMV) were detected (25,26).

After sampling, the soils were dried at room temperature, ground to dust, and diluted to 1:5 vol/vol with sterile quartz (1 to 2 mm) to reduce the competition between soil organisms and obtain soil with a structure conducive to *P. graminis* development (18). The soil-quartz mixtures were distributed in pots. Barley cvs. Carola (NORDSAAT Saatzeitgesellschaft mbH, Bohnshausen, Germany), Tiffany (Saatzeit Josef Breun GdBR, Herzogenaurach, Germany), and Tokyo (Limagrain Nickerson GmbH, Edemissen, Germany), as well as wheat cvs. Cezanne (Limagrain Verneuil Holding, Saint Beauzire, France) and Savannah (Advanta Seeds UK Ltd., Lincolnshire, UK) were used as bait plants. The seeds 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 pots, with one seed per pot. The plants were grown under controlled conditions with a 12-h photoperiod at temperatures of 12 to 14°C or 16 to 18°C (night-day) and were watered with a modified Hoagland nutrient solution at pH 7.2 (14). The plants were removed after 6

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to 19 weeks of growth to check *P. graminis* infection by PCR (13).

In addition, barley and wheat plants were collected in 2003 and 2004 from Chambon-sur-Cisse, Flavion, Gembloux, and Malines fields. A minimum of 10 plants were checked for *P. graminis* presence except for those from the Malines farmer field, from which a composite sample of plants showing mosaic symptoms was analyzed. After collecting, the roots of plants were washed individually and the plant material (roots and leaves) was stored at -20°C until further analysis.

DNA extraction. Total DNA was extracted from plant samples, which consisted of 300 mg of frozen roots, using FastDNA kit (QBIogene, MP Biomedicals, Illkirch, France) and the FastPrep system following the protocol described by Legrève et al. (13). The DNA extracts were diluted to 1:10 in Tris-EDTA solution (1 M Tris, 0.5 M Na₂EDTA, pH 8) and stored at -20°C until further analysis.

Broad-spectrum detection of *Polymyxa* species. The detection of *P. graminis* in the roots was performed from DNA extracts by PCR using the broad-spectrum detection method with Psp1 and Psp2rev primers (13) (Table 1, Fig. 1).

rDNA sequencing of *P. graminis* from barley and wheat. In order to characterize the *P. graminis* infecting barley and wheat plants (Table 2), amplified PCR products using the broad-spectrum detection method were sequenced directly or after cloning into the pCR2.1-TOPO vector for TOPO TA cloning kit (Invitrogen, Merelbeke, Belgium). The obtained plasmids were used to transform competent cells of *Escherichia coli* (One Shot cells, Invitrogen) following the manufacturer's instructions. The plasmid DNA from successfully transformed cells was extracted and purified using the QIAprep Spin Miniprep kit (QIAGEN, Antwerpen, Belgium) following the manufacturer's instructions. The sequencing was processed using the prim-

ers Psp1 and Psp2rev separately, using a Quick Start kit and CEQTM 2000 × L DNA Analysis System (Beckman Coulter, Fullerton, CA, USA). The forward and reverse sequences obtained from each sample were assembled.

Sequence comparison and determination of the conserved regions. The sequences obtained, including the internal transcribed spacers one (ITS1) region known to show specificity among the special forms (11), were aligned with published sequences of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* (accession numbers AJ311572, AJ311573, AJ311574, Y12824, and Y12826) using the ClustalW program of the European Molecular Biology Laboratory (EMBL, online). This alignment allowed the sequence variations between the two studied *P. graminis* special forms to be determined.

Restriction fragment length polymorphism (RFLP). On the basis of sequences specific to *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*, the restriction enzymes *Nru*I and *Sal*I were selected specifically to cut rDNA of *P. graminis* f. sp. *temperata* in the ITS1 region, or rDNA of *P. graminis* f. sp. *tepida* in nuclear small rDNA 18S, respectively (Fig. 1, Table 1), and were used to digest PCR products from the broad-spectrum detection of *Polymyxa* species (13). Each 25-μl reaction volume was prepared with 10.5 μl of diethyl pyrocarbonate (DEPC)-treated water, 2.5 μl 10× SuRE/Cut Buffer (Buffer B for *Nru*I and Buffer H for *Sal*I in order to obtain maximal activity of enzymes), 10 μl of amplified DNA, and 2 μl of restriction enzyme (Roche, Villvoorde, Belgium) (10 U/μl). This mixture was incubated overnight at 37°C, and the next day the enzymes were inactivated by 15 min at 65°C. After this, 2 μl of loading buffer was added and the products were loaded on 2% agarose gel in Tris Borate-EDTA buffer. The electrophoresis was performed in Sub-Cell

GT Agarose Gel Electrophoresis Systems (Bio-Rad, Nazareth, Belgium). After ethidium bromide staining, the bands of DNA were visualized using the Gel Doc 2000 (Bio-Rad).

Nested and multiplex PCR. Two primer pairs were designed on the basis of the ITS1 region specific to *P. graminis* f. sp. *temperata* or *P. graminis* f. sp. *tepida* using the "eprimer3" WEMBOSS program (8) (Fig. 1, Table 1). For the nested PCR, the broad-spectrum detection method for *Polymyxa* species was performed as described by Legrève et al. (13), except that only 0.1 μl of each primer (20 μM) was used and only 10 cycles performed. Afterward, 0.5 μl of each internal primer (PgtempN-F/PgtempN-R or PgtepN-F/PgtepN-R) was added to the tube, and the PCR was conducted under the same conditions for a further 25 cycles. The three primer pairs were also tested in multiplex PCR under the same conditions over 35 cycles.

Real-time quantitative PCR. One set of primers and Taqman probe specific to *P. graminis* f. sp. *temperata* and another set specific to *P. graminis* f. sp. *tepida* were designed using the Beacon Designer 3.0 program of Biosoft International (Corina Way, CA, USA) (Fig. 1, Table 1). The targeted region of the rDNA was moved to the ITS2 in order to increase the efficiency of the quantification of *P. graminis* f. sp. *temperata*. Real-time PCR protocol was initially optimized by varying product quantities, times, and temperatures applied in the quantitative PCR reaction. DNA was extracted from 300 mg of roots from all samples. The mixture for the real-time PCR was prepared with 12.5 μl of 2× iQ™ Supermix (Bio-Rad, Nazareth, Belgium), 2.5 μl of reverse primer at 300 nM, 2.5 μl of forward primer at 300 nM, 2.5 μl of probe at 100 nM, 2.5 μl of DEPC-treated water, and 2.5 μl of DNA extract diluted 1:10. The amplification reaction was conducted on the Bio-Rad iCycler as follows:

Table 1. Sequences of primers, Taqman probes, and restriction enzymes designed for *Polymyxa graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* detection and quantification (see Figure 1 for their location)

Designation	Sequence (5' – 3')	Detection methods ^a
Psp1	TAGACGCAGGTCATCAACCT	Broad-spectrum PCR of <i>Polymyxa</i> species (13)
Psp2rev	AGGGCTCTCGAAAGCGCAA	
<i>Nru</i> I	TCG↓CGA	RFLP for <i>P. graminis</i> f. sp. <i>temperata</i>
<i>Sal</i> I	G↓TCGAC	RFLP for <i>P. graminis</i> f. sp. <i>tepida</i>
PgtempN-F	AGCGTTGAATTGGTCTTGGT	Nested & multiplex PCR for <i>P. graminis</i> f. sp. <i>temperata</i>
PgtempN-R	TAGCCAATTCTCCCGAGTTC	
PgtepN-F	TAGCGTTGAATGGTTGTTGC	Nested & multiplex PCR for <i>P. graminis</i> f. sp. <i>tepida</i>
PgtepN-R	TTCGACTTTAGCCACCGTTT	
Pgtemp-F	GGAGTTGCAGCCCGCATG	Real-time quantitative PCR for <i>P. graminis</i> f. sp. <i>temperata</i>
Pgtemp-R	CGCCATGACGGATTGTCGTT	
Pgtemp-S	Texas red-5'AGTCAGCACGTCGGCCAAAGTCCA3'-BHQ-2	
Pgtep1-F	AATGTGGATCGTCTCTGTTGCTG	Real-time quantitative PCR for <i>P. graminis</i> f. sp. <i>tepida</i>
Pgtep1-R	CACCGTTTTGATCCAATTCGTGAA	
Pgtep1-S	FAM-5'CGGGATGGAACGCCCTCGTGGTGG3'-BHQ-1	

^a PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

an initial denaturation at 95°C for 3 min, 40 cycles each composed of (i) 95°C for 15 s and (ii) 60°C for 1 min. In each set of real-time PCR, two repetitions were performed for all tested samples.

DNA standards were prepared from PCR products from both *P. graminis* special forms and cloned in the pCR2.1-TOPO vector for TOPO TA cloning kit (Invitrogen) as detailed above, from PCR products obtained using the broad-spectrum detection of *Polymyxa* species (13), or using Psp1/ITS4 primers (ITS4 primer designed by White et al. [30] and targeting the 5' region of 28S gene; Fig. 1). The clone containing the PCR product obtained using Psp1/Psp2rev primers on wheat cv. Cezanne grown under controlled conditions on Marbais soil (accession

number AM259277, Table 2) was used as the DNA standard for *P. graminis* f. sp. *tepida*. The clone containing the PCR product obtained using Psp1/ITS4 primers

on barley cv. Tiffany grown under controlled conditions on Gembloux soil was used as the DNA standard for *P. graminis* f. sp. *temperata*. The concentration in

Table 2. Origin of *Polymyxa graminis* sequences

Cereal	Soil, year ^a	Accession number	<i>P. graminis</i> f. sp.
Barley cv. Carola	Gembloux, 04/2003*	AM259281	<i>temperata</i>
Barley cv. Carola	Gembloux, 2003	AM259280	<i>temperata</i>
Barley cv. Carola	Gembloux, 2004	AM259276	<i>temperata</i>
Barley cv. Tokyo	Marneffe, 2003	AM259279	<i>temperata</i>
Barley cv. Tokyo	Marneffe, 2004	AM259275	<i>temperata</i>
Wheat cv. Cezanne	Baisy-Thy (Genappe), 2003	AM259283	<i>tepida</i>
Wheat cv. Cezanne	Marbais, 2003	AM259277	<i>tepida</i>
Wheat cv. Cezanne	Ramillies, 2003	AM259282	<i>tepida</i>
Wheat cv. Folio	Malines, 04/2004*	AM259284	<i>tepida</i>
Wheat cv. Savannah	Marbais, 2003	AM259278	<i>tepida</i>

^a Samples marked with an asterisk were collected in fields, whereas other samples were obtained under controlled conditions.

Fig. 1. Location on *Polymyxa graminis* genome of restriction enzyme cleavage sites and primers used in broad-spectrum polymerase chain reaction (PCR) for *Polymyxa* species, in restriction fragment length polymorphism (RFLP) and nested, multiplex, and real-time PCR for *P. graminis* f. sp. *temperata* (A) and *P. graminis* f. sp. *tepida* (B) detection and quantification (see Table 1 for their sequence).

plasmid DNA (ng/μl) was measured with a spectrophotometer (22). Four serial dilutions of the standard chosen were used in each reaction for obtaining the standard curve, allowing PCR efficiency to be determined and the relative quantities of DNA in each sample tested to be calculated.

In order to evaluate the relative concentration of the DNA quantities measured by real-time PCR in the standards or in the root samples compared to the quantities of sporosori, sporosore suspensions were produced from barley and wheat roots infected by *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*, respectively, as described by Legrève et al. (14). DNA extraction and real-time quantitative PCR were then performed as described above for four serial dilutions of known quantities of sporosori.

The specificity of all primers and probes designed for nested, multiplex, and real-time PCR was assessed using BLAST software (3).

The specificity of the methods to *P. graminis* f. sp. *temperata* or *P. graminis* f. sp. *tepida* was also tested by including DNA extracts from reference isolates of *P. graminis* f. sp. *tropicalis* and *P. graminis* f. sp. *subtropicalis* (MUCL references 43177 and 43182, respectively) (11).

Microscopic observations of *P. graminis*. Root samples were stained in aniline blue lactophenol and observed microscopically in order to analyze the development stage of *P. graminis*.

RESULTS

Detection and identification of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*. *P. graminis* was detected by PCR using the broad-spectrum detection method (13) in barley and wheat plants grown on six Belgian soils. The sequencing of PCR products from these samples provided 10 sequences, which have been deposited at the EMBL under the accession numbers AM259275, AM259276, AM259277, AM259278, AM259279, AM259280, AM259281, AM259282, AM259283, and AM259284 (Table 2). These sequences were aligned with the sequences already known for *P. graminis*. The five sequences from *P. graminis* on barley were 100% homologous to the published sequence of *P. graminis* f. sp. *temperata* (accession number Y12824), except the one from an isolate from Marneffe for which one base differed from the 132 bases in the ITS1 at position 99 (1 T instead of 1 A; accession number AM259275, Table 2). The five other sequences obtained from *P. graminis* on wheat were very close to the published sequence of *P. graminis* f. sp. *tepida* (accession number Y12826), but one base was missed at the 113 position in the ITS1. For the sequence from the Marbais isolate (accession number AM259277, Table 2),

two other bases differed at positions 96 and 103 of the ITS1. This characterization study therefore revealed that the two special forms hitherto described in temperate areas are present in Belgium: *P. graminis* f. sp. *temperata* on barley plants and *P. graminis* f. sp. *tepida* on wheat.

Development of detection methods.

On the basis of the sequences obtained from our isolates and the sequences published for each special form, three detection methods specific to *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* were developed. RFLP and nested and multiplex PCR were conducted to identify the special form present in DNA extracts from the 10 samples of PCR products (Table 2). In the RFLP method, two fragments of 295 and 177 bp were obtained after the digestion of *P. graminis* f. sp. *temperata* using *Nru*I and the other two of 152 and 348 bp after the digestion of *P. graminis* f. sp. *tepida* using *Sal*I. The primers used in the nested and multiplex PCR allowed amplification of a region of 102 bp for *P. graminis* f. sp. *temperata* and a region of 128 bp for *P. graminis* f. sp. *tepida*. The sizes of the DNA fragments obtained by RFLP and nested or multiplex PCR corresponded to the expected profiles from the sequences known for these two special forms. It was shown that the three methods target the specific sequences of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* since they allowed identification of and discrimination between *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* in some samples on barley and wheat or the detection of both in mixtures (Fig. 2). In addition, no cross-reaction with *P. graminis* f. sp. *subtropicalis* and *P. graminis* f. sp. *tropicalis* was observed.

The characterization of *P. graminis* in ocula using the above methods revealed the identification of *P. graminis* f. sp. *temperata* on barley plants grown on some soils (Gembloux and Marneffe soils for example) and the identification of *P. graminis* f. sp. *tepida* on wheat grown on other soils (Baisy-Thy, Chambon-sur-Cisse, Flavion, Malines, Marbais, and Ramillies soils). In some fields at Chambon-sur-Cisse and Flavion, both special forms were detected on wheat plants (Table 3).

In order to answer whether there is host specialization of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* on barley and wheat, plants of both species were grown under controlled conditions on Belgian soils collected at Gembloux and Flavion, and on French soil collected at Chambon-sur-Cisse; the characterization of *P. graminis* special forms was done after 12 weeks of growth in the infected plants. This study revealed that different associations of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* occurred on barley and wheat plants and the frequencies of detection of each special form differed

among soils and plant species (Table 3). Both special forms were isolated from plants grown in each of the three tested soils at a very low frequency, but *P. graminis* f. sp. *temperata* was more frequent on barley than on wheat, whereas *P. graminis* f. sp. *tepida* was more often detected on wheat. This study demonstrated the specialization of *P. graminis* special forms on cereal species according to an independence test (chi-square test). In order to assess the multiplication rate of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* in barley and wheat, a real-time PCR method was developed to quantify infection by these two special forms.

Quantification of *P. graminis* special forms. The quantification of each special form by real-time quantitative PCR using specific primers and Taqman probes was assayed relative to "standard DNAs" built with specific DNA fragments of *P. graminis* f. sp. *temperata* or *P. graminis* f. sp. *tepida* cloned into plasmids. The kinetics of the fluorescence curves for four serial dilutions of the standards with DNA from *P. graminis* f. sp. *tepida* (Fig. 3) or *P. graminis* f. sp. *temperata* (data not shown) showed an efficiency of 92 to 98% and 90 to 93%, respectively. Those obtained for DNA from *P. graminis*-infected plants displayed the same profiles as shown in Figure 3 for *P. graminis* f. sp. *tepida*. No fluorescence was recorded in the presence of DNA extracted from healthy root tissues, and there was no cross-reaction between *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* or with *P. graminis* f. sp. *tropicalis* and *P. graminis* f. sp. *subtropicalis*. In addition, it was shown that the presence of DNA from the other special form did not alter the real-time PCR assay, since the quantification of *P. graminis* f. sp. *temperata* or of *P. graminis* f. sp. *tepida* in a sample prepared with a mixture of DNA from both special forms gave the same threshold cycle (CT) values as those obtained with the same quantity of DNA from the respective form used alone. This is illustrated for *P. graminis* f. sp. *tepida* in Figure 3, but it was also tested for *P. graminis* f. sp. *temperata*.

Host specialization. The proportions of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* determined by real-time quantitative PCR assays in barley and wheat plants grown on two soils were calculated by comparison with the values obtained for DNA from the clones (used as standards) and from known quantities of sporosori (Table 4). From the Chambon-sur-Cisse soil, the sporosori concentrations of *P. graminis* f. sp. *temperata* were on average ~5,000 times higher on barley than on wheat plants, and from the Gembloux soil they were on average ~500 times higher on barley. From both soils, the relative concentrations of *P. graminis* f. sp. *tepida* were on average ~100 times higher on wheat than on barley plants. The as-

assessment of the relative quantities of sporosori shows that the concentration of *P. graminis* f. sp. *temperata* on barley grown on the Gembloux soil was greater than the quantity of *P. graminis* f. sp.

tepida on wheat. This difference was less important for plants grown on the Chambon-sur-Cisse soil, for which the inoculum potential by *P. graminis* f. sp. *tepida* appeared to be higher than in the Gembloux

soil. No clear differences were observed in relation to the conditions and length of growth.

Microscopic observations of stained roots from barley and wheat plants grown

Fig. 2. **A**, Restriction fragment length polymorphism (RFLP), using *SalI* and *NruI* restriction enzymes, from polymerase chain reaction (PCR) products obtained with Psp1 and Psp2rev primers. **B** and **C**, DNA profiles produced after nested PCR for detection of *Polymyxa graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*, respectively. **D**, DNA profiles produced after multiplex PCR for identification of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*. Lanes: (1) roots of wheat cv. Cezanne grown in Flavion soil in pot (Belgium, 16–18°C, 8 weeks); (2) roots of wheat cv. Cezanne grown in Chambon-sur-Cisse soil in pot (France, 16–18°C, 8 weeks); (3) roots of barley cv. Tiffany grown in Gembloux soil in pot (Belgium, 16–18°C, 8 weeks); (4) mixture of the samples loaded in lanes 1 and 3; (5) and (6) *P. graminis* f. sp. *tropicalis* and *P. graminis* f. sp. *subtropicalis*, MUCL references 43177 and 43182, respectively; (C-) negative control = DNA extracted from root tissue of healthy barley and wheat plants grown in sterile sand; (b) blank = products used in PCR reaction with DEPC-treated water (without plant sample), and (M) size marker: EZ Load 100-bp Molecular Ruler (Bio-Rad, Nazareth, Belgium).

Table 3. Infection frequencies of *Polymyxa graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* on barley (cv. Tiffany) and wheat (cv. Cezanne) plants collected in 2004 from Chambon-sur-Cisse, Flavion, and Gembloux fields and other plants grown under controlled conditions in pots on soils collected in these three fields (16–18°C, 12 weeks of growth)^a

	Soils	Cereal	Number of plants	Frequency of <i>P. graminis</i> f. sp. (%)		
				<i>temperata</i>	<i>tepida</i>	Both
Field	Chambon	Wheat	10	0	80	20
	Flavion	Wheat	10	0	80	20
	Gembloux	Barley	13	100	0	0
Under controlled conditions	Chambon	Barley	6	83	0	17
		Wheat	10	0	90	10
	Flavion	Wheat	4	0	75	25
	Gembloux	Barley	12	100	0	0
		Wheat	5	0	80	20

^a Characterization of the *P. graminis* special forms was done using nested and multiplex polymerase chain reaction described in this study.

Fig. 3. Real-time quantitative polymerase chain reaction (PCR) assay for *Polymyxa graminis* f. sp. *tepida*. **A**, Showing the increase in fluorescence with time (Cycle) of accumulated PCR products. **B**, Standard curve obtained from results of four serial dilutions of standard sample. Samples in: (1, 2, 3, and 4) four serial dilutions of 100 to 100,000 of the standard sample used in each reaction, the clone containing rDNA of *P. graminis* f. sp. *tepida* from PCR product using Psp1/Psp2rev primers on wheat cv. Cezanne roots grown under controlled conditions in Marbais soil (accession number AM259277, Table 2); (5) roots of wheat cv. Cezanne grown in Chambon-sur-Cisse soil in pot (France, 16–18°C, 8 weeks); (6) mixed DNA of sample (5) and roots of barley cv. Tiffany grown in Gembloux soil in pot (Belgium, 16–18°C, 8 weeks); (7) roots of wheat cv. Cezanne grown in Flavion soil in pot (Belgium, 16–18°C, 12 weeks); (8) several samples: roots of barley cv. Tiffany grown in Gembloux soil in pot (Belgium, 16–18°C, 8 weeks) - *P. graminis* f. sp. *tropicalis* and *P. graminis* f. sp. *subtropicalis*, MUCL references 43177 and 43182, respectively - negative control = DNA extracted from root tissue of healthy barley and wheat plants grown in sterile sand - and blank = products used in PCR reaction with DEPC-treated water (without plant sample).

under controlled conditions were conducted. *P. graminis* was observed at all development stages on barley plants with a very good multiplication, whereas *P. graminis* was observed on wheat plants with some difficulty and only at the sporosori stage.

DISCUSSION

For some years, molecular methods have been applied to detect *P. graminis* using PCR (9,12,13,21,23,29). These methods have facilitated the identification of this root parasite, otherwise detected by

microscopy or serological methods (ELISA) (2,7,15). Molecular characterization studies also allowed *P. graminis* to be separated into special forms (11). Two of them, *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*, occur in temperate areas. They are distinguished only by their particular ribotypes, but whether this difference affects their ecology or ability to vector a particular virus is questioned. It was for discrimination and quantification of these two forms that specific techniques had to be developed. Ward et al. (29) have published a PCR method for distinguishing *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*. The methods developed in this paper allowed both precise detection and/or quantification of the forms that were present. These tools were developed on the basis of the sequences from *P. graminis* inocula, including those occurring in Belgium. Characterization of *P. graminis* present in soils from Belgium and France revealed the occurrence of *P. graminis* f. sp. *tepida* in both countries, in addition to *P. graminis* f. sp. *temperata*. Hitherto, *P. graminis* f. sp. *tepida* had been reported in Canada, France, and the United Kingdom and *P. graminis* f. sp. *temperata* in Belgium, Canada, China, France, Germany, and the United Kingdom (11,27). This is the first time that *P. graminis* f. sp. *tepida* has been reported in Belgium.

The sequences obtained on barley correspond to published sequences of *P. graminis* f. sp. *temperata*, and the sequences on wheat plants correspond to *P. graminis* f. sp. *tepida*, both with 98 to 100% homology in the ITS1 region. In order to enlarge the detection threshold of the newly developed tools, restriction enzymes for RFLP and primers for nested PCR were selected to target specific regions for each special form as well as to target common regions among inocula of the same form. In addition, the primers were selected to be usable in a unique multiplex reaction. The efficiency of these tools in detecting and discriminating the special forms by RFLP and nested and multiplex PCR was proven on DNA extracted from reference samples or infected plants. These methods demonstrated the presence of both *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* in some Belgian and French soils, and even the co-infection of some plants by both special forms. The co-infestation of the two forms in some soils had already been observed by Ward et al. (29) from a field at Kent (United Kingdom). The difference in frequency of detection of the forms on barley and wheat reveals some degree of host specificity, with *P. graminis* f. sp. *temperata* being more frequent on barley and *P. graminis* f. sp. *tepida* on wheat.

To determine the relative importance of the special forms in the tested soils and to quantify their host specificity, quantitative PCR assays were designed for each form.

Table 4. Relative quantification of *Polymyxa graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* in barley cv. Tiffany and wheat cv. Cezanne roots after growing on Chambon-sur-Cisse and Gembloux soils, estimated by real-time quantitative polymerase chain reaction

Soil	Cereal	Temperature (°C)	Week incubation	Relative DNA quantity of <i>P. graminis</i> f. sp. (per g of roots) related to 1 ng clone DNA		Estimated sporosori concentration of <i>P. graminis</i> f. sp. (per g of roots)	
				<i>temperata</i>	<i>tepida</i>	<i>temperata</i>	<i>tepida</i>
Chambon-sur-Cisse	Barley	12–14	9	94,000	0.4240	180,436	1,140
			12	63,467	0.0081	121,826	22
		16–18	9	153,333	0.2000	294,328	538
			12	47,600	0	91,370	0
			19	8,640	0.0114	16,585	31
	Wheat	12–14	9	62	20.1333	120	54,154
			12	0	53.7332	0	144,531
		16–18	9	5	26.2666	9	70,652
			12	0	1.6400	0	4,411
			19	0	0.0253	0	68
Gembloux	Barley	12–14	9	10,280	0	19,733	0
			12	18,667	0	35,831	0
		16–18	19	4,773	0	9,163	0
			9	16,933	0.0012	32,504	3
			12	26,933	0	51,699	0
	Wheat	12–14	9	45	0.0580	86	156
			12	6	0	11	0
		16–18	9	37	0.0064	70	17

The infection of *P. graminis* f. sp. *temperata* was significantly higher on barley than on wheat, and the infection of *P. graminis* f. sp. *tepida* was higher on wheat than on barley. A real difference of host specificity of both special forms was shown quantitatively. This is the first time that these two special forms occurring in temperate areas have been quantified specifically by real-time PCR and a clear segregation between them on the basis of their infection rate of barley and wheat in two soils (Chambon-sur-Cisse and Gembloux) shown. The distinct host specificity reveals particular ecological characteristics, which support the distinction of two special forms.

The microscopic observations highlighted that *P. graminis* is more easily observed on barley than on wheat, suggesting a more prolific development and multiplication on barley, but this must be confirmed in the further experiments. A morphological characterization (e.g., spore dimensions) could show differences between *P. graminis* present on barley and the one present on wheat, possibly indicating a correlation with the special form detected.

The results presented in this paper show that *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* have distinct “preferred” hosts, as suggested and demonstrated for other *P. graminis* isolates by Legrève et al. (15,16), which were later classified as different special forms (11). In view of the more important infection of *P. graminis* f. sp. *temperata* on barley and *P. graminis* f. sp. *tepida* on wheat, one should consider whether there is a specialization of the interaction between the *P. graminis* special forms, the transmitted viruses, and the host plants. It is worth noting that bymo- and furoviruses on cereals coexist in some soils, and both *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida*

have been detected in such soils. Therefore, studies are being conducted in order to better understand the ability of *P. graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* to transmit specific viruses.

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