Wheat take-all: lessons learned during a search for effective biological control

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Abstract Take-all (causal agent *Gaeumannomyces graminis* var. *tritici*) is one of the most important soil-borne diseases of wheat. Greenhouse screening of microorganisms for disease suppression was conducted, using a targeted approach that focused on the fungal genus *Trichoderma*. In spite of indications of disease suppression in preliminary assays, effective biocontrol was not observed in subsequent tests. Explanations for this apparent loss of disease suppression could include insufficient numbers of potential biocontrol agents screened during the selection process or the issue of false-positive (type 1 error) results.

Keywords take-all, *Trichoderma*, consistency, screening.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a key component of New Zealand grain production, with 450 to 500 Mt grown per annum (value of \$367-413/t depending on the purpose; United Wheatgrowers NZ Ltd.). Take-all (caused by *Gaeumannomyces graminis* var. *tritici* Walker (Ggt)) is considered the most important soil-borne disease of wheat and other cereals globally (Cook 2003). When the disease is most severe, it can result in total crop failure. Until recently, control of take-all with fungicides was not economical, but two fungicides (fluquinconazole and silthiofam), recently made commercially available, have demonstrated economically viable levels of disease suppression when applied as seed treatments, although resistance to silthiofam has already been reported (Bateman et al. 2008). To alleviate effects of take-all, some growers take advantage of the biological phenomenon take-all decline (TAD), where wheat monoculture results in reduced take-all severity due to the

accumulation of antagonistic microorganisms. This phenomenon has also resulted in take-all being one of the most studied pathosystems for biological control, with many researchers trying to introduce antagonistic *Pseudomonas* spp. at sowing to encourage biological disease suppression (Cook 2003). To date, however, there are no commercially successful biocontrol products for take-all. There have been several reports of *Trichoderma* spp. isolates reducing take-all severity (Ghisalberti et al. 1990; Duffy et al. 1997), including *T. koningii* strains found to be responsible for TAD in Western Australia (Duffy et al. 1996). From a commercial perspective, *Trichoderma* spp. are appealing as biocontrol agents, as many are prolific spore producers in culture, and they can possess a range of potential biocontrol mechanisms (Harman et al. 2004).

In the present study a Ggt inoculum rate trial was conducted to optimise disease levels to ca 60% root area infected in order to

establish a standardised biocontrol assay. This was followed by a series of greenhouse assays that screened 'targeted' microbial isolates for disease suppression. Most of the isolates were *Trichoderma* spp., with previous evidence of biocontrol, some were wheat root colonisers and some were isolates living in close association with plants under *Ggt* pressure. The aim was to select the most promising isolates for field evaluation.

MATERIALS AND METHODS

Pathogen inoculum rate experiment

Three *Ggt* isolates (cultured on autoclaved oat grain for 1 month at ambient lab conditions and dried prior to use) were used. These were: 'A3SL4' (isolated from a couch grass rhizome (*Elytrigia repens* L), Lincoln, 2002), 'H11T3 R1/3' (isolated from wheat roots, St Andrews, 2005) and 'Biomill SC3' (isolated from wheat roots, Lincoln, 2005).

A greenhouse experiment (June/July 2013, mean daily maximum temperature 19.7°C, mean daily minimum temperature 15.0°C) compared a soil (Wakanui silt loam):sand mix to a soil:vermiculite mix (both 4:1 v/v), inoculated at five initial *Ggt* inoculum concentrations (the three isolates combined, 1:1:1), including 0.01, 0.0316, 0.1, 0.316 and 1.0% (w/v) plus an un-inoculated treatment. Four wheat seeds ('Conquest') were sown per 0.9 litre capacity pot (eight replicates), which were placed in a randomised complete block design. The pots were watered to field capacity once each week to encourage disease development, with the greenhouse temperature control set to maintain a range of 15 to 25°C. Four weeks after sowing, plants from all pots were removed and roots were washed free of soil in tap water (sampled in block order). Take-all severity was assessed using a 0 to 4 scale of percent roots infected (Beale et al. 1998), where 0 = no disease, 1 = 0% to ≤10%, 2 = 10% to ≤30%, 3 = 30% to ≤60% and 4 = >60% root infection. For each pot, the average take-all score was calculated and used for statistical analyses. Plant heights and dry weights were also measured.

Greenhouse biocontrol assays

Greenhouse assays to test candidate biocontrol organisms used the same experimental set-up

detailed above, and the soil:sand mix, inoculated at 0.316% (w/v) with *Ggt* (with one exception, noted below). This inoculum amount gave an optimum disease level of 60% root area infected. Screening assays tested a total of 133 candidate biocontrol organisms. Sixty-two isolates (all *Trichoderma* spp.) were from the Bio-Protection Research Centre Culture Collection, and possessed prior evidence of biocontrol activity (various pathosystems), growth promotion, and/or rhizosphere competence (various plant systems including wheat). Seventeen *Trichoderma* spp. were from ryegrass pasture soil. Twenty *Trichoderma* spp. isolates and 34 bacterial isolates were from surface sterilised roots of wheat cultivated in commercial and research fields (healthy plants near areas of take-all infection). All potential biocontrol organisms were applied as seed coating treatments. *Trichoderma* isolates were cultured on potato dextrose agar, and conidia were harvested in water, adjusted to 8.5×10^8 conidia/ml, and 250 µl of this suspension was dispensed onto 100 wheat seeds in a Petri dish. The mixture was shaken and then left to air dry (ca 10^6 spores/seed). Bacteria were cultured in 5 ml of nutrient broth overnight and 250 µl used to coat 100 seeds as described above. Coated seeds were dried for 1 h and planted on the same day. All assays used randomised complete block designs, with one treatment per block and positive control replicates ($\sqrt{n}$ treatments) evenly distributed in each block.

Eleven assays were conducted in total, commencing in July 2013 and running almost continually through to November 2014. The greenhouse had temperature control by air heaters and thermostatically controlled vents. Over the 11 assays the average daily minimum temperature ranged from 15.0 to 16.9°C, and average daily maximum from 19.7 to 22.8°C. Assays run in the winter months received supplementary lighting (high pressure sodium lamps, 12:12 h light:dark). Assay 1 and Assay 2 tested 30 candidate biocontrol isolates each. Assay 3 re-tested the 'top' five isolates (for take-all suppression) from both Assay 1 and 2 (10 isolates total) at both 0.316 and 0.1% *Ggt* (w/v). Assay 4 re-tested the 'top' four isolates from Assay

3 at seed loading rates of 10^5 and 10^6 spores per seed. Assays 5 to 9 tested a total of 77 isolates (including the four selected for Assay 4 and 73 previously untested isolates), but were designed to operate as 'rolling' screens and analysed as a single experiment, with each isolate tested at least twice over the five assays. Assay 10 tested the 'top' five isolates from the overall analysis of Assays 5 to 9, both as individual isolates and as every pair-wise combination. Assay 11 tested the same five isolates as Assay 10, each tested at four seed inoculum rates to cover a range above and below the original inoculum rate.

Statistical analyses

The data from the pathogen rate trial (seedling emergence counts at 14 days, disease scores, plant heights and weights) were subjected to ANOVA (Genstat 17) for randomised complete block designs with a 2×5 factorial treatment structure, including two growing media and five inoculation rates ($\log_{10}$ transformed for analysis, the 0% rate was omitted). Data from the greenhouse assays were analysed using ANOVA (Genstat 17) for randomised complete block designs, and treatment means were compared using the unrestricted least significant difference LSD at $P=0.05$. For the overall analysis of Assays 5 to 9 (the rolling assays), an unbalanced ANOVA was used for analysis of the disease score data over the five assays.

RESULTS

Pathogen inoculum rate experiment

The statistically significant trends in the inoculum rate data presented in Figure 1 show that, for the parameters measured (plant emergence, disease severity, plant dry weight, plant height), there was no effect of plant growth medium at most inoculum rates. The trends also demonstrated decreasing plant emergence, increasing disease severity and corresponding decreasing plant dry weight and height, with increasing inoculum rate. Mean plant emergence over the four lesser inoculum rates was not significantly different to the 0% rate (the negative experimental control).

The 0.316% (w/w) inoculum rate (in soil:sand mix) was selected as the most suitable

for use in greenhouse biocontrol assays, as this rate produced strong but not maximum disease severity and reduced plant height and weight, but did not reduce plant emergence below the negative controls. Using a power analysis incorporating the variance data generated for the take-all disease scores, it was calculated that six replicates would be required to detect an 18% reduction in disease severity (decrease of 0.7 for the mean disease score of four plants per replicate pot) in an assay that would test 42 treatments. This was deemed appropriate for the purposes of a biocontrol assay.

Greenhouse biocontrol assays and further assays

A summary of results of each assay is presented in Table 1. The four isolates selected for further study from the first assays (Assays 1, 2 and 3, Table 1) were included in Assays 5-9 (designed to be analysed together as a single experiment), but in these they were not significantly different ($P<0.05$) to the positive control (inoculated with pathogen and planted with untreated seed) from the overall analysis (none featured in top 25% of the 77 isolates tested, when ranked by take-all root infection).

Of the isolates with predicted means significantly ($P<0.05$) less than the positive control in the overall analysis of Assays 5-9 ($n = 4$), three had significantly less disease than the controls in only one assay, and one was not significantly different from the positive control in any individual assay. Conversely, there were isolates that apparently decreased disease when Assays 5-9 were analysed separately (e.g. the isolate with least mean disease score in Assay 6) that did not fall within the most suppressive isolates overall, due to inconsistencies in suppression between assays.

None of the five isolates taken forward to further assays from Assays 5-9 (four that were significantly different to the positive controls plus the next highest ranked) demonstrated any suppression of *Ggt* root infection when re-tested, including in pair combinations or at multiple initial seed loading rates.

Figure 1 (a) Average take-all disease scores, (b) seedling plant emergence at 14 days post-sowing, (c) plant dry weight (d) and shoot height of wheat plants grown in pots with two media, at five rates of inoculation with *Gaeumannomyces graminis* var. *tritici* (mix of three pathogen strains). LSDs are for the Medium $\times$ Log₁₀ inoculum rate interactions.

DISCUSSION

This study tested 133 microbial isolates (mostly *Trichoderma* spp.) for their ability to suppress infection of wheat roots by *Ggt*. In spite of a targeted approach to the selection of isolates tested, and initial statistically significant results, no isolate demonstrated consistent disease suppression, raising questions as to how the process could be improved.

Firstly, looking at assay throughput, Campbell (1994) suggested that if BCAs have a discovery rate similar to agrichemicals, then thousands of organisms should be screened to have a good chance of success. However, Stewart et al. (2010) have detailed how creating a disease blueprint (based on pathogen lifestyle, niche and likely

BCA application strategy) can guide the selection of as few as 20–30 ‘best bet’ candidate BCAs that meet the desired criteria (from a characterised collection), an approach that will give a strong likelihood of success. This study employed the blueprint strategy, and used *in vivo* greenhouse assays as a compromise between high throughput *in vitro* lab assays (that are poor predictors of real-world biocontrol), and low-throughput and seasonally restrictive in-field screening (Pliego et al. 2011). None of the 133 candidate BCA isolates tested proved strong or consistent enough disease suppressors to warrant further study. It would appear that while the targeted approach may be successful in some pathosystems where conditions are

Table 1 Summary of results from greenhouse assays that screened fungal and bacterial isolates for suppression of infection of wheat roots by *Gaeumannomyces graminis* var. *tritici* (Ggt).

Greenhouse Assay	No. isolates tested	Ggt infection in positive control (%) ¹	Range of Ggt root infection in treatments (%) ¹	No. isolates with less disease than control (P<0.05)
Assay 1	30	66.1	44.0 – 80.0	2
Assay 2	30	58.2	41.2 – 74.2	1
Assay 3 ²	10	53.0	41.8 – 59.4	2
Assay 4 ³	4	22.9	10.0 – 16.4	2
Assay 5	37	82.0	66.4 – 86.7	6
Assay 6	39	74.2	37.7 – 72.2	30
Assay 7	39	68.0	61.5 – 80.0	0
Assay 8	39	51.2	38.4 – 74.2	1
Assay 9	37	48.3	29.7 – 65.4	1
Assays 5-9 combined	77	65.0	49.4 – 74.9	4
Assay 10	5 ⁴	25.4	20.6 – 34.4	0 ⁵
Assay 11	5 ⁶	40.1	29.8 – 59.6	0 ⁷

¹Take-all disease score values back-transformed to percent values using linear interpolation.

²Treatment means over both Ggt inoculum concentrations presented.

³In a 4×2 factorial analysis there was no effect of seed coating concentration (P=0.365), so data were analysed and presented as isolate treatments only.

⁴Tested as all ten pair combinations and all five together, as well as individually.

⁵Either individually or as a combination treatment.

⁶Each isolate tested at four seed coating concentrations.

⁷At any of the rates tested.

quite standardised (e.g. *Sclerotinia minor* Jagger disease of lettuce in nurseries (Stewart et al. 2010)), it was not useful for the Ggt/wheat pathosystem, and therefore higher throughput may have been more successful. For example, Renwick et al. (1991) tested 1800 isolates for Ggt infection suppression in tube/sand/seedling assays, and similar tube assays were used by Duffy et al. (1996) and Nasraoui et al. (2007). More compact growing systems would allow assays to be conducted under more strictly controlled conditions (e.g. in growth cabinets). There was variability in the control disease levels between assays in this study that could have been a result of differences in pot soil temperature fluctuations. Greater uniformity between assays could be achieved through stricter environmental control.

The second issue regarding the programme was the restriction of candidate BCAs. The study

mostly screened *Trichoderma* spp. While some *Trichoderma* spp. have been found to suppress take-all on wheat (e.g. Duffy et al. 1997), of the 1800 organisms screened by Renwick (1991), none of the 30 best take-all suppressors were *Trichoderma* spp. Therefore future studies on Ggt biocontrol should screen a broader range of organism types. Duffy (1997) also demonstrated that the chemistry of soils is linked to how supportive they are of a take-all suppressing *T. koningii*, and so it is possible that the soils used in the present study were not supportive of *Trichoderma* biocontrol. *Trichoderma* spp., therefore, may have always struggled to achieve strong biocontrol in the present assays, again suggesting the need for screening from a broader taxonomic range.

In this study, candidate BCAs appeared to suppress disease in some assays but not when

re-tested in others. The final area of concern was the question of whether genuine biocontrol was actually observed in the first instances. The approach employed relied upon taking forward candidates that demonstrated a statistically significant ($P < 0.05$) reduction of mean take-all root infection in assays of 30-40 isolates. However, a pitfall of using this approach was that less attention was paid to the magnitude of the effects. In many assays, maximum disease suppression was 20-30%, whereas other authors have reported suppression levels of 60% (*Trichoderma koningii* and fluorescent pseudomonads; Duffy et al. 1996) and 70% (*Pseudomonas* spp.; Nasraoui et al. 2007). This selection criterion therefore resulted in candidates being taken forward for further testing that, in hindsight, did not demonstrate strong disease suppression. Also, given the numbers of isolates tested in each screen, many isolates taken forward could have been statistical false positives (type 1 error), and not necessarily demonstrated true biological effects. This issue became evident when further testing of the selected isolates did not replicate their initial positive results. A further indication that true biocontrol may not have been observed in many assays was that the positive control means almost always occurred at the medians of the spread of treatment means. This result would be expected (considering the greater number of positive control replicates) if no treatment was having a genuine impact on disease, and simple natural variation in disease levels was being observed. The issue of whether the results of screening experiments are genuine disease suppression or simple statistical artefacts appears not to be mentioned by studies reporting successful screening of biocontrol candidates. This could be a contributing factor to why only 5% of BCAs from screens tested in the field are predicted to suppress disease (Powell & Faull 1989). These findings again reinforce the need to conduct high-throughput screening for the *Ggt*/wheat pathosystem, so that strong suppressors of disease are more likely to be found, rather than taking forward moderate disease suppressors simply because they produced statistically significant effects.

ACKNOWLEDGEMENTS

Funding for this research was provided by the Ministry for Business, Innovation and Employment Ecosystems Bioprotection Programme (LINX0804). Dr Matthew Cromey (New Zealand Institute for Plant & Food Research Ltd) provided *Ggt* isolates, and Mr David Saville provided advice on biometrical analyses.

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