EFFECTIVE CROWN ROT SCREENING METHODOLOGIES

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The principal purpose of this document is to serve as a general resource for cereal breeders wishing to screen for crown rot disease in field trials of breeding materials. We have also provided links to currently available phenotypic methods for seedling screening. qPCR-based methods for species-specific quantitative assessment of the levels of fungal mycelium in diseased tissues are also outlined. Where methods have already been described in reports or published in a refereed journal, a link to that source is provided.

Field Screening for Crown Rot

An earlier completed project in the Crown Rot Initiative, USQ00011 "Seedling and field-based phenotyping of wheat and barley" (July 2009 – June 2011) compared the performance of a range of field-based phenotyping methodologies in a Ring Test approach.

"The adult ring test was designed to compare the different screening methods for crown rot in adult plants developed at sites across Australia. The study seeks to quantify the variability in results between methods using a common set of thirty entries, comprised of wheat and barley lines with varying levels of response to crown rot infection. The ultimate aim of the project is to produce a robust and reliable method for high throughput crown rot screening. The four methods in the study each implement a differing trial structure, layout and design, together with method of infection and subsequent scoring technique. These differences are documented in the report, and results for the level of crown rot infection of thirty genotypes under each methodology are compared. In addition, material from the Wellcamp trial was assessed by most groups giving a useful comparison of rating scales without the confounding effect of experimental method." – Alison Kelly 2009 Report.

Two reports were prepared by the project's statistician, Dr Alison Kelly, one each for the 2009 and 2010 season data. These reports are available on the CAIGE website at:

http://caigeproject.org.au/images/caige/projects/crownrot_initiative/2011/crownrot_Fieldtrial_analysis2009.pdf

http://caigeproject.org.au/images/caige/projects/crownrot_initiative/2011/crownrot_Fieldtrial_analysis2010.pdf

While the analysis in these reports indicates that the symptom scoring system used had little effect on the ranking of the entries in a trial, variability within plots due to within plant variability in the degree of infection of different tillers had the most significant effect on precision.

The two field screening methods which gave the highest degree of genetic discrimination were the methods used by the crown rot team at NSW DPI in Tamworth and by the then QDAF group in Toowoomba, who are now based at the University of Southern Queensland. Descriptions of these methods have been published:

For production of inoculum (used for both seedling and field testing in Toowoomba):

Percy CD, Wildermuth GB, Sutherland MW (2012). Symptom development proceeds at different rates in susceptible and partially resistant cereal seedlings infected with *Fusarium pseudograminearum*. *Australasian Plant Pathology* **41:** 621-631.

For design, inoculation and sampling of field trials in Toowoomba, based on the unpublished methods developed by Cassandra Percy, see:

Knight NL, Sutherland MW (2015). Culm discoloration as an indicator of Fusarium pseudograminearum biomass. *Australasian Plant Pathology* **44:** 319-326.

In addition during 2014 Prof Stephen Neate and Dr Percy made available to breeding companies detailed descriptions of field-based techniques for resistance and tolerance screening. These two reports were generated as part of GRDC Project DAQ00167, a component of the Crown Rot Initiative, and are attached in Appendices 1 and 2.

The method used by the NSW DPI team at Tamworth has been described in:

Daniel R, Simpfendorfer S (2012). The impact of crown rot on winter cereal yield. In 'Proceedings of the First International Crown Rot Workshop for Wheat Improvement'. 22-23 October. (Eds. RIS Brettell, JM Nicol).

This has recently been updated in an unpublished document provided by Dr Steven Simpfendorfer which is attached in Appendix 3, with the permission of Dr Simpfendorfer.

Seedling Screening for Crown Rot

GRDC Project USQ00011 also compared seedling screening methodologies and a comprehensive report comparing the major methods used was prepared by Alison Kelly. This is available on the CAIGE website.

http://caigeproject.org.au/images/caige/projects/crownrot_initiative/2011/crownrot_Seedlingtrial_analysis2009.pdf

The more robust methods derive from the original method of Dr Graham Wildermuth:

Wildermuth GB & McNamara RB (1994). Testing wheat seedlings for resistance to crown rot caused by *Fusarium pseudograminearum*. *Plant Disease* **78:** 949-953.

A recent example is given in detail in:

Percy CD, Wildermuth GB, Sutherland MW (2012). Symptom development proceeds at different rates in susceptible and partially resistant cereal seedlings infected with *Fusarium pseudograminearum*. *Australasian Plant Pathology* **41:** 621-631.

In addition during 2014 Prof Stephen Neate and Dr Cassy Percy made available to breeding companies detailed descriptions of the current methodology for seedling screening, in the report contained in Appendix 4, generated as part of GRDC Project DAQ00167.

Quantitative Estimation of Pathogen DNA in Infected Tissues

Techniques are now available to estimate the quantity of fungal DNA, and hence fungal mycelium, present in host tissues expressing the symptoms of a disease. Using the technique of quantitative PCR (qPCR) in conjunction with DNA primers that are specific for amplifying *Fusarium pseudograminearum* DNA, it is now possible to compare the relationship between the quantity of fungus in a tissue with the severity of disease symptoms and with the magnitude of yield loss in appropriately designed trials. In particular, qPCR measurements are likely to reveal tolerant host lines. These lines will have significant levels of infection as indicated by qPCR but comparatively lower expression of disease symptoms and less severe levels of yield loss than comparable non-tolerant host lines with the same level of infection.

Primer sequences and qPCR conditions for species-specific qPCR of *F.pseudograminearum* DNA have been developed by Dr Noel Knight during the course of this project and have been published.

Knight NL, Sutherland MW, Martin A, Herde DJ (2012) Assessment of infection by the crown rot pathogen Fusarium pseudograminearum in wheat seedling tissues using quantitative PCR and a visual discoloration scale. *Plant Disease.* **96:** 1661-69.

As this is a recently developed technique, the following detailed methodology used for the qPCR analyses employed in USQ00012 has been provided by Noel Knight.

Collection, visual rating, tissue preparation and DNA extraction for qPCR from infected stem sections

A. Planting and Inoculation

Field trials were set up using a layered inoculum method developed by Dr. Graham Wildermuth and now utilised by Dr. Cassandra Percy (previously QDAF, now at USQ). Inoculum production, field maintenance and planting were described in Percy et al. (2012) and Knight and Sutherland (2015).

B. Stem Collection Procedure

1. Collect 5 plants randomly from each plot at the growth stage of choice, typically either early milk development or at harvest maturity (not recommended for qPCR assessment).
2. Keep plants in a dry, cool environment to minimise post collection hyphal growth.
3. Identify and remove the main stem from each plant.
4. Remove leaves and leaf sheaths from the main stem.
5. From the base of each main stem cut a 6 cm length (from top of sub-crown internode to 6 cm along the stem). Place into a labelled 10 mL tube.
6. Sections are kept in the freezer until rating and drying to minimise fungal growth.

C. Visual Rating

Visually rate each stem section using a 10% scale based on discolouration. Visual assessment is done on the entire 6 cm using a 10% scale (0, 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100). A cardboard rating scale with 0.6 cm marked measurements can be used to guide the ratings. Both sides of the stem need to be assessed and the approximate average taken.

D. Quantitative Polymerase Chain Reaction (qPCR)

Stem Grinding Procedure

1. Dry each 6 cm stem portion overnight at 50 °C and record the dry weight.
2. For each sample, place 6 lead shots (Size 4, Winchester, Australia) into each of two 2 mL screw cap tubes. Do not attach lid.
3. Take a 6 cm stem sample and cut into very small pieces (1-2mm) using dog nail clippers. Approximately 3 cm into each tube. Make sure each pair of tubes is correctly labelled with the sample number, these will be re-combined after grinding.
4. Place samples in open tubes in drying oven overnight at 50 °C.
5. Place caps on tubes. Grind tissues in the Fast-prep machine (MP Biomedicals, Australia) twice at 6.5 m/s for 60 sec.
6. Remove tubes from the Fast-prep machine and visually examine each tube to confirm tissues have been ground adequately.
7. Pair up tubes of the same sample again, pour out lead shots following granular convection and re-combine the samples, mixing them in the one tube.

Sub-sampling Procedure

1. Label a 1.5 mL microfuge tube with the sample number.
2. Place on the scale and zero the value.
3. Using a fine spatula sub-sample 15-20 mg of ground tissue into the empty tube.
4. Record the sub-sample weight to the nearest milligram.
5. These samples are ready for DNA extraction.

DNA extraction and qPCR

DNA was extracted as described in Knight and Sutherland (2015) and qPCR was performed as described in Knight et al. (2012).

Briefly, genomic DNA was extracted using a Wizard Genomic DNA Extraction Kit (Promega, Sydney, New South Wales) following the plant DNA extraction protocol provided with the kit. Samples were eluted into 100 µL and stored at –4°C until required.

Dual-labelled probe qPCR reactions were performed in 20-µl volumes containing autoclaved high-purity water, 0.5 units of Immolase DNA polymerase, 1× Immobuffer, 2.5 mM $MgCl_2$, 100 µM dNTPs, 150 nM *F. pseudograminearum* CAL

Fluor Gold labelled probe, 150 nM wheat FAM-labelled probe, 0.25 µM forward and reverse primers for both organisms, and 5 µl of DNA template.

Multiplex real-time qPCR was performed using clear 100-µl strip tubes and caps (Corbett Life Sciences) in a Rotor-Gene 6000. Thermal cycling was completed in less than 2 h and conditions consisted of 10 min at 95°C and then 35 cycles of 95°C for 15 s and 64°C for 1 min. The Rotor-Gene 6000 series software collected data for both reporter dyes every 0.15 s from each tube, generating a fluorescence profile for each amplification product. The threshold cycle (Ct) was recorded for each dye as the cycle at which fluorescent signal, associated with an exponential growth of PCR product, exceeded background fluorescence.
PCR controls duplicated in every assay included no template controls (NTCs) and genomic DNA standards (positive or negative) for both *F. pseudograminearum* and wheat. Tenfold serial dilutions of pure genomic wheat DNA and pure genomic *F. pseudograminearum* DNA standards were initially tested in triplicate for assessment of the standard curve and PCR efficiencies. Each experimental PCR assay with unknown samples included standards run in duplicate. Each unknown sample was assayed at least once, with a random selection of samples duplicated across separate PCR assays.

The wheat primer/probe set was designed to be included in the qPCR assay as an internal positive control. After quantification, normalised estimates of *F. pseudograminearum* colonisation of wheat tissue were obtained by dividing the quantity of *F. pseudograminearum* DNA by the original extracted sample dry weight (*F. pseudograminearum* DNA/dry weight of wheat tissue).

References

Knight NL, Martin A, Sutherland MW, Herde DJ (2012) Assessment of infection by Fusarium pseudograminearum in wheat seedling tissues using quantitative PCR and a visual discoloration scale. *Plant Disease* **96:**1661-1669

Knight NL, Sutherland MW (2015) Culm discolouration as an indicator of Fusarium pseudograminearum biomass. *Australasian Plant Pathology* **44:** 319-26.

Percy CD, Wildemuth GB, Sutherland MW (2012) Symptom development proceeds at different rates in susceptible and partially resistant cereal seedlings infected with Fusarium pseudograminearum. *Australasian Plant Pathology* **41:** 621-631.

APPENDIX 1

DAFF Crown Rot Resistance Field Testing Protocol 07/14. Neate, SM and Percy, CD

Potential Uses

Assessing fixed lines for resistance.
Determining yield loss in fixed lines.
Advancing breeding material through gene enrichment of segregating populations.
Quantitative genetics experiments to understand control of resistance.
Phenotyping for QTL identification and mapping
The field rating methods of the QDPI&F group have been used to provide phenotyping for many breeding and research groups across the country, including-

- More than 25 years of crown rot phenotyping for the Qld wheat breeding program and EGA (leading to the release of cultivars with some of the highest levels of crown rot resistance, eg EGA Wylie, Baxter and Lang).
- All crown rot phenotyping for the Northern Node of Barley Breeding Australia.
- Crown rot phenotyping for the NSW DPI's durum breeding program.
- Crown rot phenotyping for the AWCMMP and AWBMMP
- Crown rot phenotyping for USQ marker validation
- Crown rot phenotyping for Victoria's synthetic wheat program.
- Student honours and PhD theses.
- Crown rot phenotyping of NVT retentions and all cultivars appearing in variety guide
- Crown rot phenotyping for Breeding companies
- Crown rot phenotyping for UQ
- Crown rot phenotying for CIMMYT
- Identify sources of resistance.

Relevant Publications

Wildermuth GB, Purss GS (1971) Further sources of field resistance to crown rot (Gibberella zea) of cereals in Queensland. Aust J Exp Agric Anim Hus 11:455–459

Dodman RL, Wildermuth GB (1987) Inoculation methods for assessing resistance in wheat to crown rot caused by Fusarium gramineum Group 1. Aust J Agr Res 38:473–486

Wildermuth GB, McNamara RB (1994) Testing wheat seedlings for resistance to crown rot caused by Fusarium graminearum Group 1. Plant Disease 78: 949-953.

Lusted, Cassandra D (1998) An examination of the development of crown rot caused by Fusarium Graminearum Schwabe Group I in seedlings of wheat barley and oats. BSc Hons Thesis, University of Southern Queensland.

Percy, CD. Wildermuth, GB. and Sutherland, MW. (2005). Effectiveness of partial resistance sources in hexaploid wheats against Fusarium pseudograminearum isolates: 15th Biennial Australasian Plant Pathology Society Conference, 26-29 Sep 2005, Geelong, Australia.

Malligan, Cassandra D.(2009) Crown rot (Fusarium pseudograminearum) symptom development and pathogen spread in wheat genotypes with varying disease resistance. PhD Thesis University of Southern Queensland.

Neate, S.M., (2010) Crown Rot Disease of Small Grain Cereals. Fourth International Masterclass on Soil Borne Pathogens of Wheat, Eskisehir, Turkey 2010

Kelly, A (2010) Analysis of Seedling Phenotyping of Crown Rot Symptoms in Wheat and Barley. GRDC Crown Rot Ring Test Report 19pp. (2009 data analysis, available on CAIGE website)

Herde, DJ, Percy, CD., Walters, T., Davies, PA, Kelly, A, Fletcher, S and Neate SM. (2012). First International Crown Rot Laboratory and Field Workshop handout notes. Leslie Research Centre, Toowoomba, Australia.

Herde, DJ, Wildermuth GB., and Neate SM (2012) Yield loss due to crown rot in wheat in Australia's northern cropping region. Proceedings of the first International Crown Rot Workshop for Wheat Improvement, Narrabri, New South Wales, Australia

Percy, C., Herde, D., Wildermuth, G, Kelly, A., and Fletcher, S. (2012) Effective and efficient field and controlled environment screening methods to identify resistance to crown rot. Proceedings of the first International Crown Rot Workshop for Wheat Improvement, Narrabri, New South Wales, Australia.

Percy, CD. and Wildermuth, GB. and Sutherland, MW. (2012) Symptom development proceeds at different rates in susceptible and partially resistant cereal seedlings infected with Fusarium pseudograminearum. Australasian Plant Pathology, 41 (6). pp. 621-631. ISSN 0815-3191

Field Site

- To establish background inoculum level, assay the level of crown rot pre-season by intensive systematic soil sampling followed by a bioassay or inoculum molecular quantification system.
- Ideal is a high level of inoculum with little variation across the site.
- Wellcamp Research Farm. Grey vertisol, moderately cracking clay.
- A site located in an environment with wet conditions early in the season and water stress from jointing to maturity emphasises discrimination between resistance levels.
- We use other sites as required, but a comprehensive knowledge of rotations and all background diseases is necessary.

Inoculum Production

- Add millet seed to an autoclave bag
- Cover with water and soak overnight
- Drain and autoclave at 20psi for 30 minutes on 3 consecutive days
- Subculture 5 fresh, pathogenic isolates of *F. pseudograminearum* on individual Czapek-Dox Agar with 10% sucrose (Thom and Raper 1945) petri plates
- Check viability and purity, morphology and growth rate.
- Inoculate half a culture plate above into 250ml of Czapek-Dox Broth with 10% sucrose and minus agar (Thom and Raper 1945) in 500ml Schott bottles. One isolate per bottle. Shake for 7d at 150rpm at 20C
- Blend with a sterile stab mixer
- Add 30ml of blended isolate, one isolate per autoclave bag of grain, shake to mix, seal bag with sterile cotton wool/cheesecloth plug. Shake to mix
- Shake every other day for 21 days to break up clumps and encourage even colonisation
- Spread thinly on trays and dry with circulating air at 25C for two weeks, stirring regularly to break up lumps. Final inoculum should be internally colonised individual millet grains
- Test a subsample for virulence and pathogenicity under standard seedling pot test conditions. All *Fusarium* sp are unstable in storage and can change in virulence and pathogenicity, particularly with repeated sub-culturing, so that new field isolates need to be introduced regularly and all isolates need to be tested for effectiveness with each inoculum batch

Field Inoculation Method

Banded inoculum, separating the inoculum and seed in the soil, to prevent seedling blight that occurs if seed touches inoculum and to simulate natural infection as the plant grows through the inoculum.

Exact method and rate depends on aims:

- At a new site increase level and increased evenness of background inoculum by inoculating successive durum crops in seasons prior to the screening season. Once a high and evenly distributed inoculum load is present, follow with banded inoculum in the screening season.
- At a site where there is a high and evenly distributed background of inoculum, use banded inoculum in the screening season

Plot Size, Replication and Statistical Treatment

- Plot size of 3 m single rows up to 8m x 7 row plots depending on the experiment.
- One to six replicates, depending on the nature of the material.
- Randomised block; overlayed with row by column designs, spatial analysis and ANOVA with appropriate comparison of means.

Sample Number and Timing

- Number of samples taken varies from individual plants in segregating material up to 70 plants per plot.
- Sample timing varies from grain fill to maturity, depending on the experiment type.
- Currently sample 20 primary and 20 secondary tillers per plot for fixed material

Potential Resistance Standards

Genotype	Resis. level (1-9)	Notes
2-49	7	Gala/Gulyas.
Wylie	6	QT2327/Cook/QT2804
Sunco	5	Cook*3/WW15/4SUN9E-27/3Ag14
Vasco	3	3-AG-14/4* Condor //5*Oxley
Gregory	3	Pelsart/2*Batavia DH
Janz	3	3-AG-3/4*Condor//Cook
Batavia	2	Bronchus 'S'/Banks
Puseas	2	Pusa-4/Three-Seas
Bellaroi	1	920405/920274, could be replaced with any commercial durum

Disease Assessment

Stem Lesioning

The rating method is based on the number of internodes lesioned, scoring into five categories, and incorporating damage that results in a dead head.

Cat0 – no lesions
Cat1 – first internode partially lesioned
Cat2 – 1st internode fully lesioned OR 1st and 2nd internode lesioned
Cat3 – more than two internodes lesioned
Cat4 – dead head due to crown rot

Using the formula, CR Disease Severity =((no. tillers cat 1)*1.5+(no. tillers cat 2)*3.5 + (no. tillers cat.3)*6 + (no. tillers cat.4)*9) / total tillers*9 the categories are weighted to reflect crown rot damage that relates to yield, based on the progress of research from Purss to Dodman to Wildermuth.
CR severity is usually expressed as a percentage of the severity of the susceptible variety Puseas to account for variation in severity due to season.

Dead Heads

Estimating percent deadheads while quick, is a poor estimate of yield loss and should only be used in combination with stem lesion assessment. Plots with low levels of dead heads may not detect levels of crown rot resistance or tolerance

Relationship to other methods

This adult plant field test is highly significantly related to our seedling rating method, as published in r=0.78; n=28; P=<0.01.(Wildermuth and McNamara 1994),
r=0.74 in ring test which was the highest correlation between any of the methods tested (field, seedling and adult plant pot tests).

Stem lesioning is a strong predictor of yield loss (built on many years of method development), when rated with our methods. Lesioning and yield were highly significantly correlated at a grower-site field experiment in the dry 2004 (r=-0.79; n=96; P=<0.001) as well as in the wet finish of 2005 (r=-0.64; n=72; P=<0.001).

Lesioning is also significantly related to levels of soil DNA (r=0.49; n=96; P=<0.001); levels of *Fusarium pseudograminearum* isolated from the tiller bases (r=0.83; n=96; P=<0.001) and isolated from the sub-crown internode (r=0.71; n=96; P=<0.001) in 2004.

In the 2009 ring test the methods described here had the highest discrimination between genotypes of any method tested. Field (0.79) and seedling (0.82). In 2010 which was a very wet year discrimination between genotypes in the field was (0.69).

Advantages

- Well established screening method under development since the 1960's.
- Able to screen large volumes of material, over 100.000 plants have been screened in a season.
- A strong, reliable test of plant resistance (reliable in terms of crown rot experimentation, which is inherently variable).
- This field method is highly correlated to results that come from our seedling screening method.
- When plants are pulled at maturity they store well and unchanging while rating takes place.

Disadvantages

- Slow, only one test per year as it needs a growing season and summer rating to collect data.
- Expensive, due to the time consuming rating.
- Absolute disease expression is influenced by within crop rainfall and timing of water stress

APPENDIX 2

DAFF Crown Rot Field Tolerance Testing Protocol 07/14 Neate, SM and Percy, CD

Potential Uses

Determining yield loss in fixed lines due to Crown Rot.

Advancing breeding material through gene enrichment of segregating populations.

Quantitative genetics experiments to understand control of tolerance.

Phenotyping for tolerance QTL identification and mapping

Relevant Publications

Wildermuth GB, Purss GS (1971) Further sources of field resistance to crown rot (Gibberella zeae) of cereals in Queensland. Aust J Exp Agric Anim Hus 11:455–459

Dodman RL, Wildermuth GB (1987) Inoculation methods for assessing resistance in wheat to crown rot caused by Fusarium gramineum Group 1. Aust J Agr Res 38:473–486

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Herde, DJ, Wildermuth GB., and Neate SM (2012) Yield loss due to crown rot in wheat in Australia's northern cropping region. Proceedings of the first International Crown Rot Workshop for Wheat Improvement, Narrabri, New South Wales, Australia

Percy, C., Herde, D., Wildermuth, G, Kelly, A., and Fletcher, S. (2012) Effective and efficient field and controlled environment screening methods to identify resistance to crown rot. Proceedings of the first International Crown Rot Workshop for Wheat Improvement, Narrabri, New South Wales, Australia.

Percy, CD. and Wildermuth, GB. and Sutherland, MW. (2012) Symptom development proceeds at different rates in susceptible and partially resistant cereal seedlings infected with Fusarium pseudograminearum. Australasian Plant Pathology, 41 (6). pp. 621-631. ISSN 0815-3191

Fletcher S., Percy C and Kelly A. (2012) Getting more for less: Exploring the main sources of variation in measuring crown rot. Proceedings of the 7th Australasian Soilborne Diseases Conference, Fremantle, Western Australia.

Field Site Characteristics

- The level of crown rot needs to be determined pre-season by intensive systematic soil sampling using a bioassay or inoculum molecular quantification system. Below detection limit does not guarantee no crown rot at a site or patchy inoculum distribution. Soil sample results should be compared with rotation history. Several years of a weed free non-host or fallow combined with a below detection limit assay are ideal.
- Wellcamp Research Farm. Grey vertisol, cracking clay is our primary site. Other sites are used as required.
- A site located in an environment with wet conditions early in the season and water stress from jointing to maturity emphasises discrimination between crown rot tolerance levels.

Inoculum Production

- Add millet seed to an autoclave bag
- Cover with water and soak overnight
- Drain and autoclave at 20psi for 30 minutes on 3 consecutive days
- Subculture 5 fresh, pathogenic isolates of *F. pseudograminearum* on individual Czapek-Dox Agar with 10% sucrose (Thom and Raper 1945) petri plates
- Check viability and purity, morphology and growth rate.
- Inoculate half a culture plate above into 250ml of Czapek-Dox Broth with 10% sucrose and minus agar (Thom and Raper 1945) in 500ml Schott bottles. One isolate per bottle. Shake for 7d at 150rpm at 20C
- Blend with a sterile stab mixer.
- Add 30ml of blended isolate, one isolate per autoclave bag of grain, shake to mix, seal bag with sterile cotton wool/cheesecloth plug. Shake to mix
- Shake every other day for 21 days to break up clumps and encourage even colonisation
- Spread thinly on trays and dry with circulating air at 25C for two weeks, stirring regularly to break up lumps. Final inoculum should be internally colonised individual millet grains.
- Test a subsample for virulence and pathogenicity under standard conditions described in seedling pot test. All *Fusarium sp* are unstable in storage and can change in virulence and pathogenicity in storage, especially with repeated subculturing, so that new field isolates need

to be introduced regularly and all isolates need to be tested for effectiveness with each inoculum batch.

Field Inoculation Method

- Banded inoculum placed above the seed to separate the inoculum and seed in the soil, to prevent seedling blight that occurs if seed touches inoculum.

Plot Size, Replication and Statistical Treatment

- Plot size up to 8m x 7 row plots depending on the experiment and availability of crown rot free land.
- One to six replicates, depending on the nature of the material.
- Randomised block; overlayed with row by column designs, spatial analysis and ANOVA with appropriate comparison of means.
- Recently we have used a split plot design of 3 rows x 6m with plus /minus inoculum. We currently use 3m x 4 rows with plus and minus inoculum.

Sample Number and Timing

- At grain maturity 20 plants from each plot are subsampled for disease rating. After rating heads are threshed and grain is added to the machine harvested grain
- Plots are cut to a uniform size and harvested with a combine harvester.

Potential Tolerance Standards

Genotype	Resis. level (1-9)	Notes
2-49	7	Gala/Gulyas.
Wylie	6	QT2327/Cook/QT2804
Sunco	5	Cook*3/WW15/4SUN9E-27/3Ag14
Vasco	3	3-AG-14/4* Condor //5*Oxley
Gregory	3	Pelsart/2*Batavia DH
Janz	3	3-AG-3/4*Condor//Cook
Batavia	2	Bronchus ‘S’/Banks
Puseas	2	Pusa-4/Three-Seas
Bellaroi	1	920405/920274, could be replaced with any commercial durum

Disease Assessment

Currently 10 plants are sampled per 4 row x 3 m plot before harvest and stem lesioning and deadheads are recorded as described in the Field Adult Resistance Plant Test Protocol. The subsample is threshed and added to the machine harvested grain.

Relationship to other methods

Lesioning and yield were highly significantly correlated at a grower-site field experiment in the dry 2004 ($r=-0.79$; $n=96$; $P=<0.001$) as well as in the wet finish of 2005 ($r=-0.64$; $n=72$; $P=<0.001$).

Advantages

- A screening method under development and improvement since 2000's.
- This method is well correlated to results from our lesion screening method
- Cost efficient compared to the lesion rating system, because of the automated nature of the harvesting, but still requires some lesion screening to ensure loss is due to crown rot.

Disadvantages

- Not useful for individual plant selection.
- Yield loss and accuracy of data is highly dependant on seasonal conditions.
- Results can be invalidated by background inoculum especially when it is variable across a site.
- Requires new land each year as the checkerboard distribution of inoculum will take years to return to an even zero inoculum state.
- Requires preparation of large amounts of land as plots are large and it can take 2-3 seasons to prepare the land for one test

APPENDIX 3: Yield assessments (tolerance) – NSWDPI, Tamworth

Provided by Steven Simpfendorfer

	Field
Reason	Determine the yield loss to crown rot in released varieties and advanced breeding material.
Infection method	Inoculated durum grain (mixture 5 isolates) added at rate of 2 g/m row to the seed furrow at sowing. Durum grain sterilised twice at 121ºC for 60 min on two consecutive days. Macrocondial suspensions prepared in mung bean broth (grown 7 days) then used to inoculate sterilised durum grain. Grown at 25ºC for 3 weeks, air dried at 30ºC before use. Plated at this time to confirm %colonisation and ensure nil contamination. Generally target crown rot free paddocks. PreDicta B soil sample of each range taken at sowing from each trial site to establish background pathogen levels.
Size	2m x 10m plots (3 rep) or 2 x 5 m plots (4 rep)
Replication	Three replicated inoculated and uninoculated plots at each site; 4 rep is smaller plot sizes
Layout	Randomised block design
Sample timing	Maturity after plot header has cut for yield
Sample number	All plants pulled from two adjacent centre rows over 0.5m at two random locations in each plots (i.e. total of 2 m of row)

Stem browning	Rating method is based on the number of tillers with any basal browning and the extent of browning. Up to 25 random plants per plot (from metred stubble pulls) rated**:** total tillers (a), tillers with browning (b), extent (height of browning 0-3 scale; c) where 0 = no browning, 0.5 = partial browning 0-2 cm, 1 = complete browning 0-2 cm, 1.5 = complete browning 0-2 cm + partial browning 2-4 cm, 2 = complete browning 0-4 cm, 2.5 = complete browning 0-4 cm + partial browning 4-6 cm, 3 = complete browning 0-6 cm Crown rot severity = ((b/a x 100)/3) x **c** Whiteheads are a separate assessment if done, depends on expression in each season. Done on a 0-10 whole plot score where 0 = no whiteheads, 1 = approx.. 10% whiteheads in plot , 2 = approx.. 20% plot whiteheads etc. and 10 = 1005 whiteheads. Scores generally done on two dates at least 1-2 weeks apart if possible to pick up differences in varietal maturity and whitehead expression.
Agronomic data	All plots further assessed for a range of agronomic parameters including: tiller number, grain yield, screenings, protein, 1000 grain weight.
Pathology assessment	The crown and sub-crown internode of the 25 plants rated in each sample are trimmed, surface sterilised and plated on ¼ PDA + novobiocin for the recovery of *Fusarium pseudograminearum* (crown rot) and *Bipolaris sorokiniana* (common root rot). This provides incidence of infection by both pathogens.
Time involved	Sowing and harvesting minimal. Rating, processing, plating are time consuming.
Pro's	Field based assessment of both resistance (basal browning) and tolerance (yield loss). Can attribute effects to crown rot and differentiate between environmental effects. Yield loss is what growers directly see in the paddock.
Con's	Labour intensive, need consistent well trained person to conduct all visual assessments. Disease severity and yield loss varies with environment.

APPENDIX 4

DAFF Crown Rot Seedling Waterbath Resistance Test Protocol 07/14. Neate, SM and Percy, CD

Uses

Assessing fixed lines for resistance.

Advancing breeding material through gene enrichment of segregating populations.

Quantitative genetics experiments to understand control of resistance.

Phenotyping for QTL identification and mapping

The seedling rating methods of the QDPI&F group have been used to provide phenotyping for many breeding and research groups across the country, including-

- Phenotyping for the Qld wheat breeding program and EGA (leading to the release of cultivars with some of the highest levels of crown rot resistance, eg EGA Wylie, Baxter and Lang).
- All crown rot phenotyping for the Northern Node of Barley Breeding Australia.
- Crown rot phenotyping for the NSW DPI's durum breeding program.
- Crown rot phenotyping for the AWCMMP and AWBMMP
- Student honours and PhD theses.
- Phenotyping of segregating material for UQ
- Crown rot phenotyping of NVT retentions and all cultivars appearing in variety guide
- Crown rot phenotying for CIMMYT

Relevant Publications

Wildermuth GB, Purss GS (1971) Further sources of field resistance to crown rot (Gibberella zea) of cereals in Queensland. Aust J Exp Agric Anim Hus 11:455–459

Dodman RL, Wildermuth GB (1987) Inoculation methods for assessing resistance in wheat to crown rot caused by Fusarium gramineum Group 1. Aust J Agr Res 38:473–486

Wildermuth GB, McNamara RB (1994) Testing wheat seedlings for resistance to crown rot caused by Fusarium graminearum Group 1. Plant Disease 78: 949-953.

Lusted, Cassandra D (1998) An examination of the development of crown rot caused by Fusarium Graminearum Schwabe Group I in seedlings of wheat barley and oats. BSc Hons Thesis, University of Southern Queensland.

Percy, CD. Wildermuth, GB. and Sutherland, MW. (2005). Effectiveness of partial resistance sources in hexaploid wheats against Fusarium pseudograminearum isolates: 15th Biennial Australasian Plant Pathology Society Conference, 26-29 Sep 2005, Geelong, Australia.

Malligan, Cassandra D.(2009) Crown rot (Fusarium pseudograminearum) symptom development and pathogen spread in wheat genotypes with varying disease resistance. PhD Thesis University of Southern Queensland.

Neate, S.M., (2010) Crown Rot Disease of Small Grain Cereals. Fourth International Masterclass on Soil Borne Pathogens of Wheat, Eskisehir, Turkey 2010

Kelly, A (2010) Analysis of Adult Plant Phenotyping of Crown Rot Symptoms in Wheat. GRDC Crown Rot Ring Test Report 27pp. (2009 data analysis, available on CAIGE website)

Kelly, A (2011) Analysis of Adult Plant Phenotyping of Crown Rot Symptoms in Wheat. GRDC Crown Rot Ring Test Report 17pp. (2010 data analysis, available on CAIGE website)

Herde, DJ, Percy, CD., Walters, T., Davies, PA, Kelly, A, Fletcher, S and Neate SM. (2012). First International Crown Rot Laboratory and Field Workshop handout notes. Leslie Research Centre, Toowoomba, Australia.

Herde, DJ, Wildermuth GB., and Neate SM (2012) Yield loss due to crown rot in wheat in Australia's northern cropping region. Proceedings of the first International Crown Rot Workshop for Wheat Improvement, Narrabri, New South Wales, Australia

Percy, C., Herde, D., Wildermuth, G, Kelly, A., and Fletcher, S. (2012) Effective and efficient field and controlled environment screening methods to identify resistance to crown rot. Proceedings of the first International Crown Rot Workshop for Wheat Improvement, Narrabri, New South Wales, Australia.

Percy, CD. and Wildermuth, GB. and Sutherland, MW. (2012) Symptom development proceeds at different rates in susceptible and partially resistant cereal seedlings infected with Fusarium pseudograminearum. Australasian Plant Pathology, 41 (6). pp. 621-631. ISSN 0815-3191

The standard glasshouse procedures have evolved since the 1994 paper, with modifications in the ratio of soil in the three pot layers, and a changed weight of inoculum per pot. The category rating system has been replaced by the actual percentage of lesioning for genetics work, however the original system is still used for routine screening.

Soil

- Lantana clay loam topsoil from the Lockyer Valley Queensland. Low-moderate shrinking and swelling, conducive to disease development. Soils influence the ability to discriminate between crown rot seedling resistance levels, so several should be tried
- Steam Pasteurized 70 C for 30-40 minutes
- Divide soil into two. 2/3 unsieved, 1/3crush and sieve to <6mm

Inoculum Production

- Add 0.8kg wheat/0.4kg barley mix to an autoclave bag
- Cover with water and soak overnight
- Drain and autoclave at 20psi for 30 minutes on 3 consecutive days
- Subculture 5 fresh pathogenic isolates of *F. pseudograminearum* on individual Czapek-Dox Agar with 10% sucrose (Thom and Raper 1945) petri plates
- Check viability and purity, morphology and growth rate.
- Inoculate half a culture plate above into 250ml of Czapek-Dox Broth with 10% sucrose and minus agar (Thom and Raper 1945) in 500ml Schott bottles. One isolate per bottle. Shake for 7d at 150rpm at 20C
- Blend with a sterile stab mixer.
- Add 30ml of blended isolate, one isolate per autoclave bag of grain, shake to mix, seal bag with sterile cotton wool/cheesecloth plug. Shake to mix
- Shake every other day for 21 days to break up clumps and encourage even colonisation
- Spread thinly on trays and dry in fume hood at 25C for two weeks, stirring regularly to break up lumps
- Grind and sieve through 2 mm sieve, store at 5C in sealed containers.
- Test a subsample for virulence and pathogenicity under standard conditions described below. All *Fusarium sp* are unstable in storage and can change in virulence and pathogenicity in storage, especially with repeated subculturing, so that new field isolates need to be introduced regularly and all isolates need to be tested for effectiveness with each inoculum batch.

Pot Preparation Planting and Inoculating (Refer to figures 2 and 3.)

- 9cm dia x 10cm high 500ml white plastic pots without drainage holes
- Weigh out 280g of unsieved soil into white pots.
- water pots to field capacity with deionised water, level soil surface
- Add seed to pots, 13 seed per pot
- Press seed slightly into soil surface with finger, allowing seeds to remain steadfast for remaining steps in procedure.
- Add 5mls of deionised water with pipette to each pot, distributing evenly
- Weigh out 160g of sieved soil, add to pots covering seed
- Level sieved soil again
- Sprinkle 0.45 grams of inoculum of combined isolates over surface of sieved soil, making sure to evenly distribute over entire surface area of each pot
- Weigh top layer of sieved soil (40g) and add to pots, level soil, taking extreme care not to disturb inoculum layer
- Place in temperature controlled glasshouse at 25C in a root temperature tank also set at 25C
- Leave unwatered for 7 d, and then water to field capacity with deionised water daily for 14 d

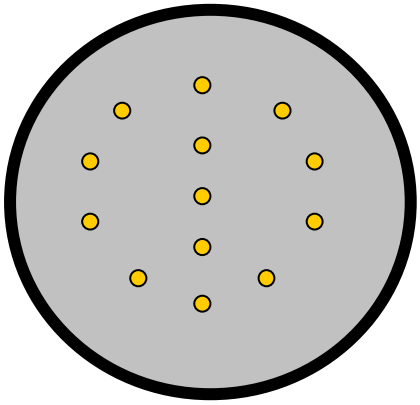

Figure 2. Top view of white pot, showing placement of cereal seed at planting.

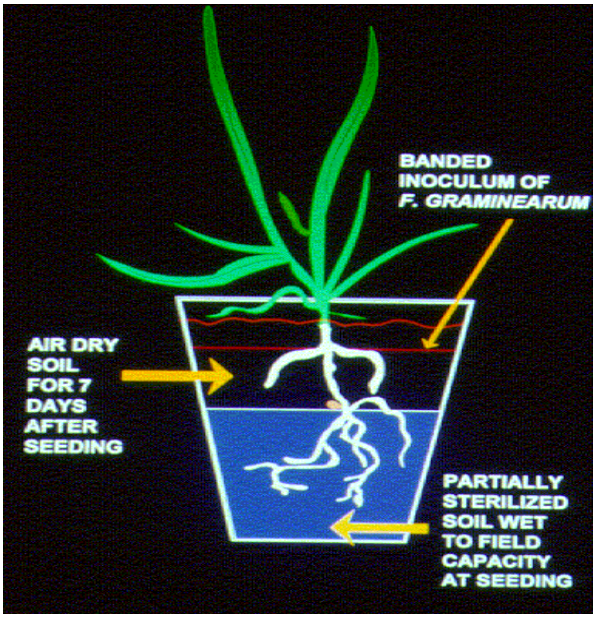

Figure 3. White waterbath pot showing banded layers of soil and inoculum. Image by G. Wildermuth

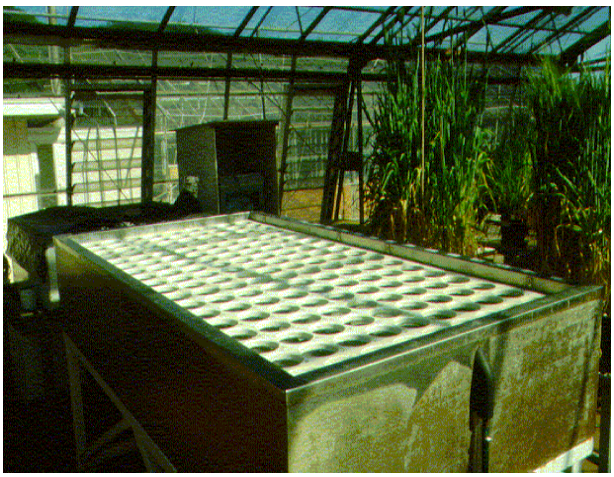

Figure 4. Waterbath with pots in position.

Potential Resistance Standards

Genotype	Resist. level (1-9)	Notes
2-49	7	Gala/Gulyas.
Wylie	6	QT2327/Cook/QT2804
Sunco	5	Cook*3/WW15/4SUN9E-27/3Ag14
Vasco	3	3-AG-14/4* Condor //5*Oxley
Gregory	3	Pelsart/2*Batavia DH
Janz	3	3-AG-3/4*Condor//Cook
Batavia	2	Bronchus 'S'/Banks
Puseas	2	Pusa-4/Three-Seas
Bellaroi	1	920405/920274, could be replaced with any commercial durum

Disease Assessment

After three weeks the seedlings are washed free of soil and the percentage of lesioning on the first three leaf sheaths are visually assessed and summed to give a score out of 300.

Replication and statistical treatment

One to six, depending on the nature of the material. Randomised block; overlayed with row by column designs, Spatial analysis and ANOVA with appropriate comparison of means.

Relationship to other methods

This seedling test is highly significantly related to our adult plant rating methods, as published in Wildermuth and McNamara (1994), ($r=0.78$; $n=28$; $P=<0.01$).

In the 2009 ring test the methods described here had the highest discrimination between genotypes of all the methods tested. Field (0.79) and seedling (0.82). In 2010 which was a very wet year discrimination between genotypes in the field was (0.69).

Advantages

- Well established screening method, in use since the 1990's.
- This seedling method is highly correlated to results from our field screening method
- Very fast (three weeks) and cost efficient compared to field screening.
- Multiple rounds of screening and generations are possible each year
- Selection on an individual plant basis is very successful with this method.
- The method mimics natural infection.
- The method is repeatable over years.

Disadvantages

- Some forms of adult plant resistance are still damaged in this strong test, and appear intermediate.
- There is evidence that some genes are most expressed at either seedling or adult growth stages, so seedling screening should be supplemented with adult plant selection.
- The requirement to control the soil moisture levels leads to daily watering in the final two weeks of the experiment.