USING TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) TO MONITOR CHANGES IN FUNGAL POPULATIONS ASSOCIATED WITH PLANTS

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SUMMARY

Terminal restriction fragment length polymorphism (T-RFLP) has been widely used as a method for analysing changes in microbial populations. Here, the technique has been further developed using *Gaeumannomyces graminis* var. *tritici* (Ggt) as a model system, to show that it can be used as a semi-quantitative measurement of fungal pathogens associated with the roots of plants, and to monitor the survival of pathogens in the soil. To provide an internal reference for measuring fluxes in Ggt populations, primers were designed to amplify products from the fungal ITS2 region and simultaneously from wheat root DNA. Peak height ratios for the fungus relative to the wheat control were then calculated and these ratios were then compared between samples to measure changes in populations. The results showed that the ratio of the Ggt TRF peak height to the wheat TRF peak height could be used as a measure of the relative amount of Ggt in a sample. These ratios correlated well with disease index scores and also with fungal biomass as assessed by determination of ergosterol content using HPLC. T-RFLP can therefore be used directly as a tool for comparison of the relative levels of Ggt and other fungi on different root samples, and by incorporating fixed amounts of wheat DNA as an internal standard into soil samples prior to DNA extraction, the same method could be used to measure fluxes in soil populations of fungi.

INTRODUCTION

Terminal-restriction fragment length polymorphism (T-RFLP) analysis is a high-throughput fingerprinting technique that has been widely used to monitor changes in microbial communities in diverse substrates including soils, sediments and water (reviewed in Schütte *et al.*, 2008). The method uses one or two fluorescently labelled oligonucleotide primers for PCR amplification and then digestion of the PCR products with one or more restriction enzymes (Liu *et al.*, 1997). This generates labelled terminal restriction fragments (TRFs) of various lengths depending on the DNA sequence of the microbe present and the enzyme used to cut the sequence. The results of T-RFLP are obtained through TRF separation by high-resolution gel electrophoresis on automated DNA sequencers. The laser scanning system of the DNA sequencer detects the labelled primer and from this signal the sequencer can record corresponding fragment sizes and relative abundances. Resulting data is very easy to analyse being presented as figures for statistical analysis, and graphically for rapid visual interpretation.

A number of different primers have been developed for the technique for analysis of fungal, bacterial, archaeal, protozoa and nematode populations (Schütte *et al.*, 2008; Edel-Hermann *et al.*, 2008), for analysis of both structural and functional genes present in a population such as those involved in nitrogen fixation or methane oxidation. There are numerous papers that detail the use of primers based on the rRNA genes for bacteria and fungi, their various merits and limitations, along with the choices of restriction enzymes for the fragment analysis, and experiments to confirm that the techniques are robust and reproducible (reviewed in Schütte *et al.*, 2008). These approaches, and in particular experiments in which artificial communities have been produced and analysed (Osborn *et al.*, 2000; Hartmann and Widmer, 2008), have also shown that peak heights can be used as a measure of relative abundance of microbes in a population within certain limitations.

The technique has also been used to study microbes associated with plants (Conn and Franco, 2004; Sakai *et al.*, 2004; Hodgetts *et al.*, 2007) and there is potential for the technique as a means of simultaneously detecting pathogens and other microbes in and on plants as a means of monitoring how populations of potentially beneficial microbes might influence the colonisation by pathogens. Conventional techniques such as culturing have limitations in that not all microbes can be grown in axenic culture (for example obligate biotrophic plant pathogens and many endophytes), and real-time PCR techniques, whilst being excellent for quantification of

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the specific microbes being tested, can only be multiplexed to allow simultaneous analysis of a small number of species at any one time. T-RFLP has the potential to be able to monitor changes in the populations of many microbes simultaneously. However, for this to be effective, it is important to confirm that the technique provides reliable semi-quantitative data, and also to have some internal reference with which to compare peak heights.

In this work, we have used the soil-borne fungus *Gaeumannomyces graminis* var. *tritici* (Ggt), which is amongst the most important diseases of UK wheat and barley (Hornby *et al.*, 1998) to show that T-RFLP can be used to semi-quantify and monitor changes in Ggt pathogen populations in the rhizosphere and on wheat roots. The ability of T-RFLP to quantify the population of Ggt on roots was validated by disease assessment assays and by detection of the fungal sterol ergosterol by HPLC, since the content of ergosterol is a particularly useful index of fungal biomass (Seitz *et al.*, 1979; Newell *et al.*, 1986, 1987, 1988; Johnson and McGill, 1990). The significance of this technique for monitoring changes in pathogen and endophyte microbial populations associated with plants is discussed.

MATERIALS AND METHODS

Fungal inoculation experiments. To assess the ability of T-RFLP to semi-quantify levels of fungal disease on plants, infection of wheat with the take-all fungus *Gaeumannomyces graminis* was established as the test system. Seed of the spring wheat cultivar Paragon was supplied by RAGT Seeds Ltd. Cambridge, UK. Under aseptic conditions, wheat seeds were washed in absolute alcohol for 2 min, rinsed in sterile distilled water (SDW) to remove the alcohol, immersed in 10% sodium hypochlorite for 3 min, and then washed 3 times in SDW and blotted dry on sterile filter paper. Wheat seeds were planted in 13 cm diameter pots (5 pots per treatment), sown at a depth of 3 cm and at a rate of 10 seeds per pot in a mixture of John Innes No.3 compost (Munro South, Wisbech, UK) and natural field soil mixed at the ratio of 9:1 (v/v). This medium contained normal soil microflora and was much more amenable to pot experiments than total field soil, which had poor drainage properties and was inconsistent as a plant growth medium. The wheat seedlings were grown in the glasshouse at 20°C day, 16°C night regime and watered twice a week. For the Ggt inoculum, infected roots were initially obtained from G. Bateman, Rothamsted Research, UK in 2005. Roots were sectioned into approximately 1 cm-length fragments and surface-sterilised as described above. Sterilised root fragments were placed on potato dextrose agar (PDA; Oxoid) containing 30 mg l^{-1} penicillin G and 133 mg l^{-1} streptomycin sulphate, and Ggt outgrowths were sub-cultured onto fresh plates as soon as possible. A single isolate was selected and routinely sub-cultured on PDA plates at 20°C and stored on PDA slants at 4°C.

For inoculation experiments, the pathogen was introduced into the growth substrate as maize meal and vermiculite inoculum. To prepare the inoculum, aliquots of 50 ml of maize meal were mixed with 450 ml vermiculite carrier and hydrated with 100 ml of sterile distilled water in 2 l conical flasks. The flasks were sealed using a cotton wool plug and foil, then autoclaved for 30 min at 120°C and 0.1 MPa. Twenty-four hours later the hydrated maize meal was shaken thoroughly, aseptically inoculated with twenty 5 mm agar plugs freshly colonised by Ggt, and incubated for 3-4 weeks at 20°C, by which time the fungus had colonised the maize meal. Flasks were shaken every other day during the incubation period to ensure that the maize meal was mixed thoroughly. Once the cultures had been incubated, the inoculum was removed and air-dried in laminar air-flow cabinets. After drying, the maize meal was ground to a coarse powder to increase the number of particles per unit mass of inoculum, and thus improve distribution of inoculum in rooting medium. The inocula were stored in airtight conditions at 4°C for up to six months. For the infection studies and monitoring survival in the soil, two concentrations of Ggt inoculum were made at the ratios of 2% and 5% (v/v) inoculum / soil-compost substrate, and compared with an uninoculated control (0%).

Disease assessment. Disease rating was assessed according to the take-all index method (Bateman *et al.*, 2004). The root systems of the sampled plants were washed free of soil, and examined against a white background while being held under water. Each sample was assessed for take-all and scored on a 0-5 scale: 0 = no disease; 1 = slight take-all, less than 10% of the root system affected; 2 = slight take-all, 11-25% of the root system affected; 3 = moderate take-all, 26-50% of the root system affected; 4 = high take-all, 51-75% of the root system affected; 5 = severe take-all, 76-100% of the root system affected. From these scores, a mean take-all index (TAI) was calculated by summing the products of the percentages of plants in each score category by the corresponding score value and dividing the total by five.

DNA extraction from roots and soil. Root segments (100 mg) were disrupted by Fastprep (QBiogene, Cambridge, UK) bead-beating for 2 cycles of 45 sec at 6.5 m s^{-1} after the inclusion of 8-10 acid washed glass beads (710-1,180 µm diameter, Sigma). DNA was then extracted using the Qiagen DNeasy kit and stored in 100 µl sterile distilled water.

To extract from soil samples, an aliquot of 100 mg rhizosphere soil was spiked with 100 mg uninfected wheat root (for use as an internal control in subsequent

semi-quantitative analysis) and was mixed in 500 µl extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 1.5 mM NaCl pH 8.0), and bead-beaten with glass beads as described above. A 1/10 volume of 20% SDS was added into the mixture, mixed on the vortex for 3 min at full speed and incubated at 65°C for 30 min, then centrifuged at 6,000 g for 10 min at 20°C with addition of a further 150 µl extraction buffer. The supernatant was collected and a half volume of 30% PEG containing 1.6 M NaCl was added, before incubation at room temperature for 1 h and centrifugation at 13,000 g for 10 min at 20°C. The pellet was re-suspended in 200 µl TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0), 13.5 µl 7.5 M potassium acetate added, and incubated on ice for 10 min prior to centrifugation at 16,000 g for 10 min at 4°C. The supernatant was collected and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), then centrifuged at 10,000 g for 10 min at 20°C. The resultant aqueous phase was incubated with RNase (0.1 mg ml^{-1}) for 20 min at 37°C, then extracted with chloroform/isoamyl alcohol (24:1) and centrifuged at 10,000 g for 10 min at 20°C. DNA was precipitated from the aqueous phase with 0.8 volume of cold isopropanol and centrifuged at 16,000 g at 4°C for 30 min. The DNA pellet was washed with 70% ethanol and centrifuged at 10,000 g for 5 min, and then this step was repeated again. Pellets were dried at 65°C, and then resuspended in 100 µl sterile distilled water.

T-RFLP analysis. Fungal primer Fitsrev (5'-ATA TGC TTA AGT TCA GCG GGT-3') located in the 28S rRNA (Ranjard *et al.*, 2001) was tagged with WellRED dyeD3 (Sigma-Proligo) and was used with the 5.8Sfor primer (5'-TCG ATG AAG AAC GCA GG-3'), which was designed for this work based on comparisons of RNA sequences for fungi from database searches. These primers amplify the ITS2 region, such that the fragments obtained for most fungi, oomyctes and plants following PCR would fall within the 60-600 bp size range detectable by the fragment analysis software. PCR was performed in 30 µl reaction volumes containing 3 µl 10 × PCR buffer (Promega), 2.4 µl 25 mM $MgCl_2$, 0.5 µl 20 pmol forward primer, 0.5 µl 20 pmol reverse primer, 0.3 µl 25 mM dNTPs, 0.3 µl *Taq* DNA polymerase (5 U $µl^{-1}$ Promega) and 1 µl template DNA in an MJ Research PTC 200 thermocycler, with PCR settings of 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 56°C for 30 s and 72°C for 1 min, completed with the final extension step of 72°C for 15 min. After amplification, PCR products were examined on 1.2% agarose gels in 1 × TBE buffer stained in ethidium bromide and visualised on an ultraviolet transilluminator. Five µl of PCR product was digested in 10 µl reaction volumes containing 1 U *Hae*III restriction enzyme and incubated at 37°C for 2 h to ensure complete digestion. Fragment analysis was performed as described in Hodgetts *et al.* (2007).

Sequence analysis. To determine the expected size of the TRFs for Ggt and for wheat, the DNA sequences were obtained by amplifying DNA from a Ggt stock culture and from an uninfected wheat plant using primers 5.8Sfor and Fitsrev. PCR products were purified with the Qiagen PCR purification kit, and sequenced directly using an ABI sequencing big dye terminator kit. The sequences of resultant PCR products were analysed with BLASTN through the National Centre for Biotechnology Information (NCBI) website (http://www.ncb.nlm.gov/BLAST), by aligning input sequences against published nucleotide sequences.

Ergosterol analysis by HPLC. Take-all infected roots were extracted following the procedure of Genney *et al.* (2000): aliquots of 100 mg infected roots were extracted in methanol using bead beating as described above to physically crush the sample at the same time. Polyvinylpyrrolidone (PVP) was added (10% w/v) to the methanol to precipitate phenolic compounds. The extract was centrifuged and the supernatant made up to 1.5 ml before being filtered through a 0.45 µm acetate syringe tip filter. A C18 reverse-phase analytical column (25 cm×0.46 cm) was utilised with a Spectra Series P100 HPLC system equipped with a Spectromonitor 3000 detector. The system was run isocratically with 100% methanol at the flow rate of 1.5 ml min^{-1}. The wavelength of the UV detector was set at 282 nm, and the peak isolated at 8 min retention time was identified as ergosterol, based on its co-chromatography and identical absorption spectrum with pure standard ergosterol (20 µg ml^{-1}).

Statistical analysis. All the data were collected from different experiments and input into Microsoft Excel 2003 for statistical analysis. Regression analysis and descriptive statistics were conducted correspondingly with different requirements in Microsoft Excel Add-In or GenStat software. In all analyses, a probability value of 0.05 or less was taken to be significant.

RESULTS

Identification of TRFs of Ggt and wheat by sequencing and T-RFLP. The expected sizes in bp of the TRF products that would be generated after *Hae*III digestion of amplification products generated using labelled Fitsrev and unlabelled 5.8Sfor were calculated from the DNA sequence of a series of cloned genes and from database searches. Based on the sequences, the predicted TRFs are 162 bp for all isolates and sequences of the take-all fungus examined both from cultures and database searches, and 86 bp for wheat, and as shown in Fig. 1, these are the actual sizes of the TRFs that were obtained experimentally on the CEQ ™ 8000 Genetic analysis system software.

Based on analysis of sequences from over 200 other soilborne fungi obtained from databases, we have found no other organisms that would produce a fragment of 162 bp following *Hae*III digestion and are therefore confident that this represents the take-all fungus.

Comparisons of Ggt disease ratings and T-RFLP peak heights. In order to confirm that the T-RFLP peak heights measure the amount of Ggt present in roots and of the disease severity, roots were inoculated with Ggt at 0% inoculum, 2% inoculum and 5% inoculum. DNA was extracted from samples from each group after 4 weeks incubation and subjected to T-RFLP analysis. Fig. 1 shows that the proportion of take-all fungal DNA to internal control increased as the percentage of inoculum used increased.

To confirm whether a correlation exists between the take-all index as a disease indicator, and T-RFLP analysis as a measure of fungal biomass, a series of experiments were conducted in which samples were assessed for their take-all index score and then used for DNA extraction and T-RFLP analysis. The peak height for the take-all fungus TRF was then divided by the peak height for the wheat control TRF to provide a ratio for each individual sample, and the results are summarised in Fig. 2. There was a moderate correlation ($P<0.001$) between the take-all index and T-RFLP analysis of root samples according to regression analysis. The data indicated that the ratio of peak heights of Ggt to the wheat control was almost 0 when the take-all index was less than 0.1, whilst the ratio increased to more than 2:1 as the take-all index increased above 0.8. The sets of data from infected root T-RFLP analysis and from take-all index assessments fitted into both a linear regression model and a polynomial regression model. Regression analysis was attempted to explain the relationships between take-all index assessments and DNA-based T-RFLP analysis. The goodness of fit of the polynomial regression model was better than the simple linear model because the R square of polynomial model is closer to 1 than the R square of the linear model.

Comparisons of T-RFLP with ergosterol content for semi-quantitative analysis of Ggt. Samples of roots infected with Ggt at 5% inoculum were collected and analysed for their ergosterol content and by T-RFLP to investigate the correlation between the two techniques for quantitative analysis. Based on the fact that Ggt was the only fungus detected in significant amounts on these roots by T-RFLP (see Fig. 1) it was assumed that most of the ergosterol detected would be from Ggt. The correlation between the content of ergosterol and the ratios of TRFs of Ggt to the wheat internal control is shown in Fig. 3, and was evaluated as being significant at $P<0.05$. The R square was very close to 1 and the goodness of fit of the model was high. This indicates that TRF peak height ratios provide a very good semi-quantitative estimation of take-all fungal biomass.

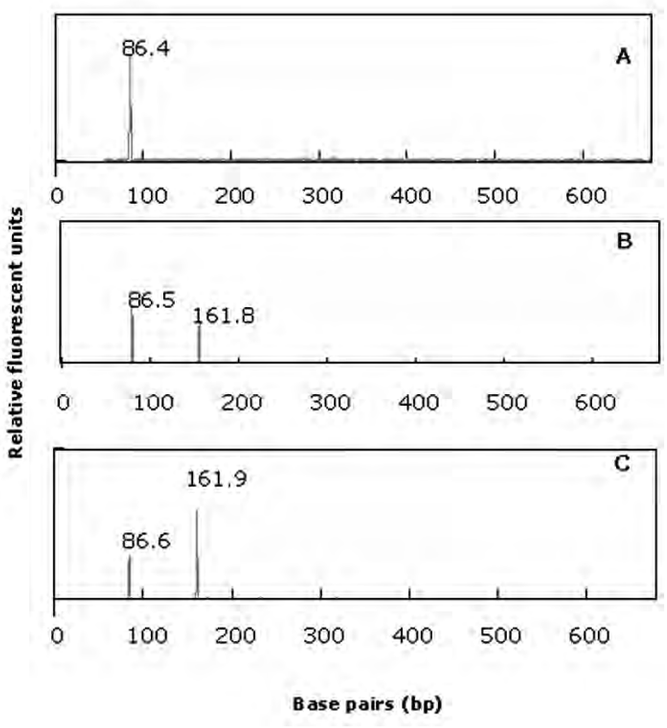

Fig. 1. TRF profiles comparing peak height ratios of Ggt and wheat**.** TRFs of root sample at (A) 0%; (B) 2%; and (C) 5% inoculum. The fragment size of 86 is for wheat as the internal standard, and the fragment size of 162 is for Ggt. The x axis gives the size in base pairs and the y axis gives the relative fluorescent units. Note the change in the ratio of the Ggt peak height to the wheat peak height as the level of inoculum increases.

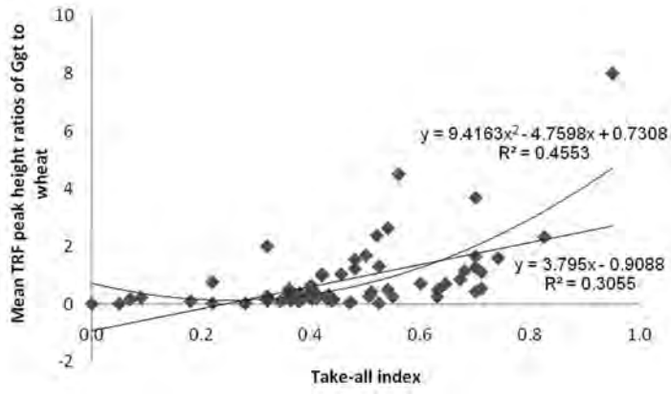

Fig. 2. Correlation between take-all index and T-RFLP analysis in root samples. Individual samples of wheat roots were assessed for % of root system affected by Ggt and the data converted to the take-all index (x axis), and DNA was extracted and used for T-RFLP analysis. Ratios between TRF peak heights of Ggt to wheat were then calculated for each sample and these ratios were plotted on the y axis.

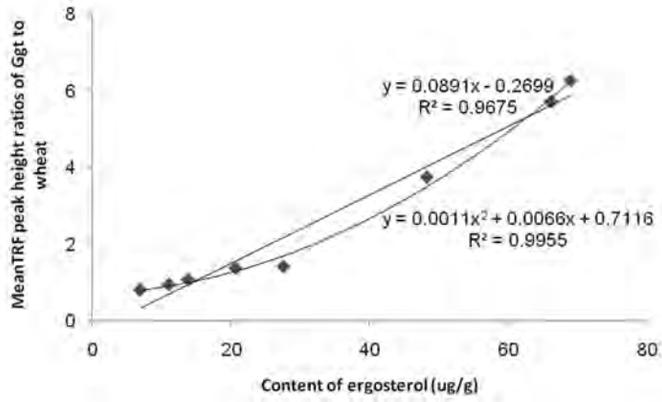

Fig. 3. Correlation between the content of ergosterol and T-RFLP analysis in root samples. Root samples were collected and divided into batches based on visual symptoms. For each batch, half of the sample was analysed for ergosterol content (x axis) and the other half used for T-RFLP analysis and ratios between TRF peak heights of Ggt to wheat were then calculated for each sample and these ratios were plotted on the y axis.

Use of T-RFLP to assess the survival of Ggt on roots and in soil over time. To assess the survival of take-all on roots over time, inocula was prepared and introduced into the soil-amended compost as described, wheat seedling roots were sampled weekly for four weeks after emergence with three replications of the experiment, and DNA extraction and T-RFLP analyses were performed. Fig. 4 shows the results obtained in which the amount of the take-all fungus in 100 mg of roots increased gradually from week 1 to week 4. In particular, the amount of Ggt went up dramatically with the 5% concentration inoculum after an incubation period of three weeks, when compared to the 2% inoculum.

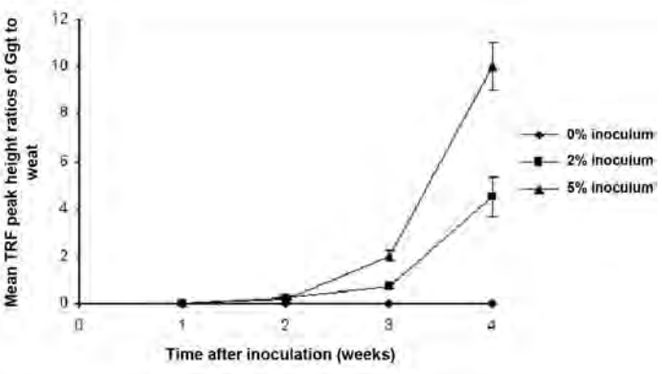

Fig. 4. Detection of Ggt DNA in the root samples at three concentrations of inocula by T-RFLP. Wheat roots were inoculated with Ggt at three different inoculum levels. DNA was extracted from roots at different time points following inoculation, DNA extracted and T-RFLP performed, and the ratios between TRF peak heights of Ggt to wheat were then calculated for each sample and the means of these ratios were plotted on the y axis. Error bars show standard deviations.

To monitor survival in soil, the soil was amended with 5% inoculum, and samples taken at 3, 7, 9, 17, 24 and 30 days for analysis. As shown in Fig. 5, the amount of take-all detected in the soil decreased dramatically between days 3 and 9 after inoculation and then declined slowly for the remainder of the experiment. One month after inoculation it was difficult to detect take-all in soil. The reason for this may be that the take-all fungus was artificially inoculated into the soil and the natural microbial community out-competed the inoculum. The regression relationship was significant at $P<0.01$ between inoculum levels and incubation periods, with an R square using the power model of 0.8231.

DISCUSSION

Most of the studies describing the use of T-RFLP to monitor microbial populations have focussed on the qualitative characterization of environmental samples, but very few have analysed the quantitative possibilities of this technique (Bruce, 1997; Liu *et al*., 1997; Osborn *et al*., 2000; Schütte *et al.,* 2008). The limitations of T-RFLP for quantitative characterization of communities have been well argued, since it is subject to drawbacks related to PCR amplifications. These problems become more evident when working with complex ecosystems in which diversity of the microorganisms is high, with differences in the ability to extract DNA from different organisms, the match of the primer binding sites (when universal primers are used), genome size, and G+C content, all of which may lead to differential amplification (Suzuki *et al*., 1998; Marsh *et al*., 2000).

In this paper, take-all infection of wheat has been used as a model system to show that T-RFLP peak height can be used as a semi-quantitative method for monitoring changes in levels of fungal plant pathogens. Primers were designed for the analysis of Ggt on wheat roots that are able to amplify from fungal DNA present in the sample and simultaneously amplify DNA from wheat. These primers amplify the ITS2 region from fungal DNA as most fungi have ITS2 regions less than 600bp, so that even if there is no restriction enzyme site for the particular enzyme that was used (*Hae*III), TRFs would be detectable in the standard 60-600bp range detectable by the fragment analysis software. By contrast, primers such as ITS1 and ITS4 (White *et al*., 1990) which amplify ITS1, ITS2 and the 5.8S rRNA gene would result in PCR products and TRFs from some fungi that are too large to detect on this software (unpublished observations).

The primers 5.8Sfor and Fitsrev used in this study were shown to produce TRFs of 162 for Ggt and 86 for

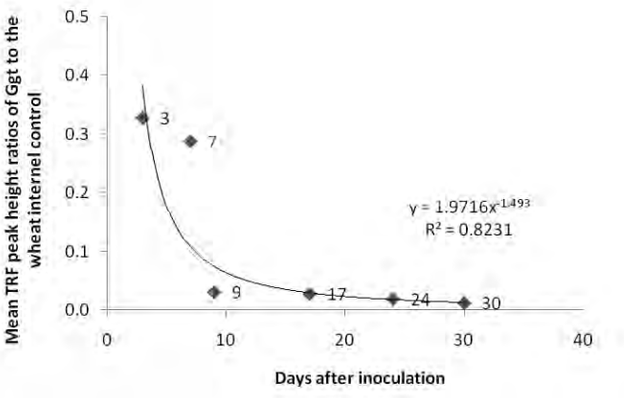

Fig. 5. Survival of the artificial inoculum of take-all in soil. Samples were taken from soil inoculated with a 5% inoculum at different days post-inoculation. 100 mg soil samples were mixed with 100 mg of wheat roots, DNA extracted and T-RFLP performed, and the ratios between TRF peak heights of Ggt to wheat were then calculated for each sample and these ratios were plotted on the y axis.

wheat when the enzyme *Hae*III was used. When peak ratios were examined and compared to disease index scores, the data indicated that the ratio of peak heights of Ggt to the wheat control was almost zero when the take-all index was less than 0.1. This would suggest that either the primers are not sensitive enough to detect Ggt at these low levels of infection or that the blackening of roots might not be a result of Ggt infection but caused by other factors, such as root rot of wheat. Conversely, when the take-all index was 0.8 or higher, the peak ratio was greater than 2, presumably as a result of whole roots being completely infected by Ggt such that little wheat DNA could be detected. Overall, the ratios were shown to correlate well with the disease index, although it is important to note that the take-all index is designed as a rapid visual assessment of symptoms and not a test to measure absolute levels of fungus present. More importantly, when T-RFLP was compared with analysis of ergosterol content as measured by HPLC, which is an objective and quantifiable measure of the amount of fungal biomass present (Seitz *et al.*, 1979; Newell *et al.*, 1986, 1987, 1988; Johnson and McGill, 1990), the correlation was highly significant, confirming that T-RFLP accurately reflected the growth of take-all fungal biomass.

Experiments were also performed on soil samples in which the wheat internal reference was added to the soil samples prior to DNA extraction. This method was used to show that the amount of Ggt in the soil declined rapidly after it had been artificially added to soil and this agrees with the findings of Herdina *et al.* (2004) that the amount of Ggt DNA detectable in artificial inoculations declined by more than 50% during an incubation period of 8 days in soil.

There has been increasingly sophisticated automation of the T-RFLP technique and more consistent and precise cross-comparisons between runs, due to the greater accuracy of in-lane comparisons. The data presented in this paper shows that it could be used as a simple and rapid method for assessing levels of Ggt, since T-RFLP is less subjective than measuring the take-all index, and easier to perform than ergosterol assays. For single species such as Ggt, it could rightly be argued that real-time PCR would give a more accurate measure of absolute levels of the fungus. However, the advantage of T-RFLP is that it will also detect DNA from other fungi present in a sample, since these will give peaks at different positions, and can therefore be used to simultaneously measure changes in populations of multiple species relative to the wheat internal reference. It has also been shown that the primers will amplify from other plant species, so the technique could be applied for the semi-quantitative analysis of fungal populations associated with plant species other than wheat.

T-RFLP bias, in that not all PCR products in a mixture are amplified with the same efficiency, is an issue that means the method cannot be used to give absolute levels of fungi present in a sample. However, the data presented here confirms the findings of others that it can be used as a rapid semi-quantitative tool for assessing the relative amounts of different microbes between samples by comparing to a fixed internal standard, with the caveat that if a TRF of a particular size is derived from more than one organism, it will be comparing this group of organisms with the standard. For example, if the ratios of two TRFs to each other are compared in one sample and give a value of 2:1, and then the ratio of the same two TRFs are compared in second sample and give a ratio of 1:1, it could be argued that the influence of factors such as the ability to extract DNA from the two organisms, match of the primer binding sites, genome size, and G+C content will be effectively the same for both samples. Therefore the ratios can be directly compared and used to indicate that there is more of the organism responsible for the first TRF in sample 1 than there is in sample 2, relative to the organism responsible for the second TRF. If one of the two organisms used is an internal reference that is present at a fixed amount in all samples, then the amount of the other organism relative to this reference can be directly compared between samples. Therefore, whilst this does not give absolute values of amounts of organisms, it does give an indication of relative amounts, and we therefore describe this as a semi-quantitative method. In previous work, we have shown that primers based on the 23S ribosomal gene can be used for T-RFLP analysis of phytoplasmas and other bacteria associated with plants, and that these primers would also amplify plant chloroplast DNA to provide an internal control into the system to guard against any potential problems of PCR

inhibitors (Hodgetts *et al.*, 2007). In this paper we have shown that primers based on fungal DNA sequences will also amplify from plant DNA to provide an internal control for fungal T-RFLP studies.

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