Pathogenicity of *Gaeumannomyces graminis* var. *tritici* increased by nitrogen applied to soil to enhance the decomposition rate of wheat residues

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Abstract Soil cores removed after harvest of a wheat crop infected with the fungus, *Gaeumannomyces graminis* var. *tritici* (*Ggt*), were amended with nitrogen and fungal saprophytes to increase decay of crop residues and subsequently reduce soil inoculum. The cores were treated with one application of 50 kg nitrogen (N) per ha, *Trichoderma* strains, or both. Cores were assessed 0, 2, 4 and 7 months after harvest. At 7 months, the crop residues had decayed to a third of their original mass, with the decay not influenced by the treatments. DNA analysis confirmed *Ggt* DNA was present in the stubble stems, crowns and roots. The pathogenicity of *Ggt* was increased by N, as shown by a 5 to 8-fold increase in take-all severity in indicator wheat seedlings planted in the N-treated cores 2 to 4 months after harvest, compared with those without N. *Ggt* remained viable in all treatments to infect wheat seedlings 7 months after harvest.

Keywords soil-borne disease, wheat, take-all, inoculum decline, *Trichoderma*.

INTRODUCTION

Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is the most important root disease of wheat (*Triticum aestivum* L.) worldwide (Hornby et al. 1998), resulting in blackened roots, and stunting and premature death of affected plants (Cook 2003). Between crops, *Ggt* survives saprophytically on crop residues, and inoculum concentrations decline as the residue decays (Hornby 1981). The amount of inoculum present in the soil when the crop is seeded affects the extent of primary infection of a susceptible host cereal such as wheat (Bailey & Gilligan 1999). Non-host break crops provide an important means of reducing *Ggt* concentrations (Hornby 1981) and their inclusion in crop rotations is an effective method for take-all management (Yarham 1981). In New Zealand, *Ggt* concentrations in the soil were reduced proportionally to the length of non-host break crops (van Toor et al. 2013), declining by 78% over 4 months following the wheat harvest (Bithell et al. 2009), but still potentially containing viable inoculum

7 months after the harvest (van Toor et al. 2016).

The rate of inoculum decline is related to the decomposition of cereal residues, which in turn are affected by air temperature, soil moisture, initial soil nitrogen (N) content and soil-borne biological activity (Douglas & Rickman 1992). The amount of respiration in the soil is indicative of biological activity. Cookson et al. (1998) found the substrate-induced respiration rate at 28 days of decay accurately predicted residue mass-loss over the first 90 days of decay. Decay rates of crop residues can be enhanced by earthworm activity, with consequent decline in *Ggt* inoculum (Hume et al. 2015), and with the addition to soil of N and fungal saprophytes. For example, degradation of the crop residues was increased with urea-N and *Trichoderma* (Sharma et al. 2012).

The present study investigated whether the addition of N and saprophytic *Trichoderma* sp. changed the rate of decomposition of wheat residues in soil cores removed from a *Ggt*-infected wheat crop. The position of *Ggt* inoculum within the crop residues in the cores at harvest and the rate of decay within the various components of the plant stems and root systems were determined in the 7 months following harvest. Earthworm population density and soil conditions were also recorded. The results were intended to provide information on methods for minimising the *Ggt* inoculum concentration in soils between cereal crops susceptible to take-all.

MATERIALS AND METHODS

Treatments

Soil cores were obtained from plots in a second-year wheat cultivar trial carried out within a commercial wheat crop. The soil had been inoculated at sowing with oat grains containing pathogenic *Ggt* isolates (Chng et al. 2005). Consequently, the cores contained residues infected with natural and known pathogenic strains of *Ggt*. Wheat in the trial was harvested on 20 February 2014, before the surrounding wheat crop was harvested on 20 March by the grower. On 5 March, four soil cores, 10 cm diameter and 15 cm deep, were removed from four replicate plots containing the wheat cultivar 'Wakanui'. A split-hinge soil corer was used to extract the cores (Kain & Young 1975). Each core was placed into a 1.5 L capacity black plastic plant pot (tapered 132 to 100 mm internal diameter, and 140 mm tall).

In the laboratory, the gaps between the core and pot were filled with coarse sand, and the pots randomly assigned to treatments of 1) untreated control, 2) nitrogen (N), 3) *Trichoderma* and 4) N plus *Trichoderma*. Sixteen pots per treatment were prepared, with four per treatment randomly assigned to be assessed at each of 0, 2, 4 and 7 months after harvest. For cores used for treatments 2 or 4, urea granules were broadcast onto each core top at the equivalent of 50 N kg/ha. The 'Trichoderma' treatment comprised *Trichoderma hamatum* (strain LU1301) and *Trichoderma* sp. "*novaeharzianum*" (strain LU1328). The strains were isolated from surface-disinfected roots of *Pittosporum* sp. and *Carex* sp. in Mid-Canterbury. The conidia were harvested from sporulating colonies grown on potato dextrose agar plates at 25°C, 12:12 light:dark, and applied at 1×10^9 conidia/m^2 in solution in equal proportions to cores for Treatments 3 and 4.

The four cores for each treatment allocated to Time 0 were retained for assessment. The remaining 48 cores were transferred to a grid of prepared holes in a pasture field at Plant & Food Research, Lincoln, Canterbury. Inserting the pots into the soil retained natural moisture and ambient soil temperature in the cores. The cores were arranged according to a randomised resolvable row-column design. Other cores pertaining to treatments not discussed here were laid out as part of the design.

Two untreated, and two *Trichoderma*-treated cores in the trial were monitored continually for temperature and soil moisture. Cores allocated to 0, 2, 4 and 7 months after harvest were assessed, respectively, on 5 March, 6 May, 23 June and 16 September 2014, being 2, 11, 18 and 30 weeks after application of treatments. For each assessment, the cores were assessed for mineral N, *Ggt* infectivity, stubble content and the number and species of earthworms.

ASSESSMENTS

Mineral nitrogen

A 14-mm diameter by 100-mm deep soil sample was removed 2-3 cm from the centre of each core and the cores were stored at 4°C in seal-lock plastic bags. The following day mineral N was extracted for 1 h from a 5-g subsample from each core, by shaking with 25 mL of 2M KCl solution for 1 h. Ammonium (NH_4-N) and nitrate (NO_3-N) in the KCl extracts were determined by standard colourimetric methods using a Lachat QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, Loveland, Colorado, USA) (Keeney & Nelson 1982; Harbridge 2007a; Harbridge 2007b).

Ggt infectivity

The wheat seedling bioassay used followed that described by Chng et al. (2004). Eight germinated seeds of the wheat 'Conquest' were sown into each core in a circle 30 mm from the centre of the core and equidistant from one another, at a depth of 20 mm. The cores were watered daily in a glasshouse set at 22°C. After 14 days, the established seedlings were assessed for the area of take-all (% severity).

Residue dissection

After wheat seedlings for the *Ggt* infectivity assessment had been removed from the cores, the stubble was separated from the soil and spray-washed over a sieve of 1.0 mm mesh. The number of plants contributing to the stubble was counted, and the stubble was then partitioned into stems trimmed to 50 mm from the crown, and the crown, large roots (>1 mm diam.) and thin roots (<1 mm diam.). The stubble parts were dried at 40°C for 72 h then separately weighed. Stubble parts from the untreated controls were frozen at –20°C until analysis for *Ggt* DNA.

Ggt DNA quantification

Roots were prepared for quantitative polymerase chain reaction (qPCR) assessments. Each dried root sample was ground in liquid nitrogen (N) using a mortar and pestle. DNA was extracted from a ≈20 mg sub-sample of the ground roots using a DNeasy Plant Minikit (Qiagen, New Zealand) following the manufacturer's instructions. The extracted DNA was suspended in 200 µL of TE manufacturer's buffer (pH 8.0).

The amount of *Ggt* DNA in each sample was quantified using the qPCR method described by Keenan et al. (2015). PCR amplifications were carried out in a CFX96 Real-Time System (BioRad). A Sybr green-based qPCR with primers Pot25SF and Pot25SR identified the samples containing inhibitors likely to affect detection of *Ggt* DNA, and these samples were omitted from the *Ggt* DNA analyses. GgtProbe 1 and primers GgtEF1 and GgtEFR1 targeting the translation elongation factor 1-alpha (EF1-α) gene from *Ggt* measured the amount of *Ggt* DNA in the cereal roots, the quantity of which was estimated using a standard curve constructed using standard regression of the cycle threshold (Ct) values against the $\log_{10}$ of the initial concentration. An undetected Ct value was assumed to mean the sample contained no *Ggt* DNA.

Earthworms

When the stubble was extracted from each soil core at each assessment time, earthworms were also removed and identified to species level using identification guides as in Schon (2016).

Soil conditions

For the period the soil cores were in the field, rainfall was accessed from NIWA's Broadfield weather station at Lincoln, Canterbury (National Institute of Water and Atmospheric Research (NIWA) 2016), within 1 km from the experiment site. Soil temperature and volumetric moisture content in four cores were recorded at 30-min intervals using a Decagon EM50 data collection system (Decagon Devices) connected to 5TM sensors. The sensors were inserted into the cores, 5 cm below the core top.

Statistical analyses

Stubble dry matter data were divided by the number of plants counted per pot, then

transformed using log transformations. These, and mineral-N were analysed with standard analysis of variance. All other data were initially analysed with a hierarchical generalized linear model approach (HGLM, Lee et al. 2006). Treatments and Time were included as fixed effects, and replicate and other potentially important layout factors as random effects The importance of the random effects were assessed with a X^2 test of the change in likelihood on dropping the effect, as implemented in GenStat's HGRTEST procedure. Fixed effects were assessed similarly, using GenStat's HGFTEST procedure. Only important random effects were retained in the final analyses. Percentage take-all severity data were analysed with a binomial-beta HGLM with fixed effects as binomial, and random effects with a beta distribution, both with a logit link. No random effects were found to be important, so none was included in the final analysis. However, the variation between samples increased with the treatment means, therefore a separate dispersion was estimated for each treatment using the Joint GLM procedure (Lee et al. 2006). Numbers of earthworms and plants per core found in residues were initially analysed with a Poisson-gamma HGLM, with logarithmic links. However, none of the potential random effects was found to be important, so these were ignored in the final analysis, which became a simple Poisson generalized linear model. For qPCR data, Ct values were first converted into quantities using parameters obtained from a standard linear regression values for the standards on the $\log_{10}$ quantities. Where the qPCR result was undetermined, the quantity was fixed at 0. Since DNA quantities are essentially counts multiplied by a molecular weight, the individual data were analysed using a Poisson-gamma HGLM with log links, a method appropriate for the analysis of counts. Fixed effects were Time and Stubble-part and their interaction. Random effects found to be important were Block (replicates), Pots, and Plant-parts within Pots. The

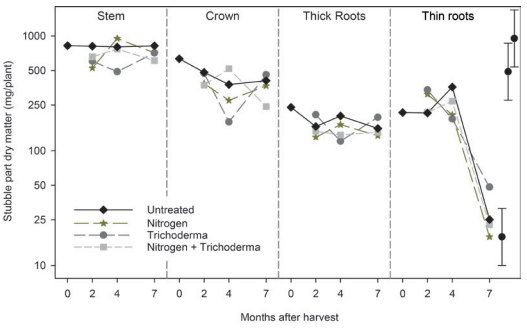

Figure 1 Mean dry matter (mg) of 0–50 mm-length stems, crowns, and thick (>1 mm diam.) and thin (<1 mm diam.) root parts of 'Conquest' wheat stubble per plant infected with *Gaeumannomyces graminis* var. *tritici* for different experimental treatments at 0, 2, 4 or 7 months after harvest in 2014. Error bars are 95% confidence intervals for selected means (lowest, highest and a mid-range mean).

analysis included an offset (McCullagh & Nelder 1989) to adjust the results to be DNA concentrations (ng/mg root DNA). The offset used was:

$$\log\left(\frac{qPCRSampleWt \times Dilution \times No.Plants}{EVol \times WtDM}\right)$$

where *EVol* is the volume into which the extracted DNA was suspended, *qPCRSampleWt* is the weight of the sample used for DNA extraction, and *WtDM* is the dry weight of the plant part from which the extraction sample was taken. Predictions (means) were made for an offset of zero.

All analyses were carried out with GenStat. Means were obtained on the transformed (or link) scale along with 95% confidence limits. These were back-transformed for presentation.

RESULTS

Wheat residues

Numbers of plants found in the stubble per core varied from 1 to 5 (mean 2.24). Treatment by time means varied between 1 and 3.5, but

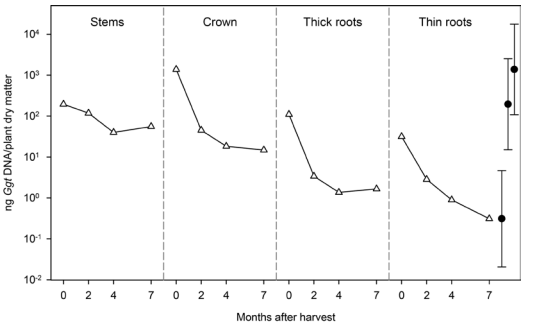

Figure 2 Mean quantities of *Gaeumannomyces graminis* var. *tritici* in 0–50 mm-length stems, crowns, and thick (>1 mm diam.) and thin (<1 mm diam.) roots of 'Conquest' wheat stubble in untreated soil cores 0, 2, 4 or 7 months after harvest in 2014. Error bars are 95% confidence intervals for selected means (lowest, highest and a mid-range mean).

this variation was not statistically significant (P=0.886). The weights of stubble parts per plant changed significantly with time (P<0.001), with the greatest degradation over 7 months occurring for thin roots, followed by thick roots and crowns (Figure 1). Treatment effects were relatively minor (P>0.05). The mean dry matter of the total stubble at harvest reduced from 1965 to 1365 mg/plant after 7 months; that of the aerial stems from 824 to 714 mg/plant and that of the buried tissue from 1087 to 555 mg/plant.

Ggt DNA

DNA analysis confirmed *Ggt* was present in the stubble. The amount of *Ggt* DNA in the parts of untreated stubble changed with time (P<0.001) (Figure 2). At harvest, most of the inoculum (mean = 1385 ng *Ggt* DNA/plant) resided in the crowns, with 32 to 195 ng *Ggt* DNA/plant in the other parts. After 7 months and per plant, 55 ng *Ggt* DNA per g tissue remained in the stem portion, 15 ng in the crown, 1.7 ng in the thick roots and to 0.3 ng in the thin roots.

Ggt infectivity

Ggt infection was detected in wheat seedling roots at 7 months after harvest in all treatments. Take-all severity in wheat seedling roots varied significantly with Time (P<0.001), with the changes over time varying between the treatments (P<0.001 for the time-by-treatment interaction). In roots of seedlings 2 months after harvest, take-all severity was greatest in soil cores treated with N alone. However, after 4 months, severity was greatest in roots of seedlings planted in cores treated with N, with or without *Trichoderma* (Table 1). By 7 months, take-all severity was similar in all four treatments.

Nitrogen

The concentration of mineral N declined from 12.6 µg/g soil at harvest to a mean of 3.4 µg/g soil at the later assessments (P<0.001), with no significant treatment effects (P>0.3 for all effects involving treatment; Table 2).

Earthworms

The numbers of earthworms did not vary strongly between the four treatments (P>0.1 for the main effect and interaction with time), but they did vary over time (P=0.023) (Table 3). No earthworms were found in the cores at harvest. After 2 months, earthworms had emerged from eggs in the cores or had migrated from the adjacent pasture into the cores, with their densities greatest in cores without the *Trichoderma* treatment. Seven months after harvest, earthworm population densities had declined to an average of 30/m^2 over all treatments. The earthworm population was dominated (96%) by *Aporrectodea caliginosa*, with the remaining 4% represented by *A. trapezoides*.

Soil conditions

Mean monthly rainfall, and temperatures and moisture in the soil cores, for the period of the experiment, are shown in Table 4.

DISCUSSION

These results suggest that cereals sown in soils

Table 1 Take-all severity (mean % of plant root area with take-all) at times 0, 2, 4 or 7 months after harvest in 2014, in roots of 'Conquest' wheat seedlings grown in soil cores containing *Gaeumannomyces graminis* var. *tritici*-infected wheat stubble and treated at harvest with 50 kg N/ha, *Trichoderma* conidia, or both (95% Confidence Limits).

Time	Untreated	Nitrogen (N)	*Trichoderma*	N + *Trichoderma*
0	3.1 (0.9,10.3)	-	-	-
2	1.3 (0.2,7.9)	5.0 (1.1,20.1)	0.1 (0.0,0.4)	0.0 (0.0,0.2)
4	0.2 (0.1,1.0)	7.8 (1.2,37.5)	1.2 (0.5,3.0)	5.3 (1.6,15.7)
7	4.3 (0.8,19.1)	1.9 (0.7,5.2)	2.9 (0.6,13.0)	1.8 (0.4,7.3)

Table 2 Available mineral nitrogen (N) in soil cores treated with 50 kg N/ha, *Trichoderma* conidia, or both, at harvest in 2014 of 'Conquest' wheat seedlings, and assessed at 0, 2, 4 or 7 months after harvest (95% confidence limits).

Time	Untreated	Nitrogen (N)	*Trichoderma*	N + *Trichoderma*
0	12.6 (6.9,22.8)	-	-	-
2	3.9 (2.1,7.1)	3.1 (1.7,5.7)	5.5 (3.0,9.9)	3.9 (2.2,7.1)
4	3.5 (1.9,6.3)	3.2 (1.7,5.7)	3.3 (1.8,6.0)	2.9 (1.6,5.2)
7	3.9 (2.2,7.1)	3.6 (2.0,6.5)	2.6 (1.4,4.7)	2.7 (1.5,4.9)

Table 3 Number of earthworms/m^2 (95% confidence limits) at times 0, 2, 4 or 7 months after harvest in 2014 of 'Conquest' wheat seedlings in soil cores containing *Gaeumannomyces graminis* var. *tritici*-infected wheat stubble and treated at harvest with 50 kg N/ha, *Trichoderma* conidia or both.

Months	Untreated	Nitrogen (N)	*Trichoderma*	N + *Trichoderma*
0	0.0 (0.0,*)	-	-	-
2	159.2 (65.4,387.6)	191.0 (84.8,430.4)	63.7 (15.6,260.0)	63.7 (15.6,260.0)
4	159.2 (65.4,387.6)	63.7 (15.6,260.0)	95.5 (30.3,301.3)	95.5 (30.3,301.3)
7	31.8 (4.4,232.9)	31.8 (4.4,232.9)	0.0 (0.0,*)	63.7 (15.6,260.0)

** Upper confidence limit for 0 cannot easily be obtained.*

Table 4 Mean monthly rainfall recorded (NIWA) nearby, and soil temperature and moisture (min, max) measured in soil cores, for the period covering the assessment times of 0, 2, 4 or 7 months after harvest of 'Conquest' wheat seedlings in 2014.

Assessments		Monthly weather data			
Months	Date	Month	Rainfall mm	Soil temp °C	Soil moisture
0	5 Mar	Mar	121.2	16.6 (8.4, 48.4)	13.9 (8.0, 24.7)
		Apr	161.2	13.8 (5.9, 24.6)	17.3 (12.3, 30.5)
2	6 May	May	44.6	9.6 (1.9, 16.0)	18.1 (15.7, 27.4)
		Jun	45.2	7.9 (2.0, 12.3)	18.6 (17.3, 25.2)
4	23 Jun	Jul	49.0	6.6 (1.9, 12.4)	19.4 (17.1, 29.0)
		Aug	15.2	7.8 (2.4, 14.2)	16.9 (14.9, 21.8)
7	16 Sep	Sep	24.6	11.0 (6.8, 17.8)	14.0 (12.3, 16.0)

containing *Ggt*-infected residues would still be susceptible to infection if sown 7 months after the harvest of a previous crop. This finding concurs with Bithell et al. (2009) who observed 22% of *Ggt* concentrations in the soil following second wheat crops remained 4 months after harvest. As indicated by DNA analysis of untreated stubble, most of the *Ggt* inoculum in stubble after 7 months was in the 50-mm length of stems above the crowns and in the crowns, having declined to negligible amounts in the roots. The rate of decomposition of the stubble was neither increased by a single application of N, nor by *Trichoderma* alone nor with N applied shortly after the crop had been harvested. Instead, the N treatments enhanced the pathogenicity of *Ggt* temporarily, as indicated by the increase in take-all severity in roots of indicative wheat seedlings 2–4 months after harvest. This may have been because the N stimulated hydrolytic enzyme production by *Ggt*, such as glucanase (Yu et al. 2007), increasing the pathogen's ability to penetrate wheat roots.

The rates of decomposition of crop residues were similar to those reported elsewhere. Weights of stems (straw) declined by 13% of their original weight over the 7 months on the soil surface, similar to that reported under similar conditions by Curtin et al. (2008). Weights of the buried parts of the stubble declined by 49% over this period, with a mean soil temperature at 50 mm deep of 10°C, in agreement with Bauer et al. (2008) who recorded 40% of the carbon in buried wheat residues being mineralised after 170 d at 9°C.

These normal decomposition rates occurred despite conditions in the cores being below optimum for residue mineralisation. Earthworms contribute to residue degradation (Hume et al. 2015), but they only colonised the soil cores initially devoid of earthworms to about a third of the earthworm population of 600/m^2 typically found in mixed cropping soils in Canterbury (Fraser et al. 1996). The mean monthly moisture content of the cores at the 50-mm depth of 17% (Table 4) was also below the optimum of 25%, as estimated from the maximum moisture recorded of 30% (H.E. Brown, Plant & Food Ltd, personal communication). The concentration of mineral N in the cores after 2 months of 3.4 µg/g soil (Table 2) was also less than expected in fallowed cropping soil (Fraser et al. 2013), which may be explained by N leaching, exacerbated by the high monthly rainfall at the start of the experiment (Table 4). These factors may explain why no increase was observed in the decomposition rates that resulted from the *Trichoderma* sp. treatment.

This study did not provide evidence that an application of N or a known fungal saprophyte could increase decay rates of wheat stubble infected with *Ggt*. However, it did show that the pathogenicity of *Ggt* may be increased with additional N, thereby increasing the risk of take-all in susceptible cereal crops sown within 2–4 months following a *Ggt*-infected host crop.

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