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Different genes can be responsible for crown rot resistance at different developmental stages of wheat and barley

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Abstract Crown rot, caused by several *Fusarium* species, is one of the most damaging diseases in wheat and barley. Growing resistant varieties has long been recognised as an integral part in effectively managing the disease. One of the factors hindering the progress of breeding for crown rot resistance is the lack of a reliable and high throughput bioassay that allows rapid and accurate assessment of large numbers of genotypes so that highly susceptible materials can be quickly rejected and potentially resistant lines identified for more focused further assessments. We developed a method which, by growing several inoculated seedlings wrapped in a single piece of moist paper towel, offers significant advantages over all of the existing methods. The new soil-less assay takes only about two weeks, requires very little space, and removes variability associated with the use of soil/potting mixes. Results from the new assay are highly reproducible and agree well with known field performances of different varieties. However, mapping studies conducted using the new soil-less assay did not detect all of the quantitative trait loci found with a soil-based assay. These results show that, although different resistance genes may all contribute to the performance of a variety, caution should be used when comparing results from different assays.

Keywords *Fusarium* diseases · Germplasm screening · QTL mapping · Inoculation methods

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Introduction

Crown rot (CR), which can be caused by many of the *Fusarium* species that also cause *Fusarium* head blight (Smiley and Patterso 1996), is a severe and chronic disease of cereals in Australia (Akinsanmi et al. 2004). Several farming practices are used in managing the disease. These include crop rotation and stubble burning to reduce inoculum load (Kirkegaard et al. 2004). However, these practices have failed to stop the spread of the disease. In fact, the CR problem has worsened dramatically in recent years (Smiley et al. 2005; Chakraborty et al. 2006; Hogg et al. 2010), most likely due to the growing trend towards conservation farming practices involving stubble retention, as CR pathogens are carried over in residues (Burgess 2005). A recent study by

Daniel and Simpfendorfer (2008) found that CR average yield losses of 25% in bread wheat, 58% in durum wheat and 20% in barley over a range of soil types. The disease occurs in many other parts of the world including North America, Argentina, Italy, South Africa, Turkey, West and North Africa (reviewed by Chakraborty et al. 2006; Hogg et al. 2010). Outside of Australia, significant yield loss from the disease occurs in the Pacific North West of USA. It was found that CR could reduce winter wheat yield by up to 35% in commercial fields and reduce barley yield by an average of 13% (Smiley et al. 2005). In addition to yield loss, CR infected plants in glasshouse assays have been shown to contain mycotoxins (Mudge et al. 2006) which may have adverse effect if present in food and feed products.

Growing resistant varieties has long been recognised as the most effective way to reduce CR damage (Purss 1966). However, varieties with high levels of resistance are not yet available. One of the difficulties in successful CR resistance breeding is the lack of a bioassay that not only provides consistent and accurate results but also allows rapid screening of large number of genotypes that are routinely involved in a breeding program. The results of significant efforts in the development of such assays are that several methods are now available. The existing methods can be classified as either field-based or glasshouse-based assays. The field based assays are routinely used for assessing reaction of mature plants (Dodman and Wildermuth 1987; Wildermuth et al. 2001), and they suffer from variation in the resident pathogen population, soil and other environmental and cultural conditions. As a result, data from field-based assays are often variable and difficult to repeat and no report is available ascribing significant QTL from field-based CR assays. The glasshouse-based assays are preferred for assessing seedling resistance. Clearly, seedling resistance may or may not reflect resistance of adult plants, although CR reactions obtained from several of the seedling-based assays show strong correlation with field rankings (Klein et al. 1985; Wildermuth and McNamara 1994; Mitter et al. 2006; Li et al. 2008). Nevertheless, seedling resistance forms an essential part in minimizing CR damage. Results from these glasshouse based assays tend to be more reproducible as factors affecting disease development are easier to control.

However, it is still not practical to use any of the existing CR assays in breeding programs. None of them can be effectively used to assess tens of thousands of lines routinely involved in a breeding program. These assays, including the seedling-based ones, all require growing seedlings or plants in soil/potting mixes for several weeks. As the containers may vary in size and shape, it is very difficult to get the same amounts/compactness of soil or potting mixes in different containers used. This leads to the different water-holding capacities of the containers. As water stress affects CR development (Li et al. 2008), the different water-holding capacities of different containers introduce additional variation which in turn further increase variability of assay results. To increase the reproducibility of these assays, multiple replications each with multiple seedlings are often used for each genotype. Further, about 45 days are usually required from seed germination to disease assessment in some of the assay systems (Mitter et al. 2006; Li et al. 2008).Thus screening large numbers of breeding lines is not only time consuming but also needs substantial glasshouse space.

By growing inoculated seedlings in a mixture of expanded clay® (Seramis, Masterfoods, Nordhofen, Germany) and Oil Dri® (Damolin; Mettmann, Germany) in a growth chamber, Babaeizad et al. (2009) successfully assessed barley seedling resistance to *Fusarium graminearum* infection 10 days after inoculation. Although the method still requires growing seedlings in containers, it offers the potential of dramatically enhancing the capacity of CR assay due to the dramatically reduced time frame of disease assessment. In pursuing a method with similar efficiency, we report here that different genes can be detected at different developmental stages or under different environments in wheat.

Materials and methods

Two *Fusarium* isolates, *F. pseudograminearum* (CS3096) and *F. graminearum* (CS3005), were used in this study. Both isolates were obtained from a CSIRO collection and they are highly aggressive according to a screening of over 650 isolates collected in field surveys from Queensland and NSW (Akinsanmi et al. 2004).

Inoculums were prepared following the method as described by Li et al. (2008). Tween 20 was added to the spore suspension to a final concentration of 0.1% v/v prior to inoculation. Seeds used for CR assessment

were selected by removing small and shrivelled kernels. The average weight of the remaining seeds was then calculated from two samples each containing 100 seeds. The selected seeds were germinated in Petri dishes on three layers of filter paper saturated with water. Newly germinated seedlings were immersed in the spore suspension for about one minute and 10 inoculated seedlings were then wrapped and grown in a single piece of moist paper towel (23.5×12.0 cm, Fig. 1). Experiments reported in this paper were all conducted in a laboratory with constant temperature (23°C) and relative humidity (65%) and with no control on either lighting duration or intensity. Seedlings were kept well watered for the first 48 hours after inoculation and then watered only when the seedlings started to wilt by dipping the paper rolls into a water bath for 30 seconds. CR severity was assessed when 'EGA Bellaroi' or 'Kamilaroi', the two most susceptible genotypes used, become severely necrotic. Depending on CR susceptibility of the genotypes used in an experiment, this takes between 7 to 14 days. CR severity was scored using a '0–5' scale as described by Li et al. (2008), where 0 representing no symptom and 5 when whole seedling completely necrotic.

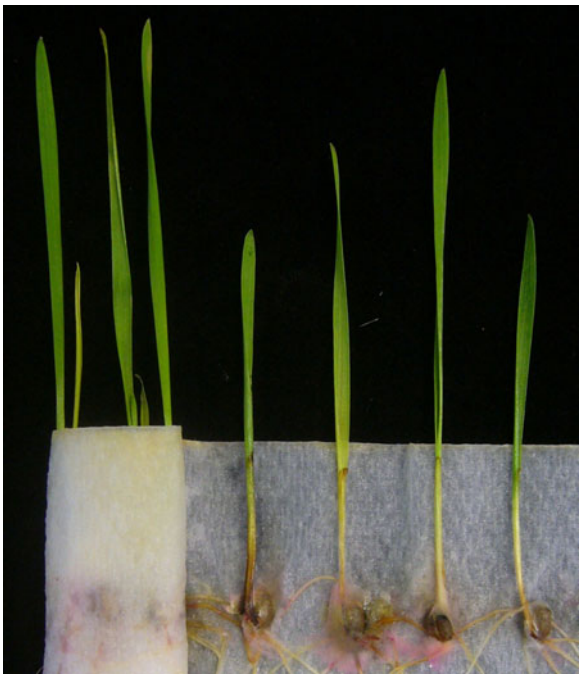

Fig. 1 Soil-less crown rot assay conducted in paper rolls. By wrapping several seedlings in a single piece of paper towel, the new assay provides unprecedented efficiency. The photo was taken five days after inoculation

Three genotypes, including '2–49' (a partially resistant bread wheat), 'Kennedy' (a susceptible bread wheat) and 'EGA Bellaroi' (a highly susceptible durum wheat), were used in a series of preliminary experiments to determine possible influences of *Fusarium* isolate (CS3005 and CS3096), concentrations of inoculum (1×10^5, 5×10^5, and 1×10^6 spores/ml, respectively), seedling stage (coleoptiles reached about 0.2 cm, 0.5 cm and 1.5 cm, respectively) and vigour. The experiments on seedling vigour were conducted by dividing a batch of seedlings into three groups. The group germinated first was selected to represent strong vigour and the group germinated last was used to represent weak vigour. The group of seedlings germinated in between was discarded. Both groups were inoculated when the majority of the coleoptiles reached about 0.5 cm.

A correlation between CR severities obtained by the new soil-less assay and field rankings was assessed using 18 genotypes (Table 1) with known field rankings (www.dpi.qld.gov.au/fieldcrops). Three independent trials each consisting of three replicates were conducted. The repeatability of the new assay was then assessed using 60 genotypes (not listed) in two independent trials, each consisting of 3 replicates. Data were subjected to analysis of variance and means were compared using Duncan's multiple range test at the $P=0.05$ level of significance.

The new soil-less assay was then exploited for mapping QTL conferring CR resistance using one barley DH population and one bread wheat population. The barley population consists of 92 doubled haploid (DH) lines generated from a cross between 'Franklin' and 'TX9425'. A linkage map was available, and a single QTL for CR resistance in a soil-based assay was known on chromosome 3H (Li et al. 2009). The barley population was assessed once with the use of two replicates with 10 seedlings in each replicate. The wheat population consists of 92 RILs (recombinant inbred lines) generated from a cross between 'Lang' and 'CSCR6'. A linkage map was also constructed for this population and two QTL for CR resistance, on chromosomes 3B and 4B respectively, were detected with a soil-based assay (Ma et al. 2010). The wheat mapping population was assessed three times, each with two replicates. Ten seedlings were used in each replicate. The average rating of the 10 seedlings in each replicate was used to represent the reaction of the line concerned. QTL analysis for CR reaction was performed following the procedure of Li et al. (2009).

Table 1 Comparison of field rankings and crown rot severity obtained from the soil-less assay on 18 wheat genotypes[a]

Genotype	Mean CR severity	Fieldranking[b]
EGA Wylie	1.48a	6
2–49	1.89a	6
Lang	2.82bc	5
Baxter	2.88bc	5
Sunco	2.95bcd	6
Sunlin	3.11bcde	4
Leichhardt	3.19bcdef	4
Kennedy	3.28bcdef	4
Strezlecki	3.34bcdef	3
Giles	3.35bcdef	3
Hartog	3.58bcdef	4
Sunvale	3.69cdef	4
Sunbri	3.70cdefg	4
EGA Stampede	3.75defg	3
EGA Bounty	3.80efg	4
EGA Ruby	3.95gh	3
EGA Bellaroi	4.52gh	2
Kamilaroi	4.95 h	2

[a] Mean CR severities followed by the same letter are not significantly different according to Duncan's multiple range test at the $P=0.05$ level of significance;

[b] field rankings were obtained from http://www.dpi.qld.gov.au/documents/PlantIndustries_FieldCropsAndPasture/Wheat_Variety_Guide_09.pdf

Specifically, the Kruskal–Wallis test was used in a preliminary analysis to detect association between markers and individual traits. A multiple QTL model (MQM) with backward elimination ($P>0.02$) was used to detect significantly associated markers as cofactors for interval mapping using MapQTL® (Version 5.0, van Ooijen 2004). For each trait 1000 random permutations identifying the LOD threshold corresponding to a genome-wide false discovery rate of 5% ($P<0.05$) were used to declare the presence of QTL. QTL maps were drawn using MapChart 2.1 (Voorrips 2002).

Results

Protocol development

Based on the preliminary experiments assessing variation in *Fusarium* isolates, inoculum concentrations, seedling stages and vigour) (not shown), the optimized protocol for the soil-less assay was designed as the following and used for all other experiments in this study. Seeds were selected by removing small and shrivelled kernels. Twenty selected seeds were used for each set of 10 seedlings in a replicate. Seeds were germinated in Petri dishes on three layers of filter paper saturated with water and only the first 10 germinated seedlings were used as a replicate. When the majority of them reached 0.5 cm, the newly germinated seedlings were inoculated by immersing in a spore suspension of $1x10^6$ spores/ml for about one minute. The 10 inoculated seedlings representing a genotype were then wrapped and grown in a single piece of moist paper towel (Fig. 1). Paper rolls containing all genotypes in a replicate were randomly placed in a container and kept in a laboratory. The inoculated seedlings were kept well watered for the first 48 hours following inoculation and then watered only when the seedlings started to wilt. Watering was carried out by dipping the paper rolls into a water bath for about 30 seconds. CR severity was assessed between 7 and 14 days after inoculation when the most susceptible genotype 'EGA Bellaroi' or 'Kamilaroi' in an experiment became severely necrotic.

Reproducibility of the new assay

The reproducibility of the new assay was assessed by testing 60 genotypes with two independent trials, each with three replicates. The average CR severities over the three replicates in the first trial ranged from 0.91 to 5.00 with an overall average of 3.33. In the second trial the severities ranged from 1.01 to 5.00 with an average of 3.42. A highly significant ($p<0.01$) and strong correlation ($r=0.91$) was obtained between CR severities of the two trials (Fig. 2).

Comparison between CR reactions of the new soil-less assay and field rankings

To compare the CR reactions based on the new soil-less assay and field rankings, 18 genotypes with known field performance (http://www.dpi.qld.gov.au/documents/PlantIndustries_FieldCropsAndPasture/Wheat_Variety_Guide_09.pdf) were assessed in three trials, each with three replicates. The three trials produced highly consistent results. CR severities of

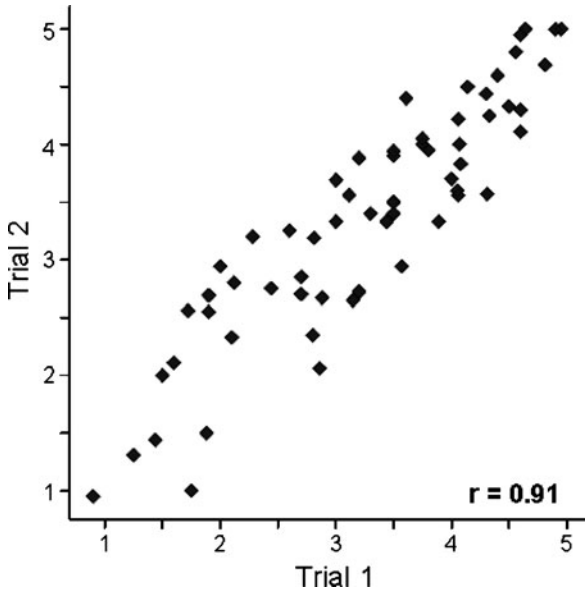

Fig. 2 Relationship between crown rot severities of 60 wheat genotypes obtained from two independent trials using the new soil-less assay, showing the high repeatability of the method. The correlation (r=0.91) is highly significant (p<0.01)

the 18 genotypes ranged from 1.31 to 5.00 with an average of 3.30 in the first trial, from 1.25 to 4.90 with an average of 3.25 in the second trial, and from 1.25 to 4.95 with an average of 3.22 in the third trial. The overall reactions of the 18 genotypes based on the soil-less assay agreed well with their respective field rankings. The two genotypes with the best field rankings, 'EGA Wylie' and '2–49', also gave the best performance in the soil-less assay, and both of the highly susceptible durum genotypes, 'Kamilaroi' and 'EGA Bellaroi' also gave the worst performance in the soil-less assay. However, the results from the soil-less assay for several genotypes with medium reactions did not agree well with their respective field rankings. These included 'Strezlecki' and 'Giles' which, compared with their field rankings, were placed higher and that of 'EGA Bounty' lower according to results from the soil-less assay (Table 1).

QTL analysis

The soil-less assay detected little difference in CR severities between the two parents of the wheat population, 'Lang' and 'CSCR6'. However, each of the two independent trials (designated as CRW-1 and CRW-2, respectively) detected clear transgressive segregation in the RIL population. CR severities of the RILs varied from 1.01 to 5.00 with an average of 3.72 in CRW-1, and varied from 0.62 to 4.83 with an average of 2.05 in CRW-2. The correlation between CR severities obtained from the soil-less assay and those from the soil-based assay was highly significant (p<0.01) but only intermediate at r=0.44 (Fig. 3). QTL analyses of the mapping data from these two trials identified a same QTL located on chromosome 4B. This QTL explained 14.7% and 15.9% of the phenotypic variance respectively in the two independent trials. When the data from the two trials were combined, the QTL explained 18.9% of phenotypic variance with a LOD value of 4.0 (Table 2).

The soil-less assay detected a clear difference in CR severities between the two parents of the barley population, with 'TX9425' more resistant than 'Franklin'. CR severities of the barley DH population, varying from 1.04 to 5.00 with an overall average of 3.91, also showed trangressive segregation. MQM analysis of the mapping data detected a single QTL on the long arm of chromosome 3H. This QTL explained 24.9% of the phenotypic variance with a LOD value of 3.5 (Table 2).

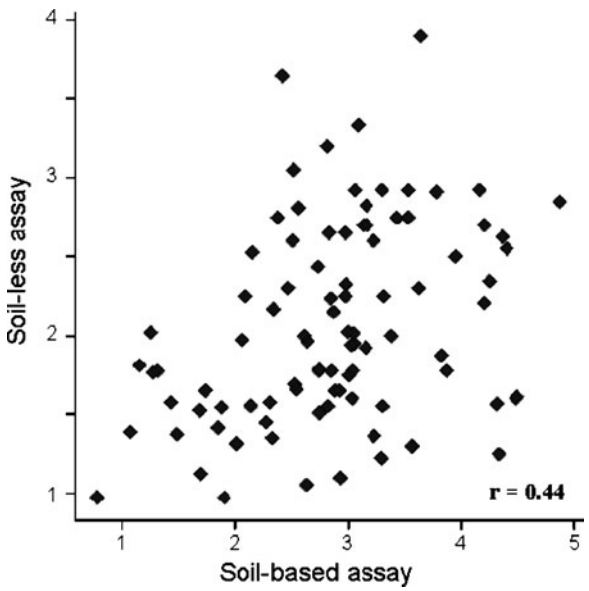

Fig. 3 Relationship between CR severities obtained from the new soil-less assay and the soil-based assay on 91 recombination inbred lines from a population of Lang/CSCR6. The correlation (r=0.44) is highly significant (p<0.01)

Table 2 QTL conferring crown rot resistance detected in a wheat recombinant inbred line population of Lang/CSCR6 and a barley doubled haploid population of Franklin/TX9425 from the soil-less assay

Population	Trial	Flanking markers	LOD	R^2(%)	Origin
Wheat	1	Barc199 & wPt-7569	3.0	14.7	Lang
	2	wPt-4918 & wPt-7569	3.4	15.9	Lang
	Combined	wPt-4918 & wPt-4918	4.0	18.9	Lang
Barley	1	bPb-4747 & bPb-6765	3.5	24.9	TX9425

Discussion

To meet the demand of breeding and pre-breeding where large numbers of genotypes need to be routinely assessed, we have attempted to improve the CR assay by reducing the time-frame required for disease assessment and/or by increasing the number of lines that can be dealt with in a given space. The new soil-less assay described in this paper dramatically enhances capacity and reproducibility of CR assessment compared to other methods currently available. Similar to the method reported by Babaeizad et al. (2009), it is quick and takes no more than 2 weeks to complete. The added advantage of the new method is that, by growing several inoculated seedlings in a single moist paper towel (Fig. 1), it does not take much space. The new method is also unique in that it removes the need of soil/potting mixes, and therefore the variability associated with growing seedlings in these media. However, this also means that the seedlings in the new assay have to more heavily rely on the endosperm for nutrient supply. Fortunately, the assay is quick and can be completed well before the depletion of nutrients from the endosperm. It takes no more than 10 days before susceptible genotypes can be meaningfully evaluated and about 14 days before severe CR can be developed for the most resistant genotypes currently available. For most of the genotypes, non-inoculated seedlings can easily survive 30 days or longer in the assay system.

The new soil-less assay detected a similar QTL to that detected using the soil-based assay of Li et al. (2008) in the barley population. The locations of the QTL from the two different assays were identical although their magnitudes differed (Table 2). The QTL detected by the soil-less assay is smaller than the one detected by the soil-based assay (Li et al. 2009). However, results obtained with the new soil-less assay on the wheat population were significantly different from those of the soil-based assay in three aspects. Firstly, the difference in CR reaction between the two parents revealed by the soil-based assay was not detectable in the soil-less assay. Secondly, the correlation between CR severities detected by the two assays was only intermediate (Fig. 3), and thirdly, of the two QTL detected by the soil-based assay (Ma et al. 2010), only the minor one on chromosome 4B remained detectable by the soil-less assay (Table 2). This QTL detected by the soil-less assay, explaining up to 18.9% of the phenotypic variance with a LOD value of 4.0, has identical location with a very similar magnitude to the one detected by the soil-based assay. The major QTL detected by the soil-based assay on chromosome 3B (Ma et al. 2010), however, was not detected with the new soil-less assay (Table 2).

To increase the capacity in the soil-based assays described by Mitter et al. (2006) and Li et al. (2008), seedlings are grown in tiny containers thus they have only very limited nutrients available. Further, water stress is applied to accelerate disease development (Li et al. 2008). Both of the limited amounts of available nutrients and water stress shorten the duration of vegetative growth. As a result, some early-maturing genotypes could reach booting or even heading stage at the time of disease assessment in these soil-based assays. The difference in plant stages is a possible reason why not all of the QTL detected by the soil-based assays were detected from the soil-less assay. These results suggest that some resistance genes may only express at certain stages of development, a phenomenon well documented for other diseases such as rust in wheat (Zhang et al. 2008). However, the current results could not rule out the possibility that the different QTL detected by the soil-less and the soil-based assays were caused by the very different growing conditions used by these assays. The different environments might have allowed the ex-

pression of genes with different mechanisms of resistance. In either case, although the new soil-less assay and those soil-based assays (Mitter et al. 2006; Li et al. 2008) all generate results correlating well with field performance, effects of different genes may be detected. Naturally, genes conferring resistance at different developmental stages or with different mechanisms could all contribute to the successful performance of a variety. The direct effect of seedling resistance is to reduce seedling fatality and thus maintains adequate plant density of a crop. It is also well known that plant density contributes to grain yield directly, but it could also reduce competition from weeds and reduce water loss through evaporation. Nevertheless, caution needs to be used when comparing disease severity results obtained from different assays. Clearly, the results obtained in this study may also apply to other disease systems. For example, although germinating seeds in *Fusarium* inoculum (Browne and Cooke 2005a) or inoculating detached leaves (Browne and Cooke 2005b; Browne et al. 2006) can be used for assessing *Fusarium* head blight resistance, the responsible genes detected by these methods may not be the same as those detected by traditional methods of assessing this disease at anthesis.

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