

Quantitative PCR to detect *Gaeumannomyces graminis* var. *tritici* in symptomatic and non-symptomatic wheat roots

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Abstract The soil-borne ascomycete *Gaeumannomyces graminis* var. *tritici* causes take-all of wheat (*Triticum aestivum*). Between host crops, *G. graminis* var. *tritici* survives saprophytically on crop debris and by infecting susceptible grass weeds or cereal volunteers. Invasion of roots in the following wheat crop results in reduced grain yield and quality. Take-all is commonly assessed in the field by visual inspection. Molecular-based methods are also available to detect *G. graminis* var. *tritici*, including a quantitative PCR (qPCR) assay that indirectly measures the amount of pathogen DNA in environmental samples. The qPCR is used as part of a commercial tool (known as PreDicta B™) to predict the risks of take-all in wheat crops prior to planting, which are dependent on the amount of *Gaeumannomyces* inoculum in field soils. Unfortunately, the costs associated with the PreDicta B™ test can be prohibitive to its use. As a result, in this study, an alternative qPCR assay was developed to measure directly the DNA of *G. graminis* var. *tritici*. The assay was shown to detect DNA of *G. graminis* var. *tritici* in both symptomatic

and non-symptomatic wheat roots, with an increase in the amount of DNA detected having a strong relationship with an increase in take-all symptoms.

Keywords Fungal pathogen · Cereals · DNA quantification

Introduction

Take-all of wheat and other cereals is caused by *Gaeumannomyces graminis* var. *tritici*, and to a lesser extent by the related fungus *G. graminis* var. *avenae* (e.g., Bithell et al. 2012b; Cook 2003). *Gaeumannomyces graminis* var. *tritici* infects roots of susceptible plants, resulting in black necrotic lesions. In severe cases, lesions spread to the stem bases leading to premature ripening of the affected plants (white heads) and a reduction in grain yield and quality. Losses of up to 60 % have been associated with *G. graminis* var. *tritici* infection of wheat in the United Kingdom (McMillan et al. 2011).

Take-all is commonly assessed in the field by visual inspection of plants. Symptoms similar to take-all on wheat roots can be caused by micro-organisms other than *G. graminis* var. *tritici* or by environmental conditions, however, leading to inconclusive identifications. *Gaeumannomyces graminis* var. *tritici* also lacks an active spore state on cereal hosts, which complicates microscopy-based diagnoses of this pathogen (Okubara et al. 2005). Consequently, time-consuming isolation of the pathogen and characterisation of fungal cultures have been required to confirm the presence of *G. graminis* var. *tritici* (Herdina et al. 1996).

While isolation and characterisation of fungal cultures is valuable for determining severity of infections and likely effects on yield, the measurement of pathogen DNA on plants or plant residues in the soil has proven less time-consuming and more quantitative. It can also provide greater

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understanding of the dynamics of inoculum multiplication in hosts or host tissues where the relationships between disease symptoms and inoculum are likely to be poor. For instance, take-all symptoms are seldom seen on rye (*Secale cereale*), but post-harvest concentrations of *G. graminis* var. *tritici* in soil can be considerably greater after rye than after wheat (Bithell et al. 2011). Indeed, quantitative PCR (qPCR) has been used to study pathogen incidence, dynamics and distribution in plant tissues during the course of infection of wheat by *Fusarium* spp., *Rhizoctonia cerealis* and *Tapesia* spp. (Okubara et al. 2005).

DNA-based assays exist to detect and quantify *G. graminis* var. *tritici*. For example, Herdina et al. (1996) developed a slot-blot hybridization technique for the semi-quantitative detection of *Gaeumannomyces graminis* var. *tritici* in infected roots and soil. Herdina and Roget (2000) also developed a quantitative DNA soil assay for detection of *G. graminis* var. *tritici*, but the assay had a low level of background signal particularly when DNA had been extracted from soils with high levels of organic matter. This was predicted to be due to non-specific DNA hybridization with the probe.

A DNA-based assay has been subsequently employed to estimate inoculum of *G. graminis* var. *tritici* and *G. graminis* var. *avenae* in soil and plant tissue (Ophel-Keller et al. 2008). This assay measures both the combined amount of inoculum DNA for these *Gaeumannomyces* species and also *G. graminis* var. *avenae* DNA alone. The amount of *G. graminis* var. *tritici* DNA is then extrapolated by subtracting the quantity measured for *G. graminis* var. *avenae* from the quantity calculated for both species. Use of the assay has demonstrated that *G. graminis* var. *avenae* is uncommon in the soils of New Zealand wheat crops (Bithell et al. 2012b). Furthermore, the assay has provided insights into the relationship between *G. graminis* var. *tritici* soil inoculum and disease severity, leading to its commercial use in New Zealand to predict the risk of severe take-all before sowing wheat crops (Bithell et al. 2012a). Nevertheless, the DNA-based diagnostic assay for detection of *Gaeumannomyces* spp. requires the use of multiple PCRs to extrapolate estimated amounts of *G. graminis* var. *tritici* rather than specifically measuring *G. graminis* var. *tritici* DNA. Furthermore, the commercialisation of the qPCR as a component of the PreDicta B test used to predict the risks of take-all in wheat crops prior to planting, can be prohibitive to its use due to the high associated costs.

This study describes the development of a qPCR-based assay to detect *G. graminis* var. *tritici* DNA in plant tissues. The qPCR can be used to quantify *G. graminis* var. *tritici* in the roots of plants with varying degrees of take-all to identify relationships between

inoculum build-up and the expression of disease symptoms.

Materials and methods

Fungal isolates and culturing

Isolates of *G. graminis* var. *tritici*, *G. graminis* var. *avenae* and other fungi were obtained from collections or researchers in New Zealand and overseas, as described in Table 1. These fungi were selected to represent species of *Gaeumannomyces* closely related to *G. graminis* var. *tritici* on wheat and where possible fungi associated with cereal cropping systems or with grasses. All isolates were routinely grown on potato dextrose agar (PDA) medium at 25 °C. For long-term storage, isolates were kept on PDA slopes at 4 °C.

DNA extraction from fungi

To extract genomic DNA from fungi, each isolate was grown in a Petri plate on PDA overlaid with cellophane (Julzar Pty Ltd), until the plate was covered by freshly growing mycelia. The mycelia were then scraped off the cellophane and genomic DNA extracted using the PureGene™ Plant Tissue DNA Purification Kit (Nalgene) following the manufacturer's instructions. DNA from each isolate was re-suspended in 200 µL of elution buffer (EB) (Qiagen). PCR was performed using the universal eukaryote primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) to confirm that the DNA could be used for PCR amplification. Reactions were performed in a total volume of 25 µL. The final concentrations of components were: 300 nM of each primer (Sigma-Aldrich), 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 0.2 mM each dNTP and 1U Taq DNA polymerase (Invitrogen). Thermal cycling was performed in a GeneAmp PCR system 9700 as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 7 min.

Design of *G. graminis* var. *tritici*-specific PCR primers

The translation elongation factor 1-alpha (*EF1-α*) gene was targeted for the design of primers to detect *G. graminis* var. *tritici* specifically. A 368-bp partial DNA sequence of *EF1-α* was amplified from each of eight isolates of *G. graminis* var. *tritici* (described in Table 1) using primers EF1-728 F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') (Carbone et al. 1999). PCR reactions were conducted as described above for amplification of the ITS region. Thermal cycling was also

Table 1 *Gaeumannomyces graminis* var. *tritici* isolates used in this study, their sources and their detection by quantitative PCR using primers GgtEFF1 and GgtEFR1 in conjunction with Taqman probe GgtEFPR1

Isolate	Source		Ct value ^a	
	Host	Country		
<i>Gaeumannomyces</i>				
<i>G. graminis</i> var. <i>tritici</i>	WF9913 ^{1,b}	Unknown	USA	24.78
	H9T3R2 ^{2,b}	Wheat	New Zealand	26.07
	H9T3R1/1.2 ^{2,b}			26.86
	Biomill ^{2,b}			24.39
	Bio4A ^{2,b}			26.09
	Bio4B ^{2,b}			26.83
	H11T3R1/3 ^{2,b}			27.01
	Bio1 ²			25.05
	Bio2 ²			24.64
	Bio3 ²			25.19
	Bio4C ²			24.89
	Bio6 ²			24.61
	Bio7 ²			24.93
	Biomill ISC3 ²			25.21
	Mal 1/8 ²			29.60
	PGG(T) ²			23.81
	PGG(T) ISC3CRI ²			24.10
	PGG ISC65R7 ²			24.27
	01/2 ²			25.30
	Ex Williams ²			24.79
	01/8 ²			24.49
	A3SL4 ^{2,b}	Couch	New Zealand	30.77
	A3RDL5 ²			22.80
	GB/NN8 ²			24.72
	GIB/NN3 ²			24.86
	S(G) ²			22.80
	<i>G. graminis</i> var. <i>avenae</i>	ABL2 ³	Turfgrass	United Kingdom
YZ1 ³		(species unknown)		ND
YZ2 ³				ND
YZ3 ³				ND
YZ11 ³				ND
Lawn2 ³				ND
PO 86/439 ³		Oats	Ireland	ND
91.A1.1 ³				ND
91.A1.2 ³				ND
91.A1.3 ³				ND
91.A5.3 ³				ND
91.A5.1 ³				ND
91.B4.3 ³				ND
9235 ³		Unknown	Unknown	ND
8929 ³				ND
8829 ³			ND	
<i>G. cylindrosporus</i>	DAR25011 ⁴	Wheat	United Kingdom	ND
	T2-2A2 ²	Prairie grass	New Zealand	ND
	T2-6A2 ²	Tall fescue	New Zealand	ND

Table 1 (continued)

Isolate		Source		Ct value ^a
		Host	Country	
	T2-6A3 ²			ND
	T2-6B1 ²			ND
	T2-6B2 ²			ND
	T2-6B4 ²			ND
	T2-13A3 ²			ND
	T2-20A1 ²			ND
	T2-20B1 ²			ND
	T2-12A1 ²	Perennial ryegrass	New Zealand	ND
<i>G. graminis</i> sp.	ICMP10358 ⁵	Oil Plant	New Zealand	ND
Other fungi				
<i>Magnaporthe grisea</i>	ICMP14481 ⁵	Rice	Thailand	ND
<i>Rhizoctonia solani</i>	RF ²	Potato	New Zealand	ND
<i>Fusarium culmorum</i>	7 ²	Wheat	New Zealand	ND
<i>F. graminearum</i>	53 ²	Wheat	New Zealand	ND
<i>F. avenaceum</i>	2B(A) ²	Wheat	New Zealand	ND
<i>F. pseudograminearum</i>	ICMP15511 ⁵	Wheat	New Zealand	ND
<i>Microdochium nivale</i>	71 ²	Wheat	New Zealand	ND
<i>Trichoderma asperellum</i>	1 ²	Unknown	New Zealand	ND
<i>Neurospora crassa</i>	ICMP6360 ⁵	Unknown	Unknown	ND
<i>Penicillium italicum</i>	1 ²	Unknown	New Zealand	ND
<i>Pythium irregulare</i>	ICMP9270 ⁵	Lucerne	New Zealand	ND
<i>Cochliobolus sativus</i>	ICMP6242	Perennial ryegrass	New Zealand	ND

^a Quantitative PCR results are given as Cycle threshold (Ct). Those samples that failed to produce a *G. graminis* var. *tritici* amplicon by a Ct of 40 were recorded as undetermined. *Gaeumannomyces graminis* var. *tritici* was considered 'not detected' (ND) in samples recorded as undetermined

^b Strains used for design of the *Gaeumannomyces graminis* var. *tritici*-specific primers GgtEFF1 and GgtEFR1, and Taqman probe GgtEFPR1

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performed as above, with the exception that the annealing temperature was 50 °C. PCR products were purified using a QIAquick PCR purification kit (Qiagen), and then sequenced in both directions with the appropriate primers (described above) (Macrogen Inc.). The *EF1-α* sequence for each strain was submitted to GenBank (accession numbers: JQ713812 to JQ713819).

The *EF1-α* DNA sequences for the eight selected *G. graminis* var. *tritici* isolates were compared with the *EF1-α* sequences from other *Gaeumannomyces* spp. as well as those of taxonomically related fungi and common wheat pathogens using ClustalW v1.83 (Thompson et al. 1997). From the resulting alignments of the *EF1-α* sequences, primers GgtEFF1 (5'-CCCTGCAAGCTTCTCTTAG-3') and GgtEFR1 (5'-GCATGCGAGGTCCCAAAA-3') and a

Taqman probe (GgtEFPR1, 5'-ACTGCACAGACCATC-3') were designed using Primer Express (Applied Biosystems) to target *G. graminis* var. *tritici* specifically. The fluorogenic MGB probe was labelled at the 3'-end with a non-fluorescent quencher and at the 5'-end with the fluorescent reporter dye 6-FAM (6-carboxy-fluorescein) (Applied Biosystems).

Quantitative PCR

Amplification of the *G. graminis* var. *tritici*-specific 106-bp *EF1-α* fragment was performed using qPCR in a total reaction volume of 20 μL. The reaction mix included 10 μL of 2× iTaq Supermix with Rox (BioRad Laboratories). The MGB probe GgtEFPR1 was added to a final concentration of 0.15 μM and the primers GgtEFF1 and GgtEFR1 to a final concentration of

0.3 μM . One hundred ng of template DNA was then added to each reaction. PCR amplifications were carried out in a StepOnePlus (Applied Biosciences) with the following steps: (i) initial denaturation at 95 °C for 3 min, (ii) 40 amplification cycles of 94 °C for 15 s, 52 °C for 20 s, and 72 °C for 20 s. Quantities of target DNA were estimated using a standard curve constructed by regressing Ct values onto the \log_{10} of the concentration of *G. graminis* var. *tritici* DNA standards. The standards were produced by 10-fold serial dilutions of genomic DNA from an initial concentration of 100 ng/ μL . The regression equation was used to calculate estimated amounts of *G. graminis* var. *tritici* target DNA in each sample. Each DNA sample and the DNA standards were amplified in duplicate, and negative and positive controls were included with each PCR assay.

Collection of plant samples and take-all assessment

Wheat plant samples at various growth stages (GS) (Zadoks et al. 1974) were collected from Chertsey in New Zealand. For each growth stage, approximately five plants were randomly selected from each of 20 locations in the crop, with locations evenly spaced on a W transect that covered the width of the paddock. The roots of each plant were washed and assessed for take-all severity on a scale of 0 to 4 (Category 0 = no take-all; Category 1 = 1 to 10 % of the root system with black necrotic lesions; Category 2 = 11 to 30 %; Category 3 = 31 to 60 %; Category 4 = 61 to 100 %). For post-harvest samples (GS 87), scoring was difficult because of degradation of senesced roots, so take-all symptoms were scored as 'none' (Category 0), 'some' (Category 1–3) or 'severe' (Category 4). The amounts of *G. graminis* var. *tritici* DNA in the roots of 2–4 plants in each take-all category at each time point were subsequently quantified by qPCR.

Extraction of total DNA from wheat roots

For extraction of total DNA from wheat roots, the entire root system of a plant was crushed in a mortar with liquid nitrogen. Approximately 100 mg of the powdered root tissue was added to a screw cap vial containing 800 μL AP1 lysis buffer (Qiagen) and 1 g of 2.3-mm steel beads. Bead-beating was performed for 1 to 3 min to macerate the root tissue and DNA was extracted using the DNeasy Plant Minikit (Qiagen) following the manufacturer's instructions. Total DNA was resuspended in 200 μL of EB (Qiagen) and stored at -80 °C before use. A sample of total DNA was extracted from each crushed root system in duplicate.

Each sample was diluted 10-fold prior to qPCR. To identify PCR inhibitors, a Sybr green-based qPCR was performed using the primers Pot25SF (5'-TCGGAATTTCGAAGCTAGAGG-3') and Pot25SR (5'-AACGTCGCTATGAACGCTTG-3') and the diluted DNA as a template. Pot25SF and Pot25SR were designed as part of this study, to target the plant 25S ribosomal

RNA gene sequence. Reactions were performed in a total volume of 20 μL including 10 μL of 2 \times iTaq Sybr Green with Rox (BioRad Laboratories) and a final concentration of 0.3 μM of each primer. The same PCR protocol described above for the *G. graminis* var. *tritici*-specific qPCR was used for amplification of the 25S ribosomal DNA sequence, with the exception that an annealing temperature of 60 °C was used.

Results

The primer pair GgtEFF1 and GgtEFR1 and the Taqman probe GgtEFPR1 were designed to amplify a *G. graminis* var. *tritici*-specific *EF1- α* fragment of 105 bp. The primer and probe sequences were 100 % identical to the complementary DNA sequences for all *G. graminis* var. *tritici* isolates. The GgtEFF1 primer had three nucleotide mismatches to the *EF1- α* sequence for *G. graminis* var. *avenae*, whereas the primers and probe had nine nucleotide mismatches to the *EF1- α* sequence for *G. cylindrosporus* (Fig. 1).

The specificity of the GgtEF primer and probe set was initially verified by performing qPCR on DNA from a collection of *G. graminis* var. *tritici* isolates. Other closely related species (e.g., *G. graminis* var. *avenae* and *G. cylindrosporus*) were also tested as well as fungi typically found growing in the same environments (e.g., *Fusarium graminearum*) (see Table 1). In these assays, only *G. graminis* var. *tritici* isolates generated an amplicon (Table 1). An ITS amplicon was produced by all DNA samples using the universal primers ITS1 and ITS4 demonstrating that PCR products could be amplified from all the DNA templates (data not shown).

The efficiency of the qPCR and the theoretical limits of detection for the assay were established by performing qPCRs on a 10-fold serial dilution of genomic DNA extracted from *G. graminis* var. *tritici*. The amplification curves (e.g., Fig. 2) showed that the *G. graminis* var. *tritici*-specific qPCR had an $R^2=0.96$ to 0.99 and a Y-intercept of approximately 35.57. *Gaeumannomyces graminis* var. *tritici* DNA was quantifiable to a minimum of 1 pg. Similar results were obtained for the calibration samples when they were used in later qPCR runs, which were carried out to quantify *G. graminis* var. *tritici* DNA in field-collected plant material. Although quantities of DNA below 1 pg could be detected, these lay outside the linear range for detection, so that accurate quantification of *G. graminis* var. *tritici* in this range was not possible. Instead, positive qPCR results for these smaller quantities of *G. graminis* var. *tritici* DNA were used only to indicate the possible presence of the pathogen.

To validate the *G. graminis* var. *tritici* qPCR assay as a tool to measure the relationships between the quantity of *G. graminis* var. *tritici* DNA detected in wheat roots and the symptoms of take-all observed in the field, plants with a range of disease categories between 0 (no take-all) and 4 (severe take-all) were tested for *G. graminis* var. *tritici* DNA. Roots

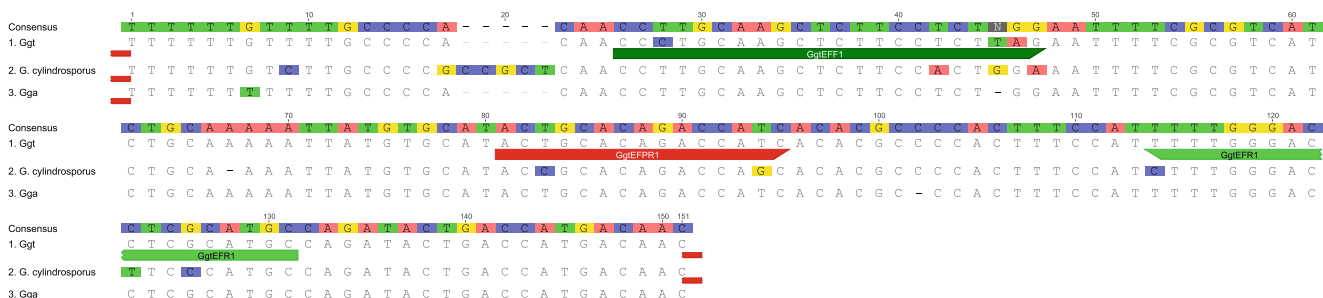


Fig. 1 Nucleotide sequence alignment of the partial *EFl-α* sequences from *Gaemannomyces graminis* var. *tritici* (Ggt), *G. graminis* var. *avenae* (Gga) and *G. cylindrosporus*. The alignment shows the location of the primers GgtEFF1 (dark green arrow) and GgtEFR1 (light green arrow) and the Taqman probe GgtEFPR1 (red arrow). This primer and

probe combination was designed for the specific detection of *G. graminis* var. *tritici*. Coloured squares show polymorphic nucleotides on the divergent sequences. A dash represents a missing nucleotide in the associated *EFl-α* sequence. The primers were designed to amplify a 105-bp fragment

with increasing severity of take-all generally showed increasing quantities of *G. graminis* var. *tritici* DNA regardless of the growth stage of the assayed plants (Spearman’s rank correlation $r=0.56$; $p<0.001$; Fig. 3). For example, wheat roots with a disease category of 4 showed the greatest quantities of *G. graminis* var. *tritici* DNA (up to 9.4×10^4 ng/g root tissue), on average five-fold greater than roots with a take-all severity score of 3. The quantities of *G. graminis* var. *tritici* DNA were, however, on average similar in samples with a disease category of 1 or 2. *Gaemannomyces graminis* var. *tritici* DNA was detected in a number of root samples collected at different times with a disease severity category of 0, although quantities were less than in plants with visible take-all symptoms.

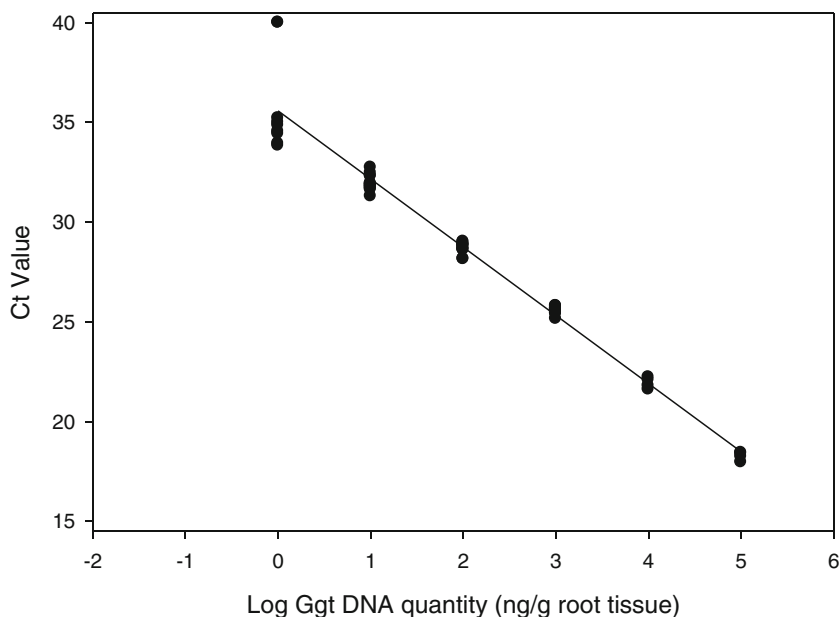
Discussion

Quantitative diagnostic assays are important tools in epidemiological studies of plant diseases as they enable the temporal

and spatial monitoring of fungal populations. In the present study, a protocol was developed for the quantitative detection of *G. graminis* var. *tritici* DNA from wheat roots. The specificity of the assay was demonstrated by successful differentiation of *G. graminis* var. *tritici* from related *Gaemannomyces* such as *G. graminis* var. *avenae* as well as other taxonomically related fungi. It was also sensitive, detecting pathogen DNA to below 1 pg in each PCR reaction. A DNA-based assay already exists to quantify *G. graminis* var. *tritici* DNA in environmental samples (Ophel-Keller et al. 2008). The assay has been used as a predictive tool for take-all in wheat crops (Bithell et al. 2012a), but its commercialisation and the associated costs limit its use for many researchers and it doesn’t detect the pathogen DNA directly. Thus, our qPCR assay provides an alternative method for the detection of *G. graminis* var. *tritici*.

A strong relationship was observed between increasing quantities of *G. graminis* var. *tritici* DNA in roots and take-all disease progression. The quantities of *G. graminis* var.

Fig. 2 Standard curve for the quantitative detection of *Gaemannomyces graminis* var. *tritici* DNA using primers GgtEFF1 and GgtEFR1 in conjunction with the Taqman probe GgtEFPR1. Black dots represent data used to establish the linear range of the qPCR. Results of qPCR are given as Cycle threshold (Ct). Those samples that failed to produce a *G. graminis* var. *tritici* amplicon by a Ct of 40 were recorded as ‘undetermined’. *Gaemannomyces graminis* var. *tritici* was considered not detected in samples recorded as undetermined



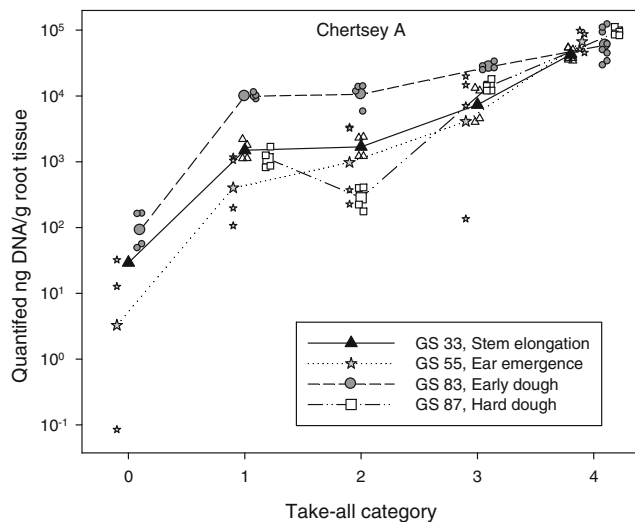


Fig. 3 Estimated quantities of *Gaeumannomyces graminis* var. *tritici* DNA (ng/g root tissue) in roots of wheat plants with increasing take-all (categories 0 to 4), which were collected at different growth stages (GS) from Chertsey, New Zealand. *Large symbols*: means for each category. *Small symbols*: individual data values

tritici DNA in roots of low severity Category 1 and Category 2 plants were similar, however. This may be due to the ectotrophic growth habit of this fungus in the early stages of root colonisation. Ectotrophic expansion of *G. graminis* var. *tritici* along the root segments would be unlikely to result in disease symptoms, yet qPCR would still detect the presence (and an increase) of pathogen DNA. Alternatively, this may result from an inability to discriminate visual symptoms for these less severe disease categories. The relative variation in the quantities of *G. graminis* var. *tritici* DNA from plants in Categories 1 and 2 was greater than for more severely affected plants.

Assigning plants within the take-all category range 0–2 was difficult late in the growth season due to their roots having already senesced. Nevertheless, there were large differences in the quantities of *G. graminis* var. *tritici* DNA detected in plants belonging to these categories. Perhaps senescing and senesced roots do not react to invasion by *G. graminis* var. *tritici* and, similarly, already blackened roots do not visibly change as inoculum further increases or degrades. The differentiation of inoculum in senescent tissue by qPCR will support future studies of *G. graminis* var. *tritici* inoculum dynamics in dead or dying tissue where take-all symptoms are hard to define. Traditionally such studies have relied on laborious isolation and culturing techniques.

Conclusion

In summary, a qPCR assay was developed that related increasing disease symptoms with increasing quantities of *G. graminis*

var. *tritici* DNA in wheat roots during take-all epidemic development. Disease assessment, however, only reflects inoculum concentrations in roots of growing plants. Thus, use of qPCR also provided a novel tool for evaluating inoculum of *G. graminis* var. *tritici* in roots of non-symptomatic plants as well as in senescent plant material. Further work is planned to examine the build-up in inoculum on roots during host plant senescence and beyond.

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