

Development of a rapid, accurate glasshouse bioassay for assessing fusarium wilt disease responses in cultivated *Gossypium* species

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A rapid glasshouse-based bioassay method to screen large numbers of cotton plants for responses to *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) was developed. Different Fov inoculum concentrations and methods of inoculation were assessed using resistant and susceptible cotton cultivars. Cotton seeds were planted directly into Fov-inoculated soil. Studies of seed germination, seedling establishment, seedling mortality and fusarium wilt symptoms (i.e. stunting, foliar symptoms and vascular browning) were performed to optimize the bioassay parameters. Growing seedlings in Fov-inoculated soils at 5×10^4 or 1×10^5 CFU g⁻¹ soil, in individual seedling tubes with 12 h at 28–30°C and 12 h at 15–18°C, gave consistent results when assessing Fov disease responses 6 weeks after inoculation. When fusarium wilt resistance ranks (FWRRs) and vascular browning index (VBI) means of 18 Australian and other cotton cultivars from the Fov glasshouse bioassay were compared against their fusarium field performance ranks (F-ranks), assessed on adult plants for cotton cultivar release, Pearson's correlation was highly significant for both comparisons. The level of congruence between field and glasshouse data indicated that this protocol should be an effective tool for large-scale screening for Fov-resistance responses in diverse germplasm and breeding populations and for advancing genetic research to develop molecular markers for Fov resistance in cotton.

Keywords: bioassay, cotton, Fov, fusarium wilt, germplasm screening

Introduction

Fusarium wilt of cotton, caused by *Fusarium oxysporum* f. sp. *vasinfectum* (Fov), has the potential to become the most economically important pathogen of cotton in Australia (Allen *et al.*, 2003). Since being first recorded in 1993, the incidence of fusarium wilt in Australian cotton crops has increased greatly, and the disease has spread rapidly to many of the cotton growing regions in New South Wales and Queensland (Reid *et al.*, 2002). Australian Fov isolates, which are in two distinct vegetative compatibility groups (VCGs) (01111 and 01112), do not appear to be related to any of the Fov races found elsewhere in the world, suggesting that they are of indigenous origin (Davis *et al.*, 1996). More recently, genetic and evolutionary studies have established that Australian Fov isolates are closely related to a lineage of indigenous *F. oxysporum* found in several areas of Australia, including the Darling Downs (Wang *et al.*, 2004).

As fusarium wilt imposes a significant constraint on cotton production in Australia, ways of managing the dis-

ease need to be developed. Evidence suggests that growing cotton cultivars with greater resistance may reduce the rate at which the pathogen increases and spreads in fields, and therefore could be an efficient way of controlling fusarium wilt (Hillocks, 1992). Despite robust improvements in the fusarium wilt resistance of Australian cotton cultivars over the last decade, cotton breeders continue to search for new sources of resistance and better ways of quantifying it. Progress has been hindered by the limited sources of resistance to Fov available within the elite Australian germplasm pool and by the lack of a suitable glasshouse screening technique to accelerate the identification of new resistant germplasm and for the screening of advanced cotton breeding lines.

A rapid and reliable glasshouse bioassay is also needed to advance the understanding of the plant's responses to Fov and resistance mechanisms, and for the identification and study of pathogenicity genes in fungal populations. Such a Fov bioassay must eliminate the chance of infection escape and ensure an accurate expression of host plant resistance or fungal pathogenicity. It must give consistent and reproducible wilting reactions that correspond with known resistance among cotton cultivars and be highly correlated with field observations under natural infection conditions (Bugbee & Sappenfield, 1968).

Various glasshouse screening methods for determining the pathogenicity of isolates of Fov and evaluating the

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resistance of cotton germplasm have been developed previously, but none have been widely adopted. The most commonly used inoculation technique involves cotton plants being grown in sterile soil, lifted and dipped in a suspension of Fov inoculum (Wiles, 1952, 1963; Harrison & Beckman, 1982; Nirenberg *et al.*, 1994). However, depending on the inoculum concentration, the length of time the plant is in contact with the inoculum and the aggressiveness of the Fov isolates used, escapes from infection are likely to occur (Wang *et al.*, 1999). Furthermore, this method is labour-intensive and the severe wounding of the root system that occurs after lifting cotton seedlings from the soil may reduce plant growth or cause early death. Additionally, it does not mimic natural infection processes because entry into the plant may be through broken roots. The inoculation of cotton by introducing spores into multiple stem punctures offers a way of reducing the probability of escapes from Fov infection; however, it only assesses resistant factors present in the stem, not the normal route for infection in crops (Bugbee & Sappenfield, 1968; Mace, 1978; Zhang *et al.*, 1993; Joost *et al.*, 1995). Other methods of inoculation include the addition of Fov agar pellets onto roots 2–3 days post-germination, under sterile conditions (Rodriguez-Galvez & Mendgen, 1995a,b), and the direct application of Fov conidial suspension to the soil (soil drenching), when cotton seedlings are 2 weeks old (Katsantonis *et al.*, 2003).

An optimal glasshouse bioassay for both testing the pathogenicity of a fungus and screening of plants for

resistance is one that produces a disease index that is solely pathogen-induced, thus mimicking the natural process of infection while eliminating non-pathogen-related stresses on the plant. Furthermore, the inoculum concentration needs to be similar and consistent across all plants being tested, and the inoculum used should be at a concentration that will produce consistent symptoms.

This study sought to establish a rapid and reliable Fov bioassay that can be used to accurately screen large numbers of individual native and cultivated cotton plants, whilst being temporally and spatially efficient. To do this, two pilot studies were done to assess three inoculation methods (soil-amended colonized substrate, soil drenching and soil-amended/wheatmeal drenching) with the aim of establishing a standard glasshouse inoculation technique ideal for genetic studies aimed at introgression of Fov resistance from the Australian native and elite cotton germplasm.

Materials and methods

Plant material

Eighteen *Gossypium* genotypes were selected, based on variation in reaction to fusarium wilt of cotton (Table 1). Of the 18 genotypes, 11 were commercial *G. hirsutum* cultivars, including Siokra 1–4, known as the Australian industry standard for Fov susceptibility, Sicot 189, designated the Australian industry standard for Fov resistance,

Table 1 Fusarium wilt resistant ranks (FWRRs) of 18 cotton (*Gossypium*) cultivars inoculated with *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) isolate 24500 in a glasshouse bioassay, plus their field-based fusarium wilt rank value (*F*-rank) information published annually by the Cotton Seed Distributors (CSD) and Deltapine Australia (W. Stiller, personal communication)

Cultivar/ Accession	Species	Origin	F-rank ^a	2006				2007				Total no. ^c
				Trial no. 1		Trial no. 2		Trial no. 3		Trial no. 4		
				NR	FWRR	NR	FWRR	NR	FWRR	NR ^b	FWRR	
Siokra 1–4 ^d	<i>G. hirsutum</i>	CSIRO-PI	7	12	1.1	12	0.5	14	0.7	12	0.8	500
Acc. 8810	<i>G. badense</i>	CSIRO-PI	130	12	2.9			14	6	12	7.3	380
Coker	<i>G. hirsutum</i>	CSIRO-PI								12	2.8	120
CSX434	<i>G. hirsutum</i>	CSIRO-PI	136	12	2.8	8	2.3					200
NM24016	<i>G. barbadense</i>	CSIRO-PI		12	1.8							120
Pima A8	<i>G. barbadense</i>	CSIRO-PI	130	12	3.4							120
Sicala 40	<i>G. hirsutum</i>	CSIRO-PI	80	12	2.6	8	2					200
Sicala 43	<i>G. hirsutum</i>	CSIRO-PI	68	12	2.6	8	2					200
Sicala 43B	<i>G. hirsutum</i>	CSIRO-PI	89	12	2.9	8	2.5					200
Sicala 45	<i>G. hirsutum</i>	CSIRO-PI	122	12	2	8	1.5					200
Sicala V-2	<i>G. hirsutum</i>	CSIRO-PI	81	12	2	8	1.5					200
Sicot 14B	<i>G. hirsutum</i>	CSIRO-PI	119	12	2.4	8	1.9					200
Sicot 189 ^e	<i>G. hirsutum</i>	CSIRO-PI	100	12	1.9	12	1.3	14	3.3	12	4.6	460
Sicot 71	<i>G. hirsutum</i>	CSIRO-PI	104	12	3.7	8	3.4	14	5.4	12	6.6	460
Sicot 80	<i>G. hirsutum</i>	CSIRO-PI	108	12	3	8	2.2					200
Sicot F-1	<i>G. hirsutum</i>	CSIRO-PI	143	12	4	12	3.7	14	6.3	12	6.2	500
TM-1	<i>G. hirsutum</i>	USDA/R. Percy		12	1.9	8	1.2					200
Gos-5511	<i>G. arboreum</i>	CSIRO-PI	189	12	9.2	8	10.0	14	10.0	12	10.0	460

^a*F*-rank calculation (http://www.csd.net.au/images/ranking_protocol_2008.pdf).

^bNumber of replications.

^cTotal number of individuals evaluated across four trials.

^dFov industry standard for susceptibility.

^eFov industry standard for resistance.

and Sicot F-1, the current superior Fov-resistant cultivar (W. Stiller, CSIRO Cotton Research Unit, Myall Vale, NSW, Australia, personal communication). All commercial Australian cotton cultivars, as well as *G. barbadense* and *G. arboreum*, were sown directly into the Fov-treated soil mixture.

Inoculum preparation

Fov wheatmeal inoculum

Wheat seeds (1 kg) were soaked in 3 L water for 18 h, then drained and autoclaved over two consecutive days at 121°C for 22 min in a slow exhaust cycle. The seed was then incubated at 25°C for 2 days and a sample plated on 25% potato dextrose agar (PDA) to screen for the presence of bacteria. Each seed lot was aseptically inoculated with 15 mL conidial suspension (10^7 conidia mL⁻¹) of a highly virulent Australian Fov isolate, VCG 01111 (acc. no. 24500), provided by Bo Wang (CSIRO-PI, Canberra, Australia) cultured on 25% (w/v) potato dextrose broth (PDB; Difco) as described below. The Fov-inoculated wheat seeds were incubated in 5-L flasks at 25°C in the dark for 3 weeks. Flasks were hand-shaken for 30 s daily to ensure complete fungal colonization of the wheat seeds. Inoculated wheat seeds were then air-dried in metal trays for 7–10 days before being coarsely ground in a commercial blender. The resultant Fov wheatmeal inoculum (4×10^7 conidia g⁻¹ substrate) was stored in paper bags at room temperature. The conidia, mycelia and chlamydospores from the wheatmeal inoculum were viable (100%) after 24 h at 25°C and remained viable for 36 months. The control treatment consisted of non-inoculated wheat seeds treated similarly, followed by autoclaving immediately prior to use.

Liquid inoculum

The Fov fungus (acc. no. 24500) was maintained in 25% glycerol at -80°C and recovered in PDB at 25°C for 4–7 days before the preparation of inocula. Approximately 1 mL of the recovered culture was used to inoculate 500 mL PDB in 2-L Erlenmeyer flasks that were incubated at 25°C on an orbital shaker at 100 rpm for 3–7 days. The PDB cultures were filtered through three layers of tissue (Kimwipes®, Kimberly-Clark®) to remove hyphal fragments and conidial concentration was determined using a haemocytometer. Conidial concentration was adjusted using sterile PDB. The inoculum was then held at room temperature (15–25°C) and used within 4 h of preparation. Autoclaved, non-inoculated PDB was used as a control treatment.

Fov colony-forming unit assay

To determine the number of Fov colony forming units (CFU), the plate count method (Koch, 1893) by serial dilution of the Fov inoculum was performed using the pour-plate technique (Miles *et al.*, 1938). A 10-fold dilution series down to 10^{-7} was prepared. One-millilitre aliquots of 10^{-5} , 10^{-6} and 10^{-7} dilution were each dis-

pensed into plates containing 20 mL liquid PDA (Nash & Snyder, 1961) media at 65°C and allowed to solidify. Plates were incubated inverted at room temperature (25°C) for 3–5 days, and colonies counted. Viable cell counts were expressed as CFU g⁻¹ for Fov-infected soil mixtures and dry Fov inoculum and as CFU mL⁻¹ for the liquid Fov inoculum. CFU counts were the number of viable propagules (chlamydospores, mycelia or microconidia) that were loose (liquid inoculum) or embedded within the soil or wheatmeal. For this reason, the data are presented as total CFU g⁻¹ medium rather than as chlamydospores, mycelia or microconidia g⁻¹.

Inoculation methods

Three inoculation methods were evaluated for their effect on fusarium wilt disease development. The methods were: (A) soil amended with Fov wheatmeal inoculum, (B) soil drenched with Fov liquid inoculum, and (C) soil drenched with Fov liquid inoculum and soil amended with sterile wheatmeal. All soil treatments used 50-mm-square forestry tubes (Garden City Plastics) containing approximately 210 mL soil mix composed of 60% compost and 40% perlite. For the soil amended with Fov wheatmeal treatments (method A), the Fov wheatmeal inoculum was incorporated into the soil mix using a cement mixer for 10 min at rates of 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 and 5×10^6 CFU g⁻¹, with the most dilute mixes prepared first, before distribution to the tubes. A wheatmeal-amended control treatment was included. For the soil drenching treatments (method B), each tube was drenched with 10 mL Fov conidial suspension at rates of 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 and 5×10^6 CFU mL⁻¹ prior to sowing seeds. Sterile PDB (10 mL) was used as a control for the non-inoculated soil mix. For the soil drenching amended with sterile wheatmeal treatments (method C), 6 g sterile wheatmeal was incorporated into 40 L soil mix for 10 min using a cement mixer. Each tube was then drenched with 10 mL Fov conidial suspension at rates of 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 and 5×10^6 CFU mL⁻¹ before the seeds were planted. PDB (10 mL) was added to the non-inoculated soil mix.

Plants were grown in a quarantine containment glasshouse and watered twice daily for 6 weeks, with 12 h at 28–30°C and 12 h at 15–18°C, as recommended by Wang *et al.* (1999). All liquid and solid waste was collected and sterilized to prevent spread of the pathogen to other glasshouses.

Experimental designs

Experiment 1

This experiment was designed to compare the three inoculation methods (A, B and C) at five Fov inoculum concentrations on three *G. hirsutum* cultivars (Sicot 189, Sicot F-1 and Siokra 1–4). The experiment was done to examine whether the method of inoculation could influence Fov disease development. The soil mixes were placed

in square forestry tubes and lightly watered for 7 days to allow Fov propagules to germinate and infest the soil mix. Immediately after this, 10 seeds of each *G. hirsutum* cultivar were planted individually. Plants were grouped in 40-cell traycycle racks holding the 10 tubes for each 'experimental unit'. The controls consisted of soil mixes with sterile wheatmeal or PDB in place of the Fov inoculum. The experiment was laid out in a row-by-column design with nine rows and 15 columns, arranged on benches in a biosafe facility at CSIRO-PI in Canberra (35°16'21.54"S; 149°6'52.86"E), with 12 h at 28–30°C and 12 h at 15–18°C (Wang *et al.*, 1999), and replicated three times. This design was generated in two steps using the spatial design search program DiGGER version 4.01a (Coombes, 2002). The first step imposed a blocking scheme of three rows by 15 columns to separate replicates. The second step was to restrict treatment interchanges to these blocks and impose a positive correlation structure on the trial between neighbouring experimental units in rows and columns, allowing for random row and column effects. Thus, the 45 experimental units in each replication consisted of all possible combinations of the three factors 'line' (Sicot F-1, Sicot 189 and Siokra 1–4), 'inoculum concentration' (control, 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6) and 'inoculation method' (A, B and C). Germination, seedling establishment, height, growth rate and vascular browning index (VBI) data were collected as described below.

Seedling germination was monitored daily from the time of sowing and germination rates calculated from the proportion of sown seeds germinated by 7 days. After the seedlings were established (showing fully expanded cotyledon leaves), heights were measured weekly. Establishment rates were calculated as the proportion of germinated seedlings reaching the expanded cotyledon stage. After 7 weeks, the stems of inoculated seedlings that were still alive were cut and rated for disease symptoms according to a VBI, based on the scale: 0 = no vascular discoloration; 1 = discoloration restricted to base of stem only; 2 = discoloration of the 'internode 0' (hypocotyl) region of the stem below the cotyledons; 3 = discoloration of stem above the cotyledons; 4 = complete vascular discoloration of stem; and 5 = plant dead (McFadden *et al.*, 2004). Plant mortality was calculated as the proportion of the total number of established seedlings that was dead.

Experiment 2

This experiment was designed to compare the glasshouse bioassay with the known field performance in fusarium-infested sites (Table 1) of 13 commercial cotton cultivars, four non-commercial lines and one diploid wild accession. The experiment was performed four times between the summers of 2006 and 2007 with Fov-amended soil containing 5×10^4 CFU g⁻¹ soil mix. Experimental units were laid out in a complete randomized design in a row-by-column matrix with $X_{(1-12)}$ rows and $Y_{(1-30)}$ columns, arranged on benches and replicated 8, 12 or 14 times (Table 1). These experimental units consisted of all possi-

ble combinations of the two factors, cultivar and replication. This design was generated in two steps using DiGGER version 4.01a. The first step imposed a blocking scheme of $X_{[(4, 8 \text{ or } 12)]}$ rows by $Y_{[(1-15)]}$ columns to separate replicates. The second step was to restrict treatment interchanges to these blocks and impose a positive correlation structure on the trial between neighbouring 'subjects' in rows and columns, allowing for random row and column effects. VBI values were determined as in experiment 1 with the following modifications: 6 = plant dead in week 5; 7 = plant dead in week 4; 8 = plant dead in week 3; 9 = plant dead in week 2; and 10 plant dead in week 1.

Glasshouse fusarium wilt scores

Fusarium wilt scores were expressed as fusarium wilt resistant rank (FWRR), which represented an index of disease severity determined from the number of plants grouped in VBI scores of 0 or 1 = A, 2 or 3 = B, 4 or 5 = C, and 6–10 = D and estimated using the following equation:

$$\text{FWRR} = 10\left(\frac{A}{N}\right) + 5\left(\frac{B}{N}\right) + 2.5\left(\frac{C}{N}\right) + 0.5\left(\frac{D}{N}\right)$$

where N = total number of plants scored per cotton line. An immune line would have a score of 10 and a completely susceptible line a score of 0.5. Based on this new scoring system for rating the severity of fusarium wilt, cotton cultivars were classified as follow: highly resistant if the FWRR values ranged from 10 to 5, moderately resistant if these values ranged from 4.9 to 2.5 and highly susceptible if FWRR ranged from 2.49 to 0.5.

Statistical analysis

Analysis of variance (ANOVA) was performed for germination, plant establishment, plant mortality, average plant height, foliar and general appearance (FGA) and VBI data, using PROC GLM (SAS Institute). To satisfy the assumption of homogeneity of variance required by ANOVA analysis, germination and plant establishment data were log-transformed before statistical analysis, using the equation $y = \log(N - X/N)$; where N is the number of seeds sown and X is the number of seeds germinated (zeros were replaced by adding 0.5 to all values prior to transformation). For all other data, log transformation was performed using the equation $y = \ln(X)$.

For all experiments, where the pattern of missing values was skewed to some treatment combinations and would have led to biased results from the ANOVA analysis, the log-transformed data were analysed using the residual maximum likelihood (REML) routine (Patterson & Thompson, 1971). The GENSTAT REML (Payne *et al.*, 2006) and PROC MIXED (SAS Institute) options were used to estimate mean values and standard errors of the differences (SED) for parameters. The Wald statistic (W) was calculated along with the relevant degrees of freedom and the probability value (compared to a χ^2 distribution) for the

fixed effects. Effects with P values of <0.05 were considered statistically significant.

The association between field and glasshouse bioassay rankings was examined using Pearson's correlation coefficient (Steel & Torrie, 1980), calculated using bivariate linear correlation analysis (SPSS v.11.0, SPSS Inc.). Similarly, associations between Fov disease response traits, VBI versus FGA, were examined using Pearson's correlation and linear regression, calculated using bivariate linear correlation and simple regression analysis (SPSS v.11.0, SPSS Inc.).

Sample size and statistical power

Performing statistical power analysis and sample size estimation is an important aspect of experimental design. These types of analysis can either be done before (a priori) or after (post hoc) data are collected (Cohen, 1988). To test the post hoc power of the analysis of variance for fusarium wilt disease responses (i.e. plant height, FGA and VBI) in experiments 1 and 2, G*POWER 3 (Faul *et al.*, 2007) was used.

Results

Fov bioassay assessments

Experiment 1

A pilot study was done to compare the effect of different Fov inoculum rates in (A) soil amended with Fov wheatmeal inoculum, (B) soil drenched with Fov liquid inoculum, and (C) soil drenched with Fov liquid inoculum and soil amended with sterile wheatmeal, on symptom development in cultivated *Gossypium* germplasm.

Seed germination and seedling establishment. The estimated variance component for row and column for the

proportion 'seed germination' and 'seedling establishment' variates was zero, so a standard analysis using ANOVA was appropriate. By comparing the P -values to the 5% level of significance, in each case, there was a significant effect of line, but no other terms were significant. This suggests that the detectable differences between groups of seed emergence and establishment counts occurred between different lines, but in none of the other groupings. Thus, this analysis indicated that effects of inoculation method (Fov spore application) or inoculation concentration (the amount of initial conidia) did not affect the germination of the cotton seed.

Average plant height and growth rate. Average plant height at week 6 and average plant growth rate (the average of the difference in height between weeks 2 and 6) was measured and the data transformed before analysis by REML. The main effects for line (Sicot F1, Sicot 189 and Siokra 1–4), inoculation method (Ino_method_A, _B, _C) and inoculum concentration (Ino) were clearly highly significant ($P < 0.001$). The interaction between Ino and Ino_method was highly significant ($P < 0.001$) for both variates. The Wald statistic showed that the highly significant interaction between Ino and Ino_method was driven by Ino_method_B, as when the latter was removed from the analysis this interaction was not significant. The distribution of the means and their confidence intervals ($P \leq 0.05$) for both growth rate and height are shown in Figures 1a,b, respectively.

Both method_A and method_C were very effective in infecting cotton seedlings, causing slow growth rate and severe plant stunting in all three cultivars, with little difference across the range of inoculum concentrations tested. Inoculation method_B, on the other hand, resulted in less stunting and was more discriminating at the higher inoculum levels (Fig. 1a,b).

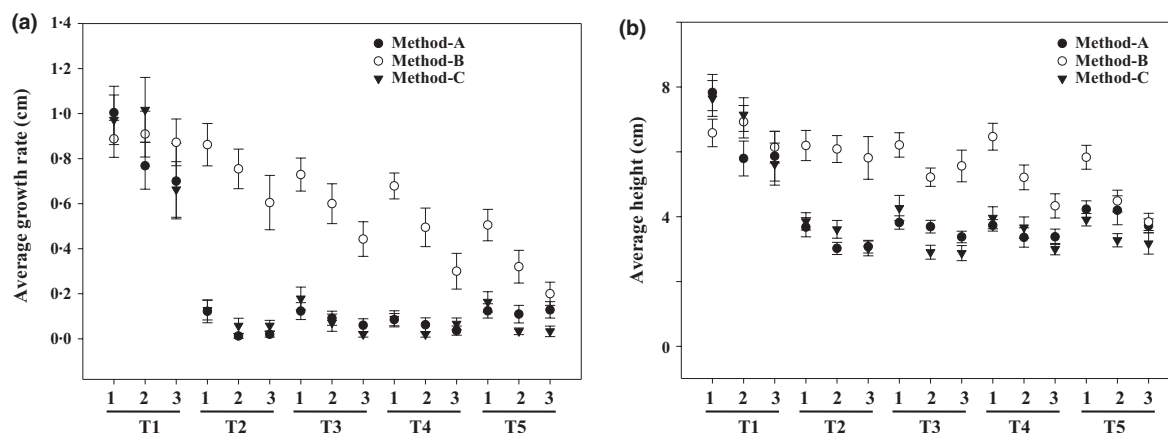


Figure 1 The effect of four *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) inoculum concentrations and three inoculation methods on average growth rate (a) and average height (b) of cotton plants. Both measurements were taken at weekly intervals (for 4 weeks) for three *G. hirsutum* cultivars: 1-Sicot F-1 (Fov resistant), 2-Sicot 189 (Fov resistant) and 3-Siokra 1–4 (Fov susceptible). T1 = control (no Fov), T2 = 5×10^4 CFU g⁻¹, T3 = 1×10^5 CFU g⁻¹, T4 = 5×10^5 CFU g⁻¹, T5 = 1×10^6 CFU g⁻¹. Method A (●): soil amended with Fov wheatmeal; method B (○): soil drenched; and method C (▼): soil drenched and amended with sterile wheatmeal treatments.

Vascular browning index (VBI). Vascular browning was measured at the end of the sixth week, after the last height measurements had been taken. An ANOVA for the VBI data showed that all main effects (i.e. line, inoculation method and inoculum concentration) were highly significant ($P < 0.001$). The ANOVA test for replicate effect showed no significant differences among replications. The means for the line effect were 2.61 for Sicot F-1 (resistant), 3.51 for Sicot 189 and 4.13 for Siokra 1–4, with a SED of 0.24. It is clear from these data that Sicot F-1 had a lower VBI score than Sicot 189, which in turn was lower than Siokra 1–4, consistent with known Fov disease responses of these cultivars under field conditions. The means for the Ino_method effect were 4.13 for method_A, 2.05 for method_B and 4.08 for method_C, with a SED of 0.24. This reflects the differences between inoculation methods applied; method_A and method_C produced comparable, high means, which translated into high Fov disease severity. Comparison with method_B suggested that the presence of the wheatmeal increased the disease severity in both treatments. It is also worth noting that Fov inoculum concentration will only have a significant effect on VBI symptoms if it exceeds 5×10^5 CFU g⁻¹ soil ($P < 0.001$).

Experiment 2

A standard cultivar resistance ranking scheme has been developed by the Australian Industry that indicates the resistance performance of cultivars relative to a nominated resistant benchmark cultivar, Sicot 189. This fusarium wilt rank value (*F*-rank), based on extensive field trials, is described in cultivar information published annually by Australia's two cotton seed companies, Cotton Seed Distributors (CSD) and Deltapine Australia. The *F*-rank of a cultivar is the proportion of surviving plants rating VBI scores of 0 and 1 relative to Sicot 189 set at 100. *F*-ranks over 100 indicate a high level of resistance to Fov. *F*-ranks similar to or below Siokra 1–4 (7) indicate high fusarium wilt susceptibility. Fusarium wilt rankings for 15 of the 18 cotton lines tested were available (Table 1; W. Stiller, CSIRO Cotton Research Unit, Myall Vale, NSW, Australia, personal communication), and were used to compare the performance of the cultivars in the bioassay.

In choosing the optimum assay protocol to assess the fusarium wilt reactions of these cultivars, the significant variates and their interactions, determined in experiment 1, were taken into account. The presence of Fov in the soil had a significant effect on Fov symptoms between the resistant and susceptible types. The bioassay reproduced comparable levels of both stunting and VBI in both experiments at Fov inoculum concentrations ranging from 5×10^4 to 1×10^5 CFU g⁻¹.

Sample size and statistical power

To test the post hoc power of the analysis (the probability that H_0 will be rejected given that in fact it is false) of variance for fusarium wilt disease responses (i.e. plant height,

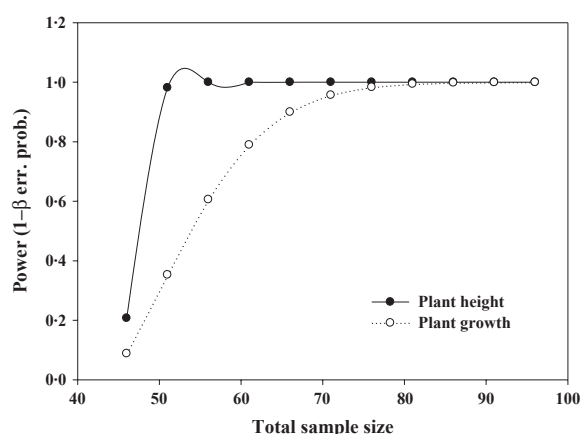


Figure 2 A graph of power analysis ($1-\beta$ error probability) as a function of effect size needed to reject the null hypothesis (H_0 : states that $\mu_{Fov\ res}^a - \mu_{Fov\ sus}^b = 0$ in cotton elite varieties under *Fusarium oxysporum* f.sp. *vasinfectum*, Fov, disease pressure) when H_1 is true. This statistical power plot was calculated as a function of the total sample size on experiment 1, for both mean plant height and mean plant growth. (a) mean Fov resistant and (b) mean Fov susceptible).

plant growth rate, FGA and VBI) in experiment 1, G*POWER 3 was used. With acceptable effect size = 0.51, alpha = 0.05, size sample = 135, group(s) = 45 (three inoculation methods $\times$ five inoculum concentrations $\times$ three lines), repetitions = 3, correlation between repeated measures (Pearson's r) = 0.89 for plant height and 0.51 for plant growth rate and non-sphericity correction = 1, power was calculated as 1.00 for plant height and 0.99 for plant growth rate. To assess the sensitivity of the experimental design, the statistical power ($1-\beta$) was plotted against sample size. The plot indicated approximately 70–90 experimental groups (Fig. 2). An experimental group in experiment 1 was the proportion of experimental material (three lines) to which a treatment (control and four Fov concentrations) using different inoculation methods (three methods) was applied. Hence, for each experimental group two replications would have been sufficient to reject a false null hypothesis (H_0).

Optimized Fov bioassay protocol

The protocol for a rapid and reliable high-throughput Fov bioassay, based on the parameters optimized in this study and those of Wang *et al.* (1999), was as follows. Approximately, 20 L steam-sterilized potting mix is inoculated by placing 9 g wheatmeal inoculum of a highly aggressive Fov isolate (24500) containing 4×10^7 CFU g⁻¹ (final concentration: between 5×10^4 and 1×10^5 CFU g⁻¹ soil mix) of Fov. The soil mix is placed in 55-mm-square forestry tubes (210 mL) and lightly watered to allow Fov propagules to germinate and infest the soil mixes overnight. Immediately after this, 10 seeds of each cotton cultivar or accession are sown in 10

individual tubes. The experiment is then laid in a row-by-column design with x (1–12) rows and y columns (1–30), arranged on benches and replicated z times (at least twice, although three or four times would be recommended) in a glasshouse at day/night temperatures of 28–30/15–18°C (Wang *et al.*, 1999). The experimental units are maintained in the glasshouse for 42 days before assessing fusarium wilt resistance by determining VBI levels. After 42 days all susceptible genotypes show fusarium wilt symptoms.

Comparison of the glasshouse-based Fov bioassay with field performance of cotton cultivars or accessions

The optimized Fov bioassay was used to screen 18 cotton lines (4920 individuals) among four trials between summer 2006 and summer 2007 in Canberra, Australia (Table 1). As in previous experiments, these bioassays produced highly reproducible results for all the replications, so only the cultivar effect was significant (data not shown). Siokra 1–4 was the most highly susceptible cultivar. Sicot F-1, Sicot 71 and acc. 8810 were moderately resistant to highly resistant, while the *G. arboreum* material was the most highly resistant cotton line. This correlated well with the fusarium wilt resistance ranking of the cotton cultivars (F -rank) for which field data were available.

A Pearson correlation was calculated to examine the relationship between glasshouse mean VBI and FWRR, and field F -ranks for the two trials conducted in 2006. There was a strong negative correlation between mean VBI and FWRR ($r = -0.743$; $P = 0.02$) indicating that the conversion formula for transforming VBI into FWRR produce an accurate representation of the Fov disease response levels in cotton. Consequently, Pearson's coefficient was calculated for determining the correlation between FWRR and field F -ranks in all four trials. The results indicated that there was a strong, positive, statistically significant correlation between FWRR and field F -ranks in all four trials ($r_{\text{trial 1}} = 0.73$, $r_{\text{trial 2}} = 0.74$, $r_{\text{trial 3}} = 0.96$ and $r_{\text{trial 4}} = 0.98$; $P = 0.01$). To estimate the reliability of the FWRR values among the four Fov trials (i.e. reproducibility of measurements), an interclass correlation coefficient (ICC) was calculated. The ICC was 0.97, indicating that 97% of the variance displayed by the FWRR values obtained in 2006 was associated with the variance displayed by the FWRR values obtained in 2007. These results confirm that the glasshouse Fov bioassay performed on young cotton plants (6 weeks old) is a good predictor of fusarium wilt resistance in adult plants under field conditions.

Discussion

An accurate, easy-to-use, reliable and robust glasshouse-based Fov bioassay for assessing the resistance of cultivated and non-cultivated *Gossypium* species was developed. The highly significant Pearson correlations

between the glasshouse (FWRR) and field disease scores (F -ranks) of 18 cotton cultivars indicate a strong link between young seedling (6-week-old) and adult cotton plant resistance, making this glasshouse-based Fov bioassay ideal for the screening and selection of fusarium-wilt-resistant cotton germplasm breeding lines. The single 55-mm-square forestry tubes combined with the bulk Fov soil inoculation using dry Fov wheatmeal inoculum at 5×10^4 to 1×10^5 CFU g⁻¹ soil, 1 day prior to sowing, was required to produce severe fusarium wilt symptoms in almost all the genotypes of the susceptible cotton cultivar Siokra 1–4. The Fov bioassay described in this study is an accurate, fast and cost-effective tool that can be used for large-scale cotton germplasm screening in temperature-controlled glasshouses, where space is limited. The statistical power analysis of sample size showed that approximately two replications (10 individuals per replication) are sufficient to obtain a high statistical power (1.00) in the analysis of variance of fusarium wilt symptoms. These results suggest that high accuracy and reliability of the Fov bioassay in producing reproducible fusarium wilt disease data are possible. Furthermore, this bioassay allowed the Fov resistance screening capacity of the glasshouse to be increased fivefold (from 720 to 3600 genotypes) relative to previous assays (McFadden *et al.*, 2004).

Currently, the most commonly used method of inoculation is root dipping (DeVay *et al.*, 1997; Wang *et al.*, 1999, 2004; McFadden *et al.*, 2004). Although, this method has proved to be an accurate and reliable means of characterizing Fov pathogenicity and, to some extent, screening cotton germplasm for resistance, its use in large-scale germplasm screening is limited. This limitation may, in part, be attributed to the fact that the root-dipping technique is sensitive to Fov inoculum potential and concentration, the length of time the plant is in contact with the inoculum, and the aggressiveness of the Fov isolates used (DeVay *et al.*, 1997; McFadden *et al.*, 2004; Becerra Lopez-Lavalle *et al.*, 2007). Furthermore, the root-dipping method is labour-intensive, time-consuming and very costly for application in large-scale germplasm screening under controlled environment conditions.

Studies conducted using this root-dipping technique have shown that the concentration of the conidial suspensions used, the inoculation time and the age of the plant when inoculated can all alter disease severity (Davis *et al.*, 1996; Wang *et al.*, 1999). These authors found that as both conidial concentration and inoculation time increased (from 1×10^5 to 1×10^8 conidia mL⁻¹ and from 1 to 25 min, respectively), so did the Fov disease index. Furthermore, Wang *et al.* (1999) found that cotton seedlings inoculated at 1 week of age always showed a higher degree of disease susceptibility than at other inoculation times, regardless of the cultivar (Wang *et al.*, 1999). These results and those of Bugbee & Sappenfield (1968) demonstrate the importance of inoculum potential of a pathogen overcoming host resistance and causing infection.

The discrepancies between the minimum and maximum concentrations of inoculum needed for disease symptoms to appear, when using the dry Fov wheatmeal substrate (this study) or by root dipping, may be caused by both the age of the root system and the length of time the cotton plants are exposed to the Fov inoculum. Cotton seedlings grown in potting mix inoculated with dry Fov wheatmeal substrate are uniformly and continuously exposed to Fov propagules distributed throughout the soil matrix. Under these conditions, the probability of cotton plants escaping inoculation by the Fov pathogen is very low. This is a plausible hypothesis, particularly because, under soil inoculation conditions, the growth and sporulation of Fov will be primarily conditioned by its competitive saprophytic ability. This is particularly important with root-infecting fungi such as Fov, which have to overcome host resistance and also compete for nutrients with other soilborne saprophytes. In the present study, the soil Fov CFU counts in experiment 1 showed that the addition of wheatmeal to the soil mixture prior to drenching with Fov liquid medium not only produced similar levels of fungal growth when compared with the dry Fov wheatmeal substrate (data not shown), but the level of Fov incidence for both treatments was also highly comparable. Furthermore, once nutrients and other resources are exhausted in the soil matrix the pathogen's conidial load falls to levels similar to those found in natural soils (approx. 5×10^3 CFU g⁻¹ soil).

In contrast, cotton plants inoculated by root dipping are only exposed to the Fov inoculum for a few minutes (1–25 min), reducing the chance of an even Fov load intake and thus Fov inoculum potential. This, in part, may explain why a higher minimum inoculum concentration (1×10^6 conidia mL⁻¹) was required to induce disease symptoms using the root-dipping method (Wang *et al.*, 1999). The resulting Fov disease pressure on the cotton plants when using this method is therefore undetermined, as there is no way of verifying the amount of conidia a plant has had the opportunity to take up. Thus, the resulting disease pressure may also vary from plant to plant within a cultivar. This implies that the severity of disease symptoms displayed by a plant may, in equal measure, be the result of the plant's natural level of susceptibility to infection, or the dose of conidia taken up by the plant. In this regard, it is worth mentioning that the conidial concentrations used for the root-dipping method are capable of overwhelming even the most resistant plant in natural conditions.

Another constraint of the root-dipping technique is the age at which a plant can be inoculated. Wang *et al.* (1999) indicated that it is important to inoculate plants at their most susceptible stage, *viz.* 1 week of age. However, because of the unavoidable mechanical damage that the roots incur as a result of this technique, it was found that 2-week-old seedlings were the most suitable plants for inoculation. These plants have relatively high and stable susceptibility, and a well-developed root system which allows them to better withstand the shock of being removed from the soil, inoculated and then replanted.

Moreover, they are easier to handle. The constraints of this technique do not easily allow seedlings to be inoculated at the optimal age. In the present study, the germination of seeds and the establishment of cotton seedling on infected soil was not affected by the method of inoculation and allowed the screening of plant material at the optimal resistant/susceptible stage.

Thus, the Fov-colonized-wheatmeal inoculation method allowed accurate screening of a large number of accessions and proved to be temporally and spatially efficient. It also permits the comparison of resistance in native and cultivated cottons. This method appears to be ideal for producing uniquely pathogen-induced responses and is logistically compatible with large-scale trials, and also avoids many of the problems associated with other inoculation methods, some of which include deliberate or unavoidable mechanical damage, the possibility of plants escaping infection during inoculation and certain other factors that can alter the plant's response to the disease.

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