The use of conventional and quantitative real-time PCR assays for *Polymyxa graminis* to examine host plant resistance, inoculum levels and intraspecific variation

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Summary

• A real-time PCR protocol based on 18S rDNA sequences was developed to provide a specific, sensitive and quantitative assay for the root-infecting virus vector *Polymyxa graminis*.

• The assay was calibrated with zoospore suspensions and inoculated roots and then shown to work with naturally infected plant roots and infested soil. Both the temperate *P. graminis* ribotypes previously described are detected but are not distinguished. DNA from related plasmodiophorids and from a range of fungi and plants was not detected.

• Different genotypes of *Triticum* were grown in a soil infested with *P. graminis* and *Soil-borne cereal mosaic virus* (SBCMV). The genotypes differed in susceptibility to *P. graminis*, the least susceptible being the *Triticum monococcum* accession K-58505.

• Conventional PCR assays and sequencing of amplified rDNA fragments showed that *P. graminis* isolates infecting wheat were mostly, but not exclusively, of ribotype II. Ribotype II was clearly associated with SBCMV transmission and seems to occur preferentially on wheat whereas ribotype I is mostly associated with barley.

Key words: diagnostics, plant resistance, *Polymyxa graminis*, real-time PCR, ribotypes, *Soil-borne cereal mosaic virus* (SBCMV), *Triticum monococcum*.

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Introduction

Polymyxa graminis Led., is an obligate root-infecting organism that was originally described from wheat (Ledingham, 1939) and which has been later found in roots of many cereals and grasses in many parts of the world. It survives in soil as clusters of thick-walled resting spores and germinates to produce biflagellate zoospores, which infect the root epidermal cells of their hosts. Within the infected cell, the *Polymyxa* cytoplasm becomes an irregularly shaped multinucleate sac (plasmodium), which then develops into a zoosporangium. Later, plasmodia develop into resting spore clusters. The most recent morphological study is that of Littlefield *et al.* (1998). The morphologically identical *P. betae* Keskin occurs in roots of sugar beet and other plants in the family *Chenopodiaceae*. *Polymyxa* is one of 10 genera in the order *Plasmodiophorales* and family *Plasmodiophoraceae* (Braselton, 1995; Dick, 2001). As discussed in a recent review (Kanyuka *et al.*, 2003), 'plasmodiophorids' were traditionally considered to be fungi but analysis of their small subunit rDNA sequences has shown that they should probably be classified within the protists and that they are not closely related to the true fungi (including the zoosporic Chytridiomycetes) or to other zoosporic plant parasites (classified as stramenopiles, which include the Oomycetes). Recent studies of the actin and ubiquitin genes confirm this classification (Archibald & Keeling, 2004).

It is unlikely that *P. graminis* does measurable damage to its host plant but it is the vector of several very important viruses, including the bymoviruses *Barley yellow mosaic virus*, *Barley mild mosaic virus* and *Wheat spindle streak mosaic virus* and the furoviruses, *Soil-borne wheat mosaic virus* and *Soil-borne cereal mosaic virus* (SBCMV) (Kanyuka *et al.*, 2003). These viruses are thought to be carried within the *Polymyxa* resting spores (Driskel *et al.*, 2004), and swimming zoospores released upon germination of resting spores have been shown to carry the viruses (Adams, 2002). Although the details have not been determined, the viruses must then move (either from zoospores or from the *Polymyxa* plasmodia) into the cytoplasm of the host plant root cells where they multiply and subsequently become systemic, causing various mosaic and streak symptoms in leaves and affecting plant growth and yield. Host plant resistance to the viruses is currently the only effective and economically practicable form of control (Kanyuka *et al.*, 2003).

Studies of nuclear 18S, 5.8S, and internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA (rDNA) regions (Ward *et al.*, 1994; Ward & Adams, 1998; Morales *et al.*, 1999; Legrève *et al.*, 2002) has shown that the two *Polymyxa* species can be clearly distinguished, and that there are several subgroups (ribotypes) of *P. graminis*. Legrève *et al.* (2002) have proposed classifying the *P. graminis* ribotypes as different *formae speciales* as there is some correlation with host range, temperature requirements and geographical origin. Two of the ribotypes are known to occur in temperate regions, including the UK, and the rDNA sequences have been used to develop *Polymyxa* spp. and *P. graminis*-specific PCR tests that allow *P. graminis* ribotype determination by amplicon size or RFLP (CAPS) analysis (Ward *et al.*, 1994; Ward & Adams, 1998; Morales *et al.*, 1999). The biological significance of the two temperate ribotypes is not clear but the majority of isolates obtained and studied have been of ribotype I and were isolated from barley, whereas most ribotype II isolates reported have been from wheat and other cereals.

Host resistance to *Polymyxa* would be a very desirable trait in cereal cultivars if a suitable source of resistance could be found. The many European barley cultivars tested so far have been highly susceptible to *P. graminis*, but there appeared to be some differences between wheat cultivars in their degree of susceptibility (Bastin *et al.*, 1989; Adams & Jacquier, 1994), although none was immune. Screening for resistance would have to take into account the different ribotypes of *P. graminis* if these are shown to be important for specificity to the host plant or to differ in their ability to transmit viruses. It would also require an efficient molecular method for the detection and quantification of *P. graminis* in plant roots.

Traditionally, detection of *Polymyxa* has relied on microscopy but more recently specific antibodies have been developed (Delfosse *et al.*, 2000; Mutasa-Göttgens *et al.*, 2000; Kingsnorth *et al.*, 2003a). PCR methods can also be exploited for diagnostic purposes. A quantitative real-time PCR method has recently been developed for *P. betae*, based on the detection of glutathione-S-transferase transcripts (Kingsnorth *et al.*, 2003b). In this paper, we report the development of a real-time PCR method for detection and quantification of *P. graminis*, in plant roots and in soil. This has then been used to examine abundance of *P. graminis* in roots of different cereal genotypes growing in infested soil. The presence of the different ribotypes was also examined using the same primers in conventional PCR-RFLP (CAPS) analysis and also by designing new primers for conventional PCR. This provides new evidence on the ability of specific *P. graminis* ribotypes to invade roots of different monocotyledonous genotypes and to transmit SBCMV.

Materials and Methods

The details of the *Polymyxa* isolates tested, together with other plasmodiophorids and some fungi used as controls are presented in Table 1. Soil samples used were: first a *P. graminis* infested viruliferous (SBCMV) soil collected from a wheat field in Kent, UK in September 2002 and stored dry at 4°C before use; second a negative control soil (free of *Polymyxa* spp.) obtained from a garden at Rothamsted Research; third two samples from Horsepool field at Woburn experimental farm that contained *P. graminis* in a bioassay (Woburn 1 and Woburn 2); and fourth one sample from the same field where no *P. graminis* was detected (Woburn 3).

DNA extraction

DNA was extracted from *Polymyxa*-infected and uninfected plants and soil samples using the method of Fraaije *et al.* (1999) except that the extraction buffer was amended to include 5 mM 1,10 phenanthroline monohydrate and 2% w/v polyvinylpyrrolidone (Zhang & Stewart, 2000). The whole plant root from each individual plant was freeze dried and ground into a fine powder using a mortar and pestle. DNA was then extracted from 30 mg of this powder. For soil samples, DNA was extracted from freeze dried aliquots of *c.* 0.25 g.

Zoospores of *P. graminis* isolate F1 from barley cv Regina were produced as described previously (Adams *et al.*, 1986), filtered through tissue paper and centrifuged at 4000 ***g*** for 10 min. The pellet was then re-suspended in 0.5 ml water and an aliquot was taken for counting. A 1 : 10 dilution series was prepared (to a dilution of 1×10^{-5}) and DNA was then extracted from 0.4 ml of each dilution by adding an equal volume of 2 × extraction buffer and following the method of Lee & Taylor (1990).

All DNA samples were quantified by a fluorometric assay as described in Fraaije *et al.* (1999), but replacing Pico Green with thiazole orange (5 μM, Aldrich, Milwaukee, WI, USA). Fluorescence was measured using a Bio-tek FL × 800 microplate fluorescence reader (Winooski, VT, USA) with excitation at 485 nm and emission measured at 528 nm.

Table 1 Organisms and isolates used to test specificity of the PCR primers

Organism	Isolate	Source
Plasmodiophorids		
Polymyxa graminis ribotype I	F1	Streatley, UK, barley
Polymyxa graminis ribotype I	F24	Basingstoke, UK, *Poa* sp.
Polymyxa graminis ribotype I	F38	Eastleach, UK, barley
Polymyxa graminis ribotype I	F42	Cambs, UK, barley
Polymyxa graminis ribotype I	F60	Lower Heyford, UK, barley
Polymyxa graminis ribotype II	F36	Prince Edward Island, Canada, wheat[1]
Polymyxa graminis ribotype II	F43	Gramat, France, barley
Polymyxa graminis ribotype II	F51	Cranbrook, UK, oats
Polymyxa graminis ribotype II	F53	Ottawa, Canada, wheat[1]
Polymyxa betae	BBES1	Broom's Barn, UK, sugar beet[2]
Polymyxa betae	F67	Nagele, Netherlands, sugar beet[3]
Polymyxa betae	F68	former Yugoslavia, sugar beet
Ligniera sp.	F69	Yolo, California, Pumpkin[4]
Plasmodiophora brassicae	N	Harpenden, UK, cabbage
Spongospora subterranea	H	UK, potato
Other microorganisms		
Alternaria alternata	HK3[5]	Zielonka, Poland, *Pinus silvestris* nursery soil
Fusarium culmorum	Fc31	Rothamsted, UK, wheat
Gaeumannomyces graminis var. *tritici*	90/2–4	Woburn, UK, wheat
Helminthosporium solani	Hs1	Rothamsted, UK, potato
Mortierella humulis	HK154[5]	Zielonka, Poland, *Fagus silvatica* soil
Mucor hiemalis	HK1[5]	Zielonka, Poland, *Pinus silvestris*
Penicilllium daleae	HK56[5]	Zielonka, Poland, *betula pendula* soil
Pythium intermedium	H23	Harpenden, UK, wheat
Trichoderma harzianum	HK3[5]	Zielonka, Poland, *Pinus silvestris* nursery soil
Trichoderma viride	HK4[5]	Zielonka, Poland, *Pinus silvestris* nursery soil

[1]From Dr D J S Barr, Ottawa, Canada
[2]From Dr E S Mutasa-Göttgens, Broom's Barn, Bury St. Edmunds, UK
[3]From Dr H Paul, CPRO-DLO, Wageningen, Netherlands, isolate 91–2
[4]From Prof. R N Campbell, UC Davis (Campbell & Sim, 1994)
[5]DNA provided by Dr H. Kwaśna

Real-time PCR analysis

Previously published sequences of 18S and ITS rDNA regions of *P. graminis* and related plasmodiophorids (Ward & Adams, 1998; Bulman *et al.*, 2001) were aligned using the GCG program PILEUP (Anonymous, 2001) to identify suitable regions for PCR assay design. Primers and probes were then chosen using Primer Express software (Applied Biosystems, Warrington, UK). Primer and probe concentrations were optimized experimentally according to the manufacturer's guide.

Assays (25 µl) were performed in MicroAmp Optical 96-well reaction plates (Applied Biosystems, Warrington, UK). The reaction mix contained 1× TaqMan Universal master mix (Applied Biosystems), 0.6 µM primer PxRealF (5′ CGT CGC TTC TAC CGA TTG GT), 0.2 µM primer PxRealR (5′ CCT TGT TAC GAC TTC TTC TTC CTC TAG T) and 0.15 µM TaqMan MGB probe PgRealP (5′ CCG GTG AAC AAT CG) which was labelled at the 5′ end with the fluorescent reporter dye FAM (6-carboxy-fluoroscein). The primers and probe were obtained from Applied Biosystems. In all cases 5 µl DNA was added to the PCR reaction. This corresponded to 100 ng DNA for samples from plant roots, 20 ng for samples from soils or 50 ng from fungal cultures. Amplification and detection were performed in a Prism 7700 Sequence Detection System (Applied Biosystems) under the following conditions: one cycle of 2 min at 50°C; one cycle of 10 min at 95°C; 50 cycles of 15 s at 95°C, 1 min at 60°C.

The cycle threshold C_t (defined as the cycle number at which a statistically significant increase in fluorescence occurs) for each PCR was automatically calculated by the ABI Prism Sequence Detector software version 1.9. For each sample, the amount of DNA was quantified using appropriate calibration curves in which C_t was plotted against the amount of target DNA. The target DNA used to generate the standards was a ribosomal DNA fragment from *P. graminis* F36, generated using PCR primers NS7 and ITS4 (White *et al.*, 1990), which was cloned into pGEM-T Easy (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. This PCR product contains about 350 bases at the 3′ end of the 18S gene and the entire 5.8S and ITS regions. Plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen, Crawley, UK) according to the manufacturer's

instructions and its concentration was determined by fluorometric assay using thiazole orange. The quantities of DNA used to generate the standard curves for real-time quantitative PCR were 0.1, 1, 10, 100, 500, 1000, 2000 and 5000 pg plasmid DNA. Quantification standards were run in each experiment and they were made in a background of water, uninfected wheat (100 ng) or uninfested soil (20 ng) as appropriate to the experiment. For all real-time PCR runs the linear correlation coefficient of the standard curve was R^2 = 0.97–0.99. Amounts of *Polymyxa* present in samples were expressed in pg plasmid DNA present per 100 ng root DNA or per 20 ng soil DNA. Samples were all tested in duplicate, in two separate PCR assays.

Conventional PCR and CAPS analysis

The primers used in real-time PCR (PxRealF and PxRealR) were also tested in conventional PCR. Each 25 μL PCR contained 25 pmol of each primer, 0.25 U *Taq* polymerase, 0.2 mM deoxyribonucleoside triphosphates, reaction buffer (10 mM Tris HCl pH 8.8 at 25°C, 50 mM KCl, 0.08% Nonidet P40, 0.1 mg ml^{-1} BSA and 1.5 mM $MgCl_2$) and 80–100 ng DNA. Cycling conditions were 30 cycles of 30 s at 94°C, 1 min at 60°C and 2 min at 72°C, followed by 10 min at 72°C. The products were analysed electrophoretically in agarose gels (2% NuSieve GTG agarose [Cambrex Bio Science, Wokingham, UK] +1% standard agarose) and the DNA detected using ethidium bromide. For CAPS analysis to determine the *P. graminis* ribotype(s) present, amplified DNA (8.5 μl) was digested using *Cfo*I and detected on agarose gels as described above. Subsequently, new primer pairs were designed to amplify the rDNA fragments only from *P. graminis* ribotype I or from ribotype II. Forward primers, Pg.F1 (5′-AAC ATG TGG ATT GTG GGC TAT GTG) and Pg.F2 (5′-ATG TGG ATC GTC TCT GTT GCT GGA), were from the ITS1 region (i.e. between 18S and 5.8S) and reverse primers, Pg.R1 (5′-AAC TCC CAT TCT CCA CAA CGC AA) and Pg.R2 (5′-CCT CAT CTG AGA TCT TGC CAA GT) were in ITS2 (i.e. between 5.8S and 26S). Primer pair Pg.F1/Pg.R1 was predicted to amplify a 292 bp fragment only from ribotype I, and primer pair Pg.F2/Pg.R2 to amplify a 430 bp fragment only from ribotype II. Cycling conditions were 1 cycle of 2 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 60°C and 30 s at 72°C, followed by 5 min at 72°C.

DNA sequence analysis

Ribosomal DNA was amplified by PCR using primers previously devised for fungal phylogenetic analysis (White *et al.*, 1990). Primers NS5 (5′ AAC TTA AAG GAA TTG ACG GAA G) or ITS5 (5′ GGA AGT AAA AGT CGT AAC AAG G) were used in combination with ITS4 (5′ TCC TCC GCT TAT TGA TAT GC) as previously described (Ward & Adams, 1998). The amplicons were cloned into pGEM-T Easy (Promega Corporation, Madison, WI, USA). Plasmid DNA was prepared from clones containing *Polymyxa* rDNA using the QIAprep spin miniprep kit (Qiagen, Crawley, UK), and sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1) using primers NS5, NS8 (5′ TCC GCA GGT TCA CCT ACG GA) and ITS4. The purified products were run at the DNA Sequencing Facility, Oxford University, UK. DNA sequences were assembled using the UNIX-based STADEN software package (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK) and further analysis used programmes in the Wisconsin (GCG) package (Anonymous, 2001).

Testing of genotypes for resistance

The following genotypes were used: (i) *Triticum aestivum* cvs: Riband, Hereward, Avalon, Claire, Cadenza, Capitaine, Galahad (ii) *Triticum durum* cv Capdur (iii) *Triticum monococcum* accessions K-38079 and K-58505 (Kanyuka *et al.*, 2004) (iv) *T. aestivum* – *Thinopyrum intermedium* chromosome $3Ag^i$ addition line L2 (Rumjaun *et al.*, 1996) and (v) *Hordeum vulgare* cv Maris Otter. Pre-germinated seeds of test plant genotypes were transplanted into 7-cm^2 plastic pots (three seedlings per pot) containing viruliferous or negative control soil mixed with sand (1 : 2), and 3.0–3.5 g l^{-1} of the controlled release fertiliser Osmocote® Plus (Scotts Europe B. V., Heerlen, The Netherlands). Two or three pots of each genotype were grown in each soil, except for the wheat-*Th. intermedium* line L2 where there was only sufficient seed for a single pot. Plants were grown for 8 wk and then assayed for SBCMV in their leaves and roots by ELISA as described by Kanyuka *et al.* (2004). Roots were also stained with 0.1% acid fuchsin (Hooper, 1986) and five randomly selected crown root segments (*c.* 2 cm long) were examined under the light microscope. The abundance of *P. graminis* resting spore clusters in each segment was scored on a scale from 0 (none) to 7 (very abundant throughout the whole segment). The remainder of the root system from each plant was then used for DNA extraction and PCR analysis.

Results

Design and testing of PCR primers and probes for real-time PCR

A section of the sequence alignment (towards the 3′ end of the 18S rDNA), with the primers (PxRealF and PxRealR) and probe (PxRealP) selected, is shown in Fig. 1. FASTA and BLAST searches of the GenBank/EMBL database indicated that the primers would be specific for *Polymyxa* species (*P. graminis* and *P. betae*) and the probe would be specific for *P. graminis* only. This was confirmed experimentally using DNA extracted from a range of fungi and uninfected plant samples. All the *P. graminis* isolates listed in Table 1 were positive using

	* 20 * 40 *
P. graminis F1	**CGTCGCTTCTACCGATTGGT**CGTCCCGGTGAACAATCGGGAGGCCGTGCT
P. graminis F51	..........G..C..A
P. betae	..........TG.....G..C...
Ligniera	.....C....T.....AT.CT.....TCG.....AT.C.T.
Spongospora	.....C....T.....AAT.CT.....ACG.....AAT.C..C
Plasmodiophora	.....C....T.....AGT.CT.....ATG.....ATT.CT.C
	60 * 80 * 100
P. graminis F1	CATGGTCAGCAATGGCCGGAGCGCGGTTGAACTTTTGTAAATTTGGACG**A**
P. graminis F51	.CA.....C.....TCC.....T...
P. betae	GC..AC.....T.AC..T...T.C.....T...
Ligniera	.G.....T.....TCC.....CCA.....GTAT
Spongospora	TGC.A.....T.CAG.T.T.T.....CCT.....GTTT
Plasmodiophora	GCC.A.....T..CG.A.T...C.....CAT.....G.TC
	* 120 * 140
P. graminis F1	**CTAGAGGAAGAAGAAGTCGTAACAAGG**TTTCCGTAGGTGA
P. graminis F51	.....
P. betae	.....
Ligniera	.....A....G.....
Spongospora	T....A....G.....
Plasmodiophora	T....A....G.....

Fig. 1 Small subunit ribosomal DNA sequences of isolates of *Polymyxa graminis* F1 (ribotype I, Y12824), and F51 (ribotype II, Y12826) aligned with those of other plasmodiophorids, *Polymyxa betae* (Y12827), *Plasmodiophora brassicae* (Y12831) and *Spongospora subterranea* (compiled from AF310889 and Y12829). Sequences are identical to *P. graminis* F1 except where shown. Base 1 corresponds to base 207 in *P. graminis* F1 sequence Y12824. The position of the forward (PxRealF) and reverse (PxRealR) primers for the *P. graminis*-specific PCR assay are shown in bold, and the position of the TaqMan® MGB probe (PxRealP) is underlined. The *Cfo*I sites, used to discriminate between ribotype I and ribotype II *P. graminis* in RFLP analysis, follow bases 46, 48, 71 and 73 on the sequence.

PCR, whereas no product was detected from any of the other organisms listed (including the *P. betae* isolates). The *P. graminis* samples tested included DNA from zoospores, resting spores and infected root material. Also no amplification was detected from uninfected plants of barley (cv Maris Otter), sugar beet (cvs. Hilma and Rex) or wheat (cvs. Michigan Amber and Cadenza). The primers were also tested in a conventional PCR assay using the same annealing temperature (60°C) as used in the real-time PCR assay and the same range of plant and fungal samples. A product was obtained from all of the *P. graminis* and *P. betae* isolates tested, but not from any of the other plant or fungal samples. An example of such a gel is shown in Fig. 2.

Quantitative real-time PCR from zoospores

After DNA extraction from a dilution series of *P. graminis* zoospores, the final volume of each preparation was adjusted so that the 5-µl samples used for the PCR reaction contained the DNA from 1×10^5 down to one zoospore. Amplification was obtained from all zoospore concentrations tested (Fig. 3); the lowest concentration (one zoospore) represents a subsample of an extract from 34 zoospores. There was a good correlation between the number of zoospores added to the PCR and the quantity of *P. graminis* DNA obtained ($R^2 = 0.98$ when $\log_{10}$ values of both were plotted; data not shown).

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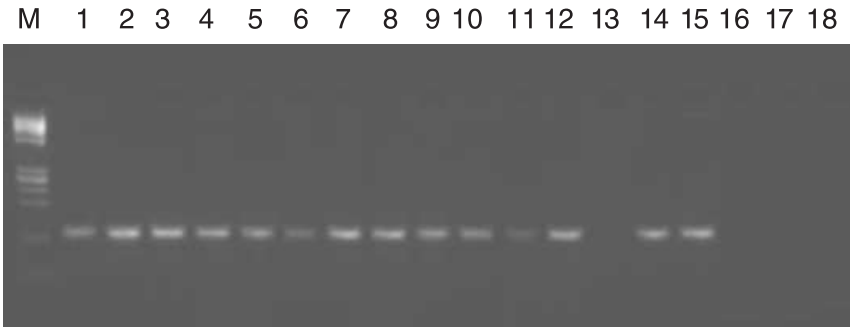

Fig. 2 Testing of various samples in conventional PCR using the *Polymyxa*-specific PCR primers PxRealF and PxRealR. Track M is the size marker ΦX174 *Hae*III, Tracks 1–12 analysis of DNA prepared from *P. graminis* isolates. Track 1, F1 from zoospores; 2, F1 from resting spores; 3, F60 from zoospores; 4, F24 from infected barley roots; 5, Rothamsted RJG0602 from infected barley roots; 6, F42 from infected barley roots; 7, F42 from infected wheat roots; 8, F53 from infected wheat roots; 9, F53 from infected barley roots; 10, F51 from zoospores; 11, F36 from zoospores; 12, Rothamsted CS508 from infected wheat roots; 13, uninfected wheat (cv Mercia); 14, *P. betae* F68 from infected sugar beet roots; 15, *P. betae* F67 from infected sugar beet roots; 16, *Spongospora subterranea*; 17, *Plasmodiophora brassicae*; 18, no DNA control.

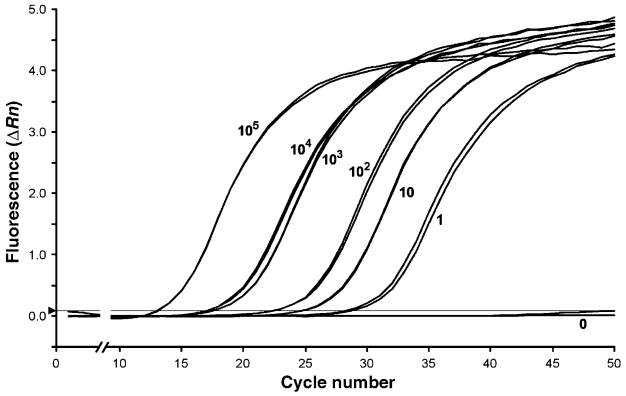

Fig. 3 Real-time PCR amplification plot of DNA samples prepared from different numbers of zoospores. Each DNA sample was tested in duplicate. The line (arrowed) is the fluorescence cycle threshold.

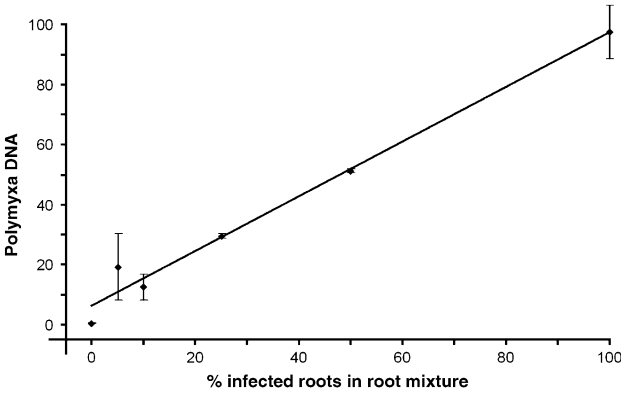

Fig. 4 Relationship between the quantity of *Polymyxa* DNA detected by real-time PCR (expressed as pg control plasmid DNA per 100 ng root DNA) and the percentage infected roots in the sample from which DNA was prepared. Wheat roots containing *P. graminis* were mixed with uninfected roots in different proportions. Two independent mixtures were prepared for each proportion and DNA samples from them were tested in duplicate in two separate real-time PCR assays. Bars show the standard deviation of each point.

Quantitative real-time PCR from infected roots

Wheat roots containing *P. graminis* were mixed with uninfected roots to give mixtures containing 100%, 50%, 25%, 10% and 0% infected roots. The infected roots were from cv Consort that had been grown in the Woburn 2 soil sample, and the uninfected roots were from cv Consort that had been grown in the Rothamsted garden soil sample. Two independent mixtures were prepared for each proportion and DNA was extracted from each of these. Each sample (100 ng DNA in 5 µl) was tested in duplicate in two separate real-time PCR assays. The amount of *P. graminis* DNA in each sample was quantified using a calibration curve generated by spiking 100 ng uninfected wheat DNA with different amounts (0.1–5000 pg) of a plasmid containing the *P. graminis* rDNA target sequence. There was a good correlation (R^2 = 0.98) between the quantity of *P. graminis* DNA detected by real-time PCR and the percentage infected roots in the sample from which the DNA was prepared (Fig. 4).

Quantitative real-time PCR from soil samples

The control soil believed to be free of *Polymyxa* was tested in a preliminary bioassay by growing wheat (cv Consort) and barley (cv Pearl) for 10 wk in a 1 : 50 soil : sand mixture. No *P. graminis* could be detected by light microscopy of stained roots or by using a conventional PCR with the *Polymyxa*-specific primers PxRealF and PxRealR. Different amounts of *P. graminis*-infected roots (20, 10, 5, 2 and 1 mg) were then mixed into aliquots (250 mg) of the soil. The infected roots were of wheat cv Consort that had been grown in the Kent soil sample. Two independent mixtures were prepared for each proportion and DNA was extracted from each of these. Each sample (20 ng DNA in 5 µl) was tested in duplicate in two separate real-time PCR assays. The amount of *P. graminis*

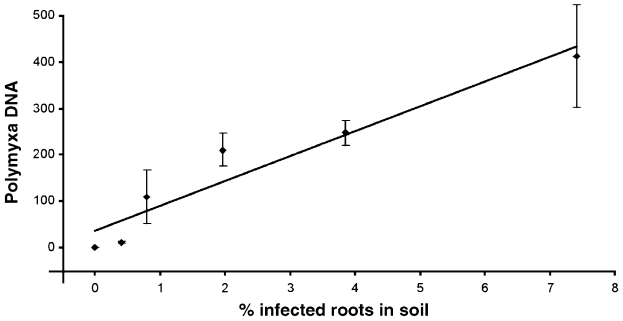

Fig. 5 Relationship between the quantity of *Polymyxa* DNA detected by real-time PCR (expressed as pg control plasmid DNA per 20 ng soil DNA) and the percentage infected roots (w/w) in the soil sample from which DNA was prepared. Roots infected with *P. graminis* were mixed with uninfested soil in different proportions. Two independent mixtures were prepared for each proportion and DNA samples from them were tested in duplicate in two separate real-time PCR assays. Bars show the standard deviation of each point. Fitted line has $R^2 = 0.92$.

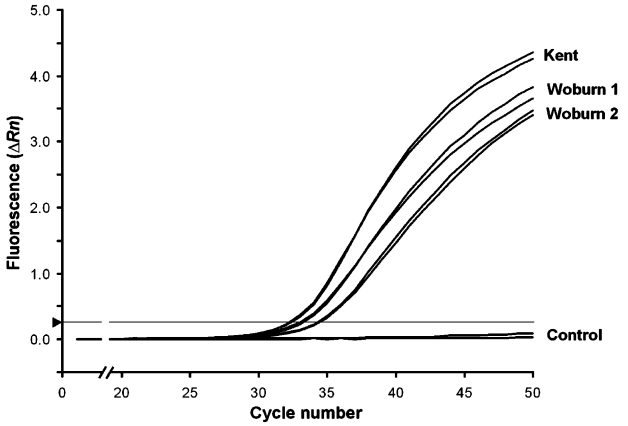

Fig. 6 Real-time PCR amplification plot of DNA extracted from field soils. Soil samples were from Kent (contains SBCMV), two from Horsepool field, Woburn Experimental Farm, Beds and a control (garden) soil without *Polymyxa graminis*. Each DNA sample was tested in duplicate. The line (arrowed) is the fluorescence cycle threshold.

DNA in each sample was quantified using a calibration curve generated by spiking 20 ng of DNA from the uninfested soil with different amounts (0.1–5000 pg) of a plasmid containing the *P. graminis* rDNA target sequence. There was good correlation ($R^2 = 0.92$) between the quantity of *Polymyxa* DNA detected by real-time PCR and the percentage infected roots in the soil sample from which the DNA was prepared (Fig. 5).

DNA was also extracted from several samples of agricultural field soils and 20 ng of each was tested using the real-time PCR assay. Three samples (two from Woburn experimental farm and the SBCMV-infested soil from Kent) that had previously been shown to contain *P. graminis* (by bioassay experiments as described above) were also positive in the real-time PCR assay (Fig. 6). There was no amplification from a further soil sample from Woburn, where no *P. graminis* had been detected by bioassay.

Evaluation of resistance to SBCMV and *Polymyxa* in different *Triticum* genotypes

P. graminis was detected in the roots of all plants both by visual assessment and by real-time PCR. There were highly significant differences between cultivars in the amounts detected (Fig. 7; $P < 0.001$ by real-time PCR, $P < 0.005$ by visual assessment from an analysis of variance). There was also a highly significant correlation between the ranking of

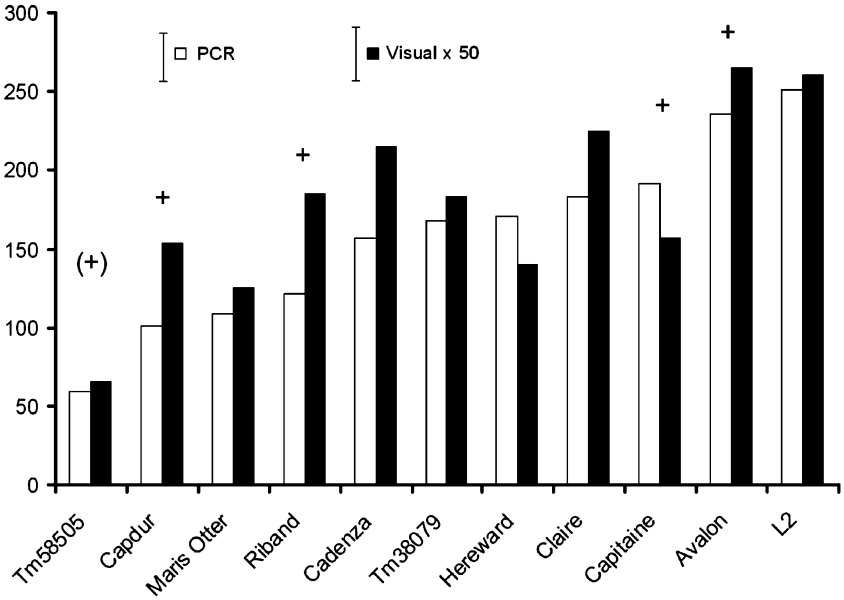

Fig. 7 *Polymyxa graminis* assessments in roots of different *Triticum* or *Hordeum* genotypes after growing in naturally infested soil. Open columns are results from real-time PCR (expressed as pg control plasmid DNA per 100 ng root DNA), closed columns are from visual (microscopic) assessments. Bars show the SEDs for comparisons between the lines (13 DF). Virus (SBCMV) was detected by ELISA in the roots of all plants except the barley cv Maris Otter and in the leaves of those plants marked +. (+) indicates a low concentration of virus and in only one of the two samples tested.

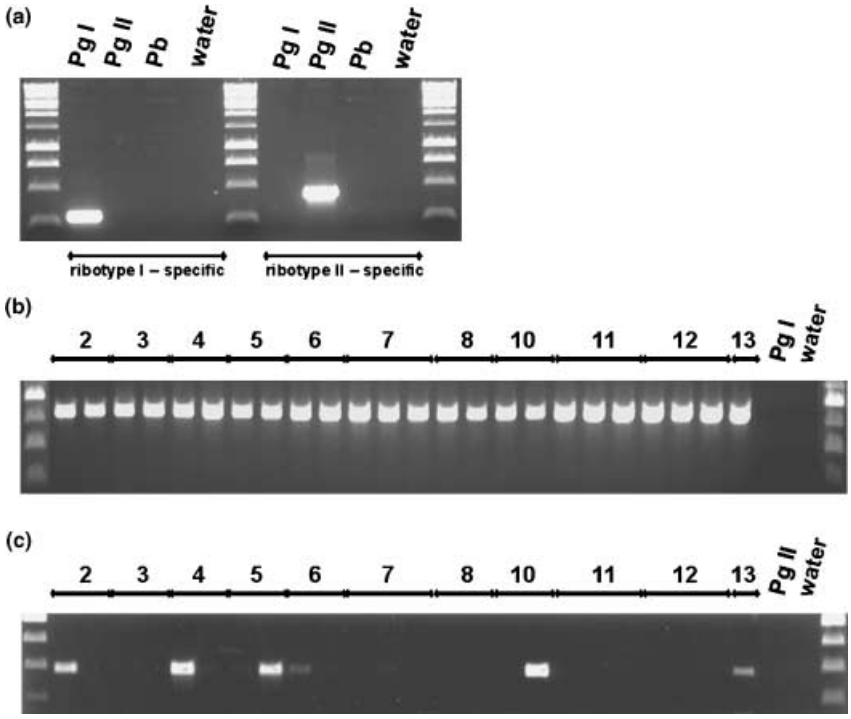

Fig. 8 Testing of plant root samples for *Polymyxa graminis* ribotypes I and II using specific primer pairs. Panel (a) shows tests of the primers on plasmids containing rDNA from *P. graminis* ribotype I (Pg I, isolate F1), ribotype II (Pg II, isolate F36) and *P. betae* (Pb, isolate F41). Panel (b) shows analysis of DNA prepared from roots of different genotypes grown in *P. graminis*-infested soil and tested with ribotype II-specific primers. Each sample is pooled from three plants in a single pot. Genotypes are: 2, wheat cv Cadenza; 3, wheat cv Hereward; 4, wheat cv Claire; 5, wheat cv Avalon; 6, wheat cv Riband; 7, *T. monococcum* K-38079; 8, *T. monococcum* K-58505; 10, barley cv Maris Otter; 11, wheat cv Capdur; 12, wheat-*Thinopyrum intermedium* chromosome $3Ag^{i}$ addition line 2. Panel (c) as panel (b) but with ribotype I-specific primers.

the cultivars by the two different methods (Spearman's Rank Correlation Coefficient = 0.665; $t_{22DF} = 4.165$; $P < 0.001$). The least *Polymyxa* was found in the *Triticum monococcum* line K-58505. SBCMV was readily detected by ELISA in the roots of all plants except the barley cv Maris Otter, but in leaf samples it was only found in the wheat cvs. Capdur, Riband, Capitaine and Avalon and, at low concentration, in *T. monococcum* K-58505.

Detection of different ribotypes of *Polymyxa graminis*

DNA from each individual plant grown in the SBCMV-infested soil was tested using conventional PCR with the *Polymyxa*-specific primers PxRealF and PxRealR. All samples were positive for *Polymyxa*, giving a DNA product of the expected size (127 bp). The ribotypes present were determined using *Cfo*I digestion of these products; ribotype I *P. graminis* gives fragments of 73 and 54 bp, ribotype II *P. graminis* gives fragments of 54, 46 and 23 bp and *Polymyxa betae* gives fragments of 79 and 46 bp (Fig. 1 and data not shown). All of the plants (with the exception of one barley plant) grown in the SBCMV-infested soil were demonstrated to contain *P. graminis* ribotype II using this assay. A few of the plants also contained *P. graminis* ribotype I but the bands for this were fainter and it was difficult to be certain whether ribotype I *P. graminis* was present. Therefore, new PCR assays were developed, designed to be specific for each of the ribotypes. Primer pairs were first tested in a PCR with plasmid DNAs containing the rDNA region of *P. graminis* ribotypes I and II as templates, and shown to be ribotype-specific as predicted (Fig. 8a). In infected root samples, ribotype I *P. graminis* was detected much more clearly using the new assay, and it was apparent that it was only present in a small proportion of the plants whereas all of them, including barley, contained ribotype II (Fig. 8b,c). In separate tests of individual plants from this experiment, trace amounts of ribotype I were found in 3/9 plants of cv Capdur, 4/9 plants of cv Capitaine, 1/6 plants of cv Hereward, 4/9 plants of *T. monococcum* K-38079 and 4/6 plants of *T. monococcum* K-58505 (data not shown).

Sequence analysis of *Polymyxa graminis* ribotypes

Ribosomal DNA sequences were determined for *P. graminis* obtained from wheat (cv Consort) that had been grown in the SBCMV-infested soil. Resting spores were purified from the roots before DNA extraction as described in Ward *et al.* (1994). A region of rDNA from the middle of the 18S gene to the 5′ end of the 26S gene was amplified using primers NS5 and ITS4 and then cloned into pGEM-T Easy. Inserts were amplified from the resulting clones using primers NS5 and ITS4 and digested using *Cfo*I to discriminate between those clones obtained from wheat and those from *Polymyxa*. Of the 23 clones analysed, 16 contained ribotype II and none contained ribotype I. Sequence was obtained, on both DNA strands, from two of the clones containing *Polymyxa* rDNA. In both cases, the sequence obtained (deposited in EMBL as accession number AJ841287) was identical to that of ribotype II *P. graminis* F51 (Y12826) over the region sequenced

(nucleotides 1–849 on the F51 sequence) except for the deletion of the A at position 491.

P. graminis rDNA sequences were also obtained from infected roots of a wheat plant (cv Consort) from Horsepool field at Woburn (Woburn 333), a wheat plant (cv Hereward) from Stackyard field at Rothamsted (Rothamsted CS508) and a barley plant (cv Siberia) from Great Knott field at Rothamsted (Rothamsted RJG0602). DNA extracted from *P. graminis*-infected roots was subjected to a PCR with primers ITS4 and ITS5, and the resulting PCR products were cloned and analysed as described in Materials and Methods. Sequence data was obtained for two clones from each sample using ITS5 as the sequencing primer. The Rothamsted CS508 and Woburn 333 samples (both from wheat) were identical to one another, and to that of ribotype II *P. graminis* F51 (Y12826) over the region sequenced (nucleotides 380–887 on the F51 sequence) except for the deletion of the A at position 491. They were also identical over the region sequenced to the *P. graminis* ribotype II obtained from the Kent wheat sample. The Rothamsted RJG0602 sample from barley was identical to that of ribotype I *P. graminis* F1 (Y12824) over the region sequenced (nucleotides 337–820 on the F1 sequence).

Discussion

Polymyxa graminis is an obligate root-infecting organism of cereals and grasses, and detecting and quantifying it, by the traditional method of microscopic examination is a skilled, time consuming and tedious task. We previously developed conventional PCR techniques to detect *Polymyxa* species (Ward *et al.*, 1994; Ward & Adams, 1998) but these could not be used for quantification. The process of quantifying target DNA has been simplified considerably in the past few years with the advent of real-time PCR (McCartney *et al.*, 2003; Ward *et al.*, 2004).

The *P. graminis*-specific real-time PCR assay developed here used primers that were specific to *Polymyxa* species (*P. graminis* and *P. betae*) with a TaqMan probe that was specific for *P. graminis* only. The specificity was confirmed experimentally using isolates of *P. betae*, the two *P. graminis* ribotypes that are known to be present in temperate regions, a range of other root-infecting and soil microorganisms (including other plasmodiophorids) and uninfected wheat, barley and sugar beet. Samples of the other *P. graminis* ribotypes (III, IV and V) were not tested as these ribotypes have been found only in tropical and subtropical regions, associated with crops such as sorghum, millet, peanut and rice, and are not known to occur in temperate regions. Analysis of sequence data from ribotype V (rice isolate, AJ010424, Morales *et al.* 1999) and ribotype III (sorghum isolate, Y12825, Ward & Adams, 1998) indicate that the real-time PCR assay should detect ribotype V but not ribotype III. There are no sequence data available in this region for ribotype IV. Real-time assays depend on detection of PCR product using fluorescent probes that are specific to the target DNA (e.g. TaqMan probes) or nonspecific DNA binding dyes such as Sybr Green (McCartney *et al.*, 2003; Ward *et al.*, 2004). We chose to use a TaqMan probe for the *P. graminis* assay, since these have the advantage of increasing specificity of the assay, and of reducing signals due to mispriming or primer-dimer formation. However, it should be possible to adapt this assay to work with Sybr Green. The assay would then be cheaper than using the TaqMan probe, and could be used for detection of *P. betae* as well as *P. graminis*. The real-time PCR with Sybr Green may therefore be suitable, for example, to screen sugar beet and related genotypes for resistance to *P. betae*. However, such an assay would not be applicable where both *P. graminis* and *P. betae* might coexist (e.g. in certain soils) and where it would be important to distinguish between them.

The real-time PCR assay was shown to be sensitive, allowing detection of a single zoospore in the PCR. It also appeared to be reliable in detecting different quantities of *Polymyxa*, whether as zoospores or as infected roots mixed with either healthy roots or uninfested soil. In a test of different *Triticum* and related genotypes, results of the assay correlated well with visual assessments. Where there were discrepancies, it is likely that the real-time PCR assay was the more accurate. This is because it is generally difficult to make quantitative assessments from microscopic examination of infected roots especially when, as with *Polymyxa*, infection is very erratically distributed in the root system. *Polymyxa* DNA was also detected in three soil samples from agricultural fields that had previously been shown to contain *P. graminis* by bioassay, while two soil samples (one from an agricultural field and one from a garden) were shown to be free of *P. graminis* using the real-time PCR assay and by bioassay. The assay is most likely to find practical application in screening plants for resistance to *Polymyxa*. Although detection in soil proved possible, there would be practical difficulties in routinely obtaining accurate assessments of inoculum concentrations as these are usually fairly low and *Polymyxa* is likely to be patchily distributed, leading to severe sampling problems. This is a common problem with assays for soil-borne pathogens as experience with take-all of cereals demonstrates (Freeman & Ward, 2004). Moreover, it is possible that the sensitivity of the test could vary depending upon the soil type.

In the host plant genotype screening experiment, all plants became infected with *Polymyxa* regardless of their virus resistance, confirming earlier results (Kanyuka *et al.*, 2004). However, some genotypes were clearly less susceptible (especially *T. monococcum* K-58505) and screening of *Triticum* genotypes will continue in an attempt to identify those that support the least multiplication of *Polymyxa* and to determine the subsequent impact on virus infection. Our results do not confirm earlier suggestions that the wheat cv Capitaine and durum wheat cv Capdur (Bastin *et al.*, 1989), and wheat-*Thinopyrum intermedium* chromosome $3Ag^{i}$ addition line 2 (Rumjaun

et al., 1996) are resistant to *P. graminis*. This could be related to the differences in the isolates or ribotypes of *P. graminis* used (see below).

The biological significance of the two temperate ribotypes of *P. graminis* is not clear but it may be significant that of 27 ribotype I isolates previously reported (Ward *et al.*, 1994; Ward & Adams, 1998; Legrève *et al.*, 2002; Šubr *et al.*, 2002; E. Ward, unpublished data), 26 were obtained from barley. By contrast, of the five reported ribotype II isolates, two were obtained from wheat, one from barley, one from rye and one from oats. In this paper we have analysed *P. graminis* from several wheat genotypes grown in soil from three locations in the UK, and in all of these plants the sole, or predominant, ribotype was type II. This was shown by the PCR assays and confirmed by rDNA sequencing that showed that all three wheat isolates were identical to one another and virtually identical to the previously published ribotype II sequence (Y12826). Wheat and barley could be infected by both *P. graminis* ribotypes and we have also observed this in semi-sterile culture experiments (Adams & Jacquier, 1994, E. Ward & M. J. Adams, unpublished). However, it seems that wheat is more commonly infected by ribotype II and barley by ribotype I of *P. graminis*. Our results also show that both ribotypes can be present in the same soil, and in the same plant. For example, the SBCMV-soil from Kent contained both ribotypes, and all tested *Triticum* and *Hordeum* sp. genotypes that were grown in it became infected with ribotype II, whilst only a few plants were also infected by ribotype I. It is not clear whether the small amount of *P. graminis* ribotype I was a result of differences between the ribotypes in their concentration in the soil or of differences in host plant susceptibility to the particular ribotype. It would be interesting to quantify the levels of each ribotype separately by quantitative real-time PCR assay, but for this new primers and probes need to be developed. All the *Triticum* plants grown in the Kent soil became infected with SBCMV (at least in their roots) and it is therefore clear that ribotype II was transmitting the virus. Further experiments will be needed to determine whether ribotype I (which is known to transmit *Barley mild mosaic virus* to barley) can also transmit SBCMV or whether there is a specific association between *P. graminis* ribotype and virus transmission. Interestingly, SBCMV was not detected by ELISA even in the roots of barley cv Maris Otter grown in the Kent soil. This is in spite of the roots of this genotype being shown to be infected with ribotype I and II of *P. graminis*. In future, it would be important to determine the exact mechanism of such a resistance to SBCMV in barley (e.g. resistance to virus transmission by *P. graminis* vs resistance to the virus). Also, experiments with controlled inoculation are needed to examine the apparent differences between barley and wheat in their susceptibility to the two *P. graminis* ribotypes. It will also be important to determine whether there is any interaction between the *Polymyxa* ribotype and the susceptibility of different host genotypes.

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