The influence of growth stage of different cereal species on host susceptibility to *Gaeumannomyces graminis* var. *tritici* and on *Pseudomonas* populations in the rhizosphere

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Abstract The dynamics of infection by *Gaeumannomyces graminis* var. *tritici* (*Ggt*), the soil-borne fungus causing the disease take-all, in wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.) and triticale (x *Triticosecale* Wittmack) was investigated at different growth stages of the cereal hosts. In the field, *Ggt* spreads along rows of plants from the inoculum sources. At harvest, *Ggt* DNA was detected up to 60 cm away from inoculum sources in all species except rye, although most of the take-all lesions occurred in roots less than 30 cm away in all species, with rye being the least affected. The greatest populations of fluorescent *Pseudomonas* sp-species were extracted from the rhizosphere of cereal roots sampled near the points of *Ggt* inoculation; were found prior to the booting growth stage in triticale and rye; and were not related to the concentration of *Ggt* in the roots. In pots in a glasshouse experiment, *Ggt* colonised seedling wheat roots to a concentration of 103 ng DNA/mg dried roots and caused 14 % take-all severity in roots during plant development. In rye, seedling roots contained *Ggt* DNA at 15 ng/mg dried root, which decreased to negligible concentrations until heading, then increased rapidly to 280 ng DNA/mg dried root at kernel development. Take-all root severity in rye increased from 1 to 50 % over that period. In a second glasshouse experiment, inoculation of roots of host plants at various growth stages with actively growing hyphae of *Ggt* showed that the pathogen was able to overcome resistance in rye plants after an establishment phase.

Introduction

Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is one of the most important root diseases of wheat worldwide (Cook 2003; Hornby et al. 1998). The pathogen infects susceptible plants, resulting in blackened roots, severe stunting and premature death (Cook 2003). Between crops, *Ggt* survives saprophytically on crop debris (Asher and Shipton 1981), and its concentration declines as the crop debris decays (Bithell et al. 2009; Hornby 1981). Soil moisture affects the rate of inoculum decline. *Ggt* can survive for long periods in dry environments (MacNish and Dodman 1973), but decline is more rapid where warm wet conditions follow harvest (Cook 2003). The amount of inoculum present in soils at crop establishment affects the extent of primary infection of susceptible host cereals (Bailey and Gilligan 1999). Soil *Ggt* DNA concentrations of <6 pg and >80 pg *Ggt* DNA/g soil before sowing are associated with low risk and high risk, respectively, of take-all in second wheat crops, when environmental conditions favour disease development (Bithell et al. 2012; Ophel-Keller et al. 2008).

Cereal species differ in susceptibility to take-all, and in their capacity to contribute to inoculum in soil after harvest. Wheat (*Triticum aestivum* L.) is highly susceptible to the pathogen, and rye (*Secale cereale* L.) is slightly less so (Rothrock 1988). Gutteridge et al. (1993) also classified wheat to be the most susceptible, rye the least susceptible, with barley (*Hordeum vulgare* L.) and triticale (x *Triticosecale* Wittmack) having intermediate susceptibility, although barley yields were less affected by take-all than those of wheat or

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triticale, because barley was at later growth stages by the time severe infection occurred.

In addition to being highly susceptible, wheat also supports rapid build-up of inoculum in soil. However, Bithell et al. (2011) found that, while few take-all symptoms were seen in rye, postharvest inoculum concentrations in soil were even higher after rye than after wheat. *Ggt* concentrations in rye roots increase rapidly at the later growth stages, and the large amounts of inoculum returned to the soil provide potential sources of infection of subsequent *Ggt*-susceptible cereal crops. Skou (1975) concluded that the ability of cereals to produce new adventitious roots was a major factor in their ability to tolerate *Ggt*, with the rate of production of new adventitious roots following attack by *Ggt* greatest in rye, less in barley, and least in wheat and triticale, differences which correlated with grain yields and root discoloration in those species.

The spread of *Ggt* infection from primary inoculum sources is influenced by the spatial positioning of host plants, since the main mechanism of disease spread is by root-to-root contact. Primary infection is more likely from inoculum just below the soil surface than at deeper depths, and secondary infection through the crown is more likely than infection through soil (Gosme and Lucas 2009). The disease is aggregated within roots and plants in line sowing more so than in broadcast sowing, with take-all severity reducing by 95 % 15 cm away from inoculation points (Gosme and Lucas 2011). Willocquet et al. (2008) showed that take-all progressed along the rows of wheat up to 20 cm from an in-row inoculum source, and seldom reached neighbouring rows. Prew (1980) concluded that the maximum spread of take-all in roots from an inoculum source was 18 cm, which occurred in 42 % of cases. Conversely, spatial analysis of take-all by Gosme et al. (2007) showed clusters of diseased plants were 2–2.5 cm diam., with secondary infections contributing only marginally to disease spread within a cropping season. A good understanding of the effects of host crops on *Ggt* concentrations is a key to better management of take-all. Knowledge of how the different hosts affect the build-up of inoculum has implications for the management of take-all in cereal crops. Aspects of *Ggt* infection in wheat and rye at different growth stages were investigated further in two glasshouse experiments.

Pseudomonas fluorescens has been implicated in suppressing take-all (Cook 2007) and may also modulate defence/stress gene expression in cereal roots (Okubara et al. 2010). This paper presents the results of a study of take-all severity and *Ggt* DNA concentrations in cereal roots in a field experiment in 2012–2013 in the South Island of New Zealand. Spread of the pathogen along crop rows from point sources of inoculum was monitored in wheat, triticale, barley and rye at different growth stages. Since *P. fluorescens* may influence the spread of *Ggt*, the populations of *P. fluorescens* in plant rhizospheres were monitored close to and away from *Ggt* inoculum sources.

Materials and methods

Field experiment

The amount of *Ggt* infection in plants as it progressed down rows from a point source of inoculum was estimated at six growth stages in four cereal species. A trial was laid out in a field previously planted in ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pasture that had been sprayed with 3 L/ha Lion® 490 DST (490 g glyphosate/L) herbicide on 30 March 2012 and cultivated by shallow ploughing and rolling. The day before sowing, 250 kg/ha Cropmaster® 15 (15 % N, 10 % P, 10 % K and 8 % S) fertiliser was broadcast on to the soil, which was power-harrowed to a medium-fine seed bed.

Seeds of 'Wakanui' wheat, 'Quench' barley, 'Amilo' rye and 'Empero' triticale, which were selected for their resistance to foliar cereal diseases (Cromey 2010), were treated with 600 mL Poncho®/tonne seed (600 g chlothianidin/L) in 10 L water to control *Yellow Dwarf Virus*-aphid vectors. The seeds were sown at 3–4 cm depth at 110 kg/ha on 8 May 2012 using a small plot drill (Oyjord) with nine coulters at 150-mm row spacings.

The plots were 6 m long and 3.4 m wide, comprising 18 rows from two adjacent drill-widths. The rows were orientated north and south. A 1.5-m wide buffer of barley 'Bumper' was sown around the trial and between the ends of each plot. In total, there were 32 main plots, with eight replicate plots for each cereal, and the main plots were arranged in a Latin rectangle, generated using CycDesign (CycDesigN v5.1, VSN International). Six rows in each plot separated by two buffer rows were allocated for sampling, with one row randomly allocated to each of six host growth stages. Thus, host growth stage constituted a split plot treatment.

Ggt-inoculated oat grains were prepared in April 2012 ((Weller and Cook 1983) from five *Ggt* isolates (H9T3 R1/1.2, Biomill SC3, A3SL4, 1201/w-1, H11T3 R1/3) collected from couch grass (*Elymus repens*) or wheat in Canterbury, and the isolates were known from earlier pathogenicity tests to be pathogenic against wheat. The inoculum was stored at 4 °C until use. On 18 May 2012 the infected kernels were mixed in equal proportions. Approximately 10 kernels were deposited in each of the six designated rows midway between the ends of each plot. The kernels were placed in a 1.0-cm diam. hole 6 cm below soil surface, where primary infection from inoculum is likely to be greatest (Gosme and Lucas 2009), and within 1 cm from where the seeds were sown, to ensure infection of host roots by *Ggt* (Gilligan and Simons 1987). The locations of the inoculation points were marked with rods.

The cereals in the plots were managed in accordance with best crop practice. Firebird® SC (400 g/L flufenacet and 200 g/L diflufenican) was sprayed at 300 mL/ha 1 week after sowing, for the control of broad-leaved weeds. The fertilizer

Ammo® 31 (30.7 % N, 14.4 % S) was broadcast onto soil at 150 kg/ha on 7 September 2012. A selective herbicide was sprayed onto foliage on 18 September 2012: 2.5 L/ha Duplosan Super® (130 g/L mecoprop-P, 310 g/L dichlorprop-P and 160 g/L MCPA as dimethylamine salt) to control wireweed (*Polygonum aviculare*) seedlings, and 800 mL/ha Proline® (250 g/L prothioconazole) to control scald on barley. Urea (46 % N) was applied at 175 kg/ha on 26 September 2012. Two hawk kites (Scare'm Hawk Kite; www.scarem.co.uk) were set up on 24 November 2012 to scare birds that were feeding preferentially on barley kernels. The trial was irrigated on 29 November, 19 December 2012 and 7 January 2013 with 28, 25 and 26 mm of water, respectively.

Assessments were conducted on six occasions for each cereal species when they had reached their designated Zadoks' growth stages (GS) (Zadoks et al. 1974) of 24 (seedling growth during winter), 39 (stem elongation), 65 flowering, 73, 87 (kernel and milk development) and 92 (dough development and ripening) (Table 1). The plots were divided into sampling areas, with the south end sampled for *Ggt* and take-all symptoms, and the north end sampled for *Pseudomonas* spp.

At each assessment in the designated sampling row for *Ggt* and take-all symptoms in each plot, four 30-cm long clear-plastic rulers were positioned on both sides of the plants, end to end from the *Ggt* inoculation point to 120 cm south of this point, and clamped in place with 'bull-dog' clamps. The plants in each 30-cm section were lifted using a garden fork inserted into the soil to a depth of 20 cm at 5 cm either side of the row. The roots of the plants were washed to remove adhering soil. They were bagged separately in labelled 5-cm segments of row at all assessment times except GS 24, when the data was combined in the 30–60, 60–90 and 90–120 cm sections. The roots for each row segment/section at each assessment were assessed for symptoms of take-all. They were then air-dried at 40 °C for 4 days and stored at −18 °C until analysis for *Ggt* DNA. *Ggt* DNA was assessed in roots of plants from four of the eight blocks (replicates).

Plants near and furthest away from the *Ggt* inoculation sources within the same row were sampled at each assessment to quantify fluorescent *Pseudomonas* spp. in the root rhizospheres. Since *Ggt* was likely to migrate equally in both directions along the row from the source of inoculation (Willocquet et al. 2008), it was assumed that plants on either side of the *Ggt* source would be infected by the fungus migrating in both directions. Hence two plants representing *Ggt*-inoculated plants were removed from the sample row within 10–20 cm north of the inoculum. Two other plants representing un-inoculated plants were removed from the same row about 200 cm from the inoculum. The plants were removed with a fork as for the sampling of the plants for take-all symptoms and *Ggt* analyses.

Because the drill coulters were not fixed, and could sway during drilling, the distance varied between the sown seed rows and the inoculum. Consequently, the inoculum was not always positioned exactly within the row of sown seed. Since *Ggt* has been reported to infect roots no more than 1.5 cm away from the inoculum point (Gilligan 1980), the distance between the middle of the sown row and the inoculum was measured on 11 January 2013 (barley and rye GS 87) for each inoculated row. This distance is referred to as the 'inoculum offset'.

Daily rainfall, soil moisture and soil temperature at 10 cm depth were recorded from 1 May 2012 to 28 February 2013 at a weather station at Lincoln, Broadfield, Canterbury (New Zealand National Institute of Water & Atmospheric Research), located 1 km from the field experiment, and downloaded from the Cliflo website (http://cliflo.niwa.co.nz/).

Glasshouse experiment 1

To better understand the dynamics of *Ggt* infection in cereal species, a pot experiment was established in November 2010 in a glasshouse set to 18 °C with natural light. 'Conquest' wheat and 'Rahu' rye seeds were sown into soil with three different concentrations of *Ggt* inoculum. The roots of the developing plants were assessed for symptoms of take-all and *Ggt* DNA concentrations at GS 15 (seedling growth), 30 (stem elongation), 50 (heading), 65 (flowering) and 71 (kernel and milk development). Four replicates were used for each of the six species x inoculation combinations for each of the five growth stages. These were laid out in a split-plot design, with growth stages as main plots (laid out as randomized blocks), and species/inoculation combinations as the split plot treatment.

Top soil (0–20 cm) collected from pasture at Lincoln, Canterbury, was passed through a 4-mm sieve and sterilised for 1 h at 110 °C. Fine sand and fertiliser were added so that 100 kg of the soil mix comprised 42 kg soil, 58 kg sand, 1053 g dolomite, 842 g Osmocote® Exact, 474 g zeolites, 368 g superphosphate, 105 g sulphate of potash and 21 g calcium nitrate. *Ggt* inoculum was prepared using the methods of Weller and Cook (1983). The pots were prepared on 1 November 2010. They contained the soil mix and the cereal seed either alone or with *Ggt* inoculum. Oat seed colonised by *Ggt* isolates A3SL4, B103 and H9T3R1/12 was macerated for 5 s in a small electric grinder, mixed into the soil, and applied to the designated inoculum pots at the rate of 2 g (low inoculum) and 80 g (high inoculum) of *Ggt*-colonised oat seed per 3.5 kg of soil mix. The pots (PB5 black polyethylene bags; 15 cm diam.×12.5 cm depth) each contained 3.5 kg (2210 mL) of soil mix. The pots were prepared by adding 2500 g of soil in the bottom of each pot (85 mm deep), adding another 700 g of soil, or inoculum plus soil, on top (30 mm deep), applying four seeds of wheat or rye to the centre of the

Table 1 Sampling dates and days after treatment for each cereal species at their designated growth stages in the field trial, Lincoln, New Zealand 2012

Assessment	Zadoks	Growth stage	Cereal	Date	DAT[a]
1	24	Seedling growth; 3rd leaf visible	Barley	20 Aug 2012	104
			Rye	20 Aug 2012	104
			Triticale	20 Aug 2012	104
			Wheat	20 Aug 2012	104
2	39	Stem elongation; flag fully emerged; ligules visible	Barley	11 Oct 2012	156
			Rye	11 Oct 2012	156
			Triticale	01 Nov 2012	177
			Wheat	12 Nov 2012	188
3	65	Mid flowering; half the spikes with anthers	Barley	23 Nov 2012	199
			Rye	23 Nov 2012	199
			Triticale	29 Nov 2012	205
			Wheat	12 Dec 2012	218
4	73	Kernel & milk development; early milk, liquid off-white	Barley	13 Dec 2012	219
			Rye	14 Dec 2012	220
			Triticale	20 Dec 2012	226
			Wheat	31 Dec 2012	237
5	87	Dough development; hard dough, holding fingernail impression	Barley	03 Jan 2013	240
			Rye	09 Jan 2013	246
			Triticale	16 Jan 2013	253
			Wheat	23 Jan 2013	260
6	92	Ripening: harvest-ripe, moisture 14 %	Barley	25 Jan 2013	262
			Rye	07 Feb 2013	275
			Triticale	14 Feb 2013	282
			Wheat	25 Feb 2013	293

[a] Days after treatment

firm surface, and adding a further 300 g of soil on top (10 mm deep). The density of the soil mix was calculated as 1.36 L/kg. The wheat and rye plants at GS 15 were harvested on 24 November 2010, then at different times after sowing when each species reached GS 30, 50, 65 and 71 (Table 3). Roots were assessed for symptoms of take-all and contents of *Ggt* DNA at each assessment.

Glasshouse experiment 2

To determine the degree of *Ggt* resistance in 'Amilo' rye relative to that of 'Wakanui' wheat, roots of these plant species were inoculated at GS 15 (seedling growth), GS 31, (stem elongation), GS 41 (early boot), GS 51 (heading), GS 71 (kernel and milk development) and GS 91 (ripening) with 9-day-old actively growing *Ggt* hyphae. Four replicates of the 12 treatment combinations were prepared and grown on benches in the glasshouse in a Latinized row-column design, constructed with CycDesign.

On 27 April 2012, the wheat and rye seeds were treated with Poncho® (600 g/L chlothianidin) at the equivalent rate of 600 mL product in 10 L water per tonne of seed, to control cereal aphids. Three days later, the seeds were rinsed and placed on wet tissue in 90 mm diameter×20 mm deep Petri dishes, and vernalised at 4 °C for 7 days (30 April to 7 May) (a prerequisite

for rye plants to grow to maturity and produce seed heads). On 7 May 2012, four vernalised wheat or rye seeds were sown to a depth of 10 mm and equidistant from one another in PB5 black-polythene bags (15 cm diameter×20 cm tall) containing 3.2 L of a sand mix comprising 0.33 m³ sand, 2 kg Osmocote, 1 kg dolomite, 600 g garden lime, 350 g superphosphate, 100 g calcium nitrate and 100 g sulphate of potash.

Seven days later, missing seedlings were replaced with seeds that had been held at 4 °C for 15 days, so that each pot had four viable plants. The glasshouse was set at 20 °C with ambient light. Plots were watered daily. On 12 June 2012, Nitrophoska Extra granules (3.0–3.6 mm diam.) containing N 12.0 %, P 5.2 %, K 14.0 %, S 6.0 %, Mg 1.2 % and Ca 5.0 % were applied to all pots remaining after the first sample at 3 g/pot (50 kg/ha). The fertiliser was to support growth of the plants until their maturity.

The wheat and rye plants at GS 15 were harvested on 31 May 2012, 24 days after sowing, then 63, 127, 136, 161 and 269 days after sowing for GS 31 41, 51, 71 and 91, respectively. At harvest, foliage was trimmed to 50 mm above the soil surface. Each polythene planter bag was cut to expose the sand and roots, and the sand was washed from the roots. The outside roots from the four washed plants from each pot were trimmed to leave c. 10 seminal roots on plants at GS 15 and c.

50 seminal roots in the subsequent growth stages. The roots were transferred to a double-layered sheet of germination paper pre-soaked in tap water. The *Ggt* isolate A3SL4 was cultured on potato dextrose agar (PDA) in a 12-h light/dark regime at 20 °C for 9 days, and kept at 4 °C until root inoculation within 5 days. Two 6-mm diam. disks of PDA containing actively growing mycelium from the *Ggt* isolate were positioned to cover the roots 150 mm below the crown of each plant. The paper was folded 20 mm in from the bottom and rolled up.

Each paper tube containing the four plants was wrapped in a plastic bag. Twenty mL of nutrient solution (945 µg/L Ca(NO$_3$)$_2$.4H$_2$0, 607 µg/L KNO$_3$, 493 µg/L MgSO$_4$.7H$_2$O, 115 µg/L NH$_4$H$_2$PO$_4$, 2.9 µg/L H$_3$BO$_3$, 1.8 µg/L MnCl$_2$.4H$_2$O, 0.22 µg/L ZnSO$_4$.7H$_2$O, 0.08 µg/L CuSO$_4$.5H$_2$O, 2.6 µg/L Fe-EDTA and 0.02 µg/L Na$_2$MoO$_4$.H$_2$O) was added to each bag to provide nutrients for growth of the plants. The *Ggt*-treated roots were incubated in the same glasshouse and the roots inspected for symptoms of take-all 11 days after inoculation, when another 20 mL aliquot of nutrient solution was added, and again 22 days after inoculation. At both assessments, the roots from each plant were counted and assessed for presence of runner hyphae, number of lesions and take-all severity (proportion (%) of roots with take-all symptoms to 200 mm below the crown. At the final assessment, a 200-mm long portion of seminal roots from immediately below the nodal roots attached to the crown of each plant was removed, washed to remove adhering agar containing *Ggt* inoculum, dried at 40 °C for 3 days and stored at −18 °C until analysis of *Ggt* DNA.

Assessments

Take-all symptoms

The numbers of seminal roots in each 5-cm segment were counted, and all roots assessed for the presence or absence of runner hyphae consistent with *Ggt* or *Phialophora*, and for take-all symptoms (Hornby 1981) under a compound microscope at 30× magnification. Take-all severity (%) was estimated as the percentage area of roots with lesions over the total area of roots (Schoeny and Lucas 1999). Roots with blackened root steeles were classed as being infected by *Ggt*. Black runner hyphae were not differentiated as being either *Ggt* or *Phialophora* spp. Brown lesions were classed as *Phialophora* spp.

*DNA extraction and **Ggt** DNA quantification*

Each dried root sample was ground in liquid nitrogen (N) using a mortar and pestle and stored in a 2-mL microtube at −20 °C until extraction for DNA. The DNA from these samples was extracted using the DNeasy Plant Minikit (Qiagen, New Zealand) following the manufacturer's instructions. The extracted DNA was suspended in 400 µL of TE manufacturer's buffer (pH 8.0). Two quantitative polymerase chain reaction (qPCR) runs were made for each sample. Samples were diluted 1:20 (0.05) with TE buffer (pH 8.0) to reduce the effects from inhibitors. Samples were added to the 96-well PCR plates in duplicate, with all samples from one replicate assigned to a single plate.

PCR amplifications were carried out in a StepOnePlus qPCR machine (Applied Biosciences). Initially, quantitative PCR amplification was performed on reaction mixes containing primers to detect plant DNA to confirm that all samples contained detectable DNA. The 20-µL reaction mix comprised 10 µL 2× iTaq Supermix master mix (BioRad) with a SYBR intercalating ds DNA dye and the primers Pot25SF – 5′TCGGAATTCGAAGCTAGAGG and Pot25SR – 5′AACGTCGCTATGAACGCTTG at a concentration of 0.3 µM each. Quantitative PCR conditions followed manufacturer's instructions (polymerase activation 95 °C for 2 min; 40 cycles of 95.0 °C for 5 s, annealing at 62.0 °C for 10 s and 72.0 °C for 15 s; terminal extension of 72.0 °C for 7 min; Hold at 10 °C). An undetermined result implied inhibitors were present, likely to affect detection of *Ggt* DNA, and the sample was omitted from the *Ggt* DNA analysis.

To measure *Ggt* DNA in the cereal roots, a qPCR assay targeting the translation elongation factor 1-alpha (EF1-α) gene from *Ggt* was used (Keenan et al. 2014). Quantitative PCR amplification was performed on a total volume of 20 µL comprising 10 µL 2× iTaq Supermix master mix (BioRad) (Rox passive reference dye), a FAM probe GgtProbe 1 FAM–5′ACTGCACAGACCATC with MGB-NFQ quencher at 0.175 µM, the primers GgtEF1 – 5′CCCTGCAAGCTCTT CCTCTTAG and GgtEFR1 – 5′GCATGCGAGGTCCCAA AA at 0.3 µM, and 2 µl of the extracted DNA solution (initial denaturation at 95 °C for 5 min; 40 cycles of 94.0 °C for 30 s, annealing at 52.0 °C for 30 s and 72.0 °C for 1 min; terminal extension of 72.0 °C for 7 min; Hold at 10 °C). All quantities of target DNA were estimated using a standard curve constructed by plotting cycle threshold (Ct) values against the log$_{10}$ of the initial concentration of duplicate or triplicate *Ggt* standards at 10, 1, 0.1, 0.01 and 0.001 ng *Ggt* DNA/µL buffer. The parameters of the standard curve were used to convert Ct values into estimated amounts of *Ggt* in the sample. An undetected Ct value was assumed to mean the sample contained no *Ggt* DNA.

Pseudomonas population analysis

Excess soil was shaken from the roots of test plants. A 2-g sub-sample of root was aseptically transferred to 20 mL sterile 0.1 M magnesium sulphate (24.6 g MgSO$_4$.7H$_2$O in 1 L RO water). The roots were vortexed four times for 15 s each followed by 10 min shaking at 180 rpm to dislodge bacterial

cells. After 10 min settling, 100-µL aliquots were serially diluted in 900 µL 0.1 M magnesium sulphate solution to obtain 1×10^1, 10^2 … 10^{10} dilutions. Twenty microlitres of each dilution was spread in triplicate onto King's B agar (King et al. 1954) modified with 20 mg/L of trimethoprim. The inverted plates were incubated at 25 °C, and the numbers of fluorescent colonies under UV light were counted at 48 and 72 h to obtain the number of colony forming units (CFU) per gram of roots.

Statistical analyses

For all measurements for all trials, some assessment was made of potential spatial patterning and of the hierarchical structure (e.g. pots within blocks). This was done by including appropriate factors (such as Blocks, Plots) as random effects in the analyses. Where such effects were found to be important, these were adjusted for in the final analyses. Because the 'inoculum offset' in the field experiment was not strongly associated with any of the measurements, it was excluded from the final analysis. For root dry weights in glasshouse Experiment 1, no spatial or hierarchical factors were found to be important, so these were analysed with simple analysis of variance, after first log-transforming the data to stabilise the variance.

All other data presented were analysed using a hierarchical generalized linear modelling approach (HGLM, Lee et al. 2006). This included fixed effects such as cereal species, inoculum presence and interactions, and, for the final analysis, any important random effects. Random effects were assessed with a X^2-test of the change in deviance on dropping the term, as implemented in GenStat's HGRTEST procedure (GenStat Committee 2013), and fixed effects similarly, using GenStat's HGFTEST procedure. In the final analyses, only the important random effects were included, but all fixed effects were included.

For take-all severity, the fixed effects had binomial distributions, and any important random effects had beta distributions, with logit links for both types of effect. For the data from the field experiment, the fitted model adjusted for the parts assessed as complete 30-cm sections by multiplying the take-all severity by 5, and increasing the binomial totals to 500 (rather than the 100 used elsewhere). Association with the inoculum offset was also assessed, by including inoculum offset as a covariate in the fixed model.

For final analyses, qPCR data were first converted into DNA quantities, using calibration regressions. Since DNA quantities are essentially molecule counts multiplied by molecular weights, the quantities were then analysed using a method appropriate for counts. For the analysis of *Ggt* DNA quantities, root counts and *Pseudomonas* counts, fixed effects were included with Poisson distributions, and random effects with gamma distributions, both with logarithmic links. For analysis of *Ggt* DNA and *Pseudomonas* data, offsets (parameter-less explanatory variables (McCullagh and Nelder 1989)

were included to adjust results as ng *Ggt* DNA/mg root, and *Pseudomonas* CFU/g root. In the *Ggt* DNA analysis from the field experiment, distances were identified to 10 cm sections rather than to the 5-cm segment, because in some instances there was only one 5-cm segment of roots present of the two possible (e.g. 10–15 cm and 15–20 cm segments were both included as being from the 10–20-cm section).

For all the measurements presented, the (predicted) means were obtained on the transformed (or link) scale (log or logit), along with 95 % confidence limits. These were back-transformed for presentation. Relationships between variables were explored graphically. All analyses were carried out with GenStat (GenStat Committee 2011).

Results

Field experiment

Take-all symptoms

The cereal × growth stage × distance ($p=0.172$) and the cereal × distance ($p=0.786$) interactions for the number of cereal roots per 5-cm segments up to 1200 cm in rows from the *Ggt* inoculation points were not statistically significant. While the growth stage × cereal ($p=0.003$) and growth stage × distance ($p<0.001$) interactions were significant, the differences between the cereals and distance only occurred after GS 39 and were inconsistent. This indicated that the number of plants sown and germination rates of plants of the different cereals did affect the early spread of *Ggt* and take-all symptoms. Runner hyphae consistent with *Ggt* or *Phialophora* were found mostly within 30 cm from the inoculation points, but these were also found as distant as 1200 cm.

Take-all was influenced by the cereal × growth stage× distance interaction (X^2_{297} =1846; $p<0.001$). Almost all take-all symptoms were found within 30 cm from the inoculation points, with the severity of take-all tending to increase with growth stage, and with exceptions (Fig. 1). The increase in take-all severity with growth stage at the inoculation point (0–5 cm segment) was strongest initially for wheat, followed by triticale. By GS 65, take-all severity in barley matched that in wheat, but had started to decrease in triticale and rye. Take-all symptoms were found in only 114 of the 2440 root samples assessed at distances beyond 30 cm from the inoculation points, but where present, severity was in most cases less than 5 %.

Ggt DNA

The concentration of *Ggt* DNA in the 5-cm segments of row generally decreased with increasing distance from the
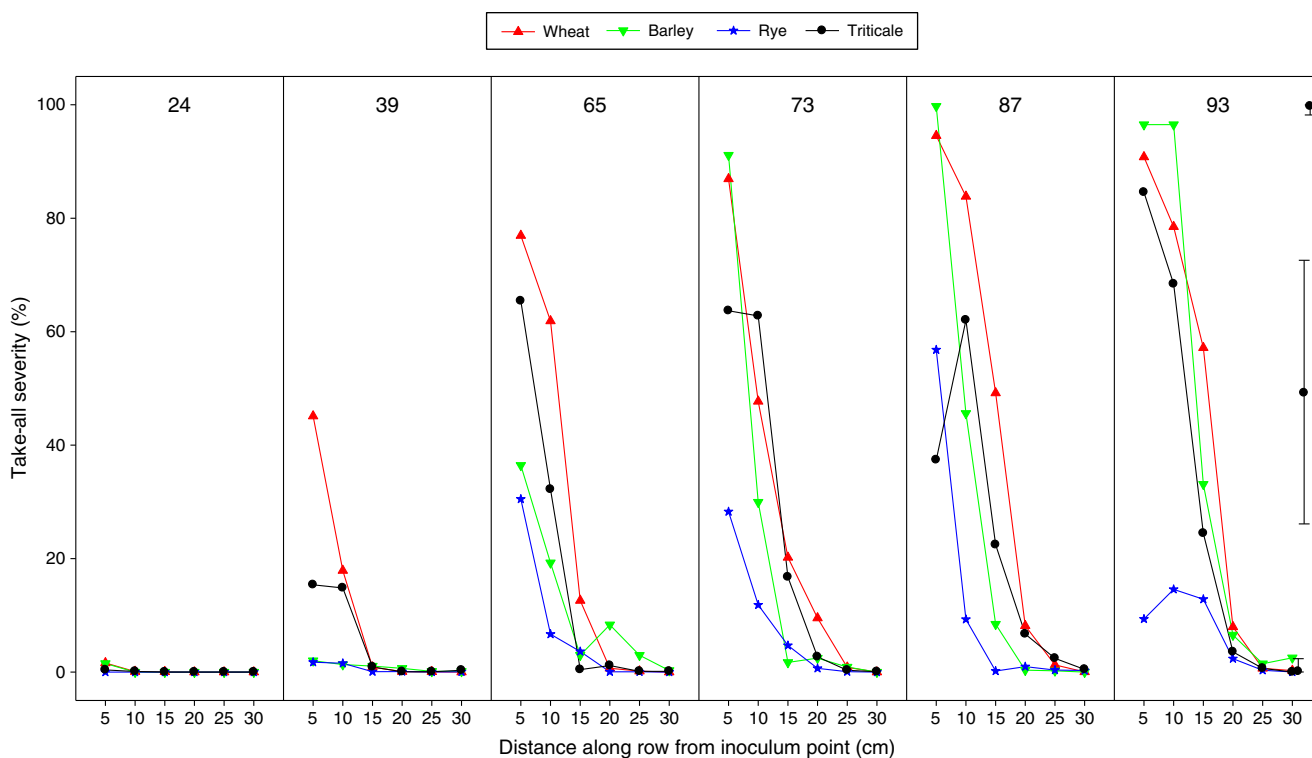
Fig. 1 Mean take-all severity in roots from each of the 5-cm root segments for wheat, barley, rye or triticale plants, at different growth stages, up to 30 cm from *Gaeumannomyces graminis* var. *tritici* inoculation points in the field trial, Lincoln, New Zealand 2012. *Error bars* show confidence limits for the smallest, largest and a mid-range of means

inoculum points, and increased with growth stage, but there was no consistent pattern of differences between the cereals or distance (cereal × growth-stage ×distance interaction; $p < 0.001$) (Fig. 2). *Ggt* concentrations in roots were greatest near the sources of inoculum, extending out to 30 cm along rows in rye at GS 24. At the later growth stages, *Ggt* in roots from the other cereals was present up to 60 cm from the inoculation points.

Increases in take-all severity in each 5-cm row segment within each 10-cm section correlated with increases in *Ggt* DNA concentration for quantities above 0.05 ng/mg root dry matter (Fig. 3). The DNA threshold for an increase in take-all severity with DNA concentration was consistent between the cereal species, at c. 1.0 ng/mg root dry matter for all except triticale, where the threshold was slightly lower. In rye, for roots with DNA above 1 ng/ mg root dry matter, expression of take-all symptoms tended to be lower for the same *Ggt* concentration than expression in the other cereals.

Fluorescent Pseudomonas

There was a strong cereal × time × *Ggt*-inoculation-point inter-action ($p < 0.001$), indicating that the pattern of change in numbers of fluorescent *Pseudomonas* with time varied both with cereal species and in the *Ggt*-inoculated/*Ggt*-free points (Fig. 4). *Pseudomonas* numbers were lowest for all four cereals

at the later growth stages, but prior to these, there was less consistency among the cereals. For all cereal species, the *Pseudomonas* populations were greater in the *Ggt*-inoculated than in the non-inoculated points until GS 73, when the popula-tions were similar in both points. For wheat and triticale, *Pseudomonas* numbers were greatest at GS 24 and declined at the other assessments. For rye and barley, the numbers of bacteria tended to increase from GS 24 to GS 65, then decline.

For each cereal species, there was no obvious relationship between estimated *Pseudomonas* numbers within 10–20 cm from the inoculation points and concentration of *Ggt* DNA (Fig. 5a). However, numbers of the bacteria were particularly high at the early cereal growth stages, when take-all severity was low (Fig. 5b).

Meteorological data

Mean seasonal temperatures for air and soil, and rain and irrigation, are presented in Table 2.

Glasshouse experiment 1

Allelopathic effects from the large amounts of oat material used as a carrier for the high *Ggt* inoculum treatments resulted in the poor establishment of plants sown in soil containing the high amounts of *Ggt* inoculum, so these data are not
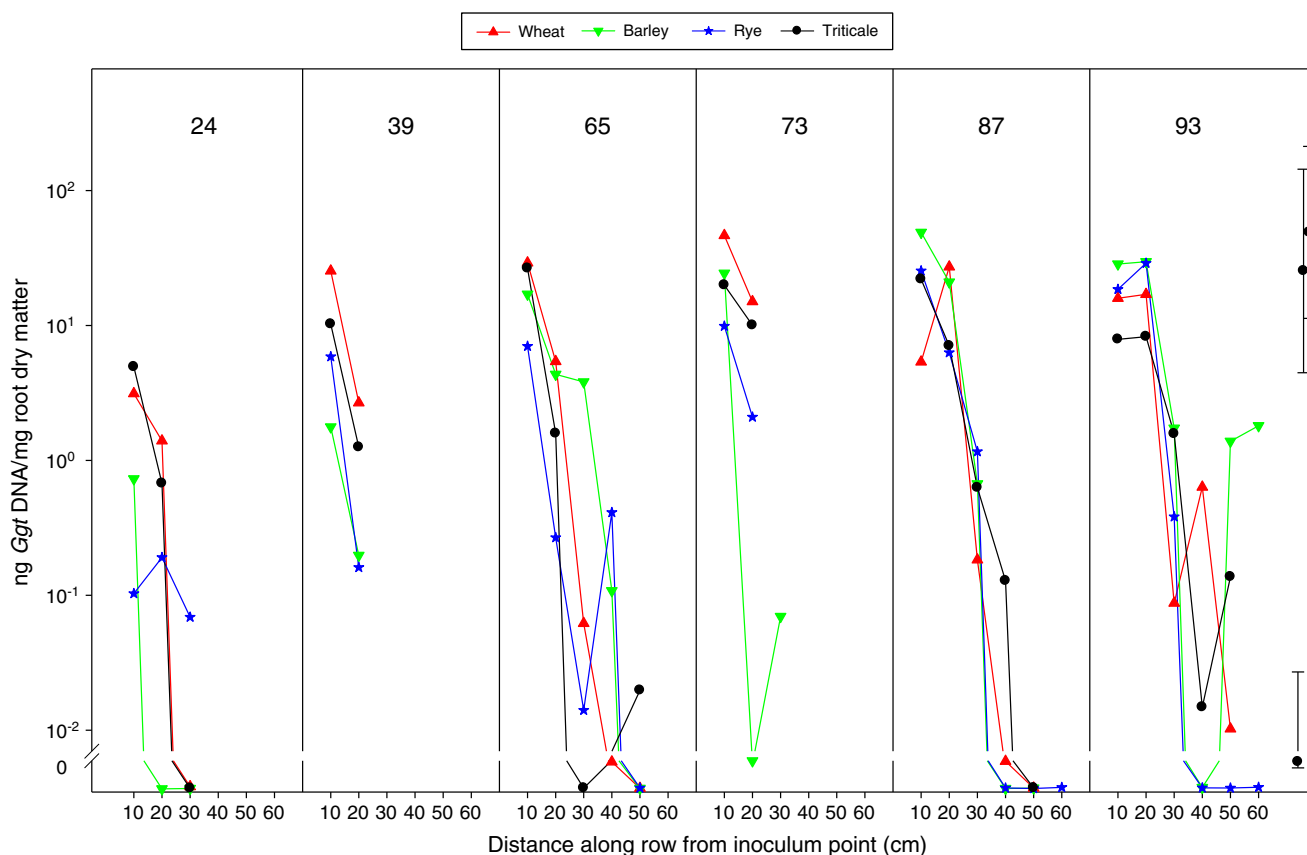
Fig. 2 Mean concentrations of *Gaeumannomyces graminis* var. *tritici* DNA in roots of wheat, barley, rye or triticale plants at different growth stages, for 5-cm segments within 10-cm sections down rows from inoculation points in the field trial, Lincoln, New Zealand 2012. *Error bars* show confidence limits for the smallest, largest and a mid-range of means

presented. Wheat and rye seeds sown into soil infected with low *Ggt* inoculum showed differences in the manner in which *Ggt* colonised the roots of the two species at the various growth stages (growth stage × cereal species interaction $p<0.001$).

In wheat, by GS 15, *Ggt* hyphae had colonised roots to a concentration of 103 ng DNA/mg dried roots, a concentration generally maintained in roots during the development of the plants and resulting in a root take-all severity of 14 % at all growth stages (Table 3). The number of roots per plant increased three-fold from GS 15 to GS 71 while the proportion of roots infected with *Ggt* remained similar for each growth stage, except for an uncharacteristic reduction at GS 30.

In rye, take-all severity was low during the early phases of growth, although runner hyphae of *Ggt* grew on root surfaces. Take-all severity was 2 % at GS 15, declining to negligible amounts until GS 65 (Table 3). Severity then increased to 8 % and at GS 71 to 50 % within large root masses. The number of roots per rye plant progressively increased from GS 15 to GS 71, while the proportion of roots infected with *Ggt* declined at GS 30 and 50 before increasing to an incidence of infection similar to wheat at the latter growth stages assessed. The concentration of extracted *Ggt* DNA mirrored the observed

severity of symptoms. At GS 15, the mean concentration was 15 ng DNA/mg dried root, decreasing to negligible concentrations until GS 50 (Table 3). Thereafter, there was rapid colonisation of roots by *Ggt*, reaching 280 ng DNA/mg dried root by the time of kernel development (GS 71). At that time, rye roots contributed approximately 200 times more *Ggt* DNA inoculum to the soil than wheat roots.

Glasshouse experiment 2

At both assessments, take-all severity on inoculated roots was consistently lower in rye than in wheat ($p<0.001$), with a consistent difference at each growth stage ($p=0.492$ and 0.708 for the growth stage by cereal interaction at the two assessments; $p<0.001$ for average changes with growth stage Table 4). Because the *Ggt* inoculum did not cause infection of the wheat control roots at GS 31 and GS 91, the data from those growth stages are not included in the results presented. Take-all severity 11 days after inoculation averaged 1.2 % in wheat and 0.2 % in rye. This increased in both species after 22 days, but was still greater in wheat (46 %) than in rye (24 %). The *Ggt* DNA concentration in wheat roots was similar to that in rye at all growth stages (Table 4).
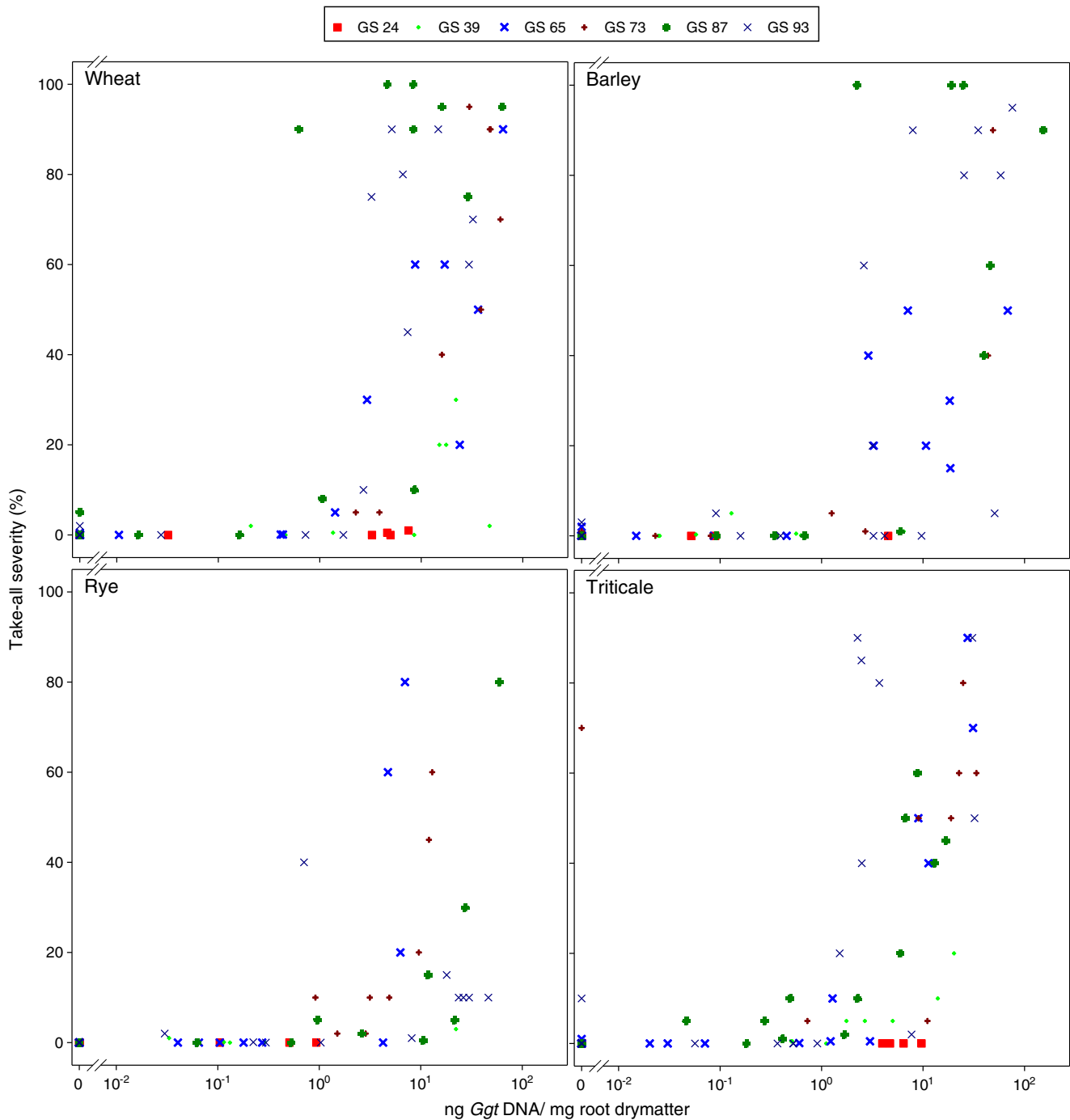
Fig. 3 Relationships between take-all severity and *Gaeumannomyces graminis* var. *tritici* DNA concentration in roots for wheat, barley rye or triticale at different growth stages in the field trial, Lincoln, New Zealand 2012

Discussion

Spread of take-all symptoms

In the field experiment, take-all symptoms were not detected beyond 30 cm from the inoculum sources. However, runner hyphae consistent with *Ggt* or *Phialophora* were observed on roots of seedlings (GS 24) of all cereal species out to the 120-

cm limit of assessment, but were more concentrated near the inoculum points. The lack of take-all symptoms distant from the inoculation points indicated that there was no background *Ggt* inoculum in the soil at the trial site, but that *Phialophora* was present. Pathogen spread was therefore assumed to be solely from the applied inoculum.

Ggt DNA was found 20 cm from the inoculation points in three of the species, and at 30 cm in rye at GS 24. At harvest,
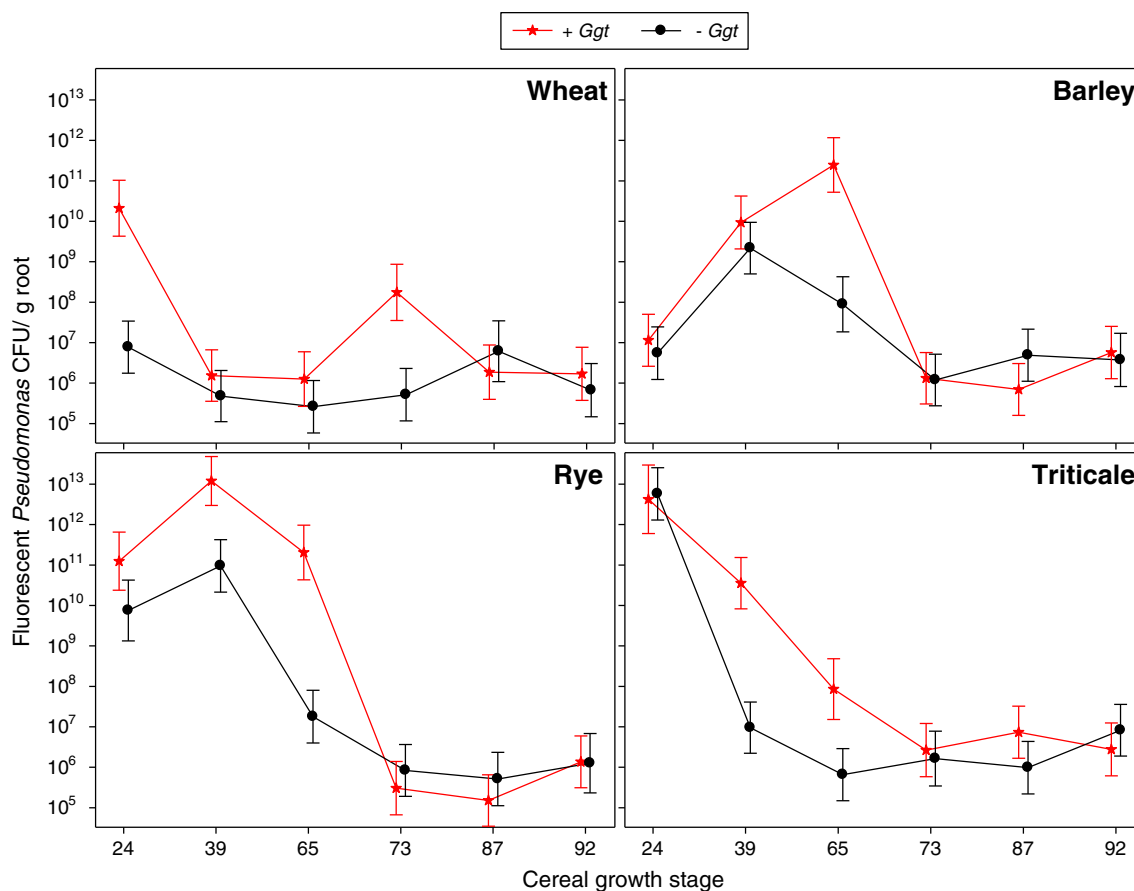
Fig. 4 Mean numbers of fluorescent *Pseudomonas* colony forming units (CFU)/g root within 10–20 cm from the *Gaeumannomyces graminis* var. *tritici* inoculation points (+*Ggt*) and away from the points (−*Ggt*) for four different cereals and different growth stage in the field trial, Lincoln, New Zealand 2012. *Error bars* are 95 % confidence limits

the amount of *Ggt* DNA was higher in those positions and there were low amounts of *Ggt* DNA out to 40 cm for wheat, rye and triticale species, and out to 60 cm for barley. Assuming an even rate of spread of *Ggt* along the rows on both directions from the inoculum point, these results show that *Ggt* inoculum could spread by 80 cm each year for wheat, rye and triticale, and 120 cm each year for barley. Although this rate of spread of take-all symptoms in wheat is in general agreement with the results of Willocquet et al. (2008), where take-all symptoms progressed steadily along roots of line-sown wheat up to 20 cm from the inoculation point, our results show that the inoculum can extend along the rows of cereals at least to 40 cm in either direction from point sources in a growing season, to provide large amounts of potential secondary inoculum to infect subsequent *Ggt*-susceptible crops.

Resistance to take-all

Take-all lesions were first observed in wheat, barley, and triticale seedling roots (GS24), and in wheat, barley, triticale, and rye at stem elongation (GS 39), intensifying in all species

at the later growth stages. Susceptibility to take-all was consistent with those reported previously (Hollins et al. 1986; Solel et al. 1990). Take-all severity in all cereal species was not strongly related to the concentration of *Ggt* in the roots and take-all symptoms occurred in all species when *Ggt* DNA exceeded 0.5 ng/g root dry matter (Fig. 3). However, take-all severity was lower in rye than in the other species for a given concentration of *Ggt* DNA in roots (Fig. 3).

The results for wheat and rye in the field experiment agree with those from the glasshouse Experiment 1, supporting the hypothesis that *Ggt* runner hyphae grow superficially on, and possibly within, rye roots before heading. The decline in *Ggt* concentrations after initial colonisation of roots may be related to the triggering of resistance in the rye host, or to its dilution by the fast-growing root system during the rapid growth of plants until head emergence (GS 50). Take-all symptoms were seldom observed in rye plants at these early growth stages, suggesting that the plants were resistant until GS 50. Since the number of roots per rye plant progressively increased during the early growth stages, while the proportion of roots infected with *Ggt* declined (Table 3), this decline in *Ggt* concentrations

Fig. 5 Relationships between
numbers of fluorescent
Pseudomonas colony forming
units (CFU) and
Gaeumannomyces graminis var.
tritici (*Ggt*) DNA concentration
(**a**) and take-all severity (**b**) in
roots of four different cereals
within 20 cm from *Ggt*
inoculation points in the field
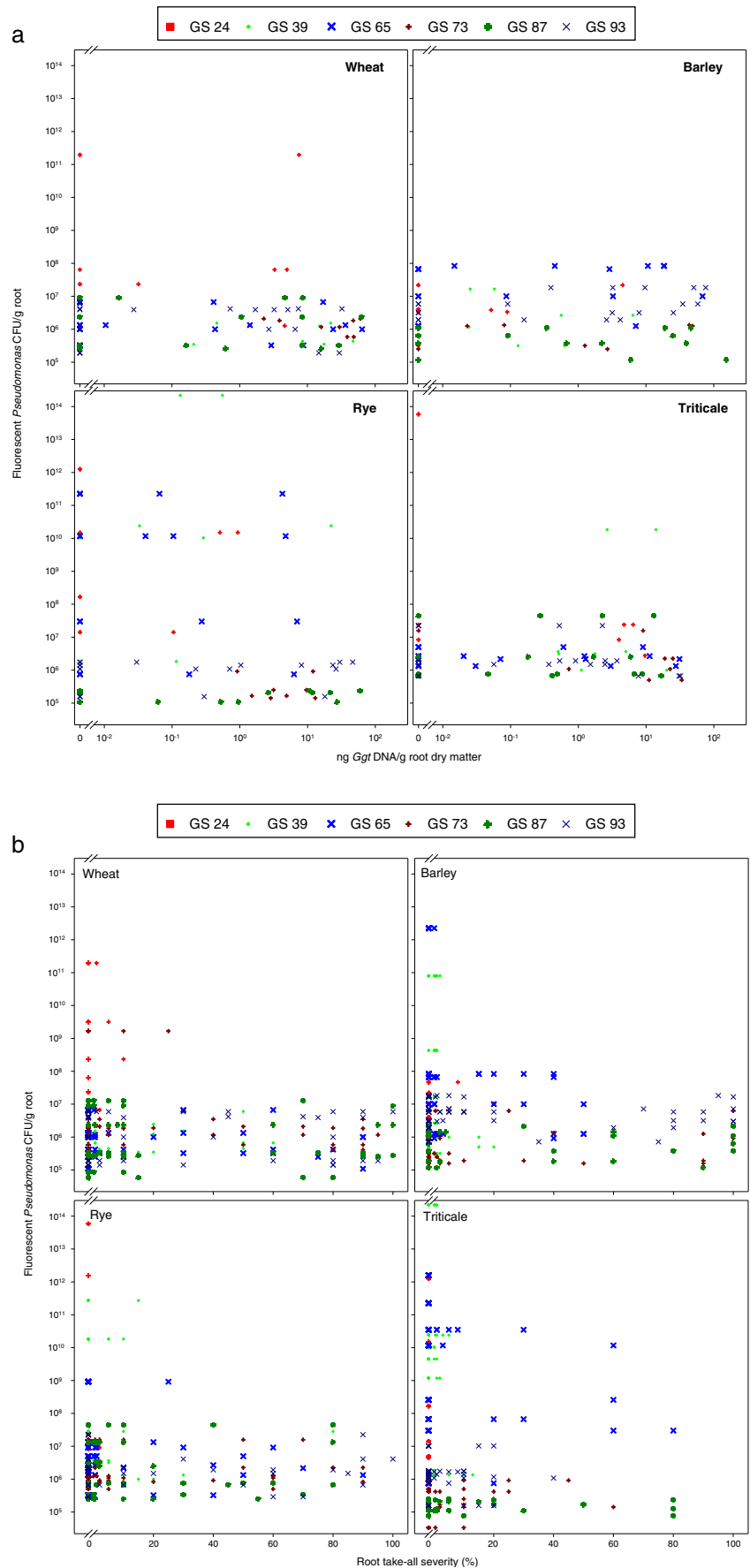trial, Lincoln, New Zealand 2012

Table 2 Mean seasonal air and soil temperatures, and rain plus irrigation, in the field trial, Lincoln, New Zealand 2012

Season	Month	Temperature (°C)		Rain + irrigation (mm)
		Air (min, max)	10-cm soil depth	
Autumn	May	9.0 (3.7, 14.4)	8.9 (5.8, 11.8)	9
Winter	Jun, Jul, Aug	7.0 (2.5, 11.5)	6.8 (2.5, 11.3)	225
Spring	Sep, Oct, Nov	10.7 (5.5, 16.1)	12.0 (6.3, 18)	172
Summer	Dec, Jan, Feb	16.6 (10.9, 22.3)	19.4 (13, 25.2)	123

after initial colonisation of roots appeared related to its dilution by the fast-growing root system. The development of disease symptoms from GS 65 was accompanied by a rapid increase in inoculum concentration, suggesting extensive internal colonisation of rye roots by *Ggt*. This occurred during anthesis and grain fill, while the plants were growing actively, suggesting that this adult plant susceptibility is not associated with the natural senescence that occurs as the grains mature. The rapid colonisation of rye roots by *Ggt* at later growth stages occurred as the numbers of roots and the root systems increased, resulting in exponential increases in inoculum quantities during grain fill. On a whole-plant basis, *Ggt* concentrations in rye roots increased 165-fold between GS 50 and GS 65 and 5-fold between GS 65 and GS 71, potentially leaving large amounts of inoculum in the soil to infect subsequent *Ggt*-susceptible cereal crops. In contrast, *Ggt* colonised the roots of wheat in the early growth stages and subsequently maintained stable *Ggt* concentrations and disease severities through to the final assessment. Deacon and Henry (1980) also observed similar responses in wheat and barley. Our results indicate that this occurred also in rye. In glasshouse Experiment 1, the greater colonisation of roots along with the more extensive root system in rye than in

wheat could result in rye plants subsequently contributing almost 200 times more *Ggt* inoculum to the soil than wheat plants. Sheng and Hunt (1991) also found that rye root growth was greater after anthesis than growth of wheat or triticale. The comparative root masses from the different cereal species could not be ascertained from the field experiment, as a significant amount of root material may have remained in the soil during harvesting of the plants

As shown in glasshouse Experiment 2, inoculation of roots of wheat and rye plants with actively growing hyphae resulted in more take-all in roots of wheat than in rye at each of the four growth stages tested. In wheat roots, take-all symptoms were observed within 11 days of inoculation, and increased with time. In rye, at all growth stages, roots were initially resistant to early colonisation from *Ggt* runner hyphae, but lesions had developed by 22 days after inoculation and resistance was overcome. This suggests that the breakdown of resistance in rye to *Ggt* after heading observed in glasshouse Experiment 1 was because *Ggt* required an establishment phase to become pathogenic, more so than the alternative hypothesis that the growth stage of plants (adult plant susceptibility) made them more susceptible to infection.

Table 3 For each growth stage (GS) and days after sowing (DAS), mean root number and masses, and amounts of *Gaeumannomyces graminis* var. *tritici* (*Ggt*) DNA and take-all severity in roots of wheat or rye plants (95 % confidence limits) in glasshouse experiment 1, Lincoln, New Zealand 2010

GS	DAS	Root dry matter (mg)		Roots (N)	Proportion of roots with *Ggt*	*Ggt* DNA (ng/mg dried root)	Take-all severity (%)
		Untreated	*Ggt*-treated	*Ggt*-treated	*Ggt*-treated	*Ggt*-treated	*Ggt*-treated
Wheat							
15	23	16 (9,28)	18 (10,30)	6.8 (3.9,11.7)	35.7 (15.3,63.2)	104 (39,274)	15.9 (4.5,43.1)
30	43	221 (128,384)	77 (44,133)	12.5 (8.0,19.6)	14.6 (5.1,35.2)	27 (10,73)	4.6 (1.1,17.1)
50	46	206 (119,357)	120 (69,207)	13.7 (9.0,20.9)	34.8 (15.4,60.8)	86 (33,228)	14.0 (3.5,42.2)
65	50	199 (115,344)	212 (122,367)	15.4 (10.3,22.8)	32.6 (13.8,59.3)	27 (10,72)	13.7 (3.6,40.8)
71	65	268 (154,464)	333 (192,578)	22.0 (15.1,32.1)	33.0 (13.8,60.4)	22 (8,59)	15.9 (4,45)
Rye							
15	23	42 (24,73)	28 (16,49)	7.9 (4.8,12.9)	16.1 (5.6,38.5)	15 (6,39)	2.4 (0.5,9.9)
30	49	484 (269,872)	437 (252,757)	24.8 (17.3,35.6)	2.4 (0.7,8.4)	1 (0,6)	0.4 (0.1,2.8)
50	65	731 (435,1228)	1039 (599,1800)	29.7 (21.4,41.4)	4.4 (1.4,13.1)	2 (1,6)	0.2 (0.0,2.0)
65	123	6803 (3926,11788)	5289 (3052,9165)	115.8 (87.9,152.4)	26.8 (11.6,50.4)	89 (34,233)	8.4 (2.0,28.8)
71	156	5081 (2932,8804)	4996 (2883,8657)	146.0 (111.6,191.0)	38.5 (18.3,63.6)	281 (107,736)	49.7 (20.1,79.6)

Table 4 Mean take-all severity and amounts of *Gaeumannomyces graminis* var. *tritici* (*Ggt*) DNA in seminal roots of wheat or rye plants after inoculation with agar plugs containing *Ggt* hyphae (95 % confidence limits), in glasshouse experiment 2, Lincoln, New Zealand 2010

Growth stage	Take-all severity (%)		*Ggt* DNA (ng/mg dried root)
	14 d	28 d	28 d
Wheat			
15	8.5 (4.2, 16.6)	53.6 (31.8, 74.1)	239.0 (60.3, 947.3)
41	6.4 (3.1, 12.7)	47.4 (25.6, 70.3)	184.9 (46.7, 733.1)
51	3.3 (1.5, 7.0)	18.2 (8.0, 36.1)	64.7 (16.3, 256.8)
71	9.8 (4.8, 18.9)	62.8 (41.3, 80.2)	258.6 (65.2, 1025.1)
Rye			
15	0.6 (0.2, 1.7)	32.8 (16.9, 53.9)	34.2 (8.6, 136.3)
41	0.7 (0.2, 2.4)	20.3 (8.6, 40.6)	203.0 (51.2, 804.8)
51	1.0 (0.4, 2.8)	14.6 (6.3, 30.3)	131.9 (33.2, 523.1)
71	1.1 (0.4, 2.8)	29.4 (14.0, 51.6)	156.0 (39.3, 618.8)

Pseudomonas

In the field experiment, during the early growth stages populations of fluorescent *Pseudomonas* in plant rhizospheres were generally greater on roots of all cereal species nearer to the *Ggt* inoculum sources than in inoculum-free soil (Fig. 4). This indicates that the bacteria favoured the rhizospheres in the presence of *Ggt*. After kernel and milk development (GS 73), however, the differences in the *Pseudomonas* populations between inoculated and non-inoculated sites disappeared, coinciding with the peak of *Ggt* DNA concentration in roots close to the inoculum points (Fig. 2). This, and the poor relationship between populations of fluorescent *Pseudomonas* and concentrations of *Ggt* in the roots in the field experiment (Fig. 5a), suggest that these bacteria were not sufficiently antagonistic to *Ggt*, or not in sufficient quantities, to reduce the population of *Ggt* or expression of take-all symptoms. Only strains producing the antibiotic 2,4-diacetylphloroglucinol (DAPG) at populations above 10^5 CFU/g root are inhibitory to *Ggt* (Kwak and Weller 2013). In addition, the build-up of fluorescent *Pseudomonas* in resident soil populations that produce antibiotics capable of suppression of *Ggt* is cultivar-specific (Mazzola et al. 2004; Okubara and Bonsall 2008). Although the *Pseudomonas* populations in the current study varied amongst cereal species (Fig. 4), this factor alone may be insufficient to select antagonistic *Pseudomonas* populations, as successive years of cropping with the same cereal species are required for the selection and proliferation of DAPG-producing *Pseudomonas* associated with take-all decline. The existence and distribution of DAPG-producing *Pseudomonas* strains in New Zealand has not yet been ascertained.

Management strategies to mitigate take-all

Results here explain the results of a previous field trial (Bithell et al. 2011) and crop surveys, which frequently showed high inoculum concentrations after crops of rye, even though take-all symptoms had not been observed in the rye crops, and severe take-all symptoms occurred in wheat crops that followed the rye. The intrinsic resistance to *Ggt* in rye roots can break down late in the growing cycle, allowing *Ggt* to proliferate. In spite of its resistance to take-all during its early growth stages, rye should not be regarded as a break crop in wheat rotations as the amount of *Ggt* inoculum in the soil is likely to be high.

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