Hort Innovation

Final Report

Improved capacity for integrated disease management of couch smut (*Ustilago cynodontis*) in turf

Project leader:

Associate Professor Andrew Geering

Delivery partner:

The University of Queensland

Project code:

TU17002

Project:

Improved capacity for integrated disease management of couch smut (Ustilago cynodontis) in turf (TU17002)

Disclaimer:

Funding statement:

This project has been funded by Hort Innovation, using the turf research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Publishing details:

ISBN 978-0-7341-4709-7

Published and distributed by: Hort Innovation

Level 7
141 Walker Street
North Sydney NSW 2060

Telephone: (02) 8295 2300

www.horticulture.com.au

Content

Summary

Couch smut is widespread in Australia, and is an issue of concern for the turf industry as it affects the vigour and appearance of green couch, the resilience of the plant to human traffic (both its tolerance to trampling and its ability to rebound from damage) and the smut spores are a public health concern, primarily by acting as allergens. Although the disease is a common around the world, there has been very little research on the disease biology and epidemiology to develop an evidence-based disease management program. In this project, the distribution of the disease across Australia was mapped by doing field surveys and by interrogating historical records held in the Australian Plant Disease Database. Using a newly developed sensitive nested PCR assay, the pathogen was shown to systemically infect all plant organs, including the underground roots and rhizomes, and therefore mowing to remove the smutted flowers would have no impact on disease incidence. Research was done to develop a rapid disease resistance screening protocol but difficulties encountered with inoculating the plants, despite using a range of techniques that are effective for other smut fungi. However, the field surveys suggested that hybrid *Cynodon dactylon* x *transvaalensis* cultivars are resistant to the disease, as couch smut was never observed in these cultivars, even when located next to a diseased paddock of pure *C. dactylon*. Systemic fungicides such as combinations of azoxystrobin and propiconazole showed great promise for the control of couch smut in glasshouse trials. An integrated disease management plan that includes a mixture of cultural practices and the use of fungicides is proposed.

Keywords

Bermudagrass, turf grass, *Cynodon*, pathology, *Ustilago, cynodontis*, smut, infection, disease management, fungicides

Introduction

Green couch (*Cynodon dactylon* (L.) Pers. and hybrids with *C. transvaalensis* Burtt Davy) is the most widely planted turfgrass in warm temperate and subtropical regions of Australia (Aldous et al., 2009; Haydu et al., 2008; Maclennan, 2018). The popularity of green couch is attributable to its lush green colour and fine texture, its hardiness, particularly in the face of abiotic stresses such as foot traffic and drought and its ability to be cut very short, which is an important trait for sporting surfaces (Wu, 2011). Green couch is widely planted in home gardens, sporting stadiums and in a range of recreational areas, including golf courses, lawn bowls greens, and public parks. Additionally, it serves an important ecological function through prevention of soil erosion at construction sites, roadside verges and landfills (Christians, 2011; Kneebone, 1966).

Couch smut, caused by the fungus *Ustilago cynodontis* (Figure 1), is among the most important of fungal diseases of green couch. Significant economic losses can occur on infected turf farms. Infection causes a reduction in the rate of stolon extension and overall dry matter production by up to 50% (García-Guzmán and Burdon (1997). The disease reduces root/shoot ratio of the infected plants, leading to reduced tolerance of turf to trampling, slowing the rate of recovery from wear(Marchelo-d'Ragga and Misaka, 2015), and reducing the amount of usage possible on a sports field (M. Hedley, pers. comm.). The weaker root and stolon system of diseased plants also creates problems for turf farmers, as there are anecdotal reports from our disease surveys of higher levels of wastage during cutting and rolling of affected paddocks as the strip of turf is prone to break at points of infection. Diseased plants have a more upright habit of growth, the leaves can appear slightly chlorotic, and the overall effect is particularly unsightly especially in the more prostrate cultivars of green couch grass where the more erect habit of growth stands out (D. Loch, pers. comm.). Clumps of infected plants also spoil the appearance of the turf, especially among more prostrate varieties. Smutted turf typically sells at lower prices for commercial applications such as stabilization of soil at construction sites and on roadsides. Furthermore, smut spores are known to cause health problems and allergies in some people (Horner et al., 1995; Levetin et al., 2016). The smut spores sully shoes and clothing when people walk and lie on diseased turf infected by *U. cynodontis*; similarly, pets can bring spores into the house on their fur.

The smut fungus *Ustilago cynodontis* (Pass.) Henn. is cosmopolitan in its distribution, found wherever suitable host plants occur (Farr and Rossman, 2019; McAlpine, 1910; Vánky, 2005; Vánky, 2012). In Australia, *U. cynodontis* is commonly found in all states and mainland territories (Figure 1) (Tran et al., 2020), having been first recorded from New South Wales in 1907 (McAlpine, 1910). Reported hosts included *C. incompletus* (blue couch), *C. aethiopicus* and *C. nlemfuensis* (stargrasses) but not *C. transvaalensis*, nor *C. dactylon* × *C. transvaalensis*. Whether *C. dactylon* × *C. transvaalensis* is truly resistant to smut infection is still an open question, as this hybrid is sterile and therefore the most obvious disease symptom, the smutted inflorescence, would not be apparent.

Despite couch smut being widespread and causing significant losses to the industry, not much is known about this pathosystem, including the pathogen infection biology, disease epidemiology, and effective disease management options. The overarching aim of this project was to develop an integrated disease management plan for couch smut for the Australian turf industry. We specifically aimed to address: (i) distribution and impact of couch smut in Australia, (iii) distribution of infection of the pathogen *U. cynodontis* within the plant, (iii) fungicides that can be used to effectively control the disease, and (iv) sources of couch smut resistance.

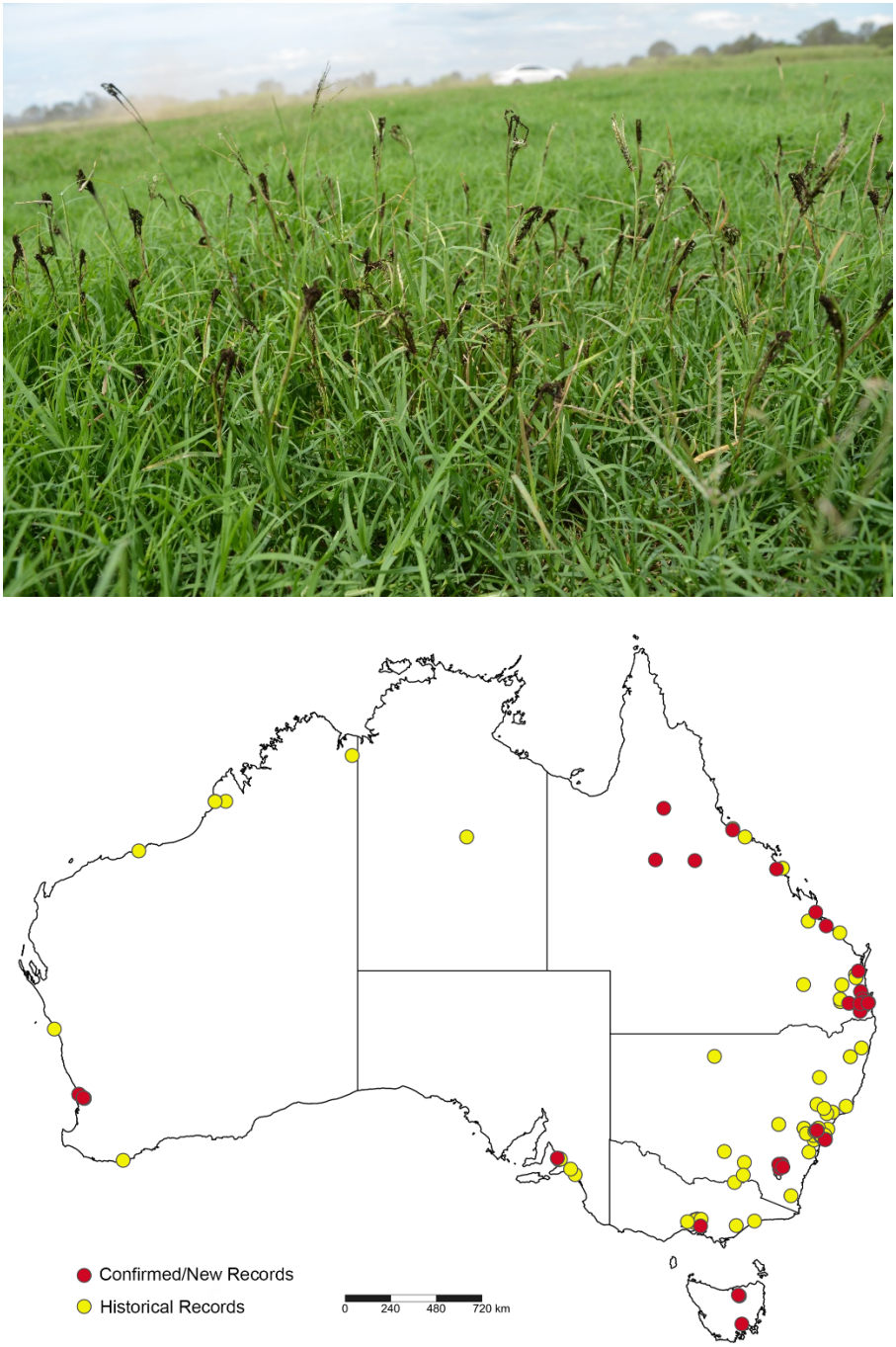

Figure 1. Top image – A green couch paddock severely infected by the couch smut pathogen, *Ustilago cynodontis*; and Bottom image - Distribution of *U. cynodontis* in Australia, yellow dots denoting historical records extracted from the Australian Plant Disease Database and red dots denoting recent records (2018–19) by the authors (Tran et al., 2020).

Methodology

Disease surveys and sample collection

Surveys were undertaken by the project team members on 13 turf farms in Queensland (Brisbane, Bundaberg, Rockhampton, MacKay), and nine farms in NSW. Turf farm managers were asked to fill in questionnaires (Appendix 1) containing requests for information on disease occurrence, management practices used to treat the disease, and observations on economic impacts. In addition, established public green spaces in QLD, NSW, WA, SA, VIC and ACT were visited by the project team members or collaborators to determine the distribution and severity of couch smut on commercially available green and hybrid couches. The Australian Plant Disease Database, which contains metadata from the major plant disease herbaria in Australia, was interrogated to identify historical records of couch smut. During the surveys, specimens were also collected for later experimental work.

Pathogen isolation and lodgment to BRIP herbarium. To obtain pure cultures of the pathogen *U. cynodontis*, teliospores produced from the smutted heads were by gently tapping spores from the smutted flowers into a 1.5-mL microcentrifuge tube containing 100 µL sterile in a laminar flow. The resulting spore suspension was shaken vigorously to homogenise the spores before streaking onto plates of Glucose Peptone Yeast Agar (GPYA) with an inoculation loop using the 16-streak plating method. Subsequently the plates were sealed and incubated at 27°C for 2 days. Once yeast colonies are visible, sub-culture single colonies from one specimen was done to obtain a single genotype isolate. The plates were sealed and incubated for 3-5 days or until use (e.g. DNA extraction).

Infected plant specimens and pure cultures of *U. cynodontis* were deposited in Queensland Plant Pathology Herbarium (BRIP).

Examination of distribution of *Ustilago cynodontis* within the plant using PCR assays

To examine the distribution of *U. cynodontis* within the plant, a diagnostic PCR assay was developed. To provide adequate sensitivity of detection, it was necessary to use a nested PCR assay format with primers targeting the internally transcribed spacer (ITS) region of the ribosomal DNA locus. The nested PCR involved two rounds of amplification with the first amplifying a larger fragment that in turn provided the template for the second round of amplification. The first primer pair was ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), which produce an amplicon of *c.* 800 bp. In the second round of amplification, a new forward primer ITSUcynoF (5' GCT AGG CGA CGG ACC GAC G 3') that is specific to *U. cynodontis* was used in combination with the ITS4 primer, producing an amplicon of *c.* 600 bp. In addition, a PCR assay targeting the host plant rDNA locus was developed to provide confidence that negative results were real and not due to PCR inhibition, poor quality DNA template or user error. For this endogenous plant gene control assay, the forward primer CynoAllTSF (5' CCG AGG GCA CGT CTG CCT 3') was paired with the ITS4 primer to amplify approximately 300 bp DNA fragments. This PCR was done in duplex with the first round of the *U. cynodontis* nested PCR.

To avoid detection of spores from smutted flowers contaminating the leaf surface but not necessarily infecting the plants, plant samples were surface-sterilized following the procedures described previously (Coombs and Franco, 2003; Massimo et al., 2015) with some modifications. In particular, the samples were first rinsed in double distilled (dd) H_2O water to eliminate surface debris, then surface sterilized by sequential immersion in 95% ethanol for 10 s, 6% bleach (LabCo bleach with 12-13% available chlorine) prepared in 0.02% Tween for 2 min, 70% ethanol for 2 min, and a final rinse in ddH_2O. After surface sterilization, DNA was extracted from approximately 2-cm long segments of different plant organs (Fig. 9) using a DNA Isolate II Plant DNA kit (Bioline, Alexandria, NSW Australia) following the manufacturer's instructions. The DNA was then used for PCR as described above.

Both rounds of the nested PCR were performed in a 10-µl reaction volume. For the first PCR, the mix contained 2.0 µl of 5× MyTaq Red reaction buffer (Bioline), 0.1 µl each of 10 mM primer (ITS1F, CynoAllTSF, and ITS4), 0.1 µl of MyTaq HS DNA polymerase at 5 U/µl (Bioline), and 1 µl of DNA extracted from plant tissues (~20 ng/µl) or nuclease-free water served as a non-template control. The following cycling

conditions were used: 3 min at 95°C; 25 cycles of 95°C for 20 s, 53°C for 20 s, and 72°C for 30 s; followed by a final extension of 72°C for 5 min. The second round PCR each reaction contains 2.0 µl of 5× MyTaq Red reaction buffer (Bioline), 0.1 µl each of 10 mM primer (ITSUcynoF and ITS4), 0.1 µl of MyTaq HS DNA polymerase at 5 U/µl (Bioline), and 1 µl of the first round's PCR products diluted 1:100, nuclease-free water served as a no-template control. The following cycling conditions were used: 3 min at 95°C; 30 cycles of 95°C for 20 s, 62°C for 20 s, and 72°C for 30 s; followed by a final extension of 72°C for 5 min. The PCR products were size fractionated on 1.5% agarose gels to examine DNA quality and size.

Efficacy of fungicides to control couch smut

Glasshouse pot trials. A glasshouse experiment was done to evaluate the efficacy of selected fungicides to cure plants of couch smut.

After a literature search, five systemic fungicides containing the following actives that are registered for the control of other fungal pathogens in turf: Azoxystrobin 95g/L, Propiconazole 155g/L, Azoxystrobin 62g/L + Propiconazole 104g/L, Triadimensol 250g/L and Iprodione 250g/L, were selected for use as treatments because these actives have been found to be effective against other smut and basidiomycete fungi (Morton & Staub, 2008, Bhuiyan et al., 2012). Additionally, a mancozeb product (Mancozeb 420g/L), a registered contact fungicide and protectant, was also included as a treatment.

Naturally infected couch grass from a Brisbane City Council park was grown in squat pots (175 mm diameter × 135 mm high) in a glasshouse and treated with the six fungicides or left untreated as a control. Each treatment was replicated four times, giving a total of four pots per treatment. Treatments were arranged in a randomized complete block design with replicates considered as blocks.

Prior to the fungicide applications, numbers of smutted and healthy inflorescences in each pot were counted to calculate baseline incidences of disease in each treatment. The tillers of the smutted inflorescences were then tagged and numbered and mowing simulated by cutting back to about five internodes (~ 10 cm) above the base of the tiller (Fig. 2). After 'mowing', the plants were treated with fungicide at the highest recommended label rate using multi-purpose hand sprayers until runoff was observed. A separate sprayer was used for each treatment and treatments repeated a second time after two months. The effectiveness of the fungicides in reducing disease symptom expression and/or infection was assessed by presence of the fungus within new growth of the tagged smutted tillers. Presence of *U. cynodontis* was assessed visually by recording binomial data of inflorescences with and without disease symptoms. The data were subjected to one way ANOVA using GenStat (20th edition, VSN International).

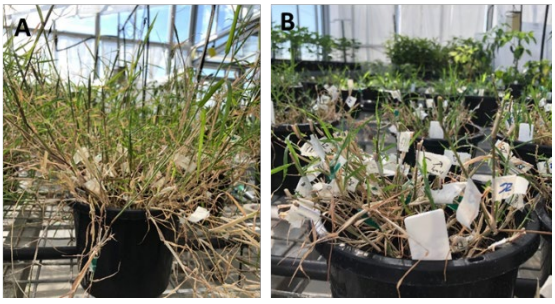

Figure 2. Smutted inflorescences of couch grass grown in pots prior to (A) and after mowing (B) but before application of fungicides.

Field trial. A field trial was established at Redlands Research Station using smutted 'Wintergreen' sourced from a commercial turf farm to test the efficacy of seven systemic fungicides, including four that showed promise from the pot trial, and three new products with different actives: Tebuconazole 240g/L, Tebuconazole 200g/L + Trifloxystrobin 100g/L, and Penflufen 227g/L; water was used at untreated control. Details of treatments are provided in Table 1. The eight treatments were arranged in a randomised complete

block design with three replicates considered as blocks (Fig. 3a). Each replicate plot was 3.75 m^2 (2.5 x 1.5 m); the experiment were marked using spot marking paint and pin markers (Fig. 3b). The fungicides were applied using a customized sprayer (Fig. 3c) at the recommended rates in 2 L of water for 3 replicate plots of each treatment. Two applications were made at one month intervals.

In order to evaluate any disease reduction resulted from fungicide applications, disease incidence was assessed prior to, at one month and two months after the first fungicide application. Disease assessment was done by counting all smutted and healthy flowers in a 0.5 m^2 quadrat in each replicate plot. After disease assessment and prior to fungicide application, the experimental plot were mown to remove smutted heads. Disease incidence was calculated as percentage of smutted flowers out of total heads counted for each treatment. Reduction in disease incidence at day 30 and day 60 after first application was estimated. The data were subjected to one way ANOVA using GenStat (20th edition, VSN International).

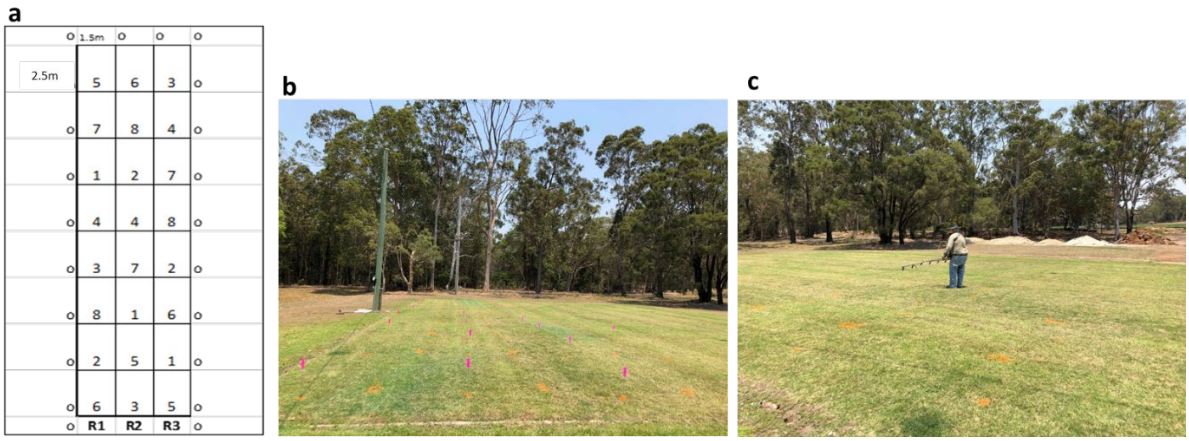

Figure 3. Experimental design for field evaluation of fungicides to control couch smut; (a) experimental map, (b) field signed with treatments marked, and (c) fungicide spray.

Table 1. Treatments used for field evaluation of fungicides against couch smut.

Treatment number	**Actives**	Application rate (mL/100 per 100m^2) *
1	n/a	n/a
2	Triadimenol 250g/L	60
3	Propiconazole 155g/L	100
4	Azoxystrobin 95g/L	60
5	Azoxystrobin 62g/L + Propiconazole 104g/L	90
6	Tebuconazole 240g/L (2 lb/gal)	60
7	Tebuconazole 200g/L + Trifloxystrobin 100g/L	20
8	Penflufen 227g/L	30

* Application rates as per product labels for curative treatments.

In vitro tests on efficacy of fungicides on germination of *Ustilago cynodontis* spores

An *in vitro* assay was done to test the inhibitory effects of the seven fungicides used in the field trial (Table 1) on germination of *U. cynodontis* teliospores. The fungicides were used at concentration of 0.5x, 1x and 2x of the recommended label rates, with four replicates per treatment.

A teliospore suspension was freshly prepared by gently tapping smutted flowers above an open Schott bottle containing sterile 0.02% (v/v) Tween 20, all within the sterile confines of a Laminar Flow cupboard. The bottle was then shaken vigorously to homogenize the spores and the concentration adjusted to 1×10^5 spores/ml using a hemocytometer. One drop (*c.* 50 µL) of the spore suspension was placed in a cavity glass slide and diluted 1:1 with fungicide at twice the desired concentration. The slides were then placed in Petri

plates lined with damp filter paper, sealed with parafilm to maintain the moisture and incubated at 27°C for 24 h. Spore germination was then measured at 48 h using a light microscope. For each treatment, 100 spores from two separate areas on each replicate plate were examined.

A two-way ANOVA with fungicide type and concentration as treatment factors and spore germination rate as the variate was done using GenStat. No significant differences were observed between concentrations of each fungicide and therefore the data across various fungicide concentrations were pooled and a one-way ANOVA performed.

Development of inoculation protocols

Germination of *U. cynodontis* spores. Prior to inoculation, germination assays were done to identify the environmental conditions (i.e. temperature and light/dark) that favor germination of spores under laboratory conditions. *U. cynodontis* spores were first harvested from a smutted flower in a laminar flow by gently tapping the inflorescence with a sterile scalpel to dislodge the spores into a container containing sterile distilled water (SDW). The resulting spore suspension was then vigorously vortexed to separate any spore lumps. The spore concentration was quantified and adjusted (serial dilution using SDW) to 4×10^4 spores/ml using a hemocytometer. Aliquots of 100 µl of spore suspension were pipetted onto sterile slides placed on surface of on 1.5% water agar (WA) plates. A cover slip was then gently placed on top of the spore drop. The WA plates were then incubated at seven temperatures (three replicate plates for each temperature treatment), starting from 4°C with 4-6°C increments (i.e. 4, 8, 12, 17, 22, 27, 32 and 37°C). Germination of spores was assessed at 12, 24 and 48 hours after incubation, using Nikon Eclipse Ci-L compound microscope (Nikon Corporation) at ×400 magnification. Spores were considered to have germinated if basidia sizes were half of the length of the spores. All germinated and ungerminated spores in three microscope views (400× magnification) randomly selected on each slide were recorded and the percentage of spore germination was calculated. The assay was replicated twice.

We also tested whether light conditions affect germination of the spores. WA plates containing slides with spores suspensions prepared as described for the temperature assay were incubated at (i) constant light, (ii) constant dark by covering the plates with aluminum foil, (iii) 12/12 light/dark by keeping the plates covered by aluminum foil for 12 hour and uncovered for 12 hour. All plates of the three treatments were incubated at 27°C, the optimal temperature for spore germination. Spore germination was assessed at 24 hours after incubation.

Inoculations of *U. cynodontis*. To develop an efficient protocol to screen for plant resistance to couch smut, we tested five different inoculation protocols, using 'Wintergreen' couch as the challenge plant as field observations suggested that it was highly susceptible to infection.

Experiment 1. A fresh teliospore suspension was freshly prepared as described above and the spore concentration adjusted to 5×10^6 spores/ml using a hemocytometer.

The mature grass plants were inoculated by spraying the spore suspensions onto the plants until runoff. Mock-inoculated controls were sprayed with 0.02% Tween 20. There were three replicate pots of grass per treatment. After inoculation, all plants were covered with plastic zip-lock bags for 48 hours to maintain dew. The treatment and control plants were then kept in separate glasshouses at ~27°C for symptom expression (Fig. 4).

To check spore germination under glasshouse conditions, 100 µL of the spore suspension used for the inoculations was pipetted on glass slides placed in a WA plates which were kept adjacent to the inoculated plants (three replicate WA plates for the inoculated and control treatments). After 24 hours of incubation, spore germination was assessed on only one replicate slide from each of the two treatments with a low germination rate of 15 and 47%. The other two replicate slides of each treatment had dried out and therefore counting for spore germination was not possible.

Two weeks after inoculation, plant samples were collected for nested PCR to determine if infection occurred, however, no *U. cynodontis* was detected. The plants have been incubated for 11 months and no smut symptoms were observed.

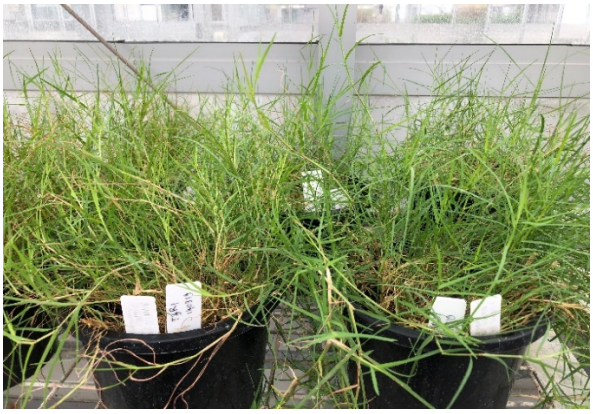

Figure 4. 'Wintergreen' pots inoculated with *Ustilago cynodontis* in glasshouse.

Experiment 2. This experiment aimed to test the hypothesis that the lack of success in infecting the plants in Experiment 1 was due to the low germination rate of the spores, which could potentially be overcome by inoculating the plants with pre-germinated spores.

To prepare inoculum, spores were collected from smutted flowers were tapped into a Schott bottle containing potato dextrose broth (PDB). The resulting spore suspension was shaken vigorously to homogenise before adjusting the concentration to ~5×10^6 spores/ml using a hemocytometer. The spore suspension was then shaken at 109 rpm, overnight at 27^o C, to stimulate germination of the spores. Prior to inoculation, the spores were examinated and a rate of 29% germination observed.

The experimental design included three treatments and three replicate plants per treatment. Treatments included (i) plants inoculated with pre-germinated spores, (ii) spore suspension prepared fresh in PDB on the day of inoculation, and (iii) control plants treated with PDB.

Spore germination under glasshouse conditions was also checked for the freshly prepared spore suspensions by placing 100-µL drops of the spore suspension on two glass slides, which were placed in a petri dish sealed with cling wrap and located adjacent to the inoculated plants. Exact data on spore germination could not be collected at 24 hours after incubation due to the spores being too dense on the slide, but a high germination rate was estimated (~70%). The inoculations were done using the methods described for the first experiment. One month after inoculation, nested PCR was performed on plant samples from all three treatments (three samples per treatment), which all came up negative. The plants were kept in the glasshouse for 8 months but no symptoms were observed.

Experiment 3. The aim of this experiment was to test if infection rates could be improved by simultaneously inoculating the two mating types of *U. cynodontis* to increase the chances of the infective stage of the fungus, the hyphal dikaryon, of forming.

In order to identify compatible mating types of *U. cynodontis*, we designed a new PCR assay using primers A1F (CCA GGC CAT CGC GAG ACA TCG G) and A1R (AGA CTT CCG TCT CCG AGA C), which target the A1 mating gene of *U. cynodontis* (Kellner et al., 2011). Fungal isolates that tested negative using this PCR were assumed to be the opposite mating type to the A1 type. Mating experiments were then set up on culture media, and successful matings adjudged to have occurred when fluffy hyphae was produced at the contact zone (Fig. 5), as described by (Digby and Wells, 1989). On the basis of these results, two isolates of *U. cynodontis*, CS112-St1-C3 and BRIP 61903-2, were selected for the inoculation experiment.

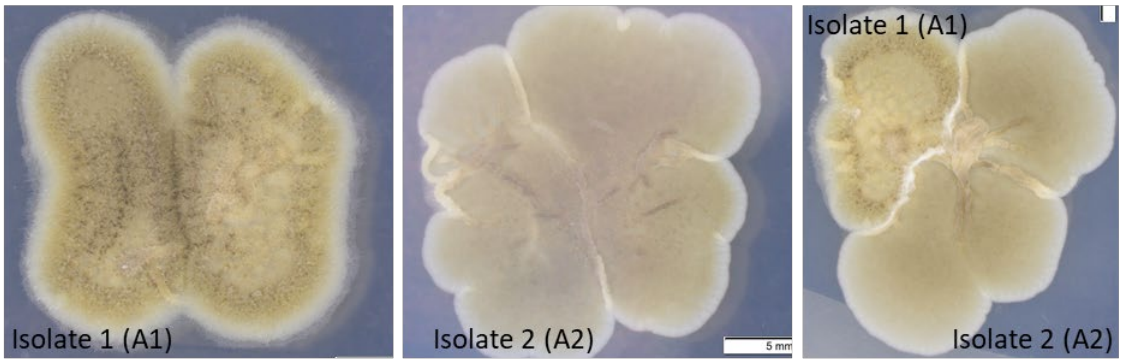

Figure 5. Mating assays of *Ustilago cynodontis in vitro*, showing no mating reaction when isolate of one mating type (A1 or A2) was grown by themselves, and an apparent positive mating reaction occurred as indicated by the fluffy hyphae produced at the contact zone when isolates of the two mating types were co-cultured.

To prepare inoculum, the selected yeast cultures of *U. cynodontis* were individually grown overnight in PDB in a rotary shaker operated at 150 rpm and a temperature of 27°C. The concentration of the resulting yeast suspension of each isolate was estimated and adjusted to ~10^6 cells/ml with ddH_2O using a hemocytometer. Equal volumes of the two isolates were mixed to give a final concentration of ~ 5 × 10^5 cells/ml prior to inoculation. The inoculations were done as described for Experiment 1. One month after inoculation, nested PCR was performed on the plant samples but again the PCR came up negative, suggesting failure of infections to develop. The plants were kept in the glasshouse for 3 months but no symptoms were observed.

Experiment 4. This experiment aimed to test whether wounding and temperature have an effect on the rate of infection. A fresh teliospore suspension at 2×10^6 spores/ml was prepared just before inoculation as described for the Experiment 1. Stolons collected from potted green couch plants were cut into two-budded segments (Fig. 6), which were wounded at the buds using a scalpel blade or left untouched as the negative control. The cuttings were inoculated using a dip inoculation method used for the sugarcane smut pathogen, *Ustilago scitaminea* (Dean, 1982) but with the following modifications. The cuttings were immersed in beakers containing either the spore suspension or 0.02% Tween 20 (untreated control) and gently shaken overnight in an incubator set at 27°C. The beakers were then taken out of the incubator and left on a laboratory bench for 1 hour. The germination rate of the teliospores in the suspensions was estimated at about 30% by examining the spores using light microscopy at 40x magnification.

The cuttings were removed from the beakers, blot-dried before being potted in 10-cm diameter pots containing commercial potting mix. The pots were then covered with a zip lock bag to maintain moisture for 72 hours before being kept in the glasshouse set at approximately 25°C or in the shadehouse at ambient temperature. There were seven cuttings grown per pot and two pots per treatment. Details of treatments are in Table 2.

Unfortunately, no symptoms of infection were observed 6-months after inoculation.

Figure 6. Two budded cuttings (a) used in dip inoculation assay (b).

Table 2. Details of treatments for dip inoculations

Treatment number	Inoculations	Temperature conditions after inoculations
1	Untreated, unwounded cuttings	~ 25°C (Glasshouse)
2		Ambient (Shadehouse)
3	Untreated, wounded cuttings	~ 25°C (Glasshouse)
4		Ambient (Shadehouse)
5	Inoculated, unwounded cuttings	~ 25°C (Glasshouse)
6		Ambient (Shadehouse)
7	Inoculated, wounded cuttings	~ 25°C (Glasshouse)
8		Ambient (Shadehouse)

Experiment 5. Seeds of *C. dactylon* have previously been infected using a vacuum-inoculation method (García-Guzmán and Burdon, 1997; Ketta and Emeran, 2020) and we attempted to repeat this method using ungerminated green couch seeds (Hortico, Clayton VIC 3168, Australia) (Fig. 7a), approximately 3-week old seedlings produced from these seeds, and 2-budded cuttings from potted cv. 'Wintergreen'.

Prior to inoculation, all plant material was surface-sterilized with 1% bleach for 30 seconds, then rinsed with reverse osmosis purified water for 1 min. Seedlings and cuttings (Fig. 7a) were either unwounded or wounded by cutting leaves near growing points (seedlings) or by wounding buds with a scalpel blade (cuttings). There were 10 treatments (Table 3), with three replicate pots per treatment; each 10-cm diameter pot was planted with 0.1 g of seeds or 10 seedlings or cuttings.

Teliospore suspensions were prepared as described previously, and used at a concentration of 2×10^6 spores/ml. The plant materials were vacuum-inoculated (Fig. 7b) with either the teliospore suspension or 0.02% Tween 20 solution as a negative control for 15 minutes at room temperature. Following inoculation, the plant materials were left in the suspensions for a further 24 hours (García-Guzmán and Burdon, 1997). Subsequently, the seeds were sown and seedlings/cuttings were planted in pots covered in zip-lock bags for 72 hours to maintain moisture (Fig. 7c). The zip lock bags were then removed and the pots were kept at ambient conditions in the shadehouse to monitor for symptom development (Fig. 7d).

None of these inoculation treatments appeared to have worked as no symptoms were observed 5 months post-inoculation.

Figure 7. Vacuum inoculation assay: (a) plant materials used for inoculation, (b) flask connected to a vacuum pump used as the inoculation apparatus, (c) potted plants covered with zip-lock bags post inoculation, and (d) zip-lock bags removed and pots kept in shadehouse for symptom development.

Table 3. Details of treatments for vacuum inoculations

Treatment number	Treatment details
1	Untreated seeds
2	Untreated, unwounded seedlings
3	Untreated, wounded seedlings
4	Untreated, unwounded cuttings
5	Untreated, wounded cuttings
6	Inoculated seeds
7	Inoculated, unwounded seedlings
8	Inoculated, wounded seedlings
9	Inoculated, unwounded cuttings
10	Inoculated, wounded cuttings

Outputs

Disease surveys

Surveys were done on 13 in QLD and nine turf farms in NSW and the farm owners or managers asked to fill in questionnaires. Additional smut specimens were collected from public spaces in the metropolitan areas of Canberra, Adelaide and Perth by Julia Kruse, a visiting scientist at the Queensland Plant Pathology Herbarium. In total, 52 isolates of *Ustilago cynodontis* were collected during the course of this project and deposited in the Herbarium (Appendix 2).

The couch cultivars grown of the farms that were surveyed were diverse, including 'Wintergreen', 'Oz Turf', 'Greenslees Park', 'TiTuf', 'Tifway', 'Agridark', 'CT-2', 'Grand Prix' and 'Stadium'. 'Wintergreen' was the most widely grown cultivar (about two-thirds of the farms surveyed), followed by 'Greenlees Park', 'C-1' and 'Santa Ana' (Table 3).

Couch smut was declared to be present or once present on 50% of the farms that were visited, with serious impacts on the quality and quantity of the turf recognized by the farmers. The farmers stated that diseased plants had poor root systems, which caused the turf rolls to break during harvest, increasing the amount of wastage. Some farmers reported that the disease significantly reduced the amount of income received for the turf as they voluntarily downgraded the status of the turf for low-end uses such as stabilizing soil on construction sites. There was widespread awareness among farmers of the symptoms and economic impact of couch smut, even on farms that were not affected by the disease.

All farmers who had dealt with the disease described attempts to control the disease with varying levels of success. Control methods included application of fungicides and rogueing of diseased plants by digging or spot-spraying with herbicides. On one farm, paddocks of green couch affected by the disease were over-sown with perennial ryegrass to stabilize the lawn mat prior to harvest. Rogueing of disease plants by chipping or hand spraying with herbicide had been used to eliminate the disease from several farms but this option was not considered economically feasible on severely affected farms. Couch cultivars such as 'Wintergreen' were considered commodities, with strong price competition between farmers, and little price incentives to produce better quality turf by controlling disease using expensive fungicides. One farmer in Yeppoon, QLD, reported success from using wettable sulphur to control the disease. There was not a strong and consistent message as to whether nutrition affected the incidence of disease.

Development of a molecular diagnostic assay for *Ustilago cynodontis*

Prior to this project, accurate diagnosis of couch smut was dependent on the observation of smutted flowers, and it was not possible to detect the pathogen in vegetative parts of the plant. The PCR primer ITSUcynoF was designed, which allowed specific detection of *U. cynodontis* when used in combination with the standard DNA bardcoding primer ITS4. While only a single round of PCR was needed to detect DNA from pure yeast cultures of the fungus, a nested PCR assay format was needed to provide the level of sensitivity required for detection of the fungal DNA *in planta* (Fig. 8).

Problems were experienced using the PCR on root or stolon samples, as soil particles are known to release chemicals that are potent inhibitors of PCR. To alert a diagnostician of 'false negative' PCR results, a PCR assay was developed using the CynoAllTSF/ITS4 primer pair to detect plant ribosomal DNA, which was successfully used to test the quality of the plant DNA extract.

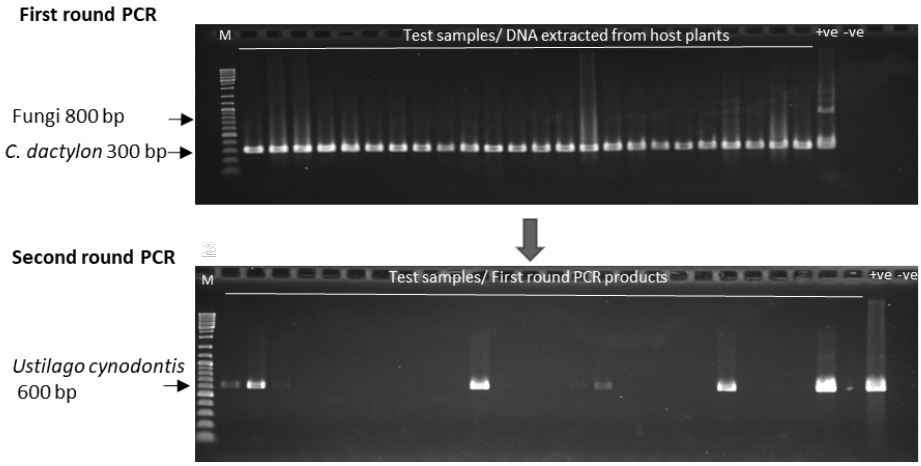

Figure 8. Gel images of the nested PCR to identify *Ustilago cynodontis* from couch grass plant.

To examine the distribution of *U. cynodontis* within the plant, eight infected plants were cut into multiple sections and each tested for infection by nested PCR. *U. cynodontis* was detected in all parts of the plant, even those parts not displaying obvious symptoms of infection (Fig. 9). These results suggest systemic infection although we cannot yet rule out multiple localized infections. To obtain conclusive evidence of systemic infection, different points of the plant will need to be inoculated and movement tracked by PCR. There were instances where some stolons tested negative for the fungus, indicating an uneven distribution of the fungus within the plant. Nevertheless, infection was sufficiently widespread, even in the roots, to suggest that mowing to remove symptomatic tissue is unlikely to control the disease.

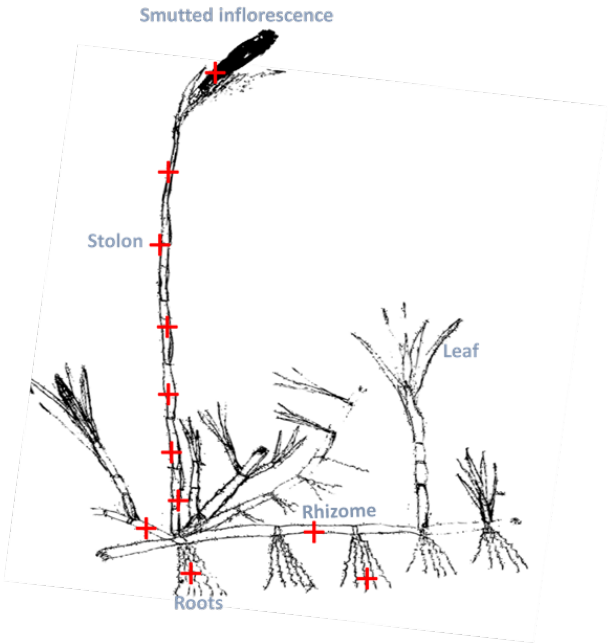

Figure 9. Distribution of the smut fungus *Ustilago cynodontis* within a green couch plant detected by nested PCR. Where the fungus was detected is indicated by an addition sign (+).

Evaluation of the efficacy of fungicides to control couch smut

Glasshouse pot trials. Prior to the applications of fungicide, the incidences of smutted inflorescence in all treatments were not significantly different (*P* = 0.976, Fig. 10A). At 2 months, the product combining the active ingredients of azoxystrobin and propiconazole had the lowest incidence of smutted inflorescences (3.8%), which was significantly lower than all other treatments and the untreated control (29.5%). Applications of triadimenol or propiconazole alone also resulted in a significant reduction in smutted inflorescences compared to the untreated control. However, disease levels in the iprodione and mancozeb treatments were not significantly different from the untreated control (Fig. 10B).

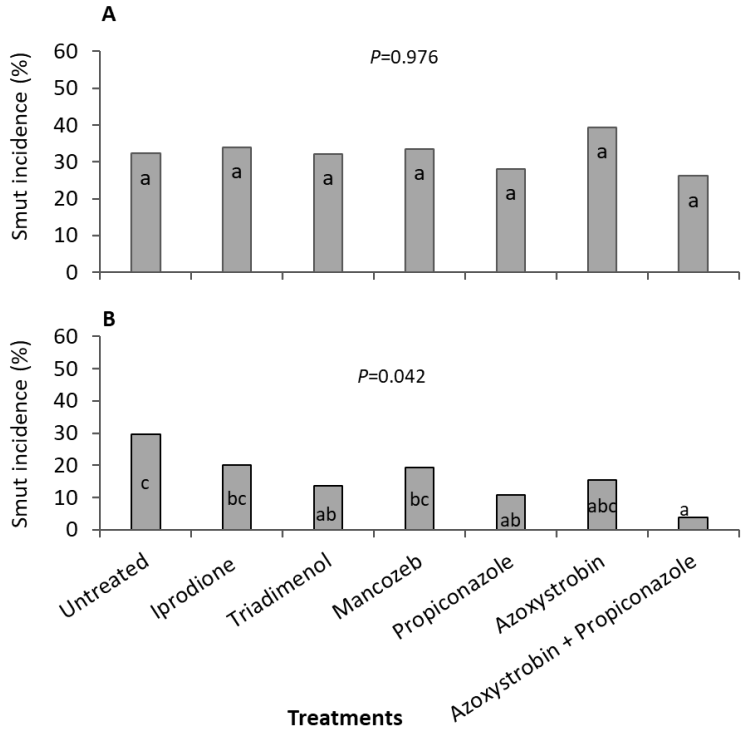

Figure 10. Incidence of smutted inflorensecences on couch grass grown in pots assessed (A) prior to and (B) 2-months after fungicide application under glasshouse conditions. Different letters indicate a significant difference using Fisher's protected least significant difference test in GenStat. Different letters on data columns within each graph indicate statistical difference between treatments (*P* = 0.54).

Field trial. No significant differences (*P*= 0.54.) in disease incidence were observed between the untreated control and the fungicide treatments (Fig. 11). Although the turf was severely affected by couch smut when sourced from the turf farm, the disease was very patchy in distribution, resulting in large variation in the incidence of disease between replicate plots, making it very difficult to analyze the results because the error bars were so large.

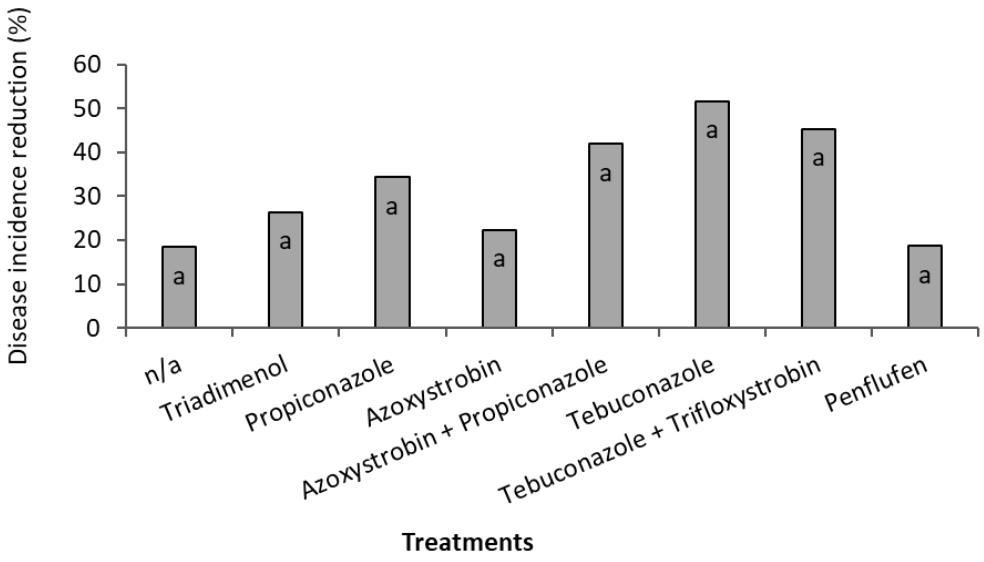

Figure 11. Reduction in smut incidence for different fungicides assessed at day 60 after the first fungicide application in field conditions. Data columns with the same letter indicate no statistical difference between treatments ($P = 0.54$).

Effect of fungicides on spore germination of *Ustilago cynodontis*

Six of the seven fungicides tested significantly inhibited spore germination, particularly the products containing the actives (i) azoxystrobin and propiconazole, (ii) tebuconazole and trifloxystrobin, and (iii) penflufen (Fig. 12). The effectiveness of azoxystrobin combined with propiconazole in controlling *U. cynodontis* is similar to that observed from the fungicide pot trial. Tebuconazole alone did not have any effect on germination of *U. cynodontis* spores.

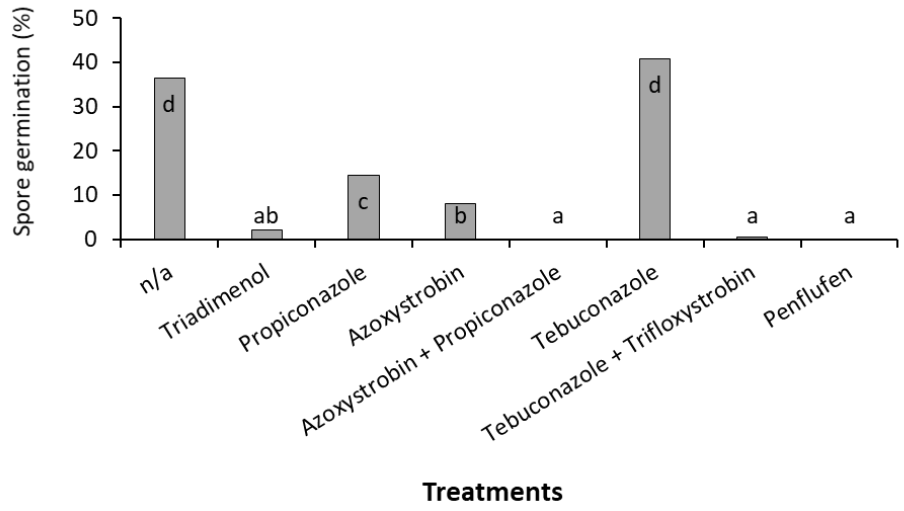

Figure 12. Effect of fungicides on germination of teliospores of *Ustilago cynodontis in vitro*. Data columns with different letters indicate statistical difference between treatments ($P < 0.01$).

Germination of spores of *U. cynodontis*. Germination of spores were observed at 12 hours and peaked at 24 hours after incubation whereas at 48 hours the basidia seemed to be detached from the spores making it difficult to be differentiated from ungerminated spores. Thus, germination data obtained at 24 hours after incubation is presented (Fig. 13).

At 4°C, no spore germination was observed but increasing rates of germination were observed as the temperature increased, reaching a peak at 27°C (Fig. 4). However, at higher temperatures, the germination rate began to decrease and no germination was observed at 37°C.

No difference in germination rates of the spores at different light/dark conditions (data not shown).

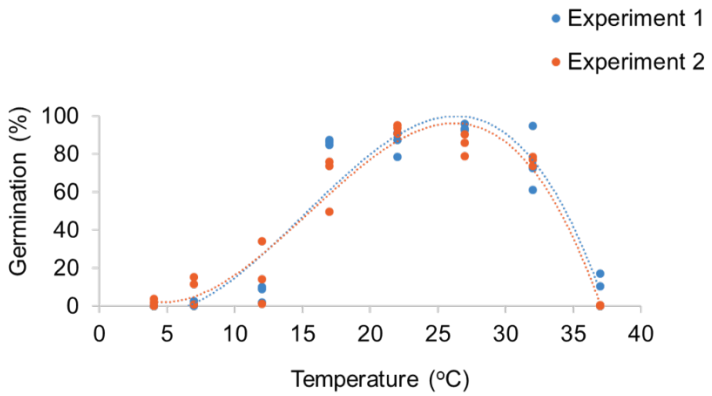

Figure 13. Effects of temperatures on germination of *Ustilago cynodontis* spores at 24 hours after incubation.

Inoculation and screening for resistance

During the course of this project, we have tested many combinations of inoculum and host plant, as well as different environmental conditions, and not had success with any inoculation method, which hindered experimentally testing resistance of green couch germplasm for couch smut.

Integrated disease management

Couch smut is a common disease that affects the performance of the turf by impacting integrity of sporting fields and binding of rolls at turf farms, causing significant losses to turf industry (García-Guzmán and Burdon, 1997; Marchelo-d'Ragga and Misaka, 2015). However, it is an under-studied disease and as such several aspects of the pathogen biology and disease epidemiology remain poorly understood, which hinders development of a disease management program. In the present work, we have put together an integrated disease management plan based on our experimental data or information collected from discussion with turf growers.

Physical & cultural practices

Many turf farmers in Australia control couch smut by rogueing diseased plants, either by spot-spraying with herbicides, or by physical removal by mechanical means such as chipping. Although rogueing is a labour-intensive method of disease control, some turf farmers have reported that they eliminated or reduced the disease incidence using this method. An important factor to maximise the effectiveness of rogueing is regular monitoring of turf for early detection the disease.

There is a mis-perception that the couch smut pathogen is only present in the inflorescences where symptoms are expressed, and therefore mowing to remove the infected parts can control the disease. We developed a sensitive and specific nested polymerase chain reaction (PCR) assay to detect *U. cynodontis* from asymptomatic parts of the plants and found that the fungus systemically infects the plants (Fig. 9). There were instances where some stolons of an infected plant tested negative for the fungus, indicating an uneven distribution of the fungus within the plant. Nevertheless, infection was sufficiently widespread, even in the roots, to suggest that mowing to remove symptomatic tissue is unlikely to control the disease.

Mowers should be cleaned when moving between paddocks to spread of the fungus, as teliopsores produced from smutted plants can be carried with the tools.

Resistant cultivars

Because an inoculation protocol has yet to be optimized, experimentally testing resistance of green couch cultivars was not possible. We have collated information from the literature, field observations and interviewing with growers. *U. cynodontis* has a narrow host range comprising closely related species of *Cynodon*, viz. *C. dactylon*, *C. incompletus*, *C. aethiopicus*, *C. nlemfuensis* and *C. plectostachyus* (Shivas and Vánky, 2001; Vánky, 2005; Vánky, 2012). In our surveys of turf farms in Queensland and New South Wales during 2018-19, we observed couch smut in tetraploid cultivars of *C. dactylon* such as 'Wintergreen', 'Windsorgreen' and 'Greenlees Park' but no disease was noted in the hybrid, triploid cultivars, even when they were growing adjacent to paddocks of infected plants (Table 3). However, failure to find disease does not constitute proof of disease resistance but is an observation warranting further investigation.

Table 3. Couch cultivars affected by couch smut during field surveys in 2018-19 in Queensland and New South Wales.

Species	Cultivar (Trademark)	Number of farms growing cultivar *	Smut present
Cynodon dactylon	Wintergreen	13	Yes
	Greenlees Park	7	Yes
	C-1 (Legend®)	5	Yes
	Grand Prix	4	Yes
	Windsor Green	4	Yes
	CT-2	2	Yes
	Variety Not Stated (Nullabor®)	1	Yes
	WGP3 (Stadium®)	1	No
	Oz-E-Green (Oz Tuff®)	3	No
	Riley's Evergreen (Conquest™)	2	No
	Gullygold	1	No
C. dactylon × *C. transvaalensis*	Santa Ana	5	No
	DT-1 (TifTuf®)	4	No
	AGRD (Agridark™)	1	No
	Tifway	1	No

* Twenty-two farms surveyed in total.

Fungicides

Our glasshouse trial and in vitro assay showed that application of Azoxystrobin + Propiconazole reduced the disease incidence. Although our field trial results were not conclusive, we have anecdotal evidence from a grower on the Sunshine Coast that Azoxystrobin + Propiconazole significantly reduced the incidence of couch smut on his farm.

Seminars, papers and other extension materials

Geering, A.D.W. (2021). Research finds mowing is an ineffective control against couch smut. Turf Australia Industry Magazine, Autumn 2021 Issue.

Geering, A.D.W. (2020). Project update: Integrated disease management of couch smut (TU17002). Turf Australia Industry Magazine, Winter 2020 Issue.

Tran, N. T., McTaggart, A. R., Drenth, A., Shivas, R. G., Loch, D. S., Kruse, J., Geering, A. D. W. (2020). Couch smut, an economically important disease of *Cynodon dactylon* in Australia. Australasian Plant Pathology 49:87-94.

Geering, A.D.W. (2019). Couch smut and buffalo yellowing investigations. Paper presented at: Turf Australia Conference 2019, Homebush, New South Wales, 30-31 May 2019.

Geering, A.D.W (2019). Improved capacity for integrated disease management of couch smut *(Ustilago cyndontis*) in turf'. Seminar presented to Turf Industry SIAP.

Geering, A.D.W. (2019). Diseases of green couch and buffalo grass. Turf Production Workshop, 13 August 2019, Riverway Stadium, Townsville, organized by Turf Queensland and Turf Australia.

Geering, A.D.W. (2019) Seminar on turf diseases presented at Asia Pacific Turfgrass Conference and Trade Exhibition 24 – 28 June, Brisbane.

Geering, A.D.W. (2019). Could fungicides be the key to fight couch smut? Turf Australia Industry Magazine, Issue 29, Summer 2019-20.
https://www.turfaustralia.com.au/public/85/files/Couch%20Smut%20Research%20article%201.pdf

Press Release, The University of Queensland (2019). 'Researchers fight to keep smut off Australia's sporting fields'. https://qaafi.uq.edu.au/article/2019/01/researchers-fight-keep-smut-off-australia%E2%80%99s-sporting-fields

Tran, N. T., McTaggart, A. R., Drenth, A., Shivas, R. G., Loch, D. S., Geering, A. D. W., (2019). Biology and epidemiology of smut (*Ustilago cynodontis*) in couch grass. *Australasian Plant Pathology Conference.* 25 – 28 NovPlease be more specific aember, Melbourne, Australia (Oral presentation).

Tran, N. T., McTaggart, A. R., Drenth, A., Shivas, R. G., Loch, D. S., Geering, A. D. W., (2019). Distribution of *Ustilago cynodontis* within couch grass plants. *TropAg Conference.* 11 – 13 November, Brisbane, Australia (Poster presentation).

TurfBreed (2019) UQ researchers putting the finger on the pulse with Couch Smut.
https://www.turfbreed.com.au/uq-researchers-putting-the-finger-on-the-pulse-with-couch-smut/

Geering, A. (2018). Smut research: Overcoming giggles to find an industry solution. Turf Australia Industry Magazine, Spring 2018 Issue.

Outcomes

Outcomes from this project are summarized as follows:

1) The surveys that were undertaken have provided a more accurate picture of the prevalence of the disease within the turf industry, and identified cultivars that are both highly susceptible and apparently resistant to the disease.
2) Knowledge generated from over a century of research on couch smut has been critically appraised and presented in a way that allows easy retrieval of information and identification of gaps in knowledge about the disease.
3) Promising results have been obtained from glasshouse experiments, which demonstrate that some systemic fungicides that are already registered for use on a range of turf diseases in Australia, could also be used to eliminate couch smut from disease-affected paddocks. Extension of the registration of these fungicides to cover couch smut should be pursued.
4) A sensitive and robust diagnostic assay for *U. cynodontis* has been developed, which for the first time allows detection of fungal hyphae in asymptomatic plant tissues. This diagnostic assay will be very

valuable for plant disease resistance screening, as it can be used to test for infection well before the appearance of symptoms such as smutted inflorescences.

5) New knowledge about pathogen biology has been obtained, which contributes to development of an integrated disease management plan. For example, systemic infection of green couch by *U. cynodontis* has been confirmed, which clearly demonstrates that mowing only disguises the disease but does not contribute to disease control.
6) While the inoculation experiments were ultimately unsuccessful, the knowledge that was generated will help guide future efforts to develop a disease resistance screening pipeline. In particular, a PCR assay to differentiate the different mating types of *U. cynodontis* is presented for the first time, which is needed when using yeast cultures as a source of inoculum.

Monitoring and evaluation

1. To what extent has the project achieved its expected outcomes?
    a. Has the literature review helped refine research questions?
    b. Has evidence been provided to support or dismiss the hypothesis that couch smut is transmitted by mowing?
    c. Are some cultivars of couch resistant to couch smut?
    d. Are there existing, registered fungicides that could help control couch smut?

A literature review on couch smut was published in the journal *Australasian Plant Pathology*, which surprisingly was the first ever review article to be done on this disease, despite the fact that the disease is found all around the world and was first described more than 100 years ago. When preparing this review, contact was made with Stephanie Digby in the USA, who had done a PhD project on *U. cynodontis* in the 1980s at the University of California, and was able to provide advice on how best to inoculate plants with the fungus.

As described earlier in this report, technical difficulties were encountered when trying to infect green couch plants with *U. cynodontis*, hence it was not possible to make conclusions about pathways of infection, nor to screen different green couch cultivars for disease resistance. However, the field surveys did provide strong circumstantial evidence that hybrid cultivars of green couch are resistant to couch smut.

2. How relevant was the project to the needs of intended beneficiaries?
    a. To what extent has the project met the needs of industry levy payers?

This project was initiated by industry levy payers in Queensland and addressed research questions that approved by the Turf Industry SIAP. In retrospect, the aims of this project were ambitious for the timeframe that was provided. Nevertheless, good progress has been made in developing an integrated pest management program for couch smut.

3. How well have intended beneficiaries been engaged in the project?
    a. Have regular project updates been provided through publication of articles in the grower magazine 'Turf Australia Industry Magazine'?
    b. Have project team members been able to directly liaise with turf growers to provide advice on disease control?
    c. Has an integrated disease management plan been widely distributed among industry members?

A large effort was made by project team members to interact with turf farmers throughout the duration of this project. The project team visited 20 different turf farms in Queensland and New South Wales, not only those in the major production areas around Sydney and Brisbane but also in smaller towns further afield such as Rockhampton and Gladstone. These farm visits usually extended for 1 ½ to 2 hours, during which time all aspects of turf pathology were discussed, not just limited to couch smut.

Results of this project were also presented on multiple occasions through various forms of media. Four articles covering project findings were published in the Turf Australia magazine, and seminars were given at the 2019 Asia Pacific Turfgrass Conference, the 2019 Turf Australia Conference, and a 2019 Turf Production Workshop in Townsville, which was jointly organized by Turf Queensland and Turf Australia. Following an approach from TurfBreed, assistance was provided to develop a fact sheet on couch smut for distribution to their member farmers.

Use of fungicides such as Azoxystrobin + Propiconazole will likely be a component of the integrated disease management plan for couch smut, but until the registration of these fungicides is extended to include couch smut, there are legal barriers to promoting this management practice widely among the turf industry.

4. To what extent were engagement processes appropriate to the target audience/s of the project?
    a. Did the project engage with industry levy payers through their preferred learning style?
    b. How accessible were extension events to industry levy payers?

A range of methods of communication were used within the project, ranging from one-to-one discussions on farms, to industry seminars and articles published in the Turf Australia magazine. The turf industry is very dispersed and therefore it is practically impossible to reach everyone but seminars were presented at two events organized by Turf Australia in Sydney and Townsville.

5. What efforts did the project make to improve efficiency?

During the course of this project, Dr Nga Tran reduced to part time on this project with the endorsement of Hort Innovation, to allow her to work on a related buffalo grass yellows project (TU19000). This project arose as a result of discussions with farmers and field observations by the team members during the couch smut surveys in the Hawkesbury Valley. A particularly severe epidemic of buffalo grass yellows was experienced in the summer of 2018/19, and this disease was rated a much higher priority at the time by the farmers compared with couch smut. By allowing Dr Nga Tran to work across two turf projects, her time was used much more efficiently, as there were considerable waiting times for results to come in from experiments being done to investigate the best methods for inoculating *U. cynodontis* and to assess the efficacy of fungicides for controlling smut disease.

Recommendations

At 2-years duration, this project was insufficiently long to fully resolve management solutions for a complex disease such as couch smut. The brevity of the project was compounded by the COVID-19 pandemic, which placed serious constraints on research activities during 2020.

Fungicides such as Azoxystrobin 62g/L combined with Propiconazole 104g/L show promise to help eradicate couch smut in a paddock but additional field trials are required to provide the data needed to extend the registration of this fungicide for use on *U. cynodontis*. Fortunately, this fungicide is already approved for use on other turf diseases in Australia and therefore the data for a new registration is not needed, just that need to extend an existing permit.

The often erratic distribution of couch smut over a patch of turf, and the apparent seasonal variation in appearance of smutted flower heads, makes design of the field trial very difficult. Rather than a traditional geometric design, a better design for a field trial to examine the efficacy of fungicides to control couch smut would be to mark out irregular plots surrounding patches of couch smut, and then examine the reduction in density of smutted flower heads within these patches after fungicide treatments.

Field observations suggest that *C. dactylon* × *C. transvaalensis* hybrids are resistant to couch smut and therefore could be used as replacement cultivars for paddocks with high incidences of the disease.

Much more fundamental research is required to understand the infection pathways for *U. cynodontis* but the scope of this work probably goes beyond the immediate applied needs of the Australian turf industry. Very likely there are fertile, pure accessions of *C. dactylon* that have natural resistance against *U. cynodontis*,

which may represent immediate replacements for susceptible cultivars such as 'Wintergreen' and 'Greenlees Park'. Alternatively, these resistant accessions could be mutated or used in traditional a plant breeding program until suitable cultivars are developed. However, this plant breeding research would require long term investment by a company or other funding body.

Refereed scientific publications

Tran, N. T., McTaggart, A. R., Drenth, A., Shivas, R. G., Loch, D. S., Kruse, J., Geering, A. D. W. 2020. Couch smut, an economically important disease of *Cynodon dactylon* in Australia. Australasian Plant Pathology 49:87-94.

References

Aldous, D.E., Haydu, J.J., Moir, R.N., 2009. Benchmarking the Australian sod industry. International Turfgrass Society Research Journal 11, 299-311.

Christians, N., 2011. Chapter 4: Warm-season grassess, Fundamentals of Turfgrass Management. John Wiley & Sons Incorporated, Hoboken, United States, pp. 59-74.

Coombs, J.T., Franco, C.M., 2003. Isolation and identification of actinobacteria from surface-sterilized wheat roots. Appl. Environ. Microbiol. 69(9), 5603-5608.

Dean, J.L., 1982. The effect of wounding and high-pressure spray inoculation on the smut reactions of sugarcane clones. Phytopathology 72(8), 1023-1025.

Digby, S., Wells, K., 1989. Compatibility and development in *Ustilago cynodontis*. Mycologia 81, 595-607.

Farr, D.F., Rossman, A.Y., 2019. Fungal Databases, U.S. National Fungus Collections, ARS, USDA.

García-Guzmán, G., Burdon, J.J., 1997. Impact of the flower smut *Ustilago cynodontis* (Ustilaginaceae) on the performance of the clonal grass *Cynodon dactylon* (Gramineae). Am J Bot 84(11), 1565-1571.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2(2), 113-118.

Haydu, J.J., Aldous, D.E., Satterthwaite, L.N., 2008. Economic analysis of the Australian turfgrass industry, Horticulture Management & Marketing,University of Florida, pp. 30.

Horner, W., Helbling, A., Salvaggio, J., Lehrer, S., 1995. Fungal allergens. Clin Microbiol Rev 8(2), 161-179.

Kellner, R., Vollmeister, E., Feldbrügge, M., Begerow, D., 2011. Interspecific sex in grass smuts and the genetic diversity of their pheromone-receptor system. PLoS Genet 7(12.e1002436), 1-17.

Ketta, H.A., Emeran, A.A., 2020. Occurrence and characterization of *Ustilago cynodontis* (Passerini) Henn., the causal agent of Bermudagrass (*Cynodon dactylon*) smut in Egypt. Canadian Journal of Plant Pathology, 1-8.

Kneebone, W.R., 1966. Bermuda grass—Worldly, wily, wonderful weed. Econ Bot 20(1), 94-97.

Levetin, E., Horner, W.E., Scott, J.A., Barnes, C., Baxi, S., Chew, G.L., Grimes, C., Kennedy, K., Larenas-Linnemann, D., Miller, J.D., 2016. Taxonomy of allergenic fungi. J Allergy Clin Immunol 4(3), 375-385.e371.

Maclennan, A., 2018. TU16001: Turf Industry Statistics and Research Project. Horticulture Innovation Australia.

Marchelo-d'Ragga, P.W., Misaka, B.C., 2015. First report of [*Ustilago cynodontis* (Henn.) Henn: Ustilaginales] the causal agent of smut of couch grass *Cynodon dactylon* (L.) Pers. in South Sudan. Inter J Agri Res Rev 3(7), 356-358.

Massimo, N.C., Devan, M.N., Arendt, K.R., Wilch, M.H., Riddle, J.M., Furr, S.H., Steen, C., U'Ren, J.M., Sandberg, D.C., Arnold, A.E., 2015. Fungal endophytes in aboveground tissues of desert plants: infrequent in culture, but highly diverse and distinctive symbionts. Microb. Ecol. 70(1), 61-76.

McAlpine, D., 1910. The smuts of Australia. Government Printer, Melbourne.

Shivas, R.G., Vánky, K., 2001. The smut fungi on *Cynodon*, including *Sporisorium normanensis* sp. nov. from Australia. Fungal Divers 8, 149-154.

Tran, N.T., McTaggart, A.R., Drenth, A., Shivas, R.G., Loch, D.S., Kruse, J., Geering, A.D.W., 2020. Couch smut, an economically important disease of *Cynodon dactylon* in Australia. Australas. Plant Pathol. 49, 87-94.

Vánky, K., 2005. The smut fungi of Ethiopia and Eritrea. Lidia 6, 93-120.

Vánky, K., 2012. Smut fungi of the world. American Phytopathological Society, St. Paul, Minnesota.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR protocols: A guide to methods and applications. Vol. 18. Academic Press, San Diego, California, pp. 315-322.

Wu, Y., 2011. Cynodon. In: Kobe, C. (Ed.), Wild Crop Relatives: Genomic and Breeding Resources, Millets and Grasses. Springer-Verlag, Berlin, Germany, pp. 53-71.

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report.

Acknowledgements

We thank the turf growers for their cooperation with the field surveys and sharing information on on couch smut. We thank Dr. Christopher Lambrides for providing the green couch germplasm and helpful discussions about the project; Dr. Dean Beasley for extracting records of *Ustilago cynodontis* from the Australian Plant Disease Database and for also assisting with generating the distribution map; the turf farm managers and growers who kindly; Dr. Julia Kruse for the help with sample collection. We are grateful to the anonymous companies for providing test samples of fungicides.

Appendices

Appendix 1. Field survey questionnaire.

Appendix 2. *Ustilago cynodontis* culture collection.

Appendix 2. Couch smut review paper.