Plant Pathology (2005) **54**, 686–698 Doi: 10.1111/j.1365-3059.2005.01252.x

Methods for studying population structure, including sensitivity to the fungicide silthiofam, of the cereal take-all fungus, *Gaeumannomyces graminis* var. *tritici*

J. Freeman*†‡, E. Ward, R. J. Gutteridge and G. L. Bateman

Wheat Pathogenesis Programme, Plant–Pathogen Interactions Division, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

Field isolates (n = 144) of the wheat take-all fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) were tested for sensitivity to silthiofam, a take-all-specific fungicide used as a seed treatment, and identified as A- or B-type by PCR–RFLP analysis of nuclear rDNA. A possible association was identified between polymorphisms in ITS2 of the nuclear rDNA and sensitivity to silthiofam. A *Ggt*-specific PCR assay was developed which simultaneously identified isolates of *Ggt* as A- or B-type, based on the polymorphisms in the nuclear rDNA. A highly significant correlation between *Ggt* type using the PCR assay and sensitivity to silthiofam was demonstrated in a collection of 358 isolates from three field experiments designed to test the effects of seed-treatment fungicides on take-all and *Ggt* populations in winter wheat. In one experiment the percentages of silthiofam-sensitive and B-type isolates were significantly less in populations from plots sown with silthiofam-treated seed in two consecutive years than in populations from plots sown with nontreated seed. However, silthiofam still provided a significant amount of control of take-all. The natural occurrence of fungicide-insensitive isolates, up to about 30% in soils in which the fungicide had never been used, is unusual. The new PCR assay provides a useful tool for studying the population structure of *Ggt*, and may provide a novel method for assessing the incidence of insensitivity to silthiofam (the target site for which has not yet been identified) in field populations of *Ggt*.

Keywords: PCR, populations, silthiofam, soilborne disease, take-all, wheat

Introduction

Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is one of the most important diseases of cereals, especially wheat, in the UK and worldwide (Freeman & Ward, 2004). In the past, control by fungicides has been a difficult proposition because the fungus is soilborne and infects roots (Bateman, 1989). However, two fungicides with different modes of action, fluquinconazole (Löchel *et al*., 1998) and silthiofam (Beale *et al*., 1998; Schoeny & Lucas, 1999), have now become available commercially for use as seed treatments specifically for controlling take-all. Fluquinconazole is a sterol biosynthesis-inhibiting triazole fungicide, whereas silthiofam has a novel mode of action and the target site has not yet been conclusively identified (see Discussion).

*To whom correspondence should be addressed.

†E-mail: jackie.freeman@bbsrc.ac.uk

‡Current address: CPI Division, Rothamsted Research.

Accepted 19 May 2005

Take-all develops in consecutive cereal crops in a characteristic way. In the UK, it often increases to a peak over 2–4 years, after which it typically declines in severity (take-all decline). Seed-treatment fungicides have the potential to decrease the losses caused by take-all, but their effective and economic use depends on accurate targeting of those crops in a sequence that will benefit from treatment (Bateman *et al*., 2003). Predicting the timing of severe disease is difficult, but necessary if chemical treatments are to be used effectively and efficiently. Some evidence has been found in previous research that the population structure of the pathogen in a wheat crop, determined using a DNA probe to identify individual isolates as one of two main genetic types (described as T1 and T2), was associated with the severity of take-all in a wheat crop grown in the following year (Bateman *et al*., 1997). Population structure was also influenced by the type of cereal crop (wheat or barley). Subsequent research, in which populations of *Ggt* were analysed in successive wheat crops on seven sites, including the three described here, found some corroboration of the association between population structure and development of take-all epidemics (Bateman *et al*., 2003). There were some inconsistencies, however, and it was considered that

the available procedures were not suitable for development as a generally applicable method of risk assessment. This was mainly because of large differences between sites in initial population structures, and large effects of unusual weather conditions that affected crop growth and epidemic development. Nevertheless, knowledge of the dynamics of field populations of *Ggt* has, potentially, a considerable value in research to understand epidemic development, but methods involving isolation of large numbers of *Ggt* colonies and characterizing them by RFLP, as above, are too laborious for routine use.

Fungicide use leads to a risk of resistance developing in the target pathogen (de Waard *et al*., 1993; Russell, 1995; McCartney *et al*., 2003). The risk may be less in populations of a soilborne pathogen such as *Ggt*, of which a large part may not come into contact with the fungicide, than for some other fungal pathogens. Also, the potential for spread of resistant alleles through and between populations of soilborne fungal pathogens is less than for those dispersed by airborne propagules. Although *Ggt* has a sexual stage, sexual hybridization is unlikely to be important in field populations, and ascospores, produced in cereal crops on stem bases and stubble, are believed to be of limited importance as a source of inoculum (Hornby *et al*., 1998; Freeman & Ward, 2004). No evidence of resistance to fluquinconazole was found in populations of *Ggt* on sites sown with fluquinconazole-treated wheat seed in four successive years (Bateman *et al*., 2003), but some variation in sensitivity of field isolates of *Ggt* to low concentrations has been reported (Russell *et al*., 2001). However, insensitivity to silthiofam occurs naturally in *Ggt* populations that have never been exposed to the fungicide, and there is variation in the level of this insensitivity (Russell *et al*., 2001; Carter *et al*., 2003; Lebreton *et al*., 2003; G.L.B., unpublished data). Research in this area is currently hampered, in the same way as the population monitoring done so far (Bateman *et al*., 1997, 2003), by the need to isolate large numbers of isolates from populations of *Ggt* in soil or on cereal roots to determine the overall level of insensitivity, and any changes in sensitivity that may occur naturally within or between cropping seasons or as a consequence of fungicide use.

Although a number of DNA-based methods for the detection and identification of *G. graminis* have been published (reviewed by Freeman & Ward, 2004), none of the published PCR-based assays was suitable for specific detection of *Ggt* and simultaneous study of its population structure. The research described here aimed to develop simpler, PCR-based diagnostic procedures to supersede the DNA probe for studying *Ggt* population structure. Silthiofam-insensitive isolates of *Ggt* had been found to occur naturally in field populations, and preliminary results reported here identify a possible association between insensitivity to silthiofam and polymorphisms in ITS2 of the nuclear rDNA. Therefore this research aimed to develop a PCR assay that identified the polymorphisms in ITS2 of the nuclear rDNA and to investigate the validity of using such a PCR assay to indicate the proportions of silthiofam-sensitive/-insensitive isolates of *Ggt* in field populations. Further aims were to determine the extent of insensitivity to silthiofam in natural populations of *Ggt*, and to investigate whether the use of silthiofam-treated seed affected the population structure of *Ggt*.

Materials and methods

Field experiments

A series of field experiments was designed to test the effects of seed-treatment fungicides on take-all in crops of susceptible winter wheat cv. Hereward, grown successively. Two of the experiments referred to here were at Rothamsted Experimental Farm (coded CS/323 and CS/508), the third was at the Scott Abbott Agricultural Trust Farm, Sacrewell, Cambridgeshire (see Bateman *et al*., 2003 for full details of the experiments). The experiments tested the effects of fluquinconazole seed treatment (as Jockey F, in which fluquinconazole is the only active ingredient, supplied by Aventis CropScience, UK), applied at 75 g a.i. per 100 kg seed, in sequences of winter wheat crops cv. Hereward, beginning with second wheat crops grown in the harvest year 1999 in CS/508 and at Sacrewell. In 2002, seed treated with silthiofam (Latitude, supplied by Monsanto, UK), applied at 25 g a.i. per 100 kg, was also tested, in split plots, in these two experiments (Table 1). CS/323 was a long-running experiment on a site exhibiting take-all decline when seed treatments were started in 1999. Silthiofam was also tested as a whole-plot treatment in successive crops in 2001 and 2002, or in 2001 only, in this experiment (Table 1).

Assessments of take-all and soil infectivity

Take-all disease and soil infectivity data are shown only for experiment CS/323, in which comparisons between nontreated and silthiofam-treated crops are made. Other take-all data from the experiments are presented elsewhere (Bateman *et al*., 2003, 2004). The plant sample to assess take-all was taken in early July 2002 at growth stage 73 (Zadoks *et al*., 1974). Ten rows 20 cm in length were dug from each plot along two parallel zigzag transects. The plants were washed thoroughly, air-dried and stored at 4°C for later assessment.

The disease assessments on stored plants were made after soaking in water. Take-all was assessed on each plant, held under water against a white background, and scored on a 0–5 scale: 0 = no disease; 1 = slight take-all, <10% of root system affected; 2 = slight take-all, 11–25% of root system affected; 3 = moderate take-all, 26–50% of root system affected; 4 = moderate take-all, 51–75% of root system affected; 5 = severe take-all, 76–100% of root system affected. This is more realistic in UK conditions than the widely used system in which category 5 has the range 61–100% (Schoeny *et al*., 1998), because yield losses tend to relate best to the upper part of this range in samples taken at this time (Gutteridge *et al*., 2003). From these scores, a mean take-all index per plot (maximum 100) was calculated by summing the products of the

Table 1 Fungicide seed treatments applied to the three field experiments from which isolates of *Gaeumannomyces graminis* var. *tritici* were obtained (all isolates from harvest year 2002)

Experiment	Year of harvest	Fungicide treatment[a] (number of replicate plots sampled[b])
Rothamsted CS/508	1999	Fluquinconazole (4)
	2000	Fluquinconazole (4)
	2001	Fluquinconazole (4)
	2002	Fluquinconazole (4), Silthiofam (4)
Sacrewell	1999	Fluquinconazole (4)
	2000	Fluquinconazole (4)
	2001	Fluquinconazole (4)
	2002	Fluquinconazole (4), Silthiofam (4)
Rothamsted CS/323	2001	Silthiofam (6), Nil (3)
	2002	Silthiofam (3), Nil (3), Nil (3)

[a]Rates of fungicide application are given in Materials and methods.
[b]At Rothamsted in CS/508, plots were 10 × 6 m in 1999, 2000 and 2001; 10 × 3 m in 2002. At Sacrewell, plots were 14 × 4 m in all years. At Rothamsted in CS/323, plots were 10 × 3 m in both years.

percentages of plants in each score category by the corresponding score value and dividing the total by 5.

Ten soil cores (5·5 cm diameter × *c.* 10 cm deep) were taken from each plot for take-all infectivity bioassays (Gutteridge & Hornby, 2003) and isolations of the take-all fungus at the same time as the plant samples (in late June or early July, growth stages 69–75, in all experiments). Each soil core was inverted into a plastic beaker, with drainage holes drilled into its base that were covered with moist coarse sand. Ten wheat seeds (cv. Hereward) were placed on the soil surface and covered with horticultural grit. The beakers were put in a controlled environment room (16 h day, day/night temperatures 15/10°C and 70% RH) where they were watered to soil capacity and then twice weekly. After 5 weeks the bioassay plants were removed from their pots, the roots were washed and the presence or absence of take-all lesions recorded on each main root axis. The mean percentage of roots infected in each soil core was determined as a measure of soil infectivity.

Fungal isolations

A panel of *Gaeumannomyces* isolates (Table 2) and an additional collection of soil and rhizosphere fungi (Table 3) were obtained.

Field isolates of *Ggt* (144) were obtained from Rothamsted Experimental Farm. Other isolates of *Ggt* (358) were obtained from soil in plots growing crops treated or not treated with silthiofam in the three field experiments. Pieces of root (*c.* 1 cm long) were cut from the upper parts of the root systems on six different plants grown in each soil core taken for the soil infectivity bioassay (see above). The root pieces were surface-sterilized for 3 min in sodium hypochlorite (*c.* 2% available chlorine), rinsed twice in sterile distilled water, and dried on filter paper in a sterile air flow. They were then placed on potato dextrose agar (PDA) containing penicillin (30 mg L^{-1}), streptomycin sulphate (133 mg L^{-1}) and chloramphenicol (50 mg L^{-1}), and incubated at 20°C for 3–6 days. Cultures resembling *G. graminis* were then transferred to fresh PDA containing the same antibiotics. Generally, three isolates were obtained after plating out six root pieces from different bait plants grown in each soil core.

Fungicide sensitivity testing

A total of 118 isolates of *Ggt* from Rothamsted experiment CS/508, 105 from Sacrewell and 135 from Rothamsted experiment CS/323, were tested for sensitivity to silthiofam. Silthiofam, formulated as 125 g L^{-1} EC (Latitude) and diluted in sterile water, was added to sterile, molten (50°C) PDA at 1 mL 100 mL^{-1}. This was poured into Petri dishes to give final concentrations of 0·5 or, usually, 1 mg L^{-1} silthiofam. Unamended agar served as the control treatment. Three 4-mm-diameter plugs from the edges of colonies of *Ggt* isolated from soil bioassay plants were placed on each plate. Three replicate plugs of the isolate were placed on three different plates for each fungicide and for control plates. Increases in colony diameter were measured after 4 days at 20°C. Isolates were recorded as insensitive to silthiofam if growth on silthiofam-amended PDA was similar to (> 95% of) that on unamended agar.

DNA extraction

Each isolate from the soil bioassay plants was subcultured into *c.* 15 mL LB broth (tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 5 g L^{-1}) in a universal bottle and grown at 22°C for 6 days. DNA extraction from LB-broth cultures was based on the method of Lee & Taylor (1990), as described previously (Ward & Bateman, 1994).

DNA was also extracted from the roots of wheat seedlings grown in sand and inoculated with either *Ggt*, *G. graminis* var. *graminis* (*Ggg*) or *Gaeumannomyces cylindrosporus* (*Phialophora graminicola*), as mycelium on pieces of agar culture, or not inoculated. Roots were removed from infected and healthy wheat plants and freeze-dried in sterile 7 mL disposable polystyrene bottles,

Table 2 Origin and molecular type of *Gaeumannomyces graminis* var. *tritici* isolates used

Isolate[a]	Origin	Year of isolation	mtSSU RFLP type[b]	ITS4/5 *Hpa*II RFLP
57·46·1	Rothamsted, UK	1997	T1	A
184	Brazil	1982	T1	A
00W4·2C	Woburn, UK	2000	T1	A
01C6·3B	Somerset, UK	2001	T1	A
50·40·3	Rothamsted, UK	1997	T1	A
00S41·3B	Peterborough, UK	2000	T1	A
00E15·4B	East Winch, UK	2000	T1	A
01L2·4C	Long Ashton, UK	2001	T1	A
00S60·4C	Peterborough, UK	2000	T1	A
00W10·3D	Woburn, UK	2000	T1	A
00W2·5A	Woburn, UK	2000	T1	A
01B6·2C	Wiltshire, UK	2001	T1	A
00R24·3·2B/2	Rothamsted, UK	2000	T1	A
00W6·5C	Woburn, UK	2000	T1c	A
00W7·4C	Woburn, UK	2000	T1c	A
93E3·2	Rothamsted, UK	1993	T1c	A
01E1·4B	East Winch, UK	2001	T1c	A
00W3·2A	Woburn, UK	2000	T2	A
92·15·4A	Rothamsted, UK	1992	T2	A
01R24·5A	Rothamsted, UK	2001	T2	A
00T6·3B	Suffolk, UK	2000	T2	B
00L3·4A	Long Ashton, UK	2000	T2	B
99S9·4B	Peterborough, UK	1999	T2	B
00E24·5C	East Winch, UK	2000	T2	A
00R11·3·3D	Rothamsted, UK	2000	T2	B
01E37·1B	East Winch, UK	2001	T2	B
01R24·4C	Rothamsted, UK	2001	T2	B
01S26·1C	Peterborough, UK	2001	T2	B

[a]All isolates were from the culture collection of G.L.B., Rothamsted Research, and all are from wheat crops.
[b]Mitochondrial small-subunit rRNA gene RFLP type.

as for fungal mycelium, then stored at room temperature. Three root pieces, *c.* 1 cm long, were placed in 2 mL screw-capped tubes (Alpha Laboratories Ltd, UK). Root pieces were ground with metal rods, as for fungal mycelium, for *c.* 1 min. This was sufficient to partially macerate tissue at the root surface, but not to obtain a powder. DNA was then extracted as for standard preparations from fungal mycelium. After resuspension in 50 μL TE, 50 μL TE containing 0·6 mg mL^{-1} RNase A was added, the tubes were incubated at 37°C for 15 min, and RNase was then removed by phenol : chloroform extraction and isopropanol precipitation.

Southern hybridization and RFLP analysis using mitochondrial rDNA probes

All isolates of *Ggt* were characterized as type T1, T1c or T2 using a mitochondrial small-subunit rDNA probe, pEG34 (Ward & Akrofi, 1994; Ward & Bateman, 1999) or pGgtMS7 (Freeman, 2004; Freeman & Ward, 2004), as described previously (Bateman *et al.*, 1997). DNA was digested with restriction enzyme *Eco*RI. The digests were separated by electrophoresis in 1% agarose gels and transferred to HybondNX nylon membranes (Amersham Pharmacia Biotech UK Ltd) by capillary blotting (Sambrook *et al.*, 1989). The AlkPhos direct kit (Amersham Pharmacia Biotech) was used for labelling and chemiluminescence detection, following the manufacturer's instructions, with a hybridization temperature of 68°C. Isolates of *Ggt* were identified as RFLP type according to the presence (type T1) or absence (type T2 and T1c) of a band representing a 4·0 kb DNA fragment, and the presence (type T1c) or absence (type T1 and T2) of a band representing a 2·5 kb DNA fragment, to which pEG34 or pGgtMS7 had hybridized.

PCR using primers ITS4/ITS5 and RFLP analysis

Consensus fungal primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G) (White *et al.*, 1990) amplify ribosomal DNA (rDNA), specifically a region of DNA stretching from the 3′ end of the 18S-like gene to the 5′ end of the 28S-like gene and including the 5·8S gene and the two internal transcribed spacer (ITS) regions. Each ITS4/ITS5 25 μL PCR contained 25 pmol of both primers, 0·25 units of MBI Taq polymerase (MBI Fermentas), buffer (10 mM Tris–HCl pH 8·8, 50 mM KCl, 0·08% Nonidet P-40, 0·1 mg mL^{-1} BSA, 1·5 mM $MgCl_2$), 0·2 mM deoxyribonucleoside triphosphates and DNA (1 μL of a 1 : 40 dilution of fungal DNA stock solution from pure cultures; 1 μL DNA or 1 μL 1 : 5 dilution of DNA from uninfected and

Table 3 Root-infecting and soil fungi used to test specificity of the *Gaeumannomyces graminis* var. *tritici* (*Ggt*)-specific PCR assay

Fungus	Isolate	Origin (or reference)
G. graminis var. *tritici* A-type	57·46·1	Wheat, Rothamsted, UK
G. graminis var. *tritici* B-type	00T6·3B	Wheat, Suffolk, UK
G. graminis var. *graminis*	01E1·3C	Wheat, Norfolk, UK
G. graminis var. *graminis*	01S52·5C	Wheat, Cambridgeshire, UK
G. graminis var. *graminis*	01W9·2A	Wheat, Woburn, UK
G. graminis var. *graminis*	01S9·5B	Wheat, Cambridgeshire, UK
G. graminis var. *avenae*	178	Bentgrass, Australia
G. graminis var. *avenae*	P086/484/1	Turf, Cheshire, UK
G. graminis var. *maydis*	W4066B	Maize, China
Gaeumannomyces cylindrosporus	01L1·2B	Wheat, Long Ashton, UK
G. cylindrosporus	89/2·1b	Wheat, Rothamsted, UK
Magnaporthe grisea	2692[a]	(Henson, 1992)
Magnaporthe poae	2562[a]	Turfgrass, USA
Gaeumannomyces caricis		*Carex*, Suffolk, UK
Gaeumannomyces incrustans	2676[a]	St Augustine grass, USA
Phialophora sp.	GP57	Wheat, Rothamsted, UK
Absidia coerulea	H1[b]	*Pinus sylvestris* nursery soil, Poland
Alternaria alternata	H23[b]	Soil, Poznan, Poland
Cladosporium herbarum	H13[b]	*P. sylvestris* nursery soil, Poland
Cladosporium cladosporioides	H36[b]	*Prunus serotina* soil, Zielonka, Poland
Cladosporium sphaerospermum	H22[b]	*P. sylvestris* nursery soil, Poland
Cylindrocarpon destructans	H9[b]	*P. sylvestris* nursery soil, Poland
Cylindrocarpon didymum	H41[b]	*Fagus sylvatica* roots, Zielonka, Poland
Epicoccum purpurascens	H30[b]	*Quercus robur* soil, Zielonka, Poland
E. purpurascens	H42[b]	*F. sylvatica* roots, Zielonka, Poland
E. purpurascens	GP0479	Maize, Cambridge, UK
Mucor hiemalis	H11[b]	*P. sylvestris* nursery soil, Poland
Mortierella humulis	H47[b]	*F. sylvatica* soil, Zielonka, Poland
Penicillium daleae	H50[b]	*Betula pendula* soil, Zielonka, Poland
Trichoderma harzianum	H3[b]	*P. sylvestris* nursery soil, Poland
Trichoderma atroviride	H25[b]	Soil, Poznań, Poland
Trichoderma virens	H21[b]	*P. sylvestris* nursery soil, Poland
T. virens	H4[b]	*P. sylvestris* nursery soil, Poland
Phoma terrestris	H52[b]	*Larix decidua* roots, Zielonka, Poland
Phoma foveata	Pf30	Potato, Rothamsted, UK
Penicillium steckii	H51[b]	*P. serotina* soil, Zielonka, Poland
Helminthosporum solani	Hs1	Potato, Rothamsted, UK
Fusarium culmorum	Fc31	Wheat, Rothamsted, UK
Fusarium culmorum	DM409[a]	(Schesser *et al.*, 1991)
Fusarium avenaceum	Fa2	Wheat, Rothamsted, UK
Fusarium oxysporum	G13	Wheat, Rothamsted, UK
Rhizoctonia solani	Rs19	Potato, Rothamsted, UK
Acremonium sp.	G17	Wheat, Rothamsted, UK
Idriella bolleyi	GP0381	Barley, Papplewick, UK
Pythium intermedium	H23	Wheat, Rothamsted, UK

[a]Isolates obtained from J. Henson, Montana State University, USA.
[b]Isolates obtained from H. Kwaśna, Agricultural University, Poznań, Poland.

infected wheat roots). Cycling conditions were 30 cycles of 94°C for 30 s, 42°C for 2 min and 72°C for 2 min, followed by a final extension of 72°C for 10 min, for amplification of DNA from pure fungal cultures. For amplification of DNA prepared from uninfected and infected wheat roots, the annealing temperature was increased to 65°C.

PCR products were digested with *Hpa*II and electrophoresed in gels containing 2% NuSeive agarose and 1% standard agarose, in 1 × TBE, containing 0·5 μg mL^{-1} ethidium bromide (Ward & Akrofi, 1994).

PCR using *Ggt*-specific primers Ggtfwd, GgtArev and GgtBrev2

Primers Ggtfwd (5′-AAG AAC ATC GGC GGT CTC GCC), GgtArev (5′-TAG CGG CTG GAG CCC GCC G) and GgtBrev2 (5′-CTA CCT GAT CCG AGG TCA ACC TAA GG) were designed during this work specifically to detect and simultaneously identify two subpopulations of *Ggt*. All three primers are used in a single PCR. Ggtfwd and GgtArev are within ITS2 of the nuclear rDNA; GgtBrev2 overlaps the junction between ITS2 and the

nuclear large subunit rRNA gene. Each 12·5 μL PCR contained 5 pmol of each of the three primers, 0·125 units of REDTaq DNA polymerase (Sigma-Aldrich), buffer (10 mM Tris–HCl pH 8·3, 50 mM KCl, 0·01% gelatin, 1 mM $MgCl_2$), 0·2 mM deoxyribonucleoside triphosphates and DNA (1 μL 1 : 100 dilution of genomic DNA stock solution). A touchdown PCR was used with an annealing temperature range of 72–67°C, decreasing by 1°C after every two cycles with 20 cycles at the minimum annealing temperature of 67°C. Cycling conditions were 30 cycles of 94°C for 30 s, annealing (as described above) for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 10 min.

DNA sequencing

The DNA sequences of gel-purified (MinElute gel extraction kit, Qiagen) ITS4/ITS5 PCR products were determined using ITS4 and ITS5 primers and the ABI Prism dye terminator cycle sequencing ready reaction kit (version 3·1) according to the manufacturers' instructions. Reactions were run either at Rothamsted on an ABI 310 automated sequencer or at the DNA Sequencing Facility, Oxford University, UK (http://polaris.bioch.ox.ac.uk/dnaseq/index.cfm) on an ABI 377 automated sequencer.

PCR primer design and DNA sequence analysis

DNA sequences were assembled using the STADEN package (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK). DNA sequence analysis and primer design were performed using the programs SEQED, PILEUP, BLAST and FASTA available in the GCG package (Genetics Computer Group, 1994). Fungal rDNA sequences of species closely related to *Ggt* were identified in the EMBL/GenBank databases by BLAST and FASTA searches. The program NET PRIMER (www.premierbiosoft.com) was used to analyse likely secondary structure, possible primer dimers and T_m of PCR primers.

Statistical analyses

Statistical comparisons were made using GENSTAT (ver. 4·22, VSN International, Hemel Hempstead, UK). Most data from field experiments were tested by ANOVA. Percentage values were usually analysed after transformation to logits. The relationship between polymorphisms in ITS2 of the nuclear rDNA and sensitivity to silthiofam was tested by regression analysis, with percentage of B-type (determined by *Ggt*-specific PCR) as the independent variable, and percentage of isolates sensitive to silthiofam as the dependent variable.

Results

Molecular methods for analysing populations of *Ggt*

Previous work had indicated that the polymorphisms detected by RFLP analysis using mitochondrial ribosomal small-subunit DNA probes, pEG34 (Ward & Gray, 1992; Ward & Bateman, 1994) or pGgtMS7 (Freeman, 2004; Freeman & Ward, 2004) would be useful in determining the structure of field populations of *Ggt* (Bateman *et al*., 1997). Because the Southern hybridization procedures involved are laborious, making routine analysis of large numbers of isolates difficult, a PCR-based assay that could detect the same polymorphisms was sought. Attempts to do this were unsuccessful because there were difficulties in amplifying DNA using consensus primers (from publications and newly designed) for the mt SSU rDNA (Freeman, 2004; Freeman & Ward, 2004). These problems probably arose because of duplication and/or insertions that have occurred in the mt SSU rDNA of *Ggt*.

An alternative PCR–RFLP assay using nuclear rDNA primers ITS4/ITS5 and restriction enzyme *Hpa*II (Ward & Akrofi, 1994) separated *Ggt* isolates in groupings (A- and B-type) roughly similar to the RFLP analysis using the mt SSU rDNA probes. Field isolates of *Ggt* (n = 144) from Rothamsted farm were tested for sensitivity to silthiofam and identified as T1, T2 or T1c mitochondrial small-subunit rRNA gene RFLP type by Southern hybridization. They were then analysed by digestion of ITS4/ITS5 rDNA PCR products with *Hpa*II to identify A- and B-type isolates. Of 68 T1 RFLP type isolates, 58 were insensitive; 45 T1c RFLP type isolates were all insensitive; and of 31 T2 RFLP type isolates, 28 were sensitive. All 28 B-type isolates were sensitive to silthiofam; 106 of 116 A-type isolates were insensitive.

PCR primer design and assay development

The DNA sequences of the ITS regions and the 5·8S rRNA genes of 28 isolates of *Ggt* (Table 2) were determined to enable the design of primers for a specific PCR that could detect the same polymorphism. ITS4 and ITS5 were used as the sequencing primers, and ITS4/ITS5 PCR products as the DNA template. The sequences of the 5·8S rRNA genes were identical for all 28 isolates, and there were few polymorphisms in the ITS regions. The sequence of isolate 00W4·2C is identical over this region to those published for *Ggt* isolates 90/2-4 (AJ010036), T2 (U17221), T5 (U17222) and rDNA type 7B isolates (AJ246151); that of 00W6·5C is identical to rDNA type 7D isolates (AJ246153); that of 00T6·3B is identical over this region to those of *Ggt* isolates 87/7-4 (AJ010037), R1 (U17219), R11 (U17220) and rDNA type 7 A isolates (AJ246150) and that of 00E15·4B is identical to an unnamed *Ggt* isolate (AF508155) (Bryan *et al*., 1995; Carter *et al*., 1999; Ward & Bateman, 1999). No sequence in the databases was identical to that of isolate 00R11·3·3D. This sequence has been deposited in the EMBL database (Accession number AJ969262).

DNA sequences of the ITS2 region of the nuclear rDNA of A- and B-type *Ggt* isolates (21 and seven isolates, respectively), and isolates of *Ggg* (Accession numbers AJ010033 and AJ010034) and *G. graminis* var. *avenae* (*Gga*) (Accession numbers AJ010031 and U17206) were aligned. Part of the alignment is shown in Fig. 1.

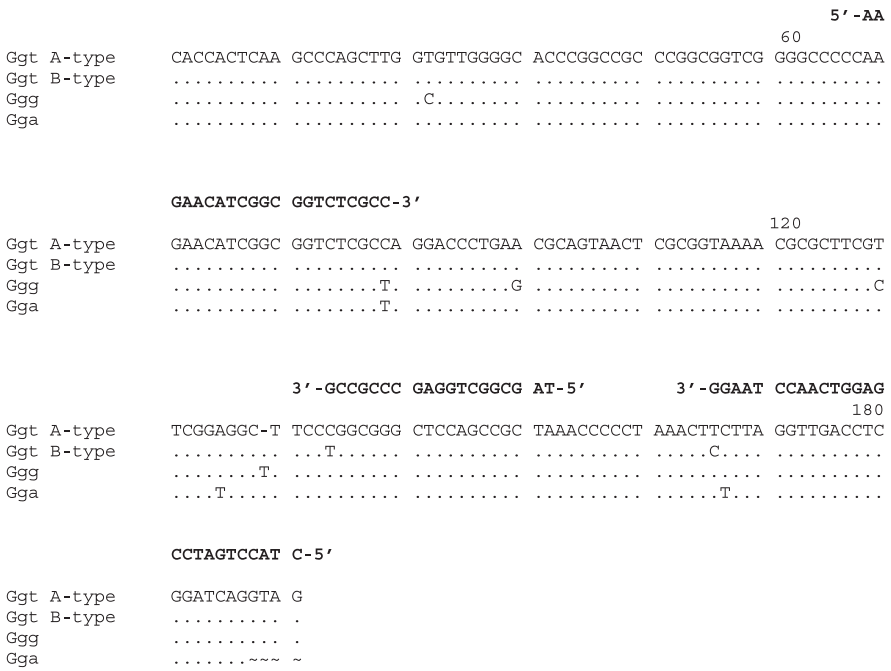

Figure 1 Alignment of the ITS2 region of the nuclear rDNA of *Gaeumannomyces* isolates used for PCR primer design. The alignment extends from the first base of ITS2 and includes the whole of ITS2 and the first 19 bases of the 5′ end of the rRNA large subunit gene. The *G. graminis* var. *tritici* (*Ggt*) A-type (21 isolates) and B-type (seven isolates) DNA sequences were all generated in this work. The *G. graminis* var. *graminis* (*Ggg*) (accessions AJ010033 and AJ010034) and var. *avenae* (*Gga*) (accessions AJ010031 and U17206) DNA sequences are from EMBL/GenBank, and are identical for each variety in ITS2. The sequences of the PCR primers designed from this alignment are shown above the alignment: the forward primer, Ggtfwd, extends from positions 59–79 of the alignment; the reverse primers, GgtArev and GgtBrev2, are the reverse complements of the sequences from positions 134–152 and 166–191, respectively.

PCR primer Ggtfwd was designed to distinguish *Ggt* from *Ggg* and *Gga* based on the polymorphism at position 79 of the alignment (Fig. 1). The reverse primers were designed to distinguish between A- and B-type isolates of *Ggt* based on the polymorphisms at positions 134 and 166 of the alignment. Ggtfwd with GgtArev, and Ggtfwd with GgtBrev2, were predicted to amplify PCR products of 93 and 132 bp, respectively.

The primer sequences were used to search the EMBL/GenBank databases using BLAST. Six accessions were identified by BLAST searches with both the forward and one of the reverse primers (GgtArev): three *Ggg* DNA sequences (U17213, U17212 and AF087685), which are all identical in the primer annealing sites and which were isolated in the USA (U17212, AF087685) and Australia (U17213) (Bryan *et al*., 1995; Zriba *et al*., 1999); one *G. graminis* var. *maydis* (*Ggm*) DNA sequence (AJ010035) and one of its anamorph, *Phialophora radicicola* (AJ010043), both isolated from maize (Ward & Bateman, 1999); and one *G. graminis* isolate (AF505659), which was not identified to variety.

The PCR assay was optimized for magnesium chloride concentration (0·5–1·5 mM), then primer concentration (0·2–1 μM), followed by annealing temperature, with other components of the PCR mix and other cycling conditions as described in Materials and methods. For optimization experiments, single isolates of *Ggt* A-type (57·46·1), *Ggt* B-type (00T6·3B), *Ggg* (01E1·3C) and *Gga* (P086/484/1) were used. A $MgCl_2$ concentration of 1·0 mM and primer concentration of 0·4 μM gave the best results. A touchdown PCR (as described in Materials and methods) was needed to obtain the required specificity. Using these conditions, specific amplification of DNA of isolates of *Ggt* was achieved whilst simultaneously determining A- and B-type of isolates of *Ggt* (Fig. 2). No amplification products using DNA of *Ggg* or *Gga* were detected, even when the gel image was very overexposed (data not shown). The *Ggt* A- and B-type isolates gave PCR products consistent with the predicted sizes of 93 and 132 bp, respectively.

The panel of 28 isolates of *Ggt* (Table 2) was used to test the *Ggt*-specific PCR assay. These isolates had previously been identified as A- or B-type by digestion of ITS4/ITS5 PCR products with *Hpa*II (Table 2). PCR conditions were as described in Materials and methods, except that 0·5 μL of a 1 : 40 dilution of genomic DNA stock solution was used as a template for all isolates. The *Ggt*-specific PCR assay successfully identified all 28 isolates as A- or B-type (Fig. 3). BLAST search results had indicated that Ggtfwd and GgtArev may amplify DNA of some isolates of other varieties of *G. graminis* (see above). DNA from *Ggt* A- and B-type isolates (one of each), four isolates of *Ggg*, two of *Gga* and one of *Ggm* (Table 3) were tested in PCR assays using ITS4/ITS5 (as a positive control) and Ggtfwd/GgtArev/GgtBrev2. DNA of all isolates was amplified by ITS4/ITS5 PCR, but only DNA of *Ggt* (A- and

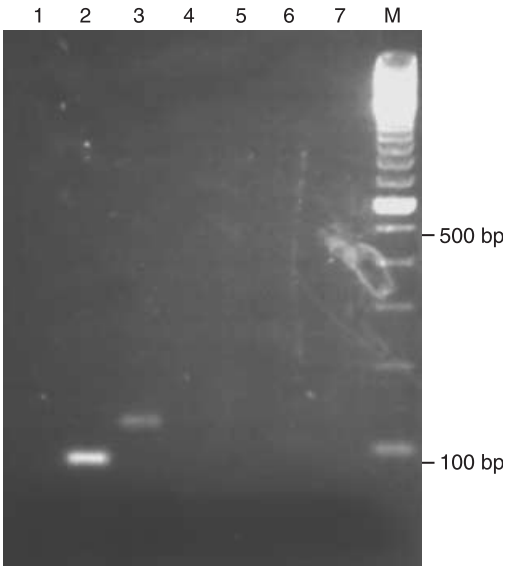

Figure 2 Touchdown PCR for specific detection of *Gaeumannomyces graminis* var. *tritici* (*Ggt*), and identification of A- and B-types of *Ggt*, with PCR primers Ggtfwd, GgtArev and GgtBrev2. Lane 1, no DNA; lane 2, *Ggt* A-type (isolate 57·46·1), 0·5 μL 1 : 40 dilution; lane 3, *Ggt* B-type (isolate 00T6·3B), 0·5 μL 1 : 40 dilution; lane 4, var. *graminis* (*Ggg*) (isolate 01E1·3C), 0·5 μL 1 : 40 dilution; lane 5, *Ggg* (isolate 01E1·3C), 0·5 μL 1 : 8 dilution; lane 6, var. *avenae* (*Gga*) (isolate P086/484/1), 0·5 μL 100 ng μL^{-1}; lane 7, *Gga* (isolate P086/484/1), 0·5 μL 575 ng μL^{-1}; lane M, 100 bp DNA ladder (Life Technologies Ltd).

B-type) and *Ggm* was amplified by the *Ggt*-specific PCR assay. DNA from a further seven isolates of *Ggg*, isolated from soil from plots growing wheat in Cambridgeshire, UK in 2002 and identified as *Ggg* by mitochondrial small-subunit rRNA gene RFLP analysis, was not amplified by the *Ggt*-specific PCR assay (data not shown).

The *Ggt*-specific PCR assay was tested on a total of 36 isolates representing 31 different species of other root-infecting and soil fungi (Table 3), with ITS4/ITS5 PCR as a positive control. DNA of all isolates was amplified by ITS4/ITS5 PCR but not by the *Ggt*-specific PCR assay.

The *Ggt*-specific PCR assay was also tested with DNA prepared from wheat roots that were noninfected, or that had been inoculated with *Ggt* (and produced symptoms of take-all), *Ggg* or *G. cylindrosporus*. The DNA from noninfected roots was not amplified by ITS4/ITS5 or *Ggt*-specific PCR (Fig. 4). DNA prepared from wheat roots inoculated with any fungus was amplified by ITS4/ITS5 PCR. DNA from noninfected wheat roots and DNA from wheat roots infected with *Ggg* or *G. cylindrosporus* were not amplified by *Ggt*-specific PCR whilst DNA from wheat roots infected with *Ggt* was, and *Ggt* isolates were correctly identified as A- or B-type.

Relationship between sensitivity to silthiofam and percentage of B-type isolates

The percentages of isolates of *Ggt*, from individual plots from the three experiments, that were B-type and sensitive to silthiofam were subjected to regression analysis. The analysis showed that the regression lines for the three experiments were coincident ($P < 0{\cdot}001$) with a straight line described by the equation $y = 26{\cdot}11 + 0{\cdot}7534x$ (Fig. 5a). The regression of percentage silthiofam-sensitive on percentage B-type using data from individual plots was highly significant, with a large percentage of the variance accounted for (Fig. 5a). The regression of percentage silthiofam-sensitive on percentage B-type using treatment means was also highly significant, and accounted for an even greater percentage of the variance (Fig. 5b).

Silthiofam sensitivity in populations of *Ggt* and effects of silthiofam on population structure

Small, representative sets of isolates from the collections made in 2000 from experiments at Rothamsted (CS/508) and Sacrewell, obtained from bait plants grown in bioassays of soil infectivity, were tested *in vitro*. All 23 isolates from Sacrewell were sensitive, but only seven out of 23 isolates from the Rothamsted site were sensitive.

In 2002, in experiment CS/508, there was a tendency for a decreased percentage of isolates recovered late in the

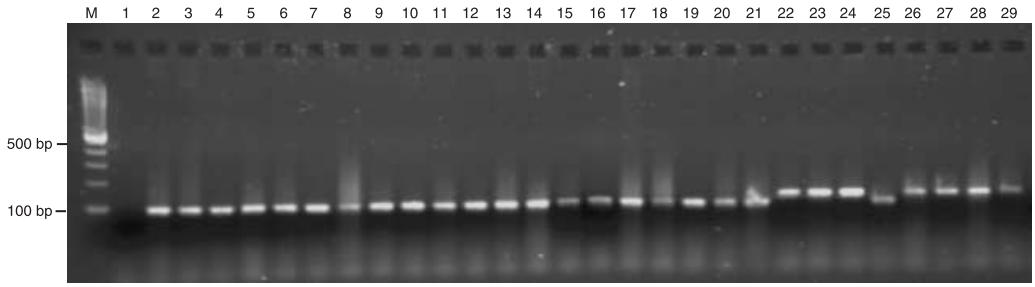

Figure 3 *Gaeumannomyces graminis* var. *tritici* (*Ggt*)-specific PCR using primers Ggtfwd, GgtArev and GgtBrev2 with 28 isolates of *Ggt* previously identified as A- or B-type by digestion of ITS4/ITS5 PCR products with *Hpa*II. Lane M, 100 bp DNA ladder (Life Technologies Ltd); lane 1, no DNA; lanes 2–21 and 25, *Ggt* A-type isolates 57·46·1, 184, 00W4·2C, 01C6·3B, 50·40·3, 00S41·3B, 00E15·4B, 01L2·4C, 00S60·4C, 00W10·3D, 00W2·5A, 01B6·2C, 00R24·3.2B/2, 00W6·5C, 00W7·4C, 93E3·2, 01E1·4B, 00W3·2A, 92·15·4A, 01R24·5A and 00E24·5C, respectively; lanes 22–24 and 26–29, *Ggt* B-type isolates 00T6·3B, 00L3·4A, 99S9·4B and 00R11·3.3D, 01E37·1B, 01R24·4C, 01S26·1C, respectively.

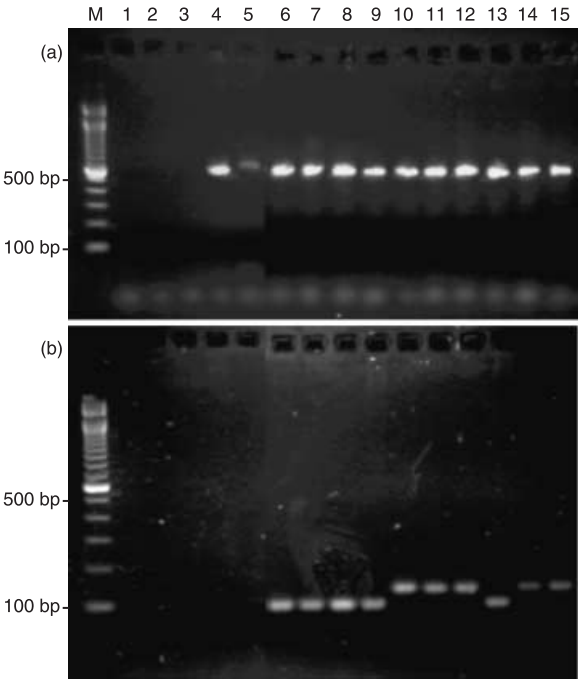

Figure 4 Amplification of DNA prepared from uninfected and take-all-infected wheat roots by ITS4/ITS5 PCR (a) and *Gaeumannomyces graminis* var. *tritici* (*Ggt*)-specific PCR (b). Lane M, 100 bp DNA ladder (Life Technologies Ltd); lane 1, no DNA; lanes 2 and 3, DNA from uninfected wheat roots; lane 4, DNA from wheat roots infected by *G. graminis* var. *graminis* (*Ggg*) isolate 01E1·3C; lane 5, DNA from wheat roots infected by *Phialophora graminicola* isolate 01L1·2B; lanes 6–9 and 13, DNA from wheat roots infected by *Ggt* A-type isolates 01E1·4B, 00W3·2A, 92·15·4A, 01R24·5 A and 00E24·5C; lanes 10–12, 14, 15, DNA from wheat roots infected by *Ggt* B-type isolates 00T6·3B, 00L3·4A, 99S9·4B and 00R11·3.3D, 01E37·1B.

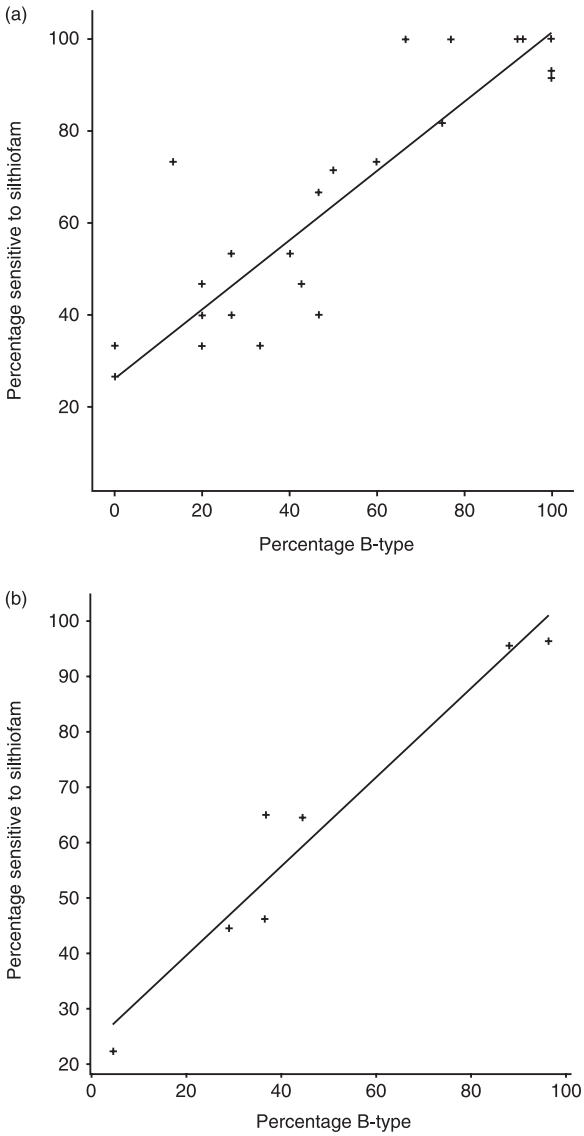

Figure 5 Relationship between percentage B-type isolates of *Gaeumannomyces graminis* var. *tritici* (*Ggt*) and percentage isolates of *Ggt* sensitive to silthiofam in individual plots (a), and using treatment means (b), from three experiments. (a) Straight line described by the equation $y = 26{\cdot}11 + 0{\cdot}7534x$ with $P < 0{\cdot}001$; variance ratio, 79·09 (df = 23); percentage variance accounted for, 76·5. (b) Straight line described by the equation $y = 23{\cdot}52 + 0{\cdot}8032x$ with $P < 0{\cdot}001$; variance ratio, 77·56 (df = 5); percentage variance accounted for, 92·7.

cropping season that were sensitive in crops that had been seed-treated with silthiofam (Table 4). However, there were no statistically significant effects of treatments, in experiment CS/508 or at Sacrewell, on the percentages of silthiofam-sensitive or B-type isolates of *Ggt* (Table 4). In experiment CS/323 there was a significant decrease in the percentage of sensitive isolates and in the percentage of B-type isolates in 2002 after treatment of two successive crops, but smaller effects after treatment of only the preceding crop were not significant (Table 5). Also in the 2002 crop, take-all was decreased significantly by silthiofam applied to the current crop, but there was no significant effect on the amount of infective inoculum in the soil (Table 5).

Discussion

Take-all disease of cereals has long attracted the interest of the agrochemical companies, but until recently there were no fungicides that could be used commercially (Bateman, 1989; Jenkyn *et al*., 2000), and management of take-all relied on husbandry. However, two fungicides have recently become available as seed treatments for controlling the disease. Fluquinconazole (a trade name of the seed-treatment formulation is Jockey®) is a sterol biosynthesis-inhibiting triazole fungicide with a relatively broad spectrum of activity (Löchel *et al*., 1998; Bateman *et al*., 2003). Silthiofam (previously MON65500, trade name Latitude®), on the other hand, has a novel and largely uncharacterized mode of action and is specific to *Ggt* (Beale *et al*., 1998; Spink *et al*., 1998; Schoeny & Lucas, 1999). Host defence responses are also enhanced following silthiofam treatment (Huang *et al*., 2001).

Table 4 Sensitivity to silthiofam and ITS2 genotype in populations of *Gaeumannomyces graminis* var. *tritici* from two field experiments in 2002 (isolates tested: 118 from Rothamsted CS/508; 105 from Sacrewell)

Experiment	Seed treatment	Logit percentage sensitive (back-transformed mean)	Logit percentage B-type (back-transformed mean)
Rothamsted CS/508	Fluquinconazole	0·48 (72·0)	–0·31 (34·7)
	Silthiofam	–0·07 (45·9)	–0·27 (36·2)
	SED[a] (3 df)	0·501	0·200
	P	0·349	0·886
Sacrewell	Fluquinconazole	1·41 (93·9)	1·10 (89·4)
	Silthiofam	1·37 (93·4)	1·38 (93·6)
	SED[a] (3 df)	0·187	0·346
	P	0·842	0·470

[a]Standard errors of differences of means.

Table 5 Effects of silthiofam seed treatments on take-all, soil infectivity and sensitivity to silthiofam and ITS genotype in populations of *Gaeumannomyces graminis* var. *tritici* in two successive crops of wheat in Rothamsted experiment CS/323 in 2002 (135 isolates tested)

Silthiofam treatment	Take-all index (0–100)	Logit percentage wheat roots infected in soil bioassay (back-transformed mean)	Logit percentage sensitive (back-transformed mean)	Logit percentage B-type (back-transformed mean)
None	78·3	0·67 (78·9)	0·33 (65·3)	–0·11 (43·8)
2001 only	78·4	0·51 (73·0)	–0·05 (47·4)	–0·43 (29·2)
2001 and 2002	62·9	0·56 (74·8)	–0·55 (24·7)	–1·43 (5·0)
SED[a] (4 df)	3·93	0·078	0·210	0·176
P	0·026	0·211	0·035	0·004

[a]Standard errors of differences of means.

Fluquinconazole is fungistatic to *Ggt*. No evidence of resistance of *Ggt* to fluquinconazole was identified in populations of *Ggt* over 4 years of study (Bateman *et al*., 2003). Fluquinconazole did not have any significant effect on the population structure of *Ggt*, measured by the percentage of the T2 mtSSU rRNA gene RFLP type, or the percentage of ITS4/ITS5 *Hpa*II B-type, over 3 years (Bateman *et al*., 2003; Freeman, 2004). Research has shown that fluquinconazole can be applied with economic effect on second or third wheat crops, but that a treated diseased crop should be followed by a break crop (or the following crop should be treated), and that treatment should not be applied during long sequences of wheat crops as it may disrupt development of the year-to-year epidemic and possibly take-all decline (Bateman *et al*., 2003, 2004).

Silthiofam is also fungistatic to *Ggt* but the target site has not yet been conclusively identified. It has been demonstrated that silthiofam inhibits ATP export from the mitochondria, possibly by inhibition of the adenine nucleotide translocator (ANT) (Joseph-Horne *et al*., 2000). As a consequence, the fungus becomes starved of ATP and a number of energy-dependent cellular functions were demonstrated to be affected, including maintenance of plasma membrane potential, glucose uptake and the biosynthesis of sterols, fatty acids, protein and RNA. The mechanism by which silthiofam affects ATP export from the mitochondria is, as yet, unknown and may be a direct or indirect effect on the ANT.

Isolates of *Ggt* that are insensitive to silthiofam occur naturally in field populations that have had no contact with the fungicide, and there are variations in sensitivity amongst isolates (Russell *et al*., 2001; Carter *et al*., 2003; Lebreton *et al*., 2003; G.L.B., unpublished data). This situation is highly unusual, and the mechanism is unknown. In this study, isolates were described as insensitive only if the mean colony diameter of the replicates on agar containing silthiofam at 1 mg L^{-1} was at least 95% of the mean colony diameter on untreated agar. Whilst the growth of most isolates of *Ggt* classified as sensitive by these criteria was almost completely inhibited by silthiofam, some isolates had apparent intermediate sensitivity (data not shown). The colony diameters of these isolates varied, even between replicated tests, and were up to 50% of those on control plates. Those isolates with intermediate sensitivity to silthiofam *in vitro* have been classified as sensitive, as the response *in vitro* is not stable and these isolates are fully sensitive when inoculated on plants grown from silthiofam-treated seed (unpublished data).

There are clearly large differences in the incidence of insensitivity of *Ggt* to silthiofam between locations and years. In six other populations sampled in southern and eastern England in 2000, the percentage of insensitive isolates ranged from 10 to 30% (unpublished data). Large

site-to-site variations in the background levels of insensitivity to silthiofam, in untreated plots, have been reported previously (Carter *et al*., 2003; Lebreton *et al*., 2003). Although, in the work reported here, there appears to have been some selection for insensitivity by the use of silthiofam, lasting until late in the crop's growing season, there is some evidence that this does not persist to the following crop. In other studies the use of silthiofam was reported to have little or no effect on the proportion of isolates insensitive to silthiofam in most cases (Carter *et al*., 2003; Lebreton *et al*., 2003), but in these reports detailed statistical analysis of the results was not presented. Therefore further research is still required to determine the effects of repeated applications to crops grown on the same site. The manufacturers currently state that the possible development of resistance to Latitude (the commercial formulation of silthiofam) cannot be excluded or predicted, and recommend that no more than three consecutive cereal crops in one rotation should be treated, as a precautionary measure (www.monsanto-ag.co.uk).

From the limited work that has been done here, and elsewhere (Carter *et al*., 2003), there is also no evidence that differences in amounts of insensitivity affect the field performance of the fungicide. Even so, the occurrence of such high incidences of naturally occurring insensitivity while the fungicide remains apparently effective is unusual and merits further study. The best method available so far for such studies involves the isolation and individual testing of large numbers of isolates. Molecular diagnostic techniques can potentially simplify this procedure.

The development of resistance to fungicides in populations of plant pathogenic fungi has been a recurrent problem in agricultural systems. Resistant alleles are often rare or nonexistent in fungal populations prior to the use of a particular fungicide, but once a resistant allele evolves in a population it can spread rapidly, especially through airborne pathogen populations, and if the selection pressure is maintained (Vendite & Ghini, 1999; McCartney *et al*., 2003;). PCR-based methods have been used to detect and monitor fungicide resistance in populations of several plant pathogenic fungi (McCartney *et al*., 2003).

All the PCR-based methods for detection and monitoring of fungicide resistance are based on detection of mutations in the fungicide target site. Until the target site for silthiofam has been identified, together with the genotype(s) responsible for the variation in sensitivity to silthiofam amongst isolates of *Ggt*, it will not be feasible to monitor the frequency of occurrence of insensitive isolates in populations of *Ggt* by DNA-based methods with absolute accuracy.

A number of DNA-based techniques have been used for the detection and identification of *G. graminis* and related species, at the species and variety level (reviewed by Freeman & Ward, 2004), some of which allow specific detection of *Ggt* and discrimination between isolates of *Ggt* by identifying different genotypes. Several of these methods divided isolates of *Ggt* into two main subpopulations. Recently two subpopulations of *Ggt*, identified by RAPD and AFLP analyses, have been reported to differ in their aggressiveness to wheat and in their relative proportions according to the number of consecutive wheat crops (Lebreton *et al*., 2003, 2004). However, initial population structures, and changes in population structure, were not consistent between two sites. In a more comprehensive study (a total of 2759 isolates of *Ggt* from 3 years at seven sites were analysed; see Introduction), large differences were also found between initial population structures, and no associations were found between changes in population structure and epidemic stage that were consistent for all sites. No simple PCR-based methods have been reported that allow specific detection of *Ggt* and discrimination between isolates simultaneously. The PCR assay described here allows specific detection of *Ggt* and simultaneous identification of two subpopulations of *Ggt* by using one forward PCR primer with two reverse PCR primers in a single PCR. The results demonstrate the need to optimize conditions for amplification by any new PCR primer pair and the possibility of detecting several single nucleotide polymorphisms in a multiplex PCR. The assay did not detect any of the other fungi tested except for *Ggm*.

The BLAST search results indicated that this assay would amplify DNA of an isolate of *Ggm*, one of *P. radicicola*, and three isolates of *Ggg*. The *Ggm* and *P. radicicola* isolates were from maize in China and Canada, respectively. *Phialophora radicicola* is probably the anamorph of *Ggm* (Ward & Bateman, 1999) and these fungi have not been well studied, possibly because they are uncommon. No other published diagnostic method, with the exception of earlier work by the authors (Ward & Bateman, 1999), has included testing for *Ggm*. One of the *Ggg* isolates (724, AF087685) could be atypical, or misidentified, as it is described as a 'take-all-causing isolate' (Zriba *et al*., 1999). The host of origin of the other *Ggg* isolates is unknown; one isolate is from the USA and one from Australia. Several studies have indicated that there is more variation within *Ggg* than among isolates of the other varieties of *G. graminis* (Elliott *et al*., 1993; Fouly *et al*., 1997; Ward & Bateman, 1999), and DNA sequence analysis of the ITS region of the nuclear rDNA has grouped isolates of *Ggg* from wheat and barley, on which they are non- or weakly pathogenic, separately from those from other hosts (Ward & Bateman, 1999). The assay described here should therefore provide a useful tool for studying population dynamics of *Ggt* on wheat. It should also be possible to use DNA prepared from infected wheat roots for population studies, eliminating the need to isolate and culture the fungus. The method was tested on a total of 352 *Ggt* isolates from three field experiments.

The method described here may provide a valid method of estimating the frequency of isolates insensitive to silthiofam in field populations of *Ggt* by monitoring polymorphisms in ITS2 using the *Ggt*-specific PCR. The regressions of percentage sensitivity to silthiofam on percentage B-type isolates were highly significant, whether the data came from individual plots or from multiple plots using treatment means. It is very unlikely that the mutations in ITS2 have a causative effect on insensitivity to silthiofam, but rather they are coupled to this effect, sharing a

common evolutionary history with the actual causative mutation, the nature of which is currently unknown. An association between the two subpopulations of *Ggt* identified by Lebreton *et al*. (2003) and silthiofam sensitivity/insensitivity was also reported, although no statistical analysis of the data was reported. At the moment it is not possible to determine whether there are any relationships between the *Ggt* subpopulations identified in the current work and that of Lebreton *et al*. (2003).

Fluquinconazole and silthiofam treatments did not result in significantly different percentages of isolates sensitive to silthiofam or of the B-type in populations in Rothamsted experiment CS/508 or at Sacrewell. However, the interpretation of this experiment needs some caution because the preceding three crops had been treated with fluquinconazole. In Rothamsted experiment CS/323, the effects of silthiofam can be monitored more clearly as fluquinconazole had not been used on any of the plots. In this experiment, the population structure of *Ggt* was affected by silthiofam, which significantly decreased percentage sensitive and percentage B-type isolates after two successive treated crops. Despite the presence of smaller proportions of silthiofam-sensitive and B-type isolates after silthiofam treatment in experiment CS/323 (especially in plots sown with treated seed in two successive years), take-all index was still significantly less where treatment was applied for two successive years than in plots with other treatments. The evidence for selection for insensitivity by the use of silthiofam is therefore variable, and the implications for take-all control of any selection that might occur, or of the differences between populations in amounts of naturally occurring sensitivity that occur, are so far unclear. The availability of the *Ggt*-specific PCR assay may simplify future studies on silthiofam insensitivity in *Ggt* populations, at least until the molecular basis for insensitivity has been elucidated, but further testing is required to verify the correlation between B-type isolates and sensitivity to silthiofam.

Acknowledgements

This research formed part of Sustainable Arable LINK project LK0907, sponsored by Defra, with additional funding from the Home Grown Cereals Authority and Aventis CropScience. We thank A. D. Todd for statistical analyses. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council.

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