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Giant Rat's Tail grass susceptibility to fungi effective in biological control of Giant Parramatta Grass

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Abstract

Sporobolus pyramidalis (Giant Rat's Tail grass - GRT) was tested in seedling and pot trials to see if inoculation with *Nigrospora oryzae* and *Fusarium* sp. would produce the same symptoms in GRT as those observed in *S. fertilis* (Giant Parramatta Grass - GPG) – blight in pot trials and crown rot in the field. Seed germination was maximal at over 90% with surface-sterilisation and alternating temperatures of 15/35°C. In seedling trials in Petri dishes, inoculation with *N. oryzae* significantly reduced height but there was no chlorosis of the youngest leaf or mortality, whereas inoculation with *Fusarium proliferatum* or with both fungi killed the seedlings. For comparison, inoculation with *N. oryzae* in GPG reduced seedling height and produced a distinctive chlorosis of the youngest leaf but did not affect mortality. In pot trials in the glasshouse with small GRT plants 8 months after inoculation with spores and then mycelium, inoculation produced no significant decrease in total or % live biomass in small GRT plants (because the controls were very mismatched). In large GRT plants, inoculation resulted in a significant decrease in total biomass with both fungi and intermediate status with either inoculant. In GPG, total biomass was unaffected by inoculation but % live biomass declined significantly with either *Fusarium* alone or with both fungi, with *N. oryzae* intermediate. Both *N. oryzae* and *Fusarium* spp. (*F. proliferatum* from all treatments and *F. chlamydosporum*, the inoculant, only from large GRT plants) were re-isolated from the plants, but not only or necessarily from those with those inoculants. *Fusarium* spp. but not *N. oryzae* were isolated from GRT seeds and field-sourced plants. The conclusion is that *N. oryzae* alone or in combination with *Fusarium* spp. present naturally even in healthy plants, should be investigated further for its biocontrol potential for GRT and other WSGs in pot and field trials.

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Introduction

Background – work on GPG

Invasive weedy unpalatable grasses constitute arguably the worst biological problems in agriculture and the environment. They out-compete both improved pasture grasses in agriculture and native grasses, which are the most threatened group of the Australian flora apart from orchids (Dept of the Arts, Heritage and Environment 2006). In agriculture, they constitute economic loss due to reduction in carrying capacity for livestock, land value, direct costs of labour and herbicides used to control them (Natural Resources and Mines 2001), and indirect environmental costs of off-target effects such as run-off into streams (Dept of the Arts, Heritage and Environment 2006) and uptake by invertebrates and other components of the food chain. Control currently relies on prevention (efforts to avoid introducing weeds into new properties), eradication (grubbing before seeding) and, if that is not possible, chemical control by broad-acre spraying or weed-wiping (Bray and Officer 2007). There are few selective herbicides for grasses within pasture, which is composed primarily of grasses. Using non-selective herbicides such as glyphosate (trade names Roundup and Zero) leaves bare ground that is even more susceptible to dominance by the weedy grasses as their seeds germinate and out-compete other grasses (Bray and Officer 2007). Using herbicides leads over time (it is estimated about 17 successive applications are needed) to herbicide resistance, which exacerbates the problem (Storrie 2007).

The weedy *Sporobolus* grasses (WSG) comprise a group of about five species that have been introduced to Australia and have invaded northern NSW and Queensland (Bray and Officer 2007). They have a current distribution of almost half a million hectares and a potential distribution of over 60% of Queensland and 30% of NSW. All are large (1-2 m tall) tussocky grasses with tough foliage that is hard for cattle to chew and digest and leads to reductions in productivity and condition (Betts and Officer 2001). All also seed prolifically from a young age, with an average-sized tussock producing about 200 seed heads annually, each distributing hundreds of tiny (1-2 mm long) seeds. WSG have been declared noxious and regionally prohibited in both states (Australian Weeds Committee 2010). Two of the most successful are *S. fertilis* (Giant Parramatta Grass – GPG) in northern NSW and southern Queensland and *S. pyramidalis* (Giant Rat's Tail grass – GRT) throughout Queensland). The most popular method of chemical control is the Group J herbicides, flupropanate and 2,2-DPA, which kill these and some other tough weedy grasses selectively but are slow to act, have long withholding periods, long residence times in soil and cannot be used with lactating cattle (Natural Resources and Mines 2001). Recently, GPG was shown to be resistant to both of these selective herbicides in the Grafton area in northern NSW (Ramasamy 2008; Ramasamy et al. 2007b; Ramasamy et al. 2008) and it is likely that such resistance has already been selected in other WSG, as in serrated tussock in Australia (McLaren et al. 2006).

An ideal solution to the problem of controlling the WSG, in particular GPG and GRT, is biological control, which has the potential to be selective and long-lasting. Selective agents are rare among grasses because of the close relationships among the grass family (Grass Phylogeny Working Group 2001), but some have been developed (Yandoc et al. 2005; Yobo et al. 2009). A search for potential biological control agents for WSG in South Africa failed to find any (Palmer et al. 2008). Recently two fungi have been observed in Australia as potential biocontrol agents for GPG: *Nigrospora oryzae* and *Fusarium* sp. In pot trials with mature tussocks of GPG at RMIT University, *N. oryzae* produced symptoms of blight, characterised by a dieback of foliage over 3-6 months and eventually death after 12 months (Fig. 1) (Ramasamy et al. 2007a; Ramasamy et al. 2011).

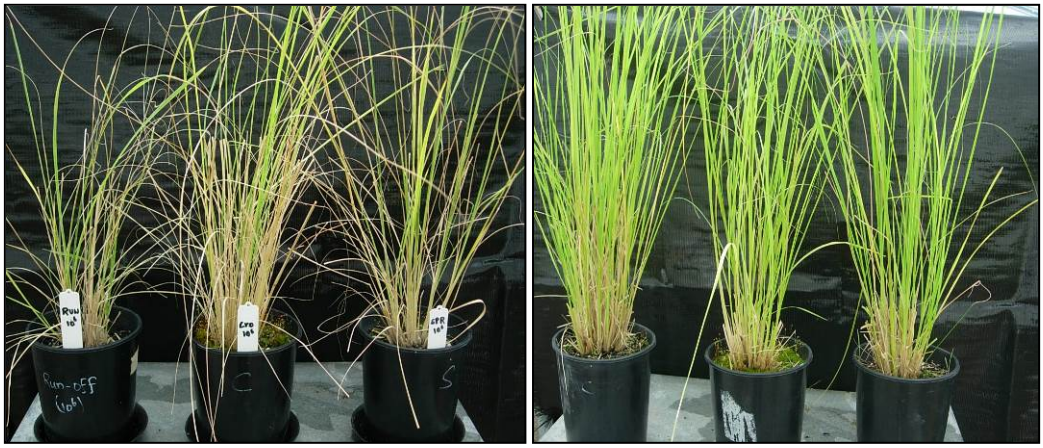


Fig. 1.1. GPG potted plants 7 months after application of 10^6 conidia/ml *Nigrospora oryzae* spores in 0.1% Tween 20 (left three pots) and control (0.1% Tween 20 alone) (right three pots). Treatments are (left to right): 1=Run-off, 2=Crown, 3= Leaves).

In the field around Grafton, NSW, the frequency of GPG declined at three properties from 88-96% in 2006 (David Officer, pers. comm.) to 22-45% in 2009 (Fig. 1a). Characteristic symptoms of dead orange leading shoots accompanied by crown rot were seen in 25-45% of plants in the field surveys in 2009 (Fig. 1b) (15).

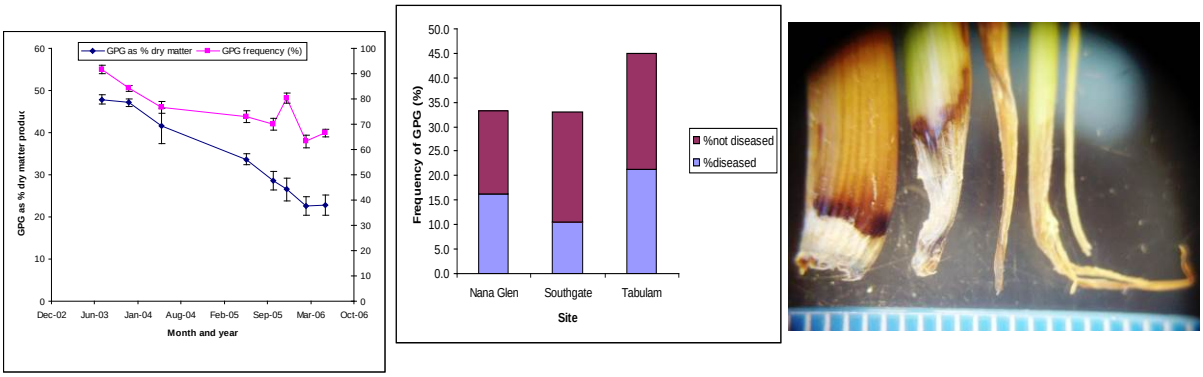


Fig. 1.2. Decline in GPG dry matter and frequency in paddocks in property at Southgate (a) from 2002-2006 and (b) further reduction in frequency and field symptoms of crown rot in 2009, (c) crown rot symptoms.

Plant density and size were also monitored in about 240 plants at three field sites (5 m x 50 m) in two properties at Southgate and Nana Glen, in early December 2009 and late April 2010. Density showed no change but diameter declined by up to 83% (Fig. 3); 32-48% of plants showed diagnostic symptoms (Lawrie 2010). Plant height increased because plants flowered rather than foliage increasing and so height was not a suitable measure.

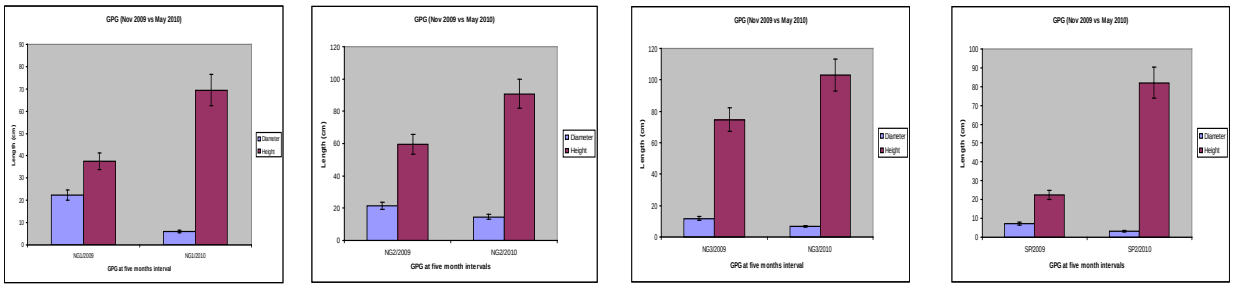


Fig. 1.3. Diameter and height of GPG plants at five month interval (Dec 2009-April 2010) in large permanent quadrats at monitoring sites near Grafton, NSW. NG1=Nana Glen site 1, NG2=Nana Glen site 2, NG3=Nana Glen site 3, SP1/2=Southgate site.

Surface-sterilisation and isolation of potential causal agents from field samples showed that the most frequently isolated potentially pathogenic fungi were *Nigrospora oryzae* , *Fusarium* sp. and *Alternaria alternata* (which is normally a secondary pathogen) (Fig. 4a) (Lawrie 2010). A synergistic syndrome between *N. oryzae* and *Fusarium* sp. seems the most likely explanation for the selective pathogenicity seen in the field. *Fusarium* sp. may be present as an endophyte (living without symptoms or effects in the outer tissues of GPG stems) and the introduction of this strain of *N. oryzae* may induce it to invade deeper into the tissues and cause the fatal crown rot.

Most recently, symptomatic plants were removed in 2010 from infected areas around Grafton and transplanted to a few properties on the Tweed River, at the northern extremity of NSW, where they were planted on the tops of sloping pasture. In the last 9 months, decline and death of GPS plants has been seen below the transplanted plants (Fig. 4b), showing that the syndrome can be induced successfully in the field.

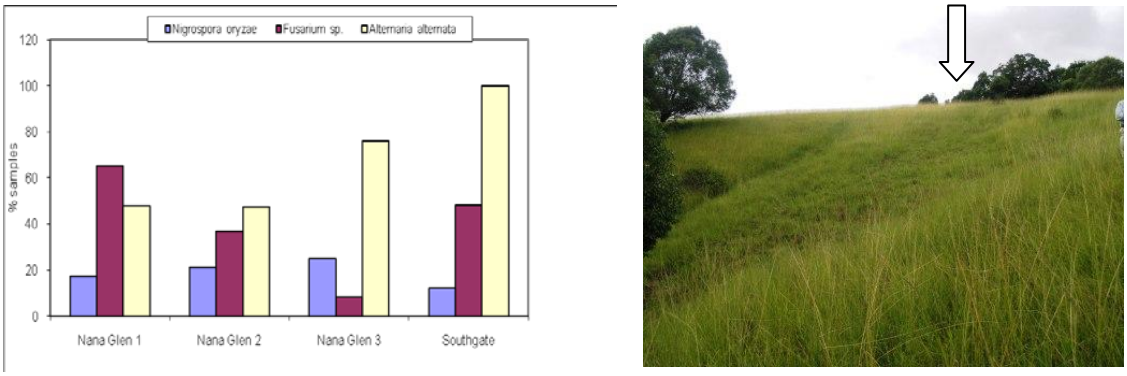


Fig. 1.4. (a) Major likely pathogens isolated from symptomatic GPG plants collected in the field in the Grafton area in early December 2009 and (b) Decline and death of GPG at Walsh Creek Reserve Road site. Arrow indicates position of GPG plant transplanted from Grafton, NSW. The tall growth on either side of the flatter area in the centre is GPG and the green growth in the flatter area is a mixture of desirable pasture grasses.

From this ongoing research, it is likely that the fungus *N. oryzae* with or without the fungus *Fusarium* sp. can be developed as successful selective biocontrol agents for GPG in the field.

This raised the question: would the same fungi have the same effect on other weedy *Sporobolus* species? Could one or more of these fungi be developed as selective biocontrol agents for the main WSG in Queensland, which is GRT?

Project objectives

1. Conduct preliminary assessment of the effects of the two fungi, *Nigrospora oryzae* and *Fusarium* sp., on GRT, to see if they produce the same symptoms as in GPG and have the same effect on reduction in plant size, in 1. seedlings and 2. potted plants in the glasshouse.

2. Assess field-collected material of GRT for the presence of either of these fungi.

Project outcomes

1. Effects of *Nigrospora oryzae* and *Fusarium* sp. on seedlings

Plant materials

Seeds of GRT were sent for testing by David Officer from Grafton, NSW, from

1. Sunbury (Date of collection- 2nd December 2009)

2. Trenayr, NSW (Date of collection- 28th May 2010)

1.1 Germination trial

The aim of this trial was to find a reliable treatment to induce germination, since seeds were highly dormant.

1.1.1 Materials and Methods

Each treatment was in duplicate for a particular type of seed.

For each treatment, two Whatman No. 1 filter papers were placed in each of two glass Petri dishes and soaked with 10 ml of sterile water. As seeds of GRT are very small and time-consuming to separate from empty glumes, a pinch of seed was used for each Petri dish rather than attempting to have a uniform number for this preliminary trial. After treatments were set up, they were incubated under either constant or alternating temperatures as shown in Tables 1 and 2.

Non-sterile treatment. Seeds were soaked in sterile tap water for 2 minutes and then transferred into the dishes. The entire procedure was not performed under clean but not aseptic conditions.

Surface-sterilisation treatment. Once prepared as above, glass Petri dishes were autoclaved. The complete procedure was performed in a laminar flow cabinet using aseptic procedures. The seeds were surface-sterilised with 50% Domestos (2.5% NaOCl with wetters) for 5 min, rinsed three times in sterile water for 5 minutes each with sterile water, transferred to glass Petri dishes and incubated in the different temperature conditions in Table 1.

Non-sterile treatment with smoke water. This was as for the non-sterile treatment but the sterile water was replaced by 10% (v/v) smoke water.

Non-sterile treatment with gibberellic acid (GA_3). This was as for the non-sterile treatment but the sterile water was replaced by 0.1 M gibberellic acid solution, prepared using sterile water. This treatment was incubated only at a constant 25°C.

Table 1. Temperature treatments for GRT seed germination trial

Temperature	Code
Constant 25ºC	A
25ºC/ 15ºC	B
37ºC/ 25ºC	C
37°C/ 15°C	X

Table 2.Treatments and codes for GRT germination trial

Location	Code	Location	Code	Treatment Conditions
Trenayr	At	Sunbury	As	25ºC
	Ast		Ass	Sterile 25ºC
	Atsm		Assm	Smoke water-25ºC
	GAt		GAs	Gibberellic acid-25ºC
	Bt		Bs	15ºC/25ºC
	Bst		Bss	Sterile-25ºC/15ºC
	Btsm		Bssm	Smoke water-25ºC/15ºC
	Ct		Cs	37ºC/ 25ºC
	Cst		Css	Sterile-37ºC/ 25ºC
	Ctsm		Cssm	Smoke water-37ºC/25ºC
	Xt 1/12/11		Xs 1/12/11	37ºC/15ºC

1.1.2 Results and Discussion

None of the Sunbury seeds germinated and so only the results for the Trenayr seeds are shown, after 2 weeks of incubation (Fig. 1). Germination varied from 0% to 93.8±1.3% and differed significantly between treatments (ANOVA, F=4.74, p=0.008; Kruskal-Wallis, H=18.69, p=0.044). The treatments with the greatest mean germination (Tukey family error tests, p=0.05) were Xt 1/12/11 (alternating 37/15°C, Fig. 2) and Cst (37/25°C, surface-sterilised). Alternating temperatures produced more germination than constant temperature. Surface-sterilisation had no effect on germination. Smoke water prevented germination. For future use, the best treatment is likely to be surface-sterilisation followed by incubation at alternating 15/35°C until seeds germinate and reach 10 mm height, at which point they should be inoculated with fungi.

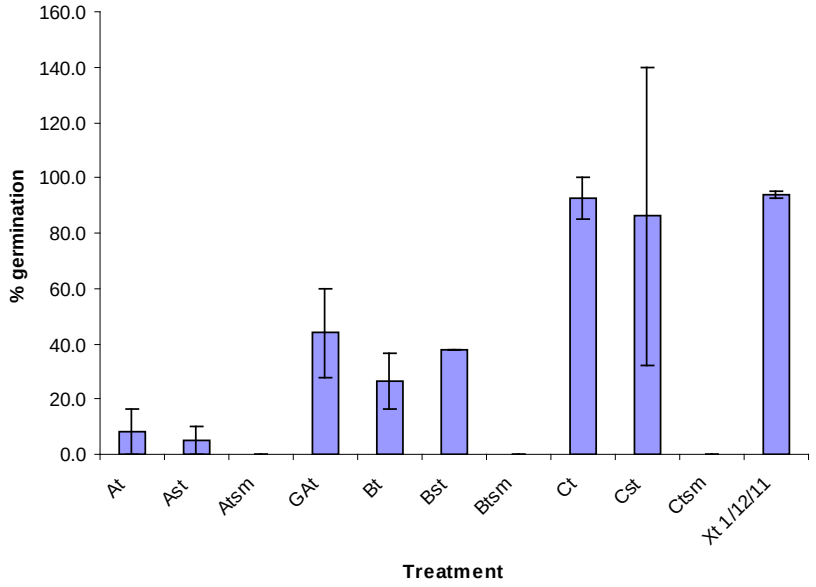


Figure 1. Effect of treatments on germination of Trenayr GRT seeds after 2 weeks of incubation in the light. Treatments were as in Tables 1 and 2. Key: A. 25°C constant, B. 25/15°C, C. 37/25°C, X. 37/15°C, s surface-sterilised, sm smoke water, G gibberellic acid. Bars are 2xSE.

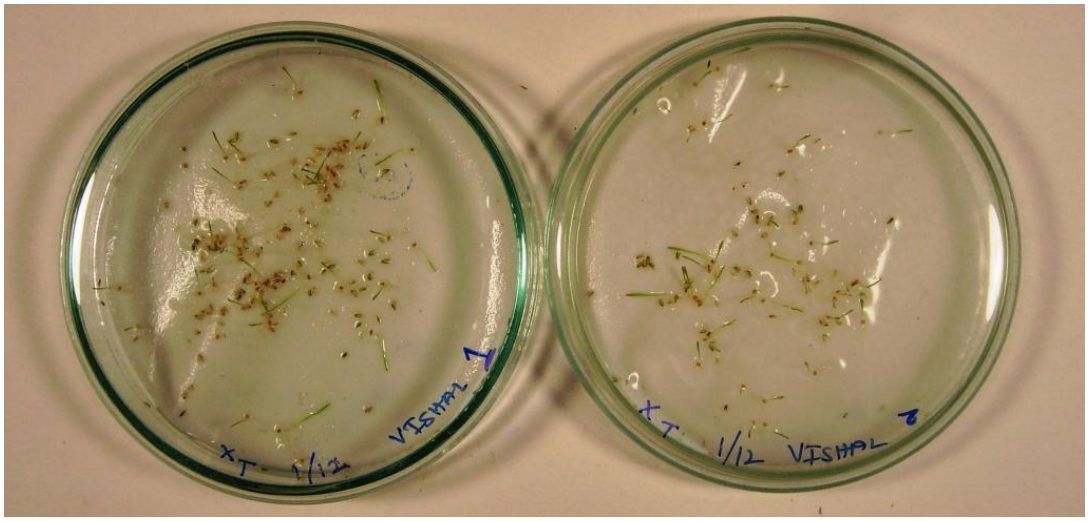


Figure 2. Germination of Trenayr GRT seeds with treatment Xt (37/15°C) after 2 weeks of incubation in the light.

1.2 Inoculation of seedlings with fungi

The aim of this part of the project was to test if cultures of the fungi *Nigrospora oryzae* and *Fusarium* sp. (previously isolated from Giant Parramatta Grass (GPG) seedlings in the RMIT glasshouses and from asymptomatic plants in the field at Grafton, NSW) could induce similar symptoms on GRT seedlings.

1.2.1 Materials and Methods

Cultures of *N.oryzae* and *Fusarium* sp.were subcultured on to separate multiple 9 cm diameter plates of V8+ juice agar (200 mL V8 juice (Campbells), 3 g $CaCO_3$, 800 mL deionised water, 15 g Davis agar, pH to 6.0-6.5) in 9 cm pre-sterilised plastic Petri dishes and incubated at 25°C in the light in a controlled growth room until growth covered the dishes and sporulation was abundant. Plates were scraped to remove the spores (conidia), suspended in 10 mL sterile deionised water per plate and the suspensions filtered through sterile muslin to remove large pieces of mycelium. Spores were counted using a haemocytometer and each suspension adjusted to 10^6 spores mL^{-1}. Seedlings of GRT from Trenayr were prepared by surface-sterilising seeds and incubating at 37°C/15°C for 4 days to break dormancy, and then grown on in a growth room at 25°C with 12-16 h light of 27 µmoles m^{-2} s^{-1} provided by 'Fluora' fluorescent tubes for 2 weeks as in 3.1 above. In the first trial, three glass Petri dishes, each containing 25-45 surface-sterilised seeds were inoculated with 10^6 spores of one of the following treatments: *N. oryzae*, *Fusarium* sp. (later identified as *F. chlamydosporum*) or an equal mixture of 10^3 spores of both fungi and incubated at 25°C in the light and inspected daily for effects, which were recorded after 2 weeks. In the second trial, four replicates were used, the isolate identified as *F. proliferatum* from the pot trials was used and seedlings were inoculated at 7 d instead of 14 d, but otherwise conditions were the same

1.2.2 Results and Discussion

Control seedlings germinated quickly (within 1 week) and all seedlings appeared equally healthy until 10 days after inoculation, after which seedlings in the inoculated treatments showed varying degrees of chlorosis and necrosis (Fig. 3a). Seedlings inoculated with *N. oryzae* were less green than the controls, but there was no clear orange colour of the youngest leaf as occurs with GPG – rather, the older leaf

became chlorotic and transparent while the younger was still green (Fig. 3b) and black clusters of *N. oryzae* spores were observed on the filter paper with the stereomicroscope (Fig. 3c). Seedlings inoculated with *Fusarium* sp. quickly showed chlorosis and necrosis and died, as did seedlings inoculated with a mixture of both species. In Experiment 1, this happened after 2 weeks but in Experiment 2, all seedlings were healthy until 10 days after inoculation, after which symptoms quickly became apparent and almost all seedlings died after 3 weeks in both the *Fusarium* and *N. oryzae* + *Fusarium* treatments (Fig. 3d-f).

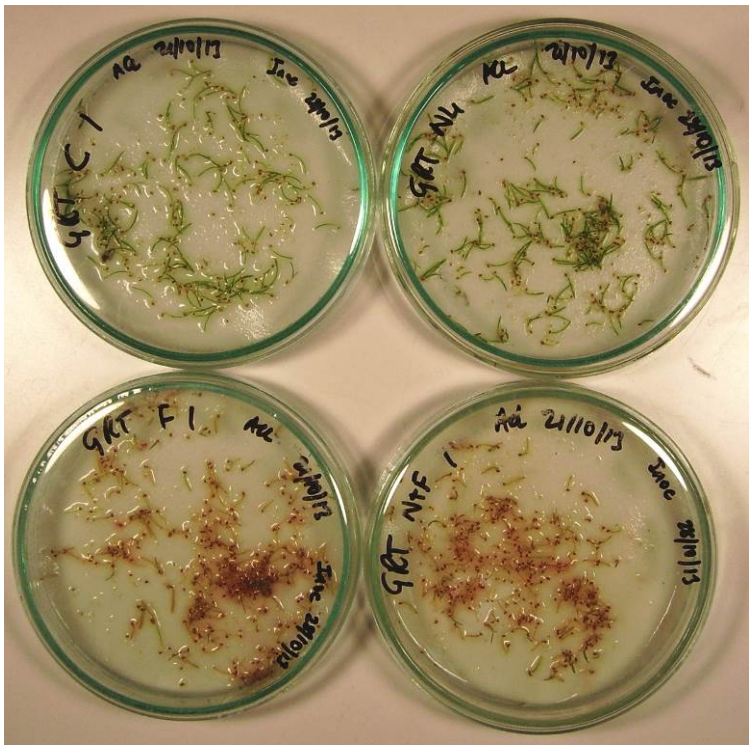


Fig. 3a. Appearance of GRT uninoculated (control) (top left), or inoculated with *N. oryzae* (top right), *Fusarium* sp. (bottom left) or both *N. oryzae* and *Fusarium* sp. (bottom right) 3 weeks after inoculation.

Fig. 3b. Appearance of GRT inoculated with *N. oryzae* 3 weeks after inoculation. Left: healthy seedlings; right: chlorotic seedlings. Both were scored as healthy in that they had an intact meristem and youngest green leaf.

Fig. 3c. Sporing of *Nigrospora oryzae* on filter paper under stereomicroscope.

Inoculation with *N. oryzae* had no effect on the percentage of healthy seedlings (in still having a green leaf and likely to grow further) (Fig. 3d) but reduced leaf length (Figs 3e-f). Inoculation with *Fusarium* sp. and with both fungi had the same effect, of killing most of the seedlings. Those that survived were either at the edge of the Petri dish or on the glass side and had thus escaped disease so far.

It seems likely that the death of the seedlings was due more to *Fusarium* spp. than to *N. oryzae*. These seedling trials are conducted under conditions that inherently favour any pathogen and so the symptoms are likely to be much more severe than with larger plants.

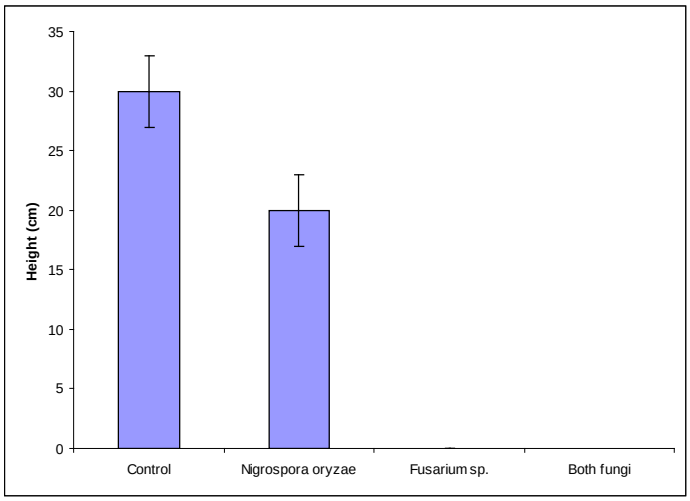


Fig. 3d. Effect of inoculation with fungi on height of GRT seedlings after 2 weeks (Experiment 1).

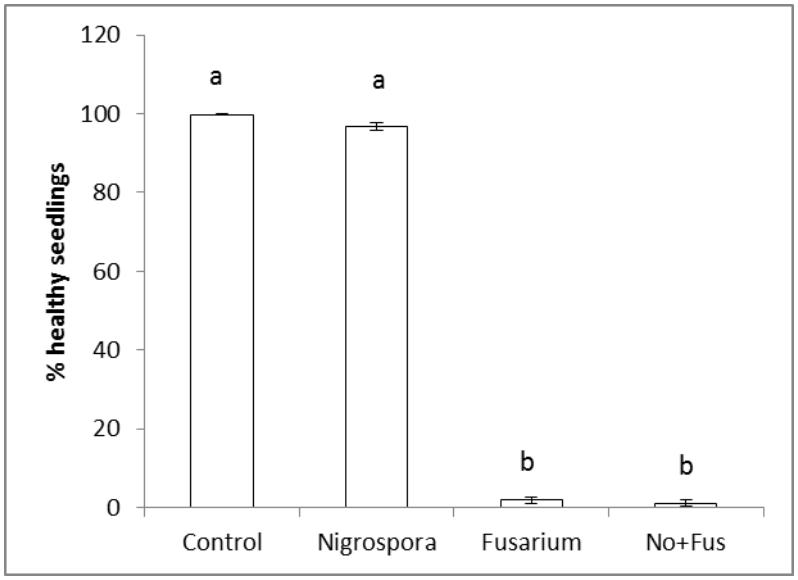


Fig. 3e. Effect of inoculation with fungi on % of healthy seedlings after 3 weeks (Experiment 2). ANOVA, F=4956.01, p<0.001. Kruskal-Wallis, H=12.80, p=0.005

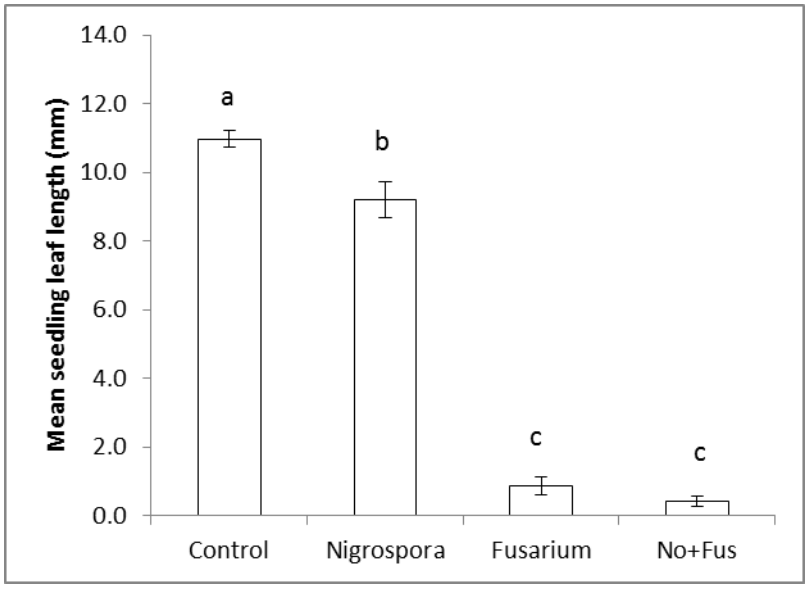


Fig. 3f. Effect of inoculation with fungi on total length of leaf (leaves) of GRT seedlings after 3 weeks (Experiment 2). ANOVA, F=279.58, p<0.001. Kruskal-Wallis, H=12.93, p=0.005

2. Effects of *Nigrospora oryzae* and *Fusarium* sp. on plants in pots

The aim of this part of the project was to assess if applying *N. oryzae* or a *Fusarium* sp. previously isolated from healthy plants in the field at Grafton to GRT plants in pots produced symptoms of blight as seen in the glasshouse or crown rot as seen in the field in GPG.

2.1 Materials and Methods

Plant materials. Pot trials were conducted with two sizes of GRT: small plants about 1 year old (previously germinated from the Trenayr, NSW, seeds) and large field-collected plants from Queensland kindly collected and sent to RMIT by Jeff in October. As a positive control, GPG plants raised from surface-sterilised seed (from Grafton - resistant to flupropanate and from Sconbiens – susceptible to flupropanate) were included.

Plant growth. The small GRT plants were grown from seed initially in a controlled temperature growth room at 25°C with lighting of 270 µmoles m^{-2} s^{-1} provided by halide lamps away from any source of contamination in the glasshouse. They were potted up into 12.5 cm diameter pots and placed in a glasshouse The large GRT plants were potted up with 2 days of arrival into 23 cm diameter pots and grown on the same glasshouse to recover; some large plants had to be split in two or three to fit the pots. GPG plants were grown from surface-steriised seed for the first 3 years in 15 cm diameter pots in the same growth room as the small GRT plants before transfer to the glasshouse and use in parallel in the trial. The glasshouse conditions were a controlled temperature of 15-25°C (winter) or 20-30°C (summer) and natural light, and no history of use with either fungus. Watering was provided by overhead sprayers controlled by automatic timers once (winter) or twice (summer) a day for 5 min.

Inoculation. Fungi were grown and spore suspensions prepared as described previously in 1.1.2. Plants were inoculated on 17 December 2012, 2 weeks after removing all dead matter above ground and trimming the foliage to 30 cm tall. Each pot to be infected was inoculated at the crown with 1 mL of suspension containing 10^6 spores mL^{-1}. This method was used because when GPG was inoculated previously this method produced as much disease as when foliage was sprayed with the suspension and it eliminated the accidental aerial dispersal of spores to uninoculated plants. Each infected pot was inoculated with 10^6 spores of one of the following treatments: *N. oryzae*, *Fusarium* sp. or an equal mixture of 10^3 spores of both fungi, and grown on in the glasshouses. Due to lack of obvious symptoms, plants were re-inoculated on 4 April with one agar cube (5 x 5 x 5 mm^3) per pot from active cultures of each fungus; dual inoculations received half-size cubes of each fungus. Dead matter was not removed at this stage as it may sustain saprophytic growth of the fungi and enable infection under vulnerable conditions. Plants of GPG were inoculated by the same method at the same time to check that symptomatic positive controls were produced by the cultures. There were two plants per treatment for the small GRT and four pots per treatment for the large GRT and the GPG; replication was limited for the small GRT due to poor survival and growth from seedlings but for large GRT and GPG by space due to competing requirements; a large new glasshouse was due to be built in 2011, then 2012, but the funding was subsequently postponed by the College of Science, Engineering and Health and is now scheduled for 2014 or 2015.

Assessment. Plants were inspected throughout growth for health (chlorosis and necrosis) and growth (height and girth). Culms were trimmed regularly to avoid seed

dispersal. Plant images were recorded on 26 July 2013 and at harvest, on 7-8 October 2013.

Biomass. To assess above-ground biomass, for each pot, on 8-9 October 2013 all above-ground plant parts were cut off at 1 cm above the soil and separated into live and dead material; each was placed in a separate paper bag and dried at 95°C for 24 h, cooled and weighed in the bag, with an average bag weight (n=5) subsequently being subtracted from the total to give the dry weight of live and dead biomass. Both total biomass and % live biomass per plant were calculated for analysis. Data were entered into spreadsheets in Microsoft Excel and the statistical program Minitab (Version 16). After checking for normal distribution, data were analysed by one-way Analysis of Variance (ANOVA) per type of plant for the effect of treatments; differences were regarded as significant if $p \leq 0.05$.

Isolations. To find the microflora associated with each type of plant and treatment and to test if the inoculants could be re-isolated, on 17-18 October 2013, a 1 cm length of live (green) and a dead (brown) culm was cut from the remaining plant material in each pot, giving 4 replicates of each of live and dead culms. Each was surface-sterilised separately in 50% Domestos (2.5% NaOCl + wetters) for 5 min and rinsed with three changes of sterile water for 2 min each. For the control and N. oryzae treatments, the culms were separated into their 4-5 imbricate parts before plating on 9 cm plastic Petri dishes of V8-juice agar (200 mL V8 juice, 3 g $CaCO_3$, 20 g Davis agar per litre) but others were plated whole on individual plates, except for small GRT, which were plated two per plate. The plates were incubated at 28°C for 14 days, when growths were recorded and examined microscopically by mounting suitable pieces in lactophenol cotton blue for identification.

As *Fusarium* species are difficult to identify with certainty by morphology, 'Fusarium' 1-4 were inoculated on 11 November 2013 on potato-sucrose agar for classical identification (Booth 1971, 1977; Burgess et al. 1988) and in glass jars containing 40 mL potato-sucrose broth for extraction of DNA and sequencing of the nuclear ribosomal internal transcribed spacer region. Fungal balls were harvested and DNA extracted on 2 December 2013 using a Qiagen DNeasy kit according to the manufacturer's instructions. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was amplified in the polymerase chain reaction (PCR) in 25 µL reactions containing 12.5 µL Promega GoTaq Green Master Mix, 1 µL ITS1 and 1 µL ITS4 primers (White et al. 1990), 8.5 µL nuclease-free sterile water and 2 µL of DNA extract. A G-Storm thermocycler was programmed with the following: 10 min 94°C, 35 cycles of: 30 s 94°C, 30 s 51°C, 1 min 72°C; and 10 min 72°C. A 5 µL aliquot of the PCR product was electrophoresed at 80-100 V on a 1.4% agarose gel in TAE (tris-acetic acid-EDTA) buffer to check that there was only one band of the expected size (~550 bp). The remainder was purified using a Qiagen PCR Purification kit according to the manufacturer's instructions. Sequencing of the ITS region was performed in 20 µL reactions according to the manufacturer's instructions using Applied Biosystems BigDye Mix 3.1 with the following reaction mixture: BigDye Mix 1 µL, 10 x buffer 2 µL, 1 µL ITS 4 primer and 14 µL of nuclease-free water in a thermocycler with the following cycles: 25 cycles of: 10 s 96°C, 5 s 50°C, 4 min 60°C. DNA was precipitated from sequencing reactions using ethanol precipitation protocol 1 according to the manufacturer's instructions, products dried overnight and sent to Micromon (Monash University) for electrophoresis and sequencing. Sequences were searched on the National Center for Biotechnology Information (NCBI)(http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) using Blastn and closest matches were recorded.

2.2 Results and Discussion

2.2.1 Appearance and biomass

All plants appeared healthy throughout growth although all had some dead leaves and culms as usual (Fig. 4). Until July, there appeared to be no obvious differences between treatments, but these were as expected, because with GPG symptoms do not appear until 3 months after inoculation and significant biomass differences were not seen until 6 months.

Fig. 4. Inoculated (left) and uninoculated (right) large plants of GRT in glasshouse.

In July 2013, it was thought that some differences between treatments in growth in the large GRT could be seen and images were recorded of all species (Figs 5-7). At this stage there were no obvious differences in the small GRT or GPG. Plants that made little growth after the culms and foliage was trimmed show this clearly, whereas those that made much growth do not.

Fig. 5. Growth of small (5" pots) plants from seed of Giant Rat's Tail Grass (GRT). C: uninoculated control, I+No: inoculated with *Nigrospora oryzae*, I+Fus: inoculated with

Fusarium sp. (field isolate from Giant Parramatta Grass - GPG), I+F+N:inoculated with both *N. oryzae* and *Fusarium* sp. on 26 July 2013

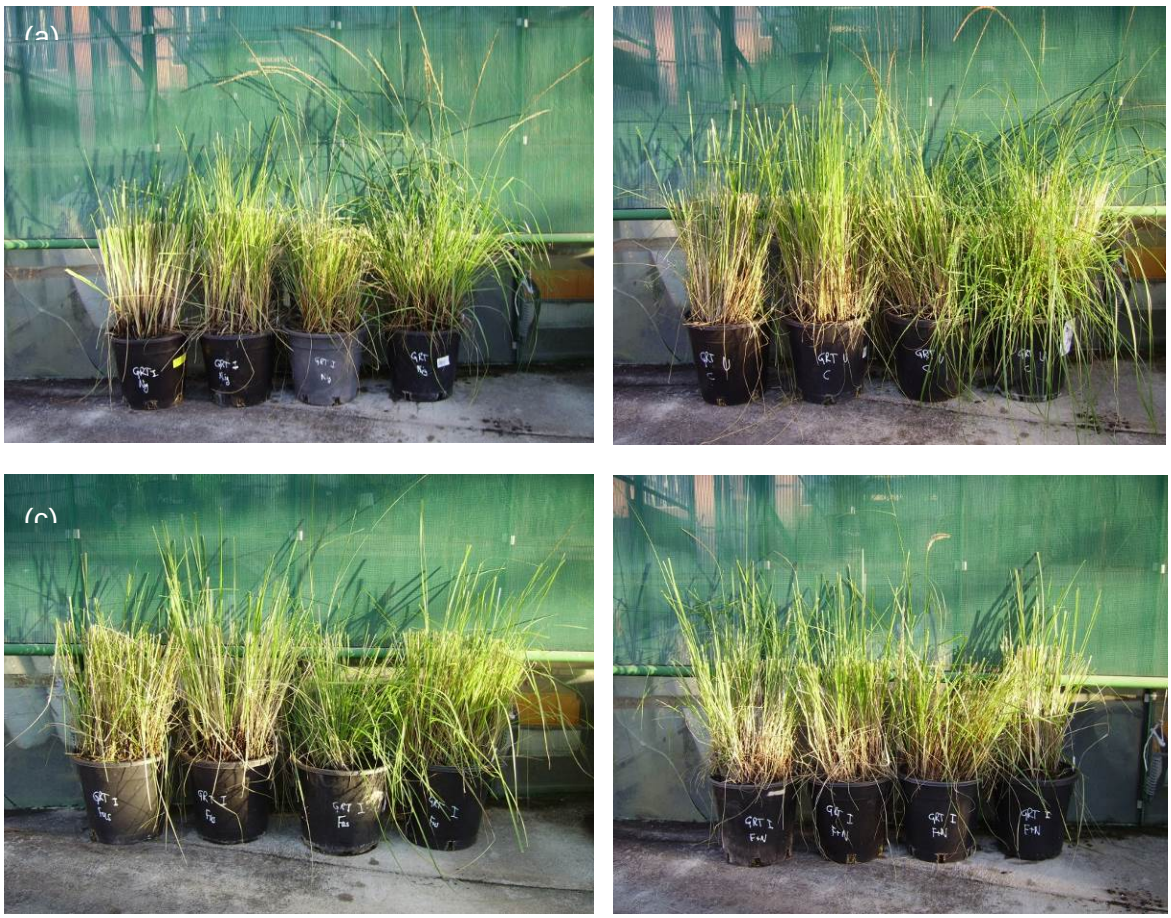


Fig. 6. Growth of large (10” pot) field-sourced plants of Giant Rat’s Tail Grass (GRT) (a) control, (b) after inoculation with *Nigrospora oryzae*, (c) after inoculation with *Fusarium* sp. (field isolate from Giant Parramatta Grass - GPG), (d) after inoculation with both *N. oryzae* and *Fusarium* sp. on 26 July 2013.

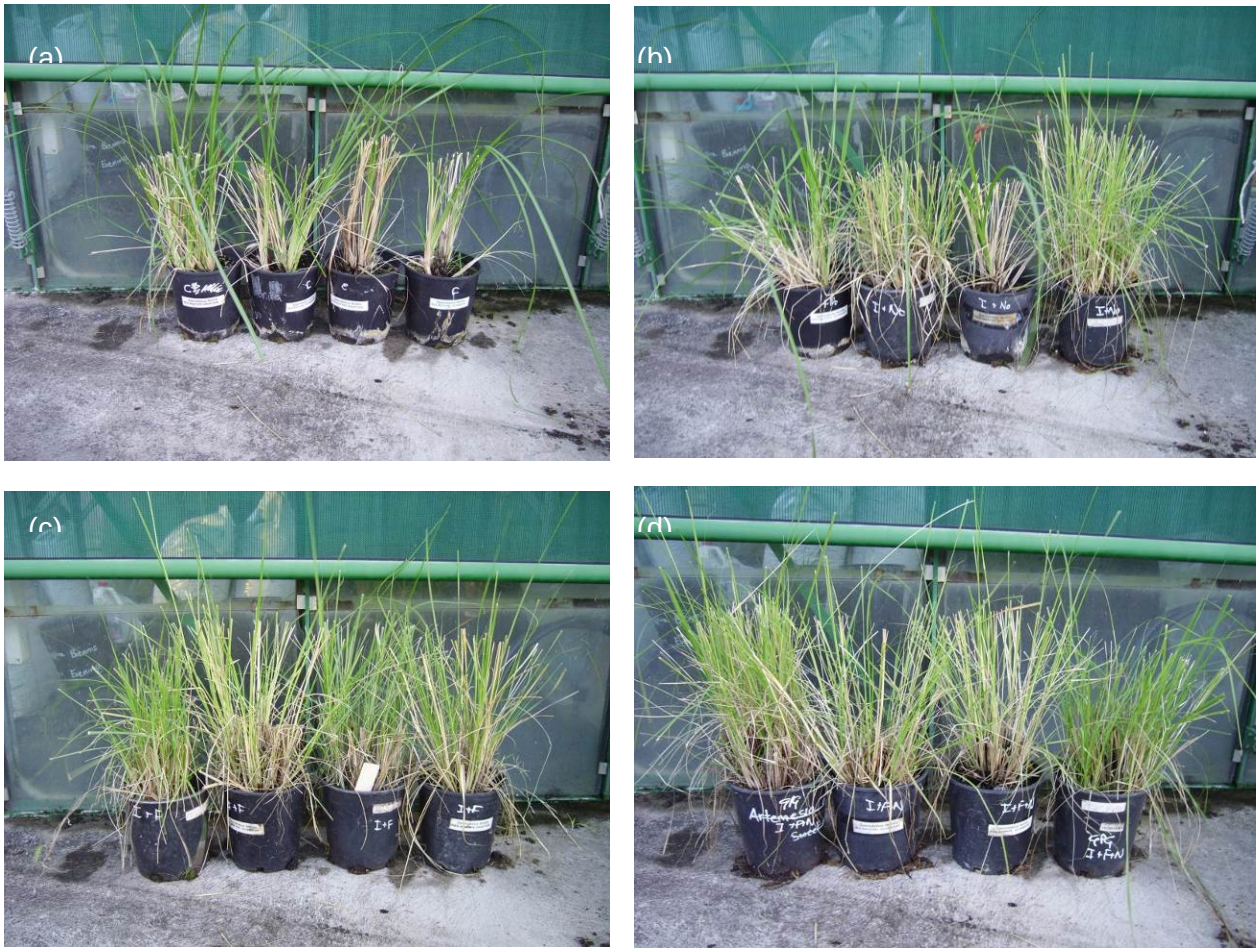


Fig. 7. Growth of medium (6” pots) seed-sourced plants of Giant Parramatta Grass (GPG) (a) control, (b) after inoculation with *Nigrospora oryzae*, (c) after inoculation with *Fusarium* sp. (field isolate from Giant Parramatta Grass - GPG), (d) after inoculation with both *N. oryzae* and *Fusarium* sp. on 26 July 2013.

At harvest in October 2013, in the small GRT plants, there was no significant difference in % live biomass (ANOVA, F=0.05, p=0.891) or total biomass (ANOVA, F=0.27, p=0.844) with treatment (Figs 8-9). The large variation in the sizes of the duplicate controls made it impossible to find such a difference statistically, although the plant treated with *N. oryzae* appeared smallest (Fig. 8). The low survival of transplanted seedlings reduced the replication to less than desirable and greater replication may have shown differences.

Fig. 8. Small GRT plants at harvest. Left to right, 2 pots of each of the treatments: control, *Nigrospora oryzae*, *Fusarium* sp., and both fungi together.

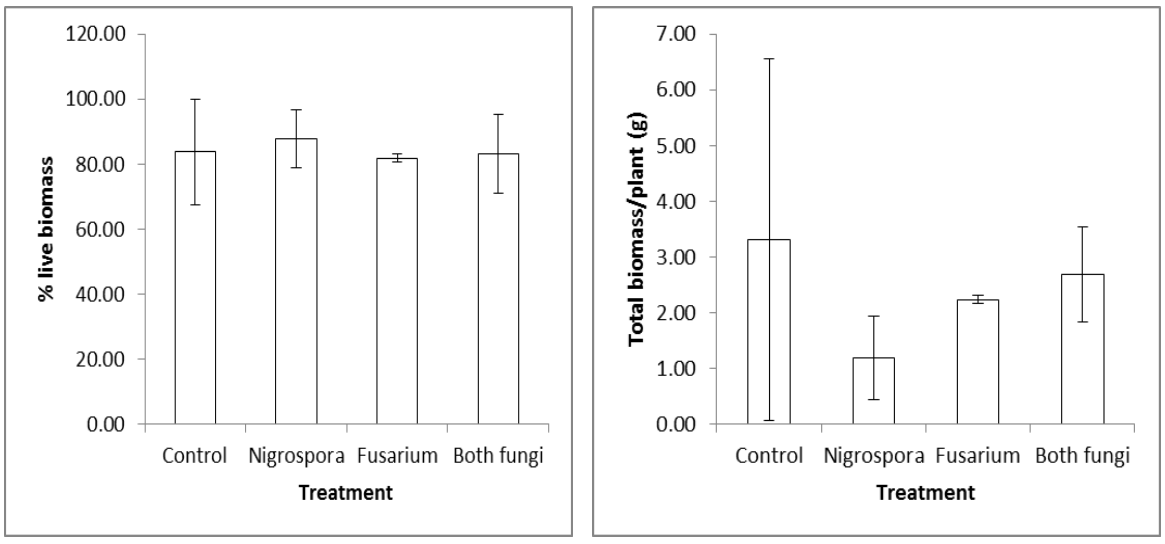


Fig. 9. Biomass of small GRT at harvest. Left: % live biomass per plant; right: total biomass per plant.

In the large GRT, there was no significant difference in % live biomass (ANOVA, F=0.13, p=0.943) but there was a significant difference in total biomass (ANOVA, F=4.51, p=0.024) with treatment (Figs 12-13). The total biomass for the both fungi treatment was significantly less than that for the control; the *N. oryzae* and the *Fusarium* treatments were intermediate. The total biomass for the both fungi treatment was only 45%, the *N. oryzae* treatment 75% and the *Fusarium* treatment only 59% of the control. There appeared to be more death in the trimmed culms in the inoculated treatments, in that many had not resprouted, whereas most had resprouted in the control treatment, but this was not assessed quantitatively. The centres of the inoculated tussocks showed signs of rot and fungal hyphae on the rotting culms (Fig. 14).

Fig. 12. Large GRT plants at harvest. Left to right, 4 pots (2 visible, 2 behind) of each of the treatments: control, *Nigrospora oryzae*, *Fusarium* sp., and both fungi together.

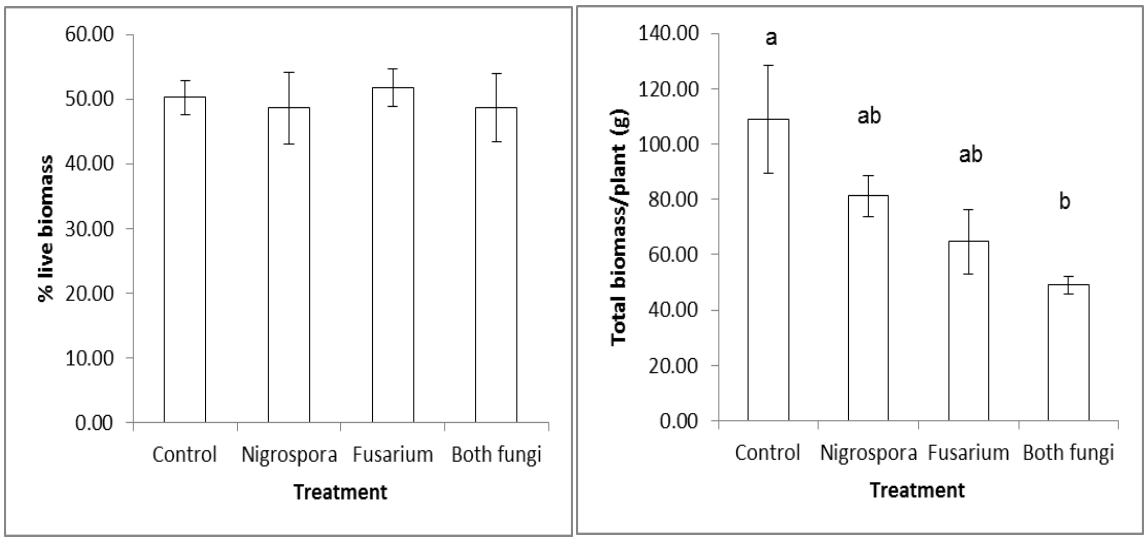


Fig. 13. Biomass of large GRT at harvest. Left: % live biomass per plant, right: total biomass per plant.

Fig.14. Fungal hyphae associated with rot on culms in centre of tussocks of large GRT. Top: inoculated with *Nigrospora oryzae*; bottom: inoculated with *Nigrospora oryzae* and *Fusarium* sp.

GPG responded differently from GRT. In GPG, there was a significant difference in % live biomass (ANOVA, F=9.70, p=0.002) but not in total biomass (ANOVA, F=0.94, p=0.453) with treatment (Figs 10-11a). The % live biomass for the *Fusarium* only and the both fungi treatments were the same and both were less than that for the control; the *N. oryzae* treatment was intermediate. The decrease in % live biomass is similar to previous results with *N. oryzae*, although in previous experiments the difference has been significant. The decrease with *Fusarium* sp. and both fungi together has not been tested previously.

Fig. 10. GPG plants at harvest. Left to right, 4 pots (2 in front, 2 behind) of each of the treatments: control, *Nigrospora oryzae*, *Fusarium* sp., and both fungi together.

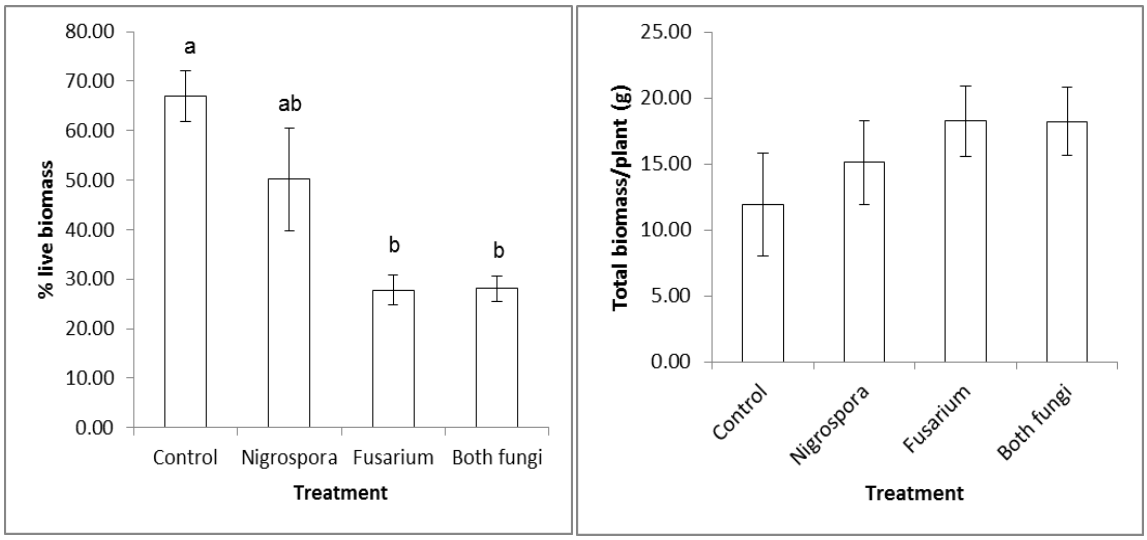


Fig. 11a. Biomass of GPG at harvest. Left: % live biomass per plant, right: total biomass per plant.

Regrowth after harvest of large GRT plants

Regrowth from GRT plants 5 weeks after harvest appeared to vary with treatment (Figs 11b) and so this regrowth was harvested and measured. The biomass of the regrowth varied in the same way as the original harvest, in that both fungi together reduced biomass and each alone was intermediate (Fig. 11c) (ANOVA, F-5.15, p=0.017). This suggests that regrowth is less vigorous after slashing and so would reduce the robustness of GRT in the field. There was no effect on the proportion of live tillers among treatments (Fig. 11c) (ANOVA, F-1.52, p=0.260), suggesting that it was the amount of growth made rather than the number of tillers responding that was different.

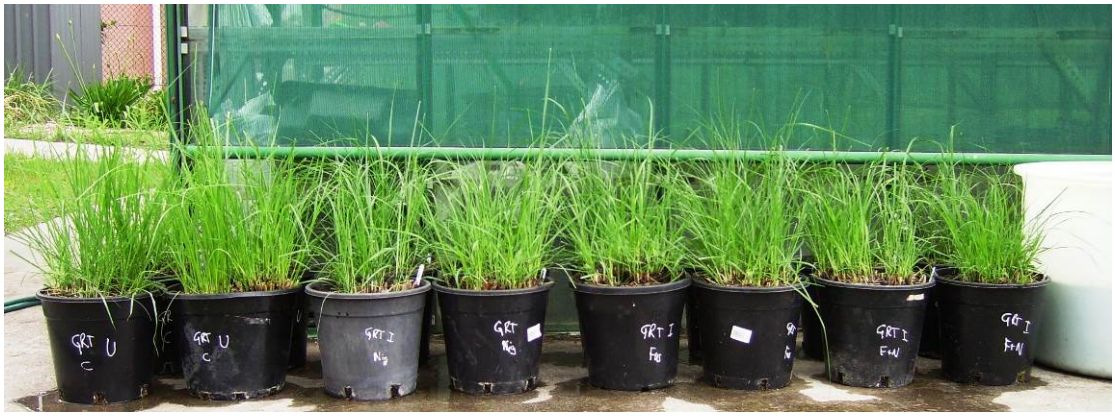


Fig. 11b. Regrowth in GRT plants 3 weeks after harvest. Left to right, 4 pots (2 in front, 2 behind) of each of the treatments: control, *Nigrospora oryzae*, *Fusarium* sp., and both fungi together.

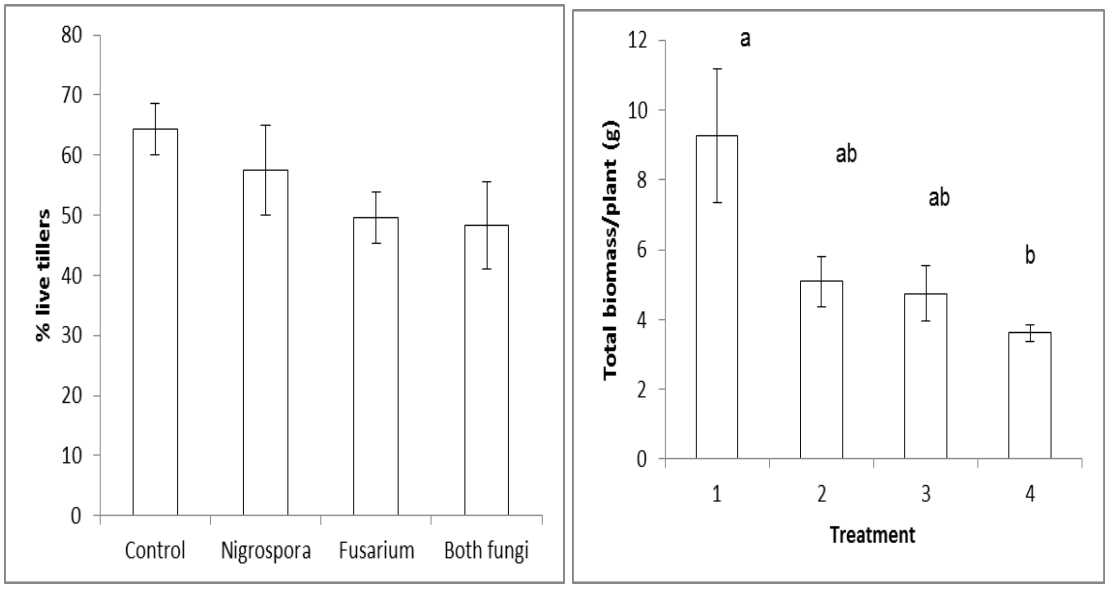


Fig. 11c. Biomass of GRT regrowth 5 weeks after harvest. Left: % live tillers per plant, right: total shoot regrowth biomass per plant.

2.2.2 Isolations

Fungi, bacteria and nematodes were isolated from the base of both green and brown culms of all plants (Figs 15-18). Generally more fungal than bacterial colonies were isolated (Tables 3-6). A total of 26 sporing fungi was isolated in 60 colonies as well as several fungi that were not sporing, and four morphologically distinguishable bacteria were also isolated in 26 colonies (Table 3). Though efforts were made to protect the small GRT and GPG plants from contamination in a growth room before the experiment, the large GRT started with a full complement of normal soil flora from Queensland and many of the fungi isolated are those normally associated with plant materials, e.g. *Epicoccum, Monilia*, in particular grasses, e.g. *Bipolaris.Alternaria*, as well as soils, e.g. *Humicola, Torulomyces.* Most of these are saprophytes or at most facultative pathogens.

Of the fungi isolated, only the following eight are likely to be primary pathogens (Tables 4-6): *Bipolaris* (leaf spots were observed at harvest), all four *Fusarium* types, *Geniculosporium*, *Nigrospora* and *Thielaviopsis*. Of these, those most likely to produce the crown rot symptom are *Fusarium* and *Nigrospora*.

The isolated fungi included both fungi used as inocula. *N. oryzae* was only isolated from large GRT (Table 3a) but the large number of specimens overgrown by bacteria and nematodes may have hidden further infection. Similar problems have arisen in previous work with GPG, in which both *Nigrospora* and *Fusarium* spp. have been re-isolated in both glasshouse and field experiments, with *Fusarium* spp. in the majority.

Fusarium spp. were isolated frequently from all treatments, including the controls, and from both live and dead culms (Table 3a), though more may have been present. Isolates were divided on morphology into four types and, because of the complexities of identifying *Fusarium* species, sequencing was performed to identify all four isolates to species (Table 3c). The suggested identities were checked thoroughly against the morphology of the same isolates on potato-sucrose broth; all matched the expected features except for ‘Fusarium 3’, as the isolate subcultured simultaneously for sequencing had been overgrown by *N. oryzae*. Ideally, more time would be allowed for repeated subculture and purification before sequencing.

F. proliferatum was isolated from culms of all plant species, whether live or dead (Table 3a), whereas *F. chlamydosporum* was isolated only from large GRT plants, in which central crown rot was evident. The initial inoculum used was identified morphologically as *F. chlamydosporum*, but it is possible that it was a mixed culture with *F. proliferatum*, or *F. proliferatum* may have been present on/in in all plants, as an endophyte or saprophyte. Any future experiments should include initial testing for microflora associated with the plants. *F. proliferatum* was pathogenic to GRT seedlings (Fig. 3) and both it and *F. chlamydosporum* could be purified as single-spore cultures for further experimentation to establish if each can cause disease individually if desired, though it is likely that both will be present and available in most Queensland pasture soils infested with WSG (Burgess et al. 1988). *F. proliferaturm* is present in eastern Australia (Burgess et al. 1988) and *F. chlamydosporum* was reported as common in soils throughout eastern Australia and ‘abundant’ in grassland soils in central Queensland, as well as being isolated from plants (Burgess et al. 1988). Also, Dr Sethu Ramasamy isolated six species of *Fusarium* from GRT from Queensland and northern NSW during an RIRDC-funded project in 2010-2011 (*F. proliferatum, F. equiseti, F. sporotrichioides, F. chlamydosporum, F. oxysporum* and *F. graminearum* – all identified by sequencing as well as morphologically).

F. proliferatum is a puzzling organism because it has a very wide range of plant hosts and occurs widely in a variety of habitats (both natural and agricultural) around the world (Proctor et al. 2010). It is part of a species complex that also includes other species that infect grasses and it seldom appears to occur as the only *Fusarium* species in nature (Leslie et al. 2004). Strains also differ in the amount and types of mycotoxin produced, which may influence the amount of symptoms noted (Leslie et al. 2004). Specific strains may infect *Sporobolus* species with/without disease symptoms and toxin production may be modified by challenge with other pathogens, predators or environmental conditions, as is known in other toxin-producing fungi.

F. chlamydosporum has been implicated in root rot in *Coleus* (in conjunction with the bacterium *Ralstonia* (previously *Pseudomonas*) *solanacearum)* (Singh et al. 2013), reduction in seed germination in a variety of weeds (Mohler et al. 2012), head blight in wheat in Iran (Chehri et al. 2011) and mycotoxin contamination of wheat in Kenya (Wagacha et al. 2010), among others. By contrast, *F. chlamydosporum* has been suggested for biocontrol of groundnut (peanut) rust as it reduced pustule size and urediniospore germination (Mathivanan and Murugesan 2000). It too is seldom isolated without other *Fusarium* species.

Both *N. oryzae* and *F. proliferatum* have been found as endophytes in *Jatropha curcas*, a tropical crop plant in India (Kumar and Kaushik 2013) and *N. oryzae* and *F. oxysporum* as endophytes in *Smallanthus sonchifolius* (Yacon) in South America (Rosa et al. 2012). *N. sphaerica* and *F. oxysporum* were present as endophytes of both endemic (*Bothriocloa macra*) and introduced (*Hyparrhenia hirta*) grasses in northern NSW, mainly from the stem and leaf (White and Backhouse 2007). In many other plants, both a *Nigrospora* and a *Fusarium* species are reported as endophytes but without deleterious symptoms in the plant, including rice (Fisher et al. 1992).

In GRT and GPG, the most likely hypothesis is that either a *Fusarium* species is initially present asymptomatically as an endophyte and that the addition of *N. oryzae* coupled with conducive growth conditions induces pathogenicity by either or both. The *Fusarium* species may not be specific - any *Fusarium* species may act in the same way. Alternatively, both fungi may colonise endophytically and it is only when they contact one another that an antagonistic reaction that may involve mycotoxin production is induced and results in crown rot, as a plant may be colonised simultaneously by several endophytes that have no interaction (White and Backhouse 2007). Antagonistic effects could be tested directly by agar plate challenges between *N. oryzae* and each of the *Fusarium* species. Similar methods could be used to test culture filtrates.

Ideally, each inoculant would be isolated only from the treatment in which it was used, but with an incubation of months rather than weeks in a glasshouse, such an expectation would be unrealistic. Isolating at intervals over the first few weeks may produce this result, but both *N. oryzae* and *Fusarium* sp. have abundant conidia that are spread by wind and water - in this regard, one of the problems with conducting field trials on GPG-infested pastures in NSW has been spread of the disease to the control blocks (9). It is recommended that tracing the inoculants be done over time in in the crown in future to find out what happens. The fungi could be followed by both classical isolation techniques and by using specific primers for the inoculants on DNA extracts from the crown tissues expected to be infected.

Many of the specimens produced bacteria and nematodes rather than fungi and may have prevented the pathogenic fungi from growing out (it would have been desirable to leave them longer before discarding). This could be the result of inadequate surface-sterilisation and it is recommended to double the time in NaOCl to 10 min, to pull apart the imbricated leaves in the base of the culm before surface-sterilisation

and to isolate on to media with and without an antibacterial compound such as streptomycin (or chloramphenicol + gentamycin) to avoid this in future. Most of the bacteria are expected to be saprophytic as all were bacilli and bacteria are not recorded as pathogens of *Sporobolus* species. This is perhaps too facile an assumption, however, as root rot in Coleus is caused by a complex of F. chlamydosporum with R. solanacearum and so it might be worthwhile isolating the major bacterial types and identifying them, most efficiently by sequencing the 16S rDNA.

All the nematodes were saprophytic (no stylet) and are normal soil fauna, but they were important in spreading the bacteria and possibly fungi on isolation plates. They should not be ignored, as their large numbers and prevalence across virtually all treatments means that they could play a role in triggering pathogenicity. Most species ingest bacteria, fungi or both and play a role in the breakdown of organic matter. Their high numbers in the imbricated leaf bases in the culm sections suggests that they may be important in the effect of inoculation and further experiments should be considered with and without nematicides.

Table 3a. Summary of fungi isolated from GRT and GPG (for details,see Tables 4-6).

GRT small		GRT large		GPG	
Live	**Dead**	**Live**	**Dead**	**Live**	**Dead**
		Acremonium	*Acremonium*		
			Aureobasidium		
		Alternaria?		*Alternaria alternata*	
					Arthrinium
				Aureobasidium	
		Basidiomycete			
			Basidiomycete *(Rhizoctonia?)*		
	Bipolaris			*Bipolaris*	
Epicoccum					
		Exophiala			
Fusarium proliferatum	*Fusarium proliferatum*	*Fusarium proliferatum*	*Fusarium proliferatum*	*Fusarium proliferatum*	*Fusarium proliferatum*
		Fusarium chlamydosporum	*Fusarium chlamydosporum*		
			Humicola		
Nigrospora oryzae/ Stachybotrys elegans		*Nigrospora oryzae/ Stachybotrys elegans*		*Nigrospora oryzae/ Stachybotrys elegans*	
Torulomyces indicus				*Torulomyces indicus*	
Geniculosporium					
			Microsporum		
		Monilia	*Monilia*	*Monilia*	
Nigrospora oryzae					
		Phoma			
Rhodotorula	*Rhodotorula*	*Rhodotorula*			
Scedosporium	*Scedosporium*		*Scedosporium*		
					Scopulariopsis
			Sporothrix		
			Thielaviopsis		
	Torulomyces indicus				*Torulomyces indicus*
		Trichoderma	*Trichoderma*		
			Yeast (large)		

Table 3b. Summary of bacteria isolated from GRT and GPG (for details, see Tables 4-6).

GRT small		**GRT large**		**GPG**	
Live	**Dead**	**Live**	**Dead**	**Live**	**Dead**
		Bacilli white			
		Bacilli hyaline		Bacilli hyaline	Bacilli hyaline
Bacilli cream	Bacilli cream	Bacilli cream		Bacilli cream	Bacilli cream
Bacilli orange					
Bacilli pink	Bacilli pink	Bacilli pink	Bacilli pink	Bacilli pink	Bacilli pink
		Bacilli yellow	Bacilli yellow	Bacilli yellow	Bacilli yellow

Table 3c. ITS sequences and closest matches for Fusarium 1-4.

'Fusarium'	Sequence length (bp)	% match	Closest match	Closest accession no.
1	530	99	*Fusarium proliferatum*	HM769951.1
2	534	99 98	*Fusarium* sp. NSC-13 *Fusarium chlamydosporum**	GU257906.1 KC778406.1*
3	550	95	*Stachybotryis bisbyi* (current correct name *S. elegans*)#	AF081480.2
4	585	98	*Monocillium indicum* (current correct name *Torulomyces indicus*)	GQ169328.1

*This is the closest sequence of a named species of *Fusarium* in the database. #Since the fungus was inoculated into media the agar cultures have been overgrown by *N. oryzae* and so *N. oryzae* has been recorded as isolated originally.

Table 4. Isolations from small GRT 18/10/13 (scored 27/10/13) (re-examined 10/12/13) P=pathogenic, S=saprophytic

Treatment	Live/dead	Type of growth	Colour	Texture	No. bits colonised	Total no.	% bits colonised	Identification	Role
Control	Live	Fungus	White	Fluffy, spreading	1	4	25	*Fusarium proliferatum*	P/S
		Fungus	White/grey blobs	fluffy	1	4	25	*Epicoccum*	S
		Bacterium	Cream	Smooth	1	4	25	Bacilli cream	S
		Bacterium	Orange	frilly	2	4	50	Bacilli orange	S
	Dead	Fungus	White	Fluffy, spreading	2	4	50	*Fusarium proliferatum*	P/S
		Fungus	White	Fluffy, spreading	1	4	25	*Nigrospora oryzae/ Stachybotrys elegans*	P/S
		Bacterium	Cream	Smooth	3	4	75	Bacilli cream	S
Nigrospora	Live	Bacterium	Cream	Smooth	4	4	100	Bacilli cream	S
	Dead	Fungus	White	velvety	2	4	50	*Fusarium proliferatum*	P/S
		Fungus	White	Fluffy, spreading	1	4	25	*Torulomyces indicus*	S
		Fungus	white-grey	fluffy, black blobs	1	4	25	*Nigrospora oryzae*	P/S
		Fungus	white	fluffy	1	4	25	*Geniculosporium*	P/S
		Fungus	olive-green	velvety	2	4	50	*Bipolaris*	P/S
Fusarium	Live	Fungus	Grey-green	fluffly	1	4	25	*Scedosporium*	S
		Bacterium	Cream	Smooth	3	4	75	Bacilli cream	S
	Dead	Fungus	Grey-green	fluffly	1	4	25	*Scedosporium*	S
		Yeast	Pink	Smooth	1	4	25	*Rhodotorula*	S
		Bacterium	Cream	Smooth	2	4	50	Bacilli cream	S
No+Fus	Live	Bacterium	Cream	Smooth	4	4	100	Bacilli cream	S
	Dead	Bacterium	Cream	Smooth	4	4	100	Bacilli cream	S

Table 5. Isolations from large GRT 17/10/13 (scored 24/10/13) (re-examined 10/12/13). P=pathogenic, S=saprophytic

Treatment	Live/dead	Type of growth	Colour	Texture	No. bits colonised	Total no.	% bits colonised	Identification	Role
Control	Live	Fungus	White	Fluffy, spreading	3	18	17	*Fusarium proliferatum*	P/S
		Fungus	White-pink	Fluffy-floccose	5	18	28	*Monilia*	S
		Fungus	Grey-white	Fluffy	3	18	17	*Alternaria*?	
		Bacterium	Cream	Smooth	6	18	33	Bacilli cream	S
		Bacterium	Yellow	Smooth	4	18	22	Bacilli yellow	S
		None			2	18	11		
	Dead	Fungus	Green	Powdery	8	19	42	*Trichoderma*	S
		Fungus	White-pink	Fluffy-floccose	2	19	11	*Monilia*	S
		Fungus	Cream-grey	Velvety-greasy	1	19	5	*Aureobasidium*	S
		Fungus	White	Fluffy, spreading	6	19	32	*Fusarium proliferatum*	P/S
		Fungus	Grey-green	Velvety	3	19	16	*Scedosporium*	S
		Fungus	White-pink	Velvety, black bits	1	19	5	*Humicola*	S
		Bacterium	Cream	Smooth	2	21	10	Bacilli cream	S
		Bacterium	Yellow	Smooth	2	20	10	Bacilli yellow	S
Nigrospora	Live	Fungus	White	Fluffy-floccose	2	19	11	*Fusarium proliferatum*	P/S
		Fungus	White/red-purple	Fluffy, spreading	4	19	21	*Fusarium chlamydosporum*	P/S
		Fungus	Green	Powdery	1	19	5	*Trichoderma*	S
		Fungus	Olive-green	Velvety-greasy	3	19	16	*Exophiala*	S
		Fungus	white	slimy	2	19	11	*Acremonium*	S
		Fungus	White	Fluffy	2	19	11	Torulomyces indicus	S
		Bacterium	Cream	Smooth	12	19	63		S
		Nematodes	cream	slimy	6	19	32		S
	Dead	Fungus	White	wefts, spreading	2	4	50	Basidiomycete (clamp connections)	
		Fungus	Black	velvety, on stem	4	4	100	*Thielaviopsis?*	P/S
		Fungus	white	Fluffy, spreading	2	4	50	*Nigrospora oryzae*	P/S

		Yeast	white	scurfy	2	4	50	Large yeast	S
		Bacterium	cream	Smooth	1	4	25	Bacilli cream	S
		Nematodes	cream	slimy	1	4	25		S
Fusarium	Live	Fungus	white-grey	flat-fluffy	1	4	25	*Fusarium proliferatum*	P/S
		Fungus	Grey-white	fluffy	1	4	25	*Alternaria?*	S
		Fungus	White-grey	Fluffy, strands	1	4	25	*Phoma*	P/S
		Fungus	white	wefts, spreading	1	4	25		
		Bacterium	cream	Smooth	3	4	75	Bacilli cream	S
		Nematodes	cream	slimy	1	4	25		S
		Algae	cream	slimy	1	4	25	Diatoms	A
	Dead	Fungus	white	Fluffy, spreading	3	4	75	*Fusarium proliferatum*	P/S
		Fungus	Grey-white	flat	1	4	25	*Microsporum*	S
		Fungus	White, red reverse	Fluffy, spreading	1	4	25	*Fusarium chlamydosporum*	P/S
		Nematodes	cream	slimy	3	4	75		S
No+Fus	Live	Fungus	white	fluffy-floccose	2	4	50	*Fusarium proliferatum*	P/S
		Fungus	Black	in bacterial sludge	1	4	25		
		Fungus	Grey-white	fluffy	1	4	25	*Alternaria?*	
		Fungus	white	fluffy	1	4	25		
		Yeast	white	smooth	1	4	25	Large yeast	S
		Bacterium	cream	Smooth	3	4	75	Bacilli cream	S
		Nematodes	cream	slimy	3	4	75		S
	Dead	Fungus	green	Powdery	1	4	25	*Trichoderma*	S
		Fungus	white	floccose	1	4	25	*Sporothrix*	S
		Bacterium	cream	Smooth	3	4	75	Bacilli cream	S
		Nematodes	cream	slimy	1	4	25		S

Table 6. Isolations from GPG 17/10/13 (scored 27/10/13) (re-examined 10/12/13) P=pathogenic, S=saprophytic

Treatment	Live/dead	Type of growth	Colour	Texture	No. bits colonised	Total no.	% bits colonised	Identification	Role
Control	Live	Fungus	white	fluffy	4	4	100	*Fusarium proliferatum*	P/S
		Fungus	grey-white	fluffy	1	4	25	*Bipolaris*	P
		Bacterium	cream	smooth	1	4	25	Bacilli cream	S
		Bacterium	yellow	smooth	1	4	25	Bacilli	S
	Dead	Fungus	white	fluffy	3	4	75	*Fusarium proliferatum*	P/S
		Fungus	grey-white	fluffy	1	4	25	*Alternaria?*	P?
		Bacterium	cream	smooth	2	4	50	Bacilli cream	S
		Bacterium	yellow	smooth	1	4	25	Bacilli yellow	S
		Nematodes	cream	slimy	1	4	25		S
Nigrospora	Live	Fungus	white	fluffy	3	4	75	*Fusarium proliferatum*	P/S
		Bacterium	cream	smooth	2	4	50	Bacilli cream	S
		Bacterium	yellow	smooth	1	4	25	Bacilli yellow	S
		Nematodes	cream	slimy	1	4	25		S
	Dead	Fungus	white	fluffy	1	4	25	*Fusarium proliferatum*	P/S
		Fungus	grey-white	fluffy	1	4	25	Alternaria	P
		Bacterium	cream	smooth	4	4	100	Bacilli cream	S
		Nematodes	cream	slimy	2	4	50		S
Fusarium	Live	Fungus	white	fluffy	1	4	25	*Fusarium proliferatum*	P/S
		Fungus	white	fluffy-floccose	2	4	50	*Nigrospora oryzae*/ *Stachybotrys elegans*	P/S
		Bacterium	cream	smooth	4	4	100	Bacilli cream	S
	Dead	Fungus	white-pink	velvety	1	4	25	*Fusarium proliferatum*	P/S
		Fungus	white-pink	floccose	2	4	50	*Monilia*	S
		Fungus	dark grey	velvety	1	4	25	Arthrinium	S
		Bacterium	cream	smooth	2	4	50	Bacilli cream	S
		Nematodes	cream	slimy	1	4	25		S
No+Fus	Live	Fungus	white	flat-fluffy	1	4	25	*Fusarium proliferatum*	P/S

		Fungus	white	fluffy	1	4	25	*Stachybotrys elegans*	P/S
		Fungus	white	fluffy, wefts	2	4	50	Basidiomycete?	
		Bacterium	cream	smooth	1	4	25	Bacilli cream	S
	Dead	Fungus	white-pink	velvety	1	4	25	*Fusarium proliferatum*	P/S
		Fungus	white	fluffy	1	4	25	*Torulomyces indicus*	S
		Fungus	white	fluffy, wefts	2	4	50	Basidiomycete?	
		Fungus	white	fluffy	1	4	25	*Scopulariopsis?*	S
		Bacterium	cream	smooth	2	4	50	Bacilli cream	S

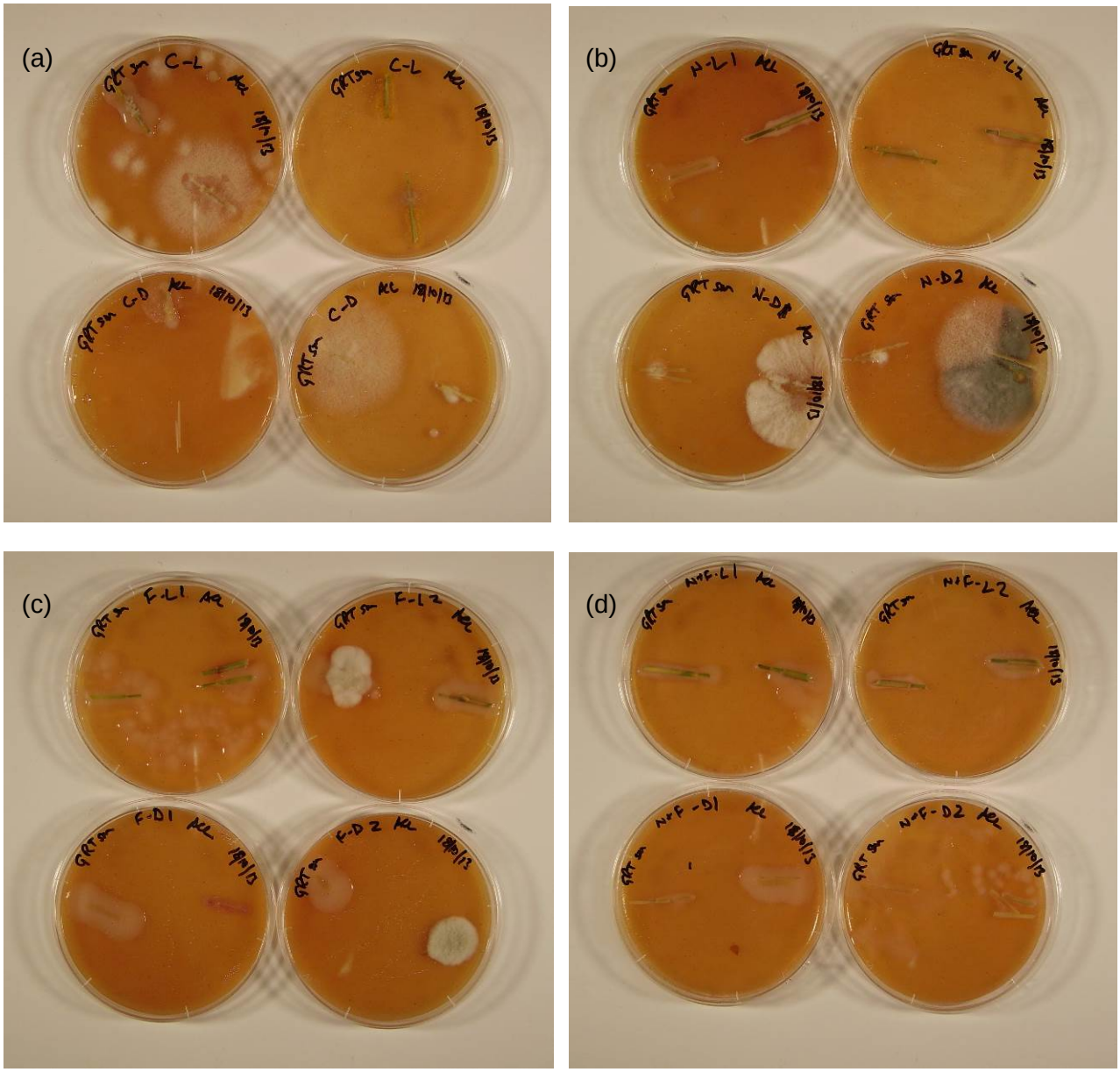


Fig. 15. Growths isolated from small GRT plants after harvest. (a) control, (b) inoculated with *Nigrospora oryzae*, (c) inoculated with *Fusarium* sp., (d) inoculated with both *Nigrospora oryzae* and *Fusarium* sp.

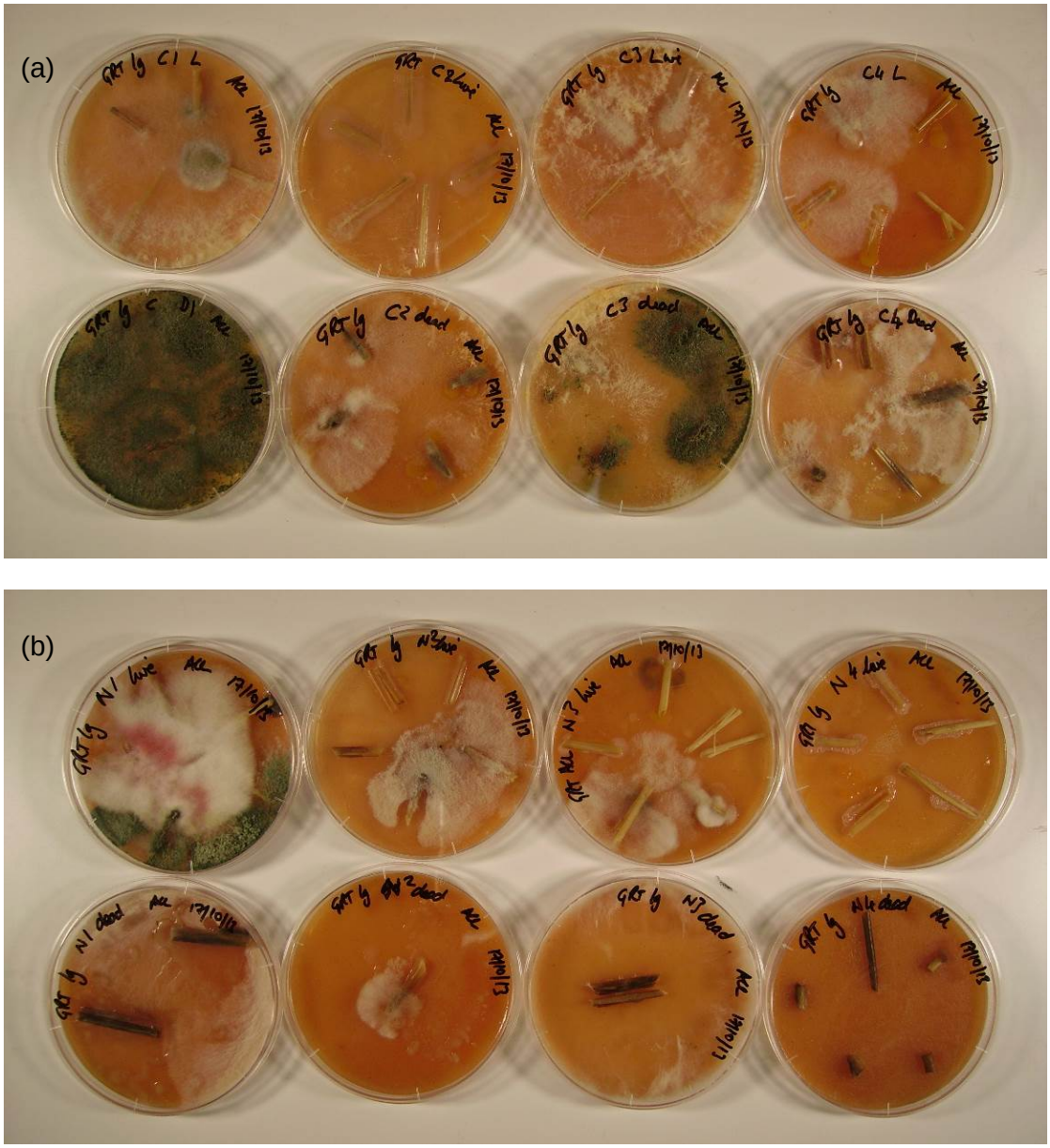


Fig. 16a. Growths isolated from large GRT plants after harvest. (a) control, (b) inoculated with *Nigrospora oryzae*.

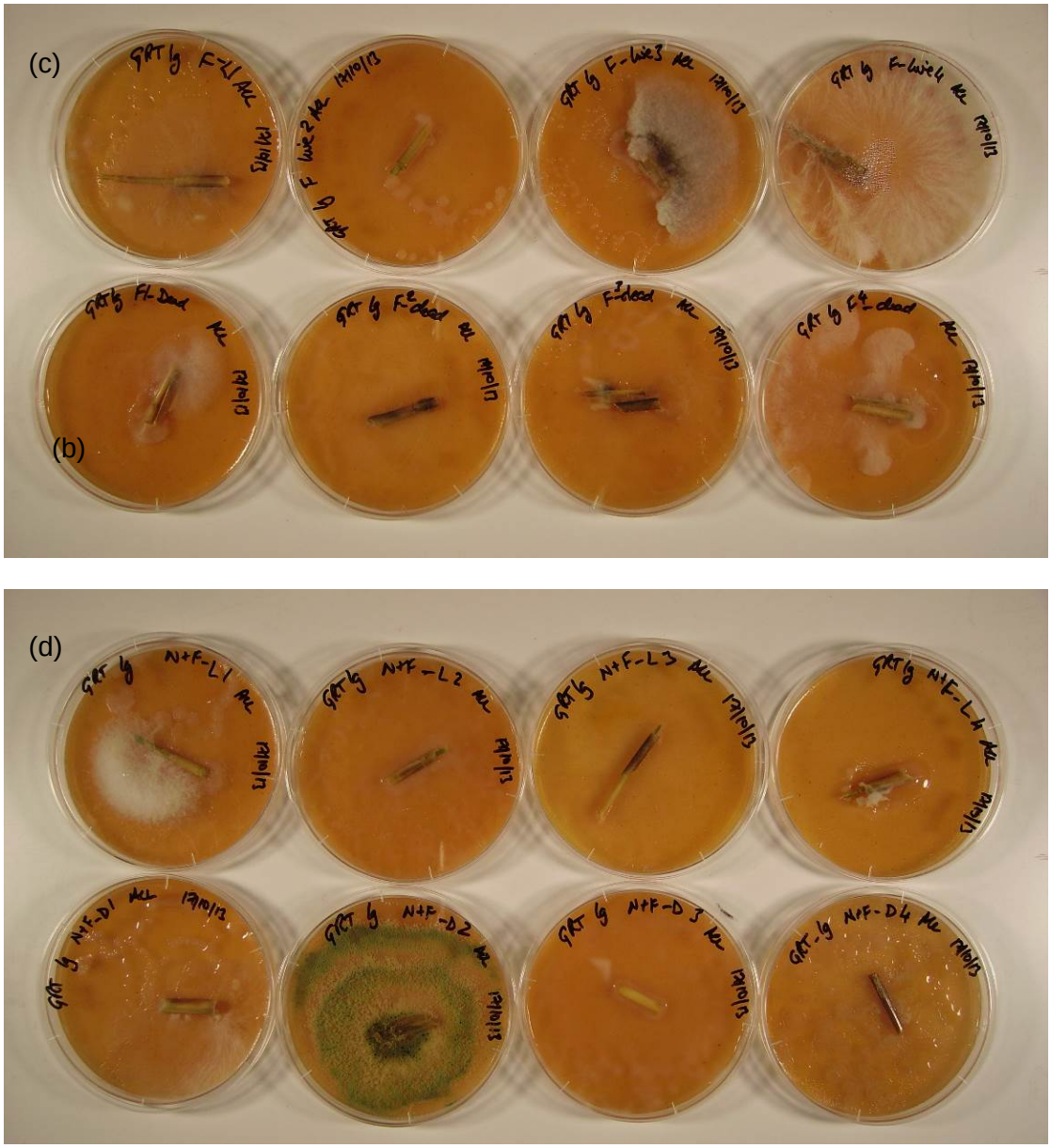


Fig. 16b. Growths isolated from large GRT plants after harvest. (a) control, (b) inoculated with *Nigrospora oryzae*.

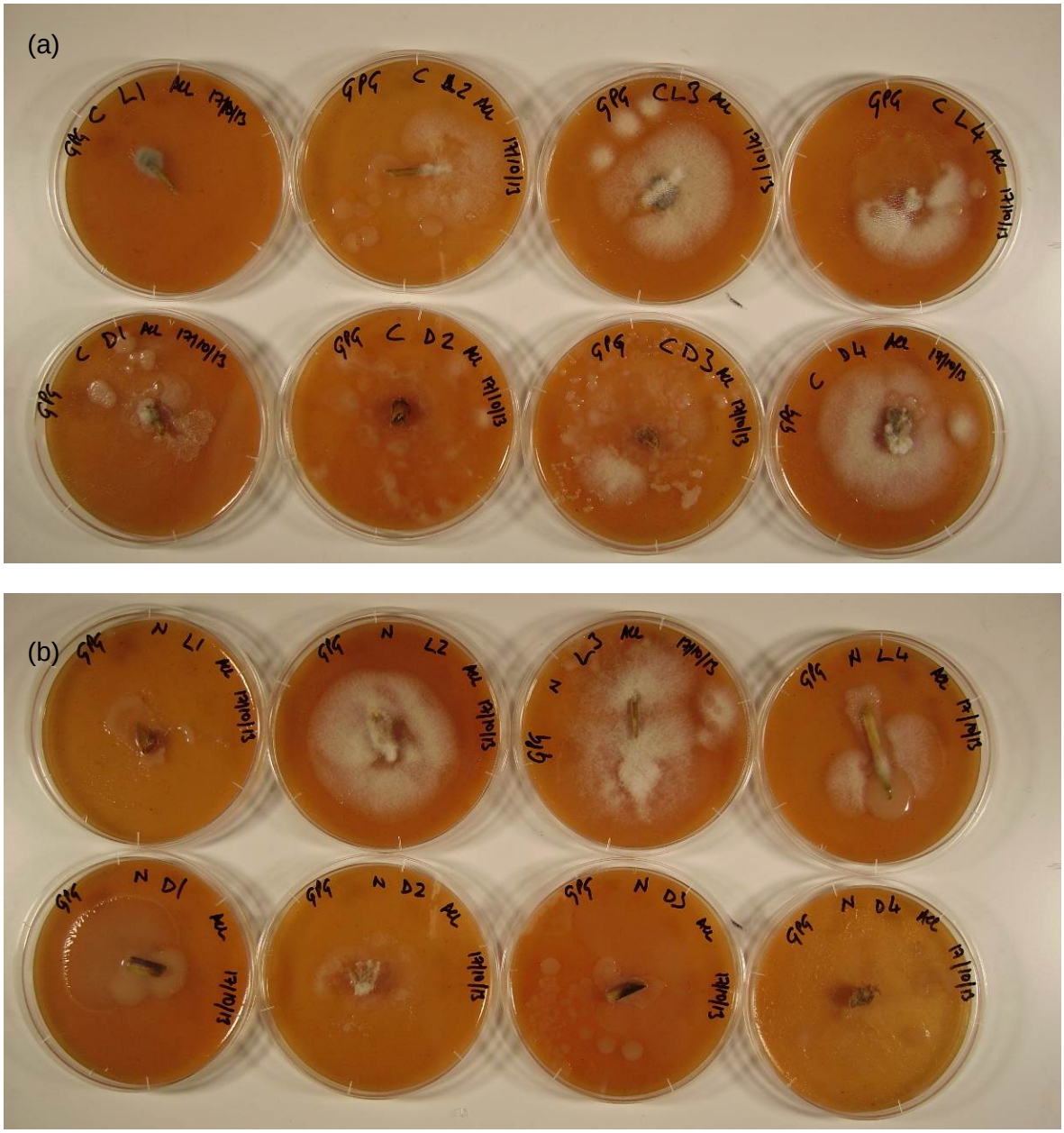


Fig. 17a. Growths isolated from GPG plants after harvest. (a) control, (b) inoculated with *Nigrospora oryzae*.

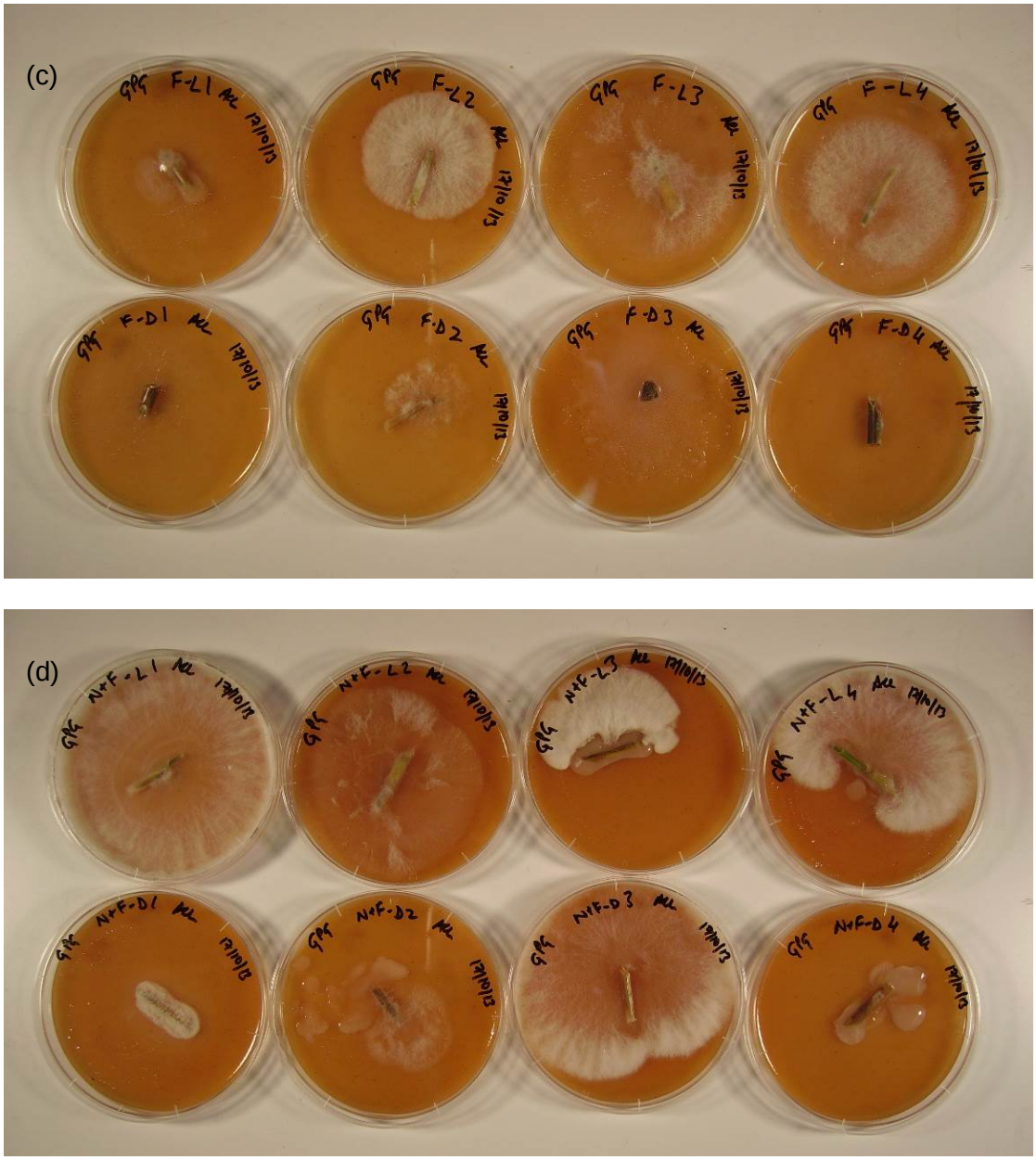
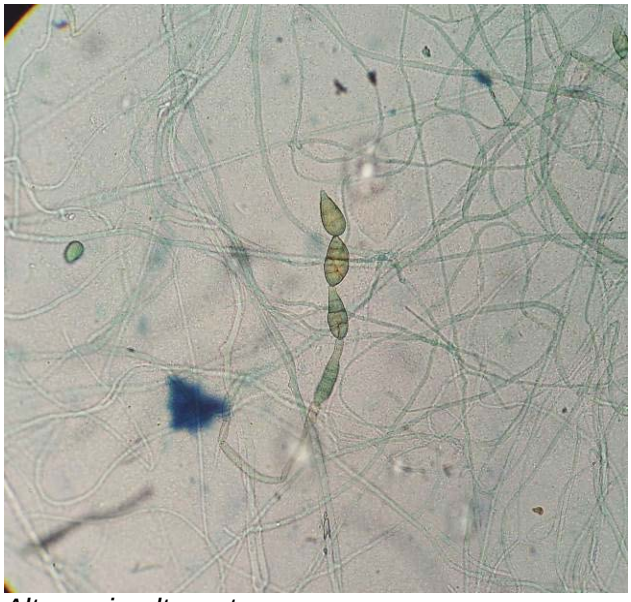
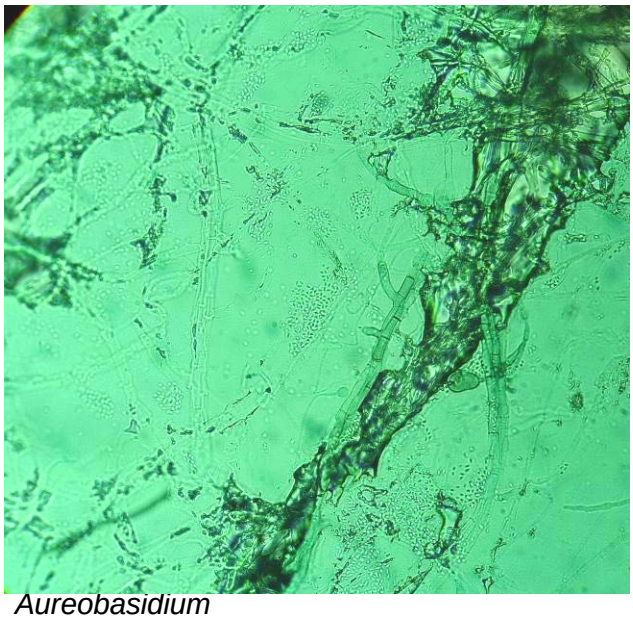


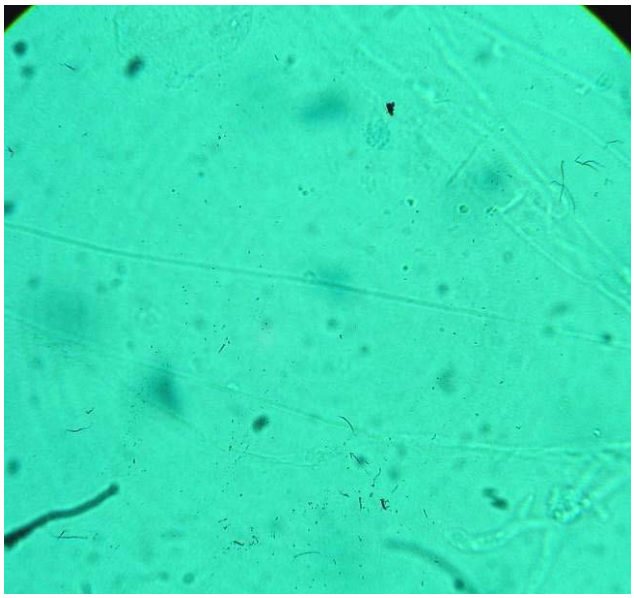
Fig. 17b. Growths isolated from GPG plants after harvest. (c) inoculated with *Fusarium* sp., (d) inoculated with both *Nigrospora oryzae* and *Fusarium* sp.



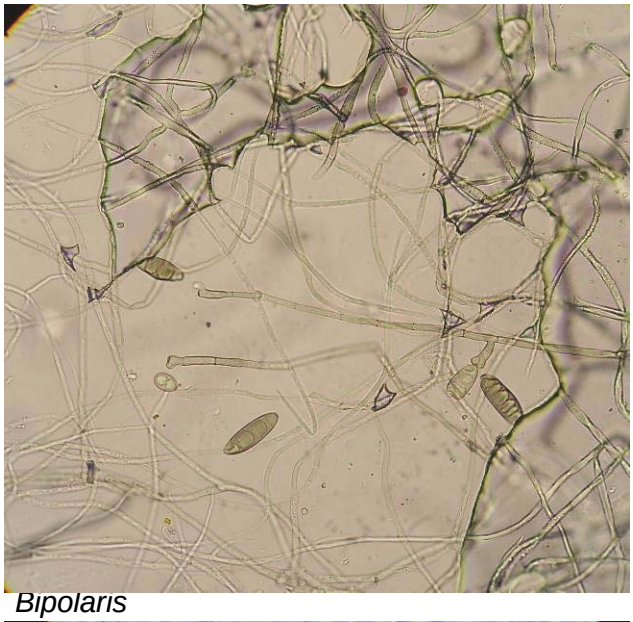
Alternaria alternata



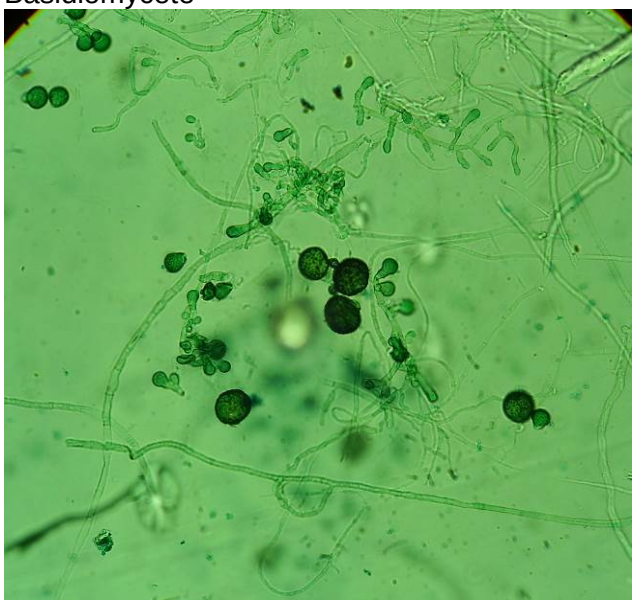
Aureobasidium



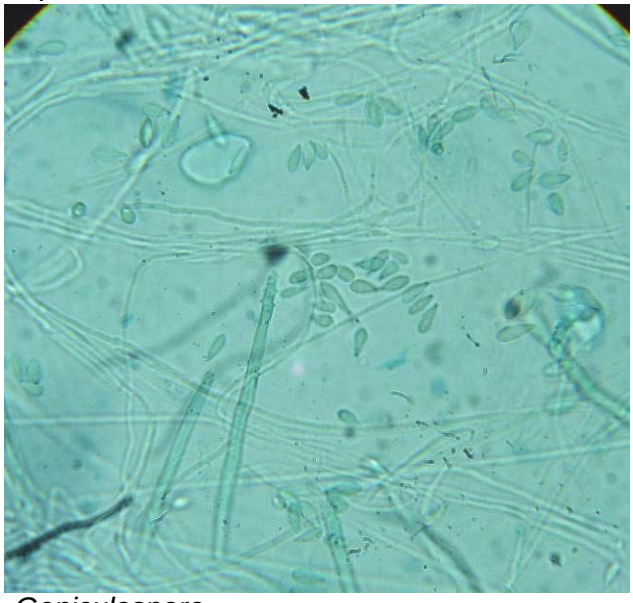
Basidiomycete



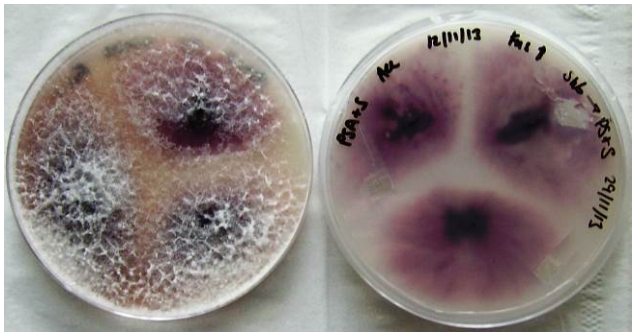
Bipolaris

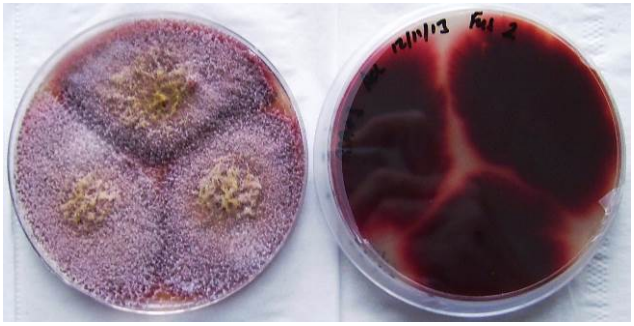


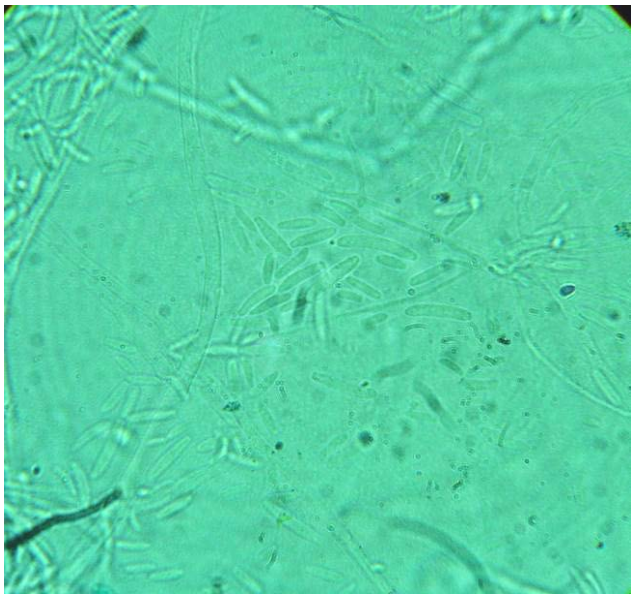
Epicoccum

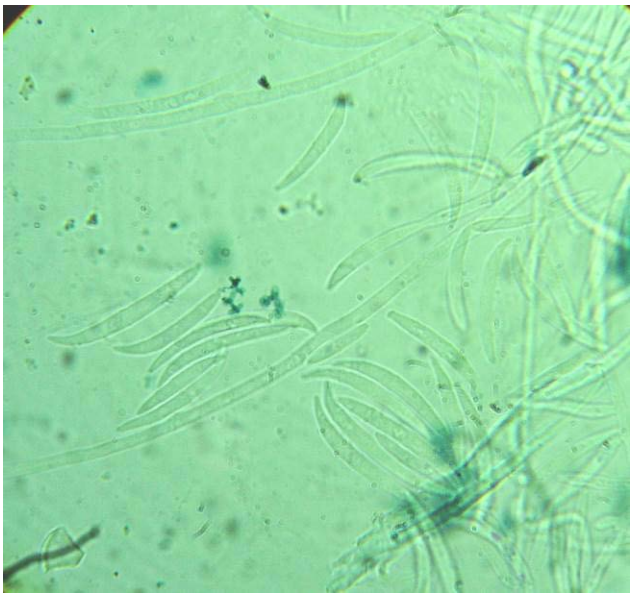


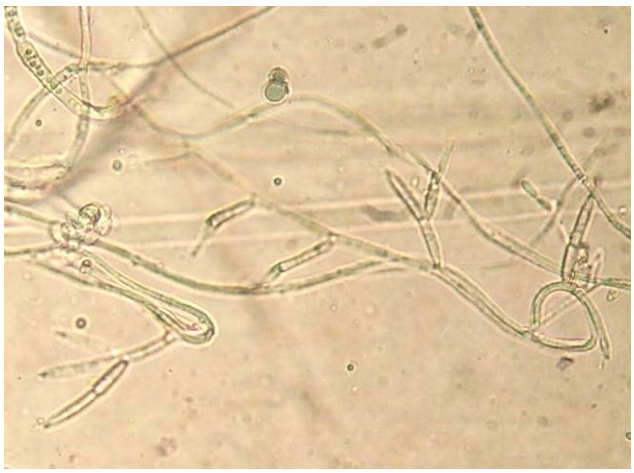
Geniculospora

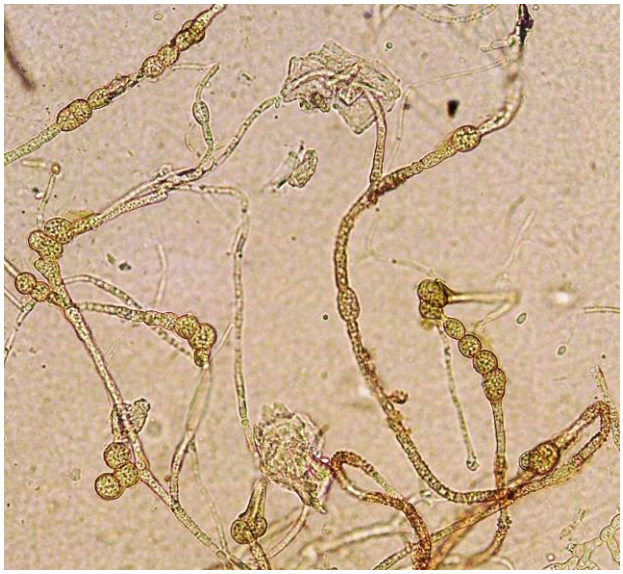


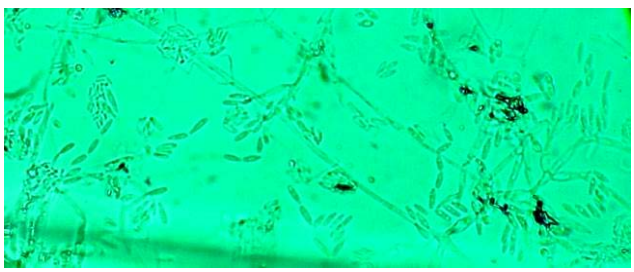






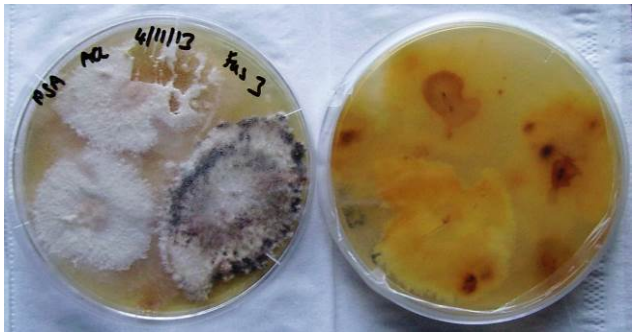


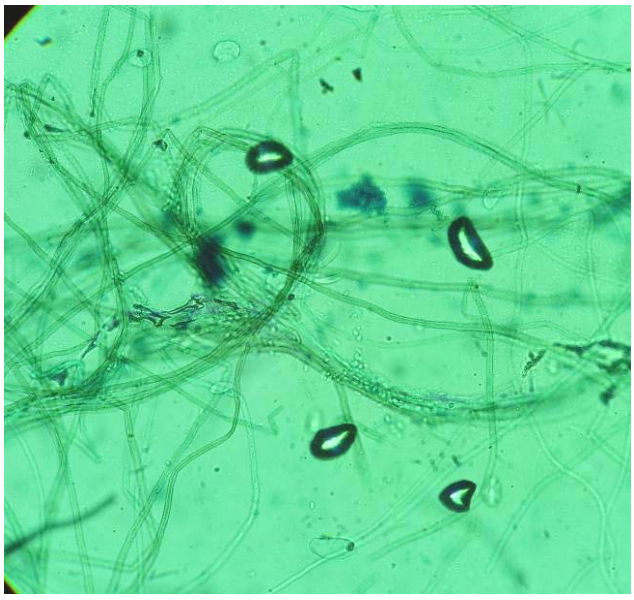


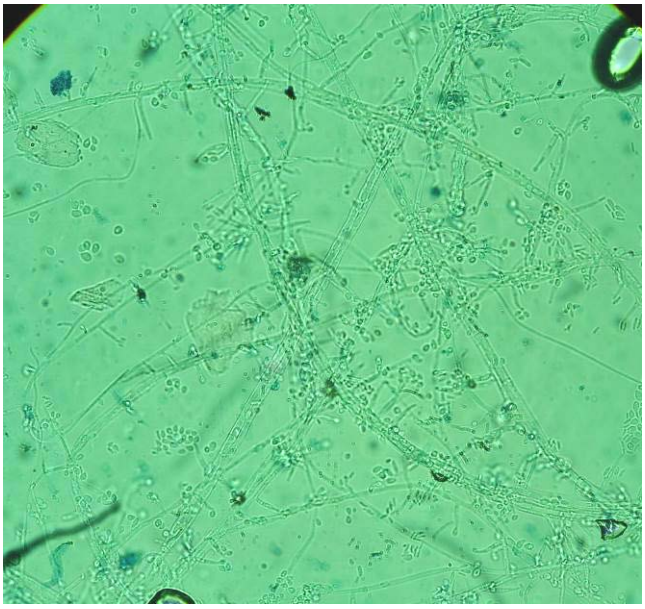


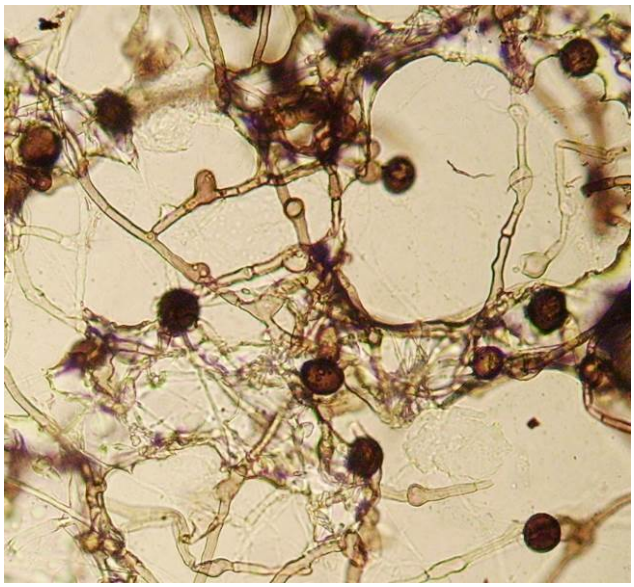
'*Fusarium* 1' = *F. proliferatum* (sequencing)

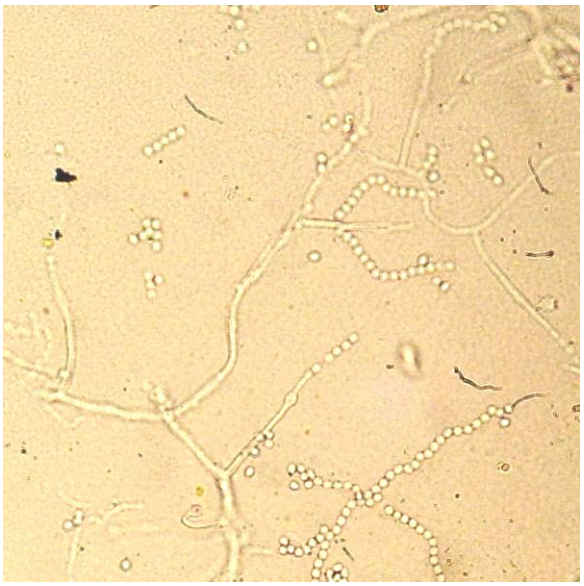
'*Fusarium 2*' =*F. chlamydosporum*





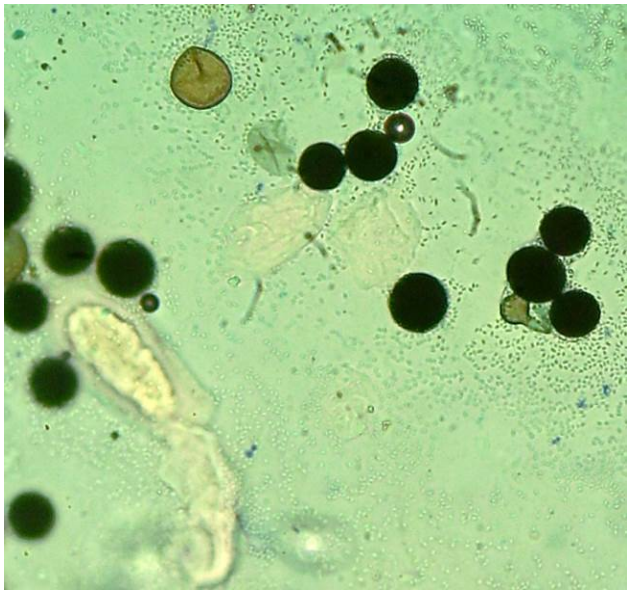




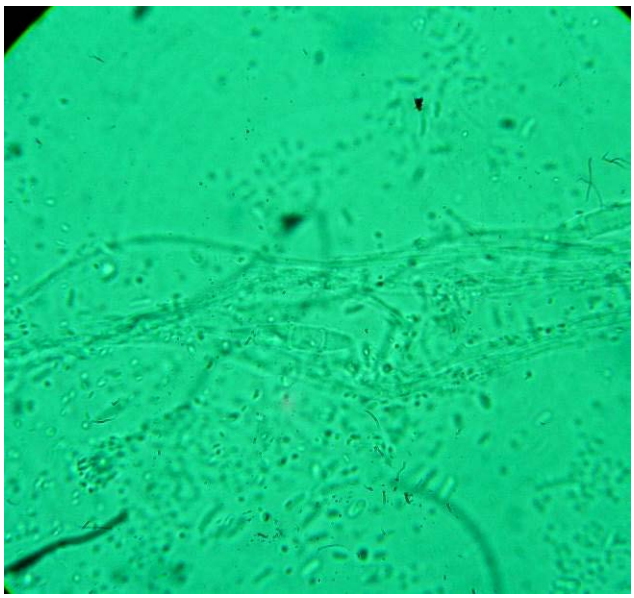


'*Fusarium* 3' = *Stachybotrys bisbyi* (sequencing)

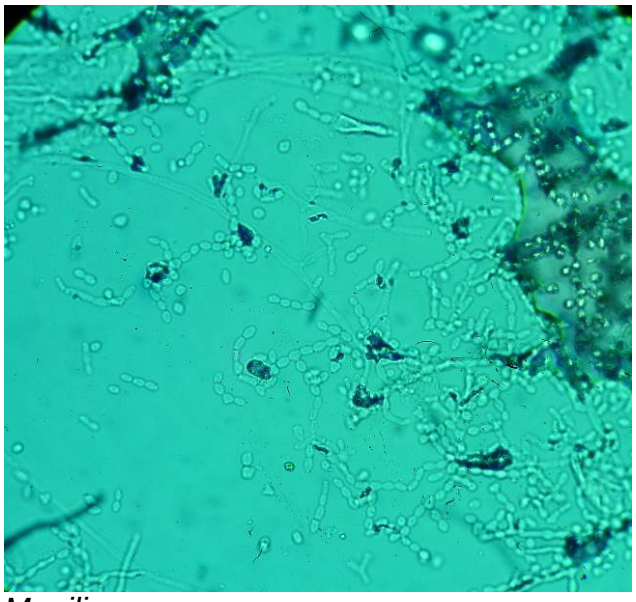
'*Fusarium* 4' = *Monocillium indicum* (seq.)



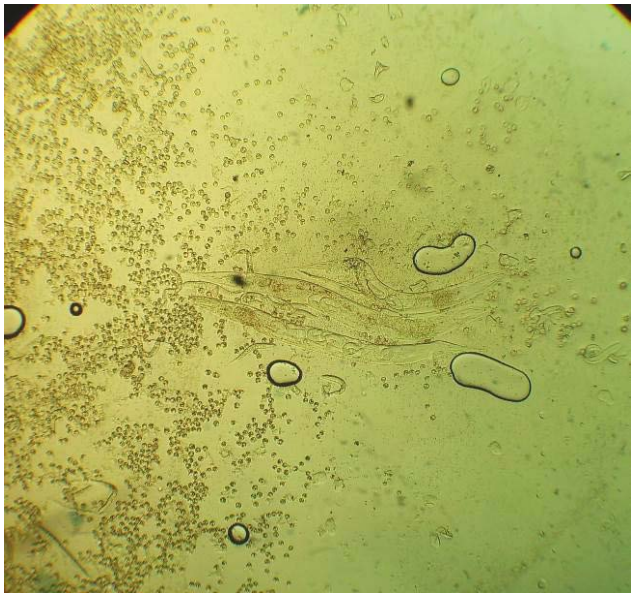
Humicola



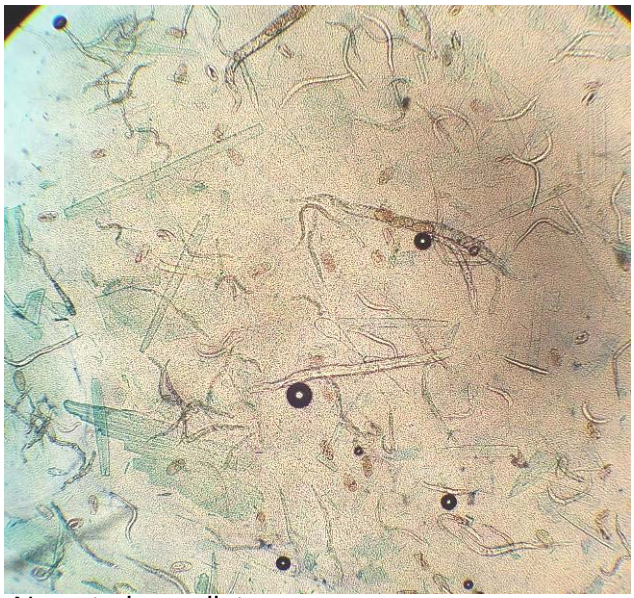
Microsporum



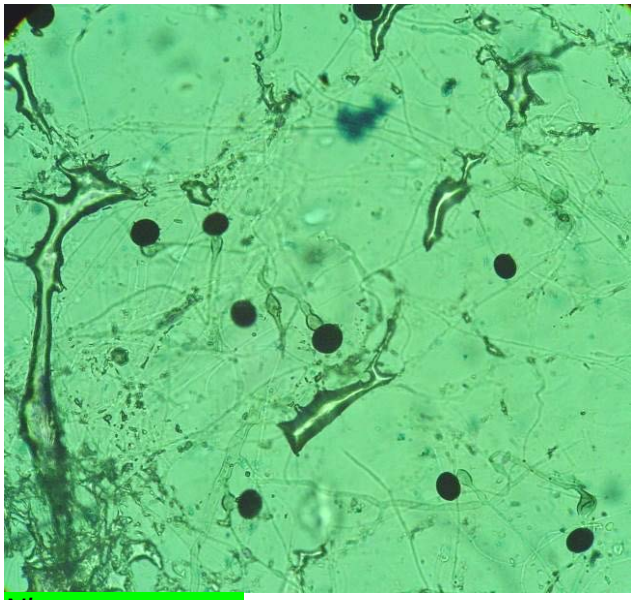
Monilia



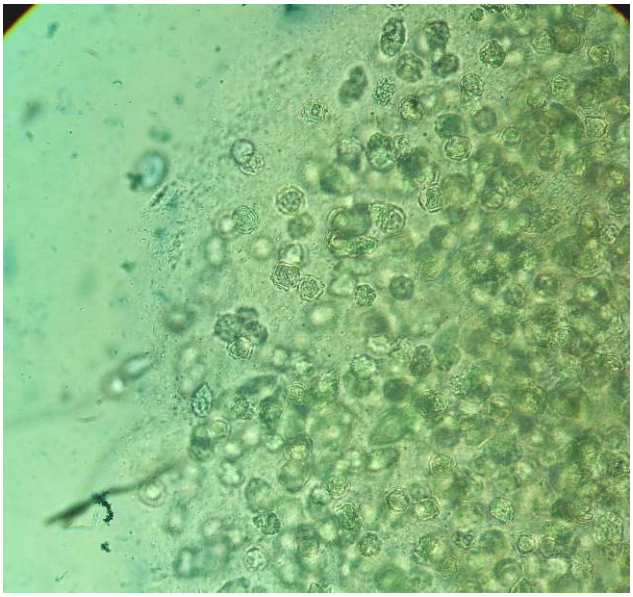
Nematodes



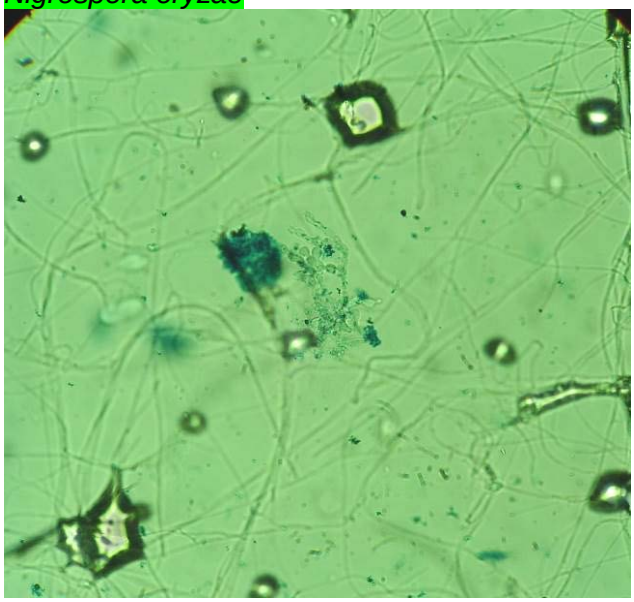
Nematodes + diatoms



Nigrospora oryzae

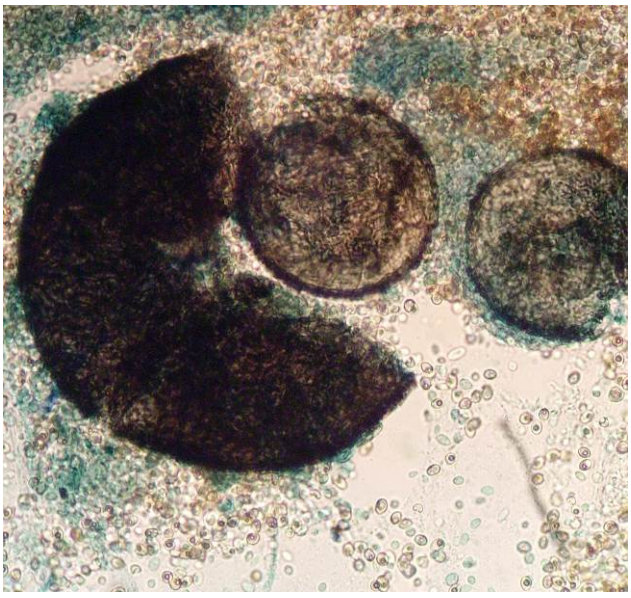


Rough blobs

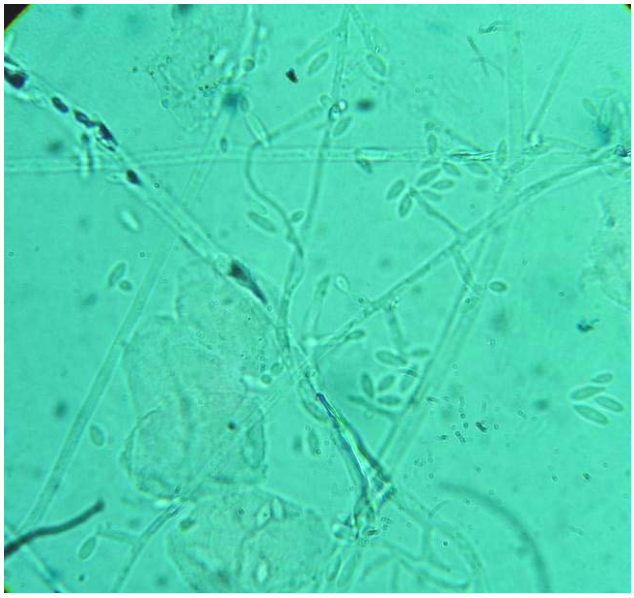


Scopulariopsis

Scedosporium – annelloconidia (V8A)

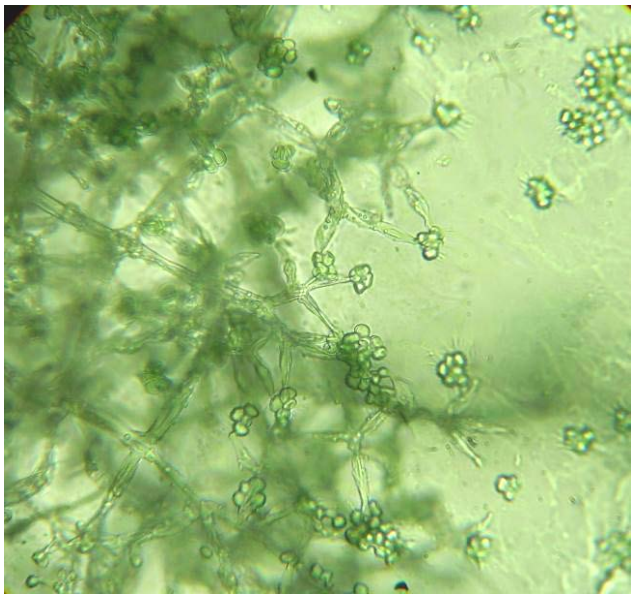


Scedosporium–cleistothecia (PDA)



*Sporothrix***?**

Thielaviopsis



Trichoderma

Fig. 18. Microscopic appearance of some fungi isolated from live and dead culms of GPG and GRT.

3. Assess field-collected material of GRT for the presence of either of these fungi

The aim of this section was to find out if either field-collected seeds or large field-collected plants naturally harboured *N. oryzae* or *Fusarium* spp.

3.1 Seeds

Unsterilised and surface-sterilised seeds used in the germination trial in Section 1.1 were examined for the growth of fungi around them on the filter paper used. Several fungal growths were evident around the seeds (mainly ungerminated ones) and spreading over the surrounding filter paper (Fig. 19). Fungi observed comprised mainly *Fusarium* spp., *Rhizopus oryzae*, *Penicillium* spp. and *Alternaria alternata*, but no *N. oryzae* was seen. Contamination was seen with both seed sources, although it was more common with the ungerminated Sunbury seeds. A typically rich wine-red *Fusarium* species was seen on almost all plates with unsterilised seeds.

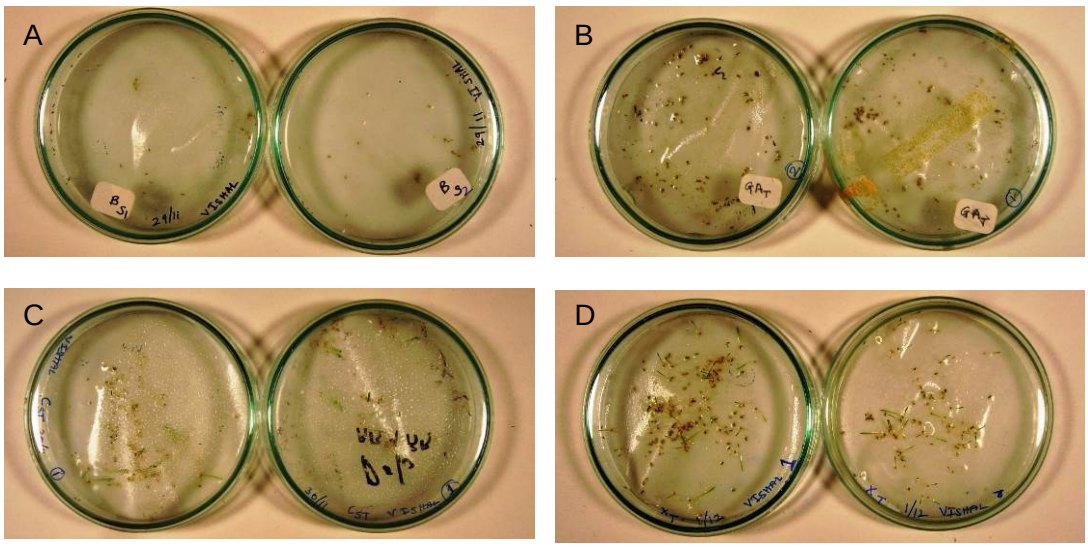


Fig. 19. Unsterilised GRT seeds from germination trial showing contamination with *Fusarium* (all) and *Alternaria alternata* (A). A, C Sunbury seed; B, D Trenayr seed.

3.2. Large plants

Individual tillers were excised (three per pot) as plants were potted up. The basal 2.5 cm was surface-sterilised as for seeds and plated out in three pieces on V8+ agar at 25°C. A variety of microorganisms was seen, including fungi, yellow and white bacteria and saprophytic nematodes (Fig. 20). The fungi were similar to those seen on seeds. The *Fusarium* isolates were stored for later DNA analysis and comparison with the inoculant. There are likely to be at least two *Fusarium* species as the sequence primed at more than one point; these may exist as endophytes causing little or no harm to the growing plant but attacking and living saprophytically on dead matter in old culms and leaves. The absence of *N. oryzae* from the isolates is not conclusive – it could have been present at low frequency or have been suppressed by overgrowths of bacteria or other fungi. Mites contaminated some plates early and they were discarded without waiting for slower fungi to grow out.

Fig. 20 Fungi isolated from pieces of field-collected GRT. The red colours are due to *Fusarium* spp. and the main grey-black fungus is *Alternaria alternata*.

The conclusion from this series of experiments is that *Fusarium* spp. are common in both seeds (externally) and large plants (perhaps internally). This suggests that the presence of *Fusarium* sp. may be necessary for any effect of *N. oryzae* and that *N. oryzae* may modify the status of *Fusarium* spp. and causes them to attack the plant instead of existing as endophytes. This may be similar to the change in status of *F. pseudograminearum* in cereals, in which environmental conditions or attack by another pathogen triggers the *Fusarium* sp. to become pathogenic instead of endophytic and cause a crown rot. Both *Fusarium* and *N. oryzae* were found as field isolates in inoculated plants of GPG and *Fusarium* sp. was also common in uninoculated plants; more than one *Fusarium* species was isolated, suggesting that there is no specific relationship of *N. oryzae* with a particular species of *Fusarium*.

Overall conclusions from the project

The main objectives of the project have been achieved. GRT seedlings in Petri dishes were reduced in leaf length by *N. oryzae* and killed by *F. proliferatum* or both fungi together within 3 weeks. GRT decreased in total biomass over 8 months in a glasshouse trial when inoculated with *N. oryzae* and *Fusarium* sp. even in large field-grown plants in pots, and the effects of the fungi were additive. This contrasted with GPG, in which there was a decline in % live biomass but not total biomass, but in which the effect of the *Fusarium* sp. alone was equal to that of both fungi together. Although 8 months may seem a long time to show effects, faster effects may be seen in infested pastures, where surrounding desirable grasses are likely to compete for nutrients and light and exacerbate the decline of the GRT and GPG, as seen in inoculated sites in the Tweed Valley, in which infected GPG has been replaced by palatable grasses without exposing bare soil (Fig.1.4).

Both fungal inocula were re-isolated from the plants but not necessarily from those inoculated. Seeds and plant materials from the field had *Fusarium* spp. but not *N. oryzae*, though the sample numbers were small and a more thorough survey, such as that carried out by Dr Sethu Ramasamy, would probably find it. F. proliferatum was the Fusarium species isolated constantly from all treatments, including the controls, suggesting its importance. *F. chlamydosporum*, the *Fusarium* inoculum from northern NSW, was only isolated from large GRT plants, which showed obvious crown rot. This suggests that the disease triangle operates: interactions between the fungi, the host plant and the environment. *Fusarium* spp. and *N. oryzae* may both be endophytic initially but under the right conditions one or both becomes pathogenic and results in the symptomatic crown rot. Alternatively, *N. oryzae* may act as a primary pathogen but is then overwhelmed by *Fusarium* spp. once the plant is under attack, or *N. oryzae* may act to stress the plants or endophytic *Fusarium* spp. in such

a way as to increase their virulence and cause disease. The constant isolation of both a *Fusarium* species and *N. oryzae* from both symptomatic and asymptomatic plants suggests a connection.

Recommendations

This is only one instance of an experiment with GRT, *N. oryzae* and *Fusarium* sp. and has been run at a very small scale as a pilot to test if inoculation produced any effect, which it has. This should be followed up with a larger project that includes, successively:

- Testing antagonistic reactions between *N. oryzae* and *Fusarium* spp. and possibly their mycotoxins (starting with *F. profileratum* and *F. chlamydosporium*) in agar plates. This would show if there is any reaction and any specificity with respect to *Fusarium* species.

- repeating this type of pot trial with a range of sizes and provenances of field-collected GRT plants to test if these results are replicable. Using field-collected large plants is preferable to testing with small axenic plants where the biocontrol agents have no competition or predation.

- expanding the range of WSGs to be tested to see if more species are susceptible.

- conducting this type of experiment with desirable pasture plants, e.g. grasses and clovers to test if they too are affected. This is a priority and essential before any field trial. Some limited testing has been conducted, so far without any apparent effects of inoculation with *N. oryzae*.

- field trials with inoculation by spores vs planting infected plants (as for GPG by Officer et al. 2012) to test if the agent(s) is(are) effective in the field. I have already been contacted by several graziers keen to provide field sites for testing.

Budget

This project was a joint initiative of the School of Applied Sciences (SAS), RMIT University, and MLA whereby 50% of the total cost of $9,000 was paid by each party. The SAS sum was paid and expended in full before the MLA sum arrived. The full MLA agreed sum of $4,500 was paid as Milestone 1 at the commencement of the project. Therefore no tax invoice needs to be prepared and sent. A financial acquittal will be prepared by the School of Applied Sciences finance and sent via RMIT R&I in January 2014, as the finance team is not processing any further items for 2013. I also still have ~$200 yet to be invoiced to RMIT for recent sequencing by Micromon before that acquittal is prepared.

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