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1 **Identification of novel QTLs for resistance to crown rot in the doubled**

2 **haploid wheat population 'W21MMT70' x 'Mendos'**

3

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20 **Abstract**

21 Crown rot (causal agent *Fusarium pseudograminearum*) is a fungal

22 disease of major significance to wheat cultivation in Australia. A doubled

haploid wheat population was produced from a cross between line 'W21MMT70', which displays partial seedling and adult plant (field) resistance to crown rot, and 'Mendos', which is moderately susceptible in seedling tests but partially resistant in field trials. Bulked segregant analysis (BSA) based on seedling trial data did not reveal markers for crown rot resistance. A framework map was produced consisting of 128 microsatellite markers, four phenotypic markers, and one sequence tagged site marker. To this map 331 previously screened AFLP markers were then added. Three quantitative trait loci (QTLs) were identified with composite interval mapping across all of the three seedling trials conducted. These QTLs are located on chromosomes 2B, 2D, and 5D. The 2D and 5D QTLs are inherited from the line 'W21MMT70', whereas the 2B QTL is inherited from 'Mendos'. These loci are different from those associated with crown rot resistance in other wheat populations that have been examined, and may represent an opportunity for pyramiding QTLs to provide more durable resistance to crown rot.

Key words

Triticum aestivum - *Fusarium pseudograminearum* – QTL mapping – disease resistance

Crown rot (causal organism *Fusarium pseudograminearum*) is a significant fungal disease of wheat in Australia (Backhouse et al. 2004) and elsewhere (Paulitz et al. 2003, Balmas 1994, Marasas et al. 1988), particularly in production regions where stubble of previous cereal crops is retained and water stress late in the growing season is common. Symptoms of the disease in seedlings include browning of the coleoptile, subcrown internode and basal leaf sheaths. In adult plants, a honey brown discoloration of the tiller bases is typical followed by whiteheads (heads containing no grain) or varying degrees of shrivelled grain (Klein et al. 1991). It has been estimated that losses due to crown rot cost the Australian cereals industry $56 million annually (Brennan and Murray 1998).

Crown rot is a difficult disease to manage, as *F. pseudograminearum* survives between wheat crops on infected wheat stubble or grass weeds (Wildermuth et al. 1997). In the last 15 years the disease has become more prevalent in many Australian growing environments, due to the adoption of minimum tillage, stubble retention practices in farming systems. Evidence from the North-West United States indicates that incidence and severity of the disease is increasing there also (Smiley et al. 2005). The majority of cultivars currently grown in Queensland are moderately or highly susceptible to the disease (Wildermuth et al. 2001). Current control methods focus on crop rotation and the planting of partially resistant varieties such as ‘Sunco’, ‘Baxter’, and ‘Lang’ (Wildermuth et al. 2004).

However, even partially resistant cultivars can suffer yield losses if planted in soil where the level of inoculum on stubble residues is high, and when environmental conditions favour the pathogen.

Breeding programmes are seeking to develop varieties which express improved levels of crown rot resistance in the field in a range of environmental conditions. Phenotypic disease assessments of genetic variation in field trials, which are generally made at harvest, are time-consuming, labour intensive and suffer from significant environmental effects on disease expression. A seedling test is available which correlates well with the field performance of many genotypes, however there is a subset of cultivars which show moderate susceptibility in seedling trials but partial resistance as adult plants in the field. For these reasons the coupling of molecular techniques with conventional breeding (marker-assisted selection) has the potential to more rapidly and reliably identify genomic regions from various sources that contribute to resistance, and should greatly increase the efficiency of selecting such resistance sources.

There are currently only two previous reports of molecular markers identified for resistance to crown rot. Wallwork et al. (2004), using a bulked segregant analysis, reported markers for a crown rot resistance QTL in a cross between the moderately field resistant cultivar 'Kukri' and the susceptible cultivar 'Janz'. In a more detailed mapping study, Collard et al.

(2005) have recently identified both major and minor QTLs for seedling resistance to crown rot in the partially resistant wheat line ‘2-49’.

We have been screening for alternative sources of partial resistance with the goal of pyramiding additional QTLs together with those identified in ‘2-49’ to gain improved levels of resistance. This screening has identified significant variation for seedling resistance between the parents of a ‘W21MMT70’ x ‘Mendos’ doubled haploid (DH) population, originally constructed for unrelated studies of quality attributes (Kammholz et al. 1998). In this report the phenotypic and molecular analysis of seedling resistance in this population indicates that several previously undescribed QTLs contribute to resistance.

Materials and Methods

Plant materials: A wheat x maize induced doubled haploid population consisting of 95 lines of wheat (*Triticum aestivum* L.) was produced at the Leslie Research Centre from a cross between ‘W21MMT70’ and ‘Mendos’ by Kammholz et al. (1998). The Western Australian experimental line ‘W21MMT70’ displays partial seedling and adult plant (field) resistance to crown rot, whereas the Australian cultivar ‘Mendos’ is susceptible in seedling trials, but displays partial adult plant resistance.

Seedling disease assessment: Three seedling trials were carried out in a growth cabinet (25˚C, 60% humidity, 12-hour photoperiod) at the University of Southern Queensland in 2001, and in glasshouse tests at the Leslie Research Centre in 2003 and 2005. Phenotyping was conducted according to the method of Wildermuth and McNamara (1994). Briefly, 13 seeds of each genotype were sown in pots containing steam/air treated soil (70°C, 30 minutes) inoculated with *Fusarium pseudograminearum*. After 21 days, each of the first three leaf sheaths from 10 seedlings per pot were rated for disease severity using a five point scale whereby: 0 = no infection; 1 = 0-25%; 2 = 25-50%; 3 = 50-75%; and 4 = 75 – 100%. The values obtained for each leaf sheath were added to give an overall score out of 12. Due to space constraints in the growth cabinet, each repeat of the 2001 trial contained only single pot entries of each genotype, but was replicated three times over a four month period. In the 2003 and 2005 trials entries were replicated twice and four times respectively. All trials included the susceptible check cultivar 'Puseas', and disease severity ratings of the doubled-haploid lines were converted to a percent (%) 'Puseas' scale. Data from seedling trials were analysed using SPSS version 12.0.1 (SPPS Inc., 1989-2003).

DNA extraction: DNA was extracted from 3-5 leaves of 14-day-old seedlings as described by Cakir et al. (2003). Quantification was carried out

1 on Agarose gels, and DNA diluted to a concentration of 10ng/μL prior to
2 use in PCR.
3
4 **Bulked segregant analysis:** Bulked segregant analysis (BSA; Michelmore
5 et al. 1991) was initially conducted to identify putative crown rot resistance-
6 associated markers. Two DNA bulks were constructed, one by combining
7 equal amounts of DNA from 15 resistant lines and one by combining DNA
8 from 14 susceptible lines. These decisions were based upon phenotypic
9 results obtained from the 2001 seedling trial. Bulks were included when
10 screening for polymorphism between parental lines. Three hundred and
11 ninety (390) microsatellite (SSR) primer pairs were used to determine
12 polymorphism between parents and bulks. The SSR sources and sequences,
13 amplification conditions, and electrophoresis protocols are described in
14 Collard et al. (2005).
15
16 **Molecular mapping:** Ma (2000) produced a linkage map of the
17 'W21MMT70' x 'Mendos' population consisting of a total of 407 markers
18 including AFLP (331), RAPD (59), SSR (14), and phenotypic markers (3).
19 Because chromosomal locations of these markers were largely unknown, a
20 framework microsatellite map consisting of 128 SSR, one sequence-tagged-
21 site (STS66-3B), the phenotypic markers of Ma (2000; awns, *GluB3* and
22 *GluD3*) and a phenotypic marker for *Sr36* (data kindly provided by Dr.

Harbans Bariana), was produced using the program Map Manager QTX (Manly et al. 2001) with a stringency of p=0.01. The previously screened AFLP markers were manually added to the framework map and their best location was determined by using the links report generated by Map Manager QTX. Due to the much reported problem of reproducibility of RAPD markers (see for example Jones et al. 1997), these were not included for mapping. Chi-squared tests for segregation distortion of markers were carried out, and markers showing distortion were noted but not removed prior to map construction and QTL detection.

QTL detection: One thousand (1000) permutation tests at 2cM intervals were carried out to determine likelihood ratio statistic (LRS; equal to LOD x 4.61) significance thresholds for QTL detection for all trials. Composite interval mapping (using default parameters) for seedling resistance to crown rot was carried out using Windows QTL Cartographer version 2.0 (Wang et al. 2001-2004). MapChart (Voorrips 2002) was used for graphical presentation of linkage groups and QTLs.

Results

Phenotypic analysis

Means of parental and doubled-haploid lines, as well as the range of the population from each seedling trial are given in Table 1. Both the 2001 and

2005 trials were not normally distributed (Shapiro-Wilk test $p<0.05$) and hence all trials were subject to square root transformation to satisfy assumptions of normality for ANOVA. Levene's test revealed that the requirement for homogeneity of variance was met ($p>0.05$). The Tukey honestly significant difference procedure showed that both glasshouse trials were not significantly different from each other, but were significantly different to the growth cabinet trial. The population mean was higher (greater susceptibility to crown rot) when the trials were carried out in the growth cabinet. The distribution of mean severity ratings from each of the seedling trials of the DH lines and parents are shown in Figures 1a, 1b, and 1c. In each trial, 'W21MMT70' displayed partial resistance to crown rot, whereas 'Mendos' seedlings were susceptible.

Bulked segregant analysis

A total of 390 microsatellite markers were screened across parents and bulks. Of these, 163 (41.9%) identified polymorphisms between W21MMT70 and Mendos. The microsatellites were selected for their genome coverage, with 52 (31.9%) present in the A genome, 64 (39.3%) present in the B genome, and 47 (28.8%) present in the D genome. Eleven primer pairs showed banding patterns in the bulks that suggested they might be associated with resistance. However, marker analysis of the individuals within the bulks, constructed based on results from the 2001 trial, did not

indicate any consistent linkage with crown rot resistance. Consequently a mapping approach was adopted in an attempt to identify QTLs.

Molecular map

One hundred and fifteen (115) of the 163 polymorphic markers were scored on the DH population, and these were combined with phenotypic data for *Sr36*, and the SSR and phenotypic markers of Ma (2000) in order to produce a framework linkage map consisting of 133 markers. Of the 331 AFLP markers, 159 were manually distributed amongst the framework map. After addition of the AFLP markers 27 linkage groups were formed, and all were able to be assigned to chromosomes on the basis of consensus maps (Somers et al. 2004, Appels 2003). Due to gaps, chromosome 2A was separated into three linkage groups, while chromosomes 2D, 3B, 5A, and 7A were separated into two linkage groups. All 21 chromosomes contained markers, although some (particularly chromosomes 3D, 4D and 6D) were less densely mapped than others. The total map distance was 2204cM (data not shown).

Segregation distortion

Segregation distortion was observed for 13.5% of the markers. Of these, 64.9% were AFLP markers. Deviation from the expected 1:1 ratio was shown in 13 of the 27 linkage groups. With exception of some markers on

1 2B and 5D, the distorted loci were not clustered. In the case of chromosome
2 2B, the distorted loci favoured the 'Mendos' parent, whereas for
3 chromosome 5D, distorted loci favoured the 'W21MMT70' source.
4
5 **QTLs for seedling resistance to crown rot**
6 Using composite interval mapping, eight QTLs for seedling resistance to
7 crown rot were found (Table 2). Of these eight QTLs, only three (on
8 chromosomes 2D, 2B, and 5D) were consistently present in all three
9 seedling trials. The remaining QTLs were identified in only one
10 (chromosomes 4A, 5A, and 6A) or two (chromosomes 1A and 3B) of the
11 three seedling trials. Of the QTLs observed in all three seedling trials, the
12 5D and 2D QTLs were inherited from the resistant parent 'W21MMT70',
13 whereas the 2B QTL was inherited from the susceptible parent 'Mendos'.
14 Interval maps and likelihood ratio statistic (LRS) plots for the 2B,
15 2D, and 5D QTLs are shown in Figure 3. The 2B QTL (Figure 3a) had
16 maximum LRS values of 26.06 (2001 trial) 18.9 (2003 trial) and 31.6 (2005)
17 trial. This QTL explained between 13.2% and 19.9% of the phenotypic
18 variance. The 2D QTL was suggestive in both the 2001 and 2003 trials
19 (LRS of 10.7 and 11.3 respectively), and significant in the 2005 trial (LRS
20 17.0). This QTL explained 10.2% of the phenotypic variance in 2005. The
21 5D QTL was highly significant in 2001 (LRS 43.5, explaining 28.1% of the
22 phenotypic variance), significant in 2003 (LRS 13.6, explaining 13.8% of

the phenotypic variance), and only suggestive based upon the 2005 data (LRS 8.1, explaining 4.8% of the phenotypic variance).

The effect of various combinations of alleles at the 2B, 2D, and 5D QTL are shown in Figure 2. The doubled-haploid lines with all 3 resistance alleles (the 2B allele from 'Mendos', and the 2D and 5D alleles from 'W21MMT70') had a mean severity rating of 44.4%. This value is 28.4% lower than the population mean of 62%, and 54.5% lower than lines having susceptible alleles at all three loci (97.6% 'Puseas').

Discussion

The continuous distribution of disease severity ratings from all three seedling trials, as well as the presence of transgressive segregants, indicates that crown rot resistance is a quantitative trait. The population mean of the growth cabinet trial was higher than each of the glasshouse trials, and this may indicate that this environment was more conducive to development of disease symptoms. In addition to the inclusion of the check cultivar 'Puseas' (the cultivar against which % disease severity was expressed) line 2-49 was also included in each of the seedling trials (data not shown). Line 2-49 is recognized as one of the best sources of resistance to crown rot currently available (Wildermuth et al. 2001). In comparison to line '2-49' (mean disease severity of 42.2% across the 3 trials), 'W21MMT70' had a mean severity rating of 53%.

1 In an attempt to rapidly and efficiently identify genomic regions
2 associated with seedling resistance to crown rot, BSA was carried out using
3 microsatellites of known location in the wheat genome. The lines chosen
4 for BSA were identified from the 2001 growth cabinet trials and prior to
5 subsequent trials. While this approach identified a number of potential
6 candidate locations, when the individuals of the bulks were analysed,
7 linkage to crown rot resistance could not be confirmed. Most successful
8 reports of the use of BSA to identify molecular markers linked to traits of
9 interest have involved qualitative traits such as powdery mildew resistance
10 (Xie et al. 2004), common bunt resistance (He and Hughes 2003), and leaf
11 rust resistance (Cherukuri et al. 2003). When used in studies on quantitative
12 traits, the BSA technique appears most useful at identifying loci with large
13 phenotypic effects (Cook et al. 2004), although in the current study, even
14 the 5D QTL with the largest effect based on the 2001 phenotypic data (LRS
15 46.2) was not detected by BSA. This inability to detect QTLs by
16 conducting BSA based on one years' data possibly reflects the between-year
17 variability that is evident in the phenotypic data.
18 In the mapping study, a number of markers (13.5%) displayed
19 segregation distortion. Often in reports on the construction of linkage maps,
20 distorted loci are removed prior to map construction (e.g. Ubi et al. 2004,
21 Román et al. 2002). Distorted markers have been reported to cause
22 inaccuracies in the linkage distances calculated between markers (Cloutier

1 et al. 1997), hence the decision is often made to exclude them. However,
2 Hacket and Broadfoot (2003) have conducted simulation studies that
3 suggest distorted segregation ratios have little effect on map construction.
4 Of particular interest, distortion was observed in the regions of
5 chromosomes 2B (in favour of the 'Mendos' allele) and 5D (in favour of the
6 'W21MMT70' allele) where QTLs were found. Segregation distortion on
7 chromosome 2B has been previously reported in a 'Sunco' x 'Tasman'
8 mapping population (Kammholz et al. 2001). The authors explain the
9 distortion as being caused by the presence of an alien introgression from
10 *Triticum timopheevi* that is present in 'Sunco'. 'Mendos' also contains this
11 introgression, and this may be the cause of the distortion in this population
12 as well. Faris et al. (1998) reported the occurrence of three regions of
13 segregation distortion on chromosome 5D in an *Aegilops tauschii* (the D
14 genome donor in bread wheat) cross, although comparison between these
15 regions and that of the current study are difficult to make due to a lack of
16 common markers. Apart from those on 2B and 5D, most of the other
17 distorted markers were not clustered, and it is acknowledged that markers
18 displaying mild segregation distortion may simply be the result of less than
19 optimal numbers of individuals for genotyping.

20 Of the eight QTLs identified, only three (the 2B, 2D, and 5D QTLs)
21 were consistent across all seedling trials. This finding highlights the
22 significant environmental variation exhibited when screening for resistance

1 to crown rot, and supports the role of molecular markers as valuable tools to
2 aid breeding programs in the selection of resistant materials. The other five
3 QTLs identified were only minor in their effect, and as these QTLs were
4 only identified in one or two of the three seedling trials, further studies need
5 to be carried out in order to confirm their putative correlation with crown rot
6 resistance.

7 Only two other studies have reported the discovery of molecular
8 markers associated with resistance to crown rot. Wallwork et al. (2004)
9 have identified a QTL on chromosome 4B that explained up to 48% of the
10 phenotypic variance from a cross between the moderately resistant cultivar
11 'Kukri' and the susceptible cultivar 'Janz'. Collard et al. (2005) reported
12 two major QTLs derived from '2-49' on chromosomes 1A and 1D as well as
13 up to four potential minor QTLs, including a 4B locus in the same region as
14 that identified by Wallwork et al. (2004). The molecular map produced in
15 the current study was sufficient to enable comparisons with the QTLs
16 reported in Wallwork et al. (2004) and Collard et al. (2005). Of the loci
17 identified in this current study, only the 1A QTL appears to coincide with
18 any previously identified region. A comparison of flanking markers from
19 Collard et al. (2005) and the current study reveals that this QTL is located in
20 a similar region of chromosome 1A as that found in line '2-49'. As this
21 marker-trait association is only suggestive in two of the three seedling trials

in the ‘W21MMT70’ x ‘Mendos’ DH population, further work is required to confirm its significance.

The identification of the QTL on chromosome 2B from the susceptible parent (‘Mendos’) is consistent with the observation of trangressive segregants in the seedling trials. Indeed, other studies on disease resistance have shown that significant QTLs may be inherited from susceptible parents (Collard et al. 2005; Steiner et al. 2004). ‘Mendos’ is an Australian wheat cultivar that has not been widely grown since 1969, when *Sr36* and certain other additional genes it possessed for resistance to stem rust were overcome (Zwer et al. 1992). The QTL for crown rot resistance on chromosome 2B is in close proximity to *Sr36*, which indicates that this QTL is located on the introgression from *Triticum timopheevi* – an introgression that contains a number of important disease resistance genes (Brown-Guedira et al. 2003, Bariana et al. 2001, Tao et al. 2000). Strong evidence has been provided for the clustering of disease resistance genes within chromosomes (Dilbirligi et al. 2004), and the close association of the 2B crown rot QTL with *Sr36* may indicate that this QTL is positioned within such a cluster. As is the case for black-point resistance in wheat (Lehmensiek et al., 2004), screening for *Sr36* resistance may also be a promising strategy for identifying a useful level of crown rot resistance in pedigrees that utilize the *T. timopheevi* introgression.

This is the second mapping study of molecular markers linked to partial crown rot resistance in seedlings. Three consistent QTLs were identified on chromosomes 2B, 2D, and 5D in each of the three seedling trials conducted. These QTLs differ from the major QTLs previously described by Collard et al. (2005), and thus represent potential for pyramiding QTLs for the improvement of wheat affected by this economically important disease. We are currently screening individuals from a '2-49' x 'W21MMT70' DH population to test the hypothesis that these two resistance sources can be combined to produce higher levels of resistance to crown rot.

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Table 1. Means and ranges for crown rot disease severity (expressed as the square-root percentage of the susceptible check cultivar Puseas) for parental lines and doubled haploids. Population means sharing the same letter are not significantly different (Tukey HSD, p>0.05).

Table 2. QTLs for crown rot resistance detected using composite interval mapping in three seedling trials. Chromosome location, likelihood ratio statistic (LRS) value, percent phenotypic variance explained (VE), significance level (SL), and the parent contributing the resistance allele (Source) are shown.

Figure 1. Histograms of crown rot severity ratings of the 'W21MMT70' x 'Mendos' wheat population from the 2001 (a) 2003 (b) and 2005 (c) seedling trials. Disease severity is expressed as the square-root percentage of the susceptible check cultivar 'Puseas'. Parental means are indicated by filled ('W21MMT70') and unfilled ('Mendos') arrows.

Figure 2. Mean disease severity (% of 'Puseas') of doubled-haploid lines with combinations of alleles from the three QTL regions. In all instances, differences were significant between lines carrying a QTL contributing to resistance and those without (Students t-test, p<0.05).

Figure 3. Interval maps (cM) of the 2B (a), 2D (b), and 5D (c) QTL found in all three seedling trials. Chromosome regions exceeding the suggestive threshold are indicated by filled vertical bars. AFLP markers are not included for the purpose of clarity.

TABLE 1.

Table 1. Year	W21MMT70	Mendos	Population Mean	Population Range
2001	7.56	10.22	8.56[a]	5.24-11.48
2003	6.62	9.40	7.11[b]	3.99-11.10
2005	7.62	9.65	7.42[b]	3.82-11.38

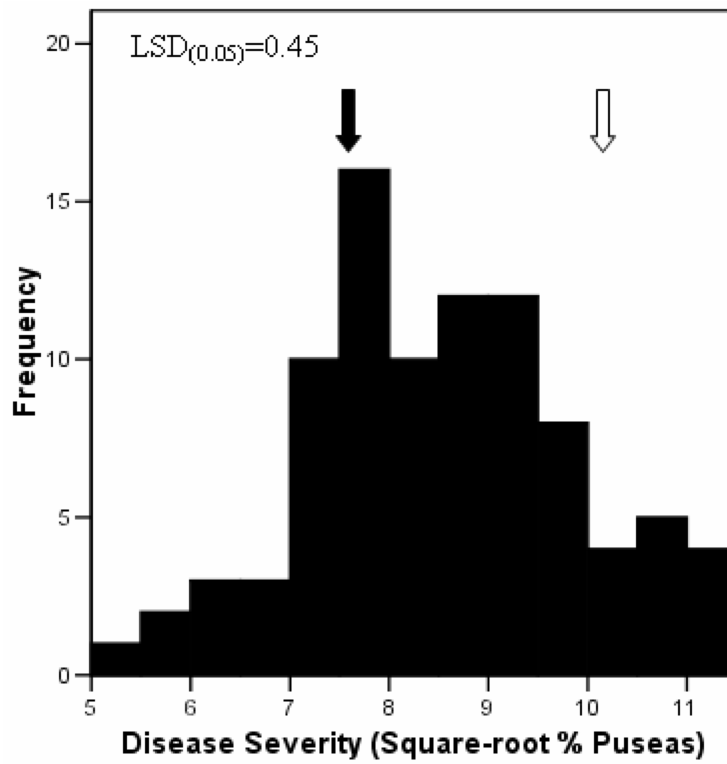
TABLE 2.

Chromosome	2001			2003			2005			Source
	LRS	VE	SL[1]	LRS	VE	SL	LRS	VE	SL	
1A	7.8	4.9	Sg	3.3	2.2	NS	8.3	4.8	Sg	‘Mendos’
2B	26.0	13.2	HS	18.9	13.0	HS	31.6	19.9	HS	‘Mendos’
2D	10.3	4.8	Sg	11.3	7.7	Sg	17.0	10.2	S	‘W21MMT70’
3B	1.8	0.8	NS	8.2	5.0	Sg	13.7	8.1	Sg	‘W21MMT70’
4A	1.9	0.8	NS	0.8	0.6	NS	9.3	5.2	Sg	‘Mendos’
5A	9.9	6.2	Sg	3.5	2.5	NS	0.9	0.7	NS	‘Mendos’
5D	43.5	28.1	HS	13.6	13.8	S	8.1	4.8	Sg	‘W21MMT70’
6A	3.4	1.6	NS	9.3	5.9	Sg	0.8	0.4	NS	‘W21MMT70’

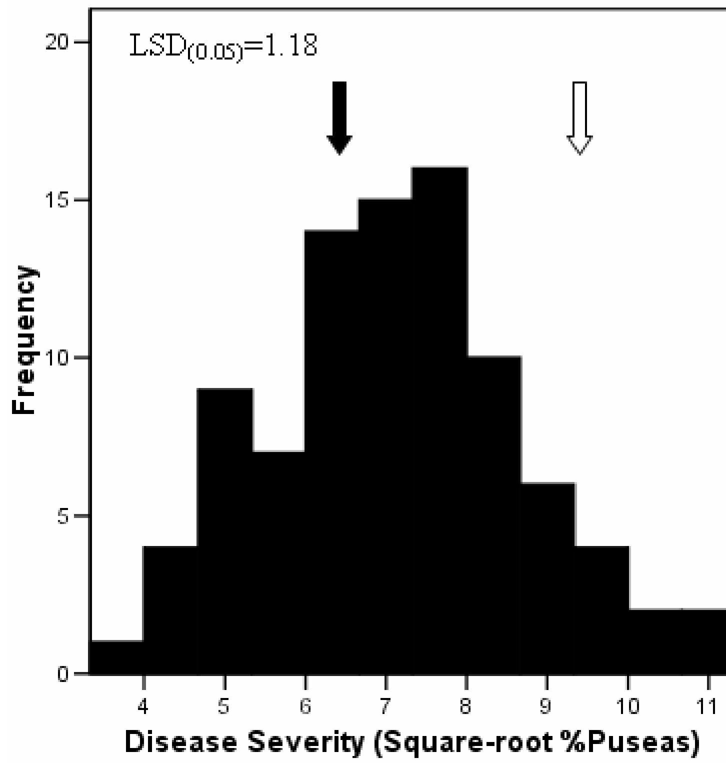
[1] Significance level determined by 1000 permutation tests for each seedling trial. NS – not significant; Sg – suggestive (LRS greater than 7.8, 7.9, and 7.8 for the 2001, 2003, and 2005 trials respectively; S – significant (LRS greater than 14.5, 13.5, and 18.2 for the 2001, 2003, and 2005 trials respectively; HS – highly significant (LRS greater than 20.6, 18.2, and 22.3 for the 2001, 2003, and 2005 trials respectively).

FIGURE 1.

a) 2001 Seedling Trial



b) 2003 Seedling Trial



c) 2005 Seedling Trial

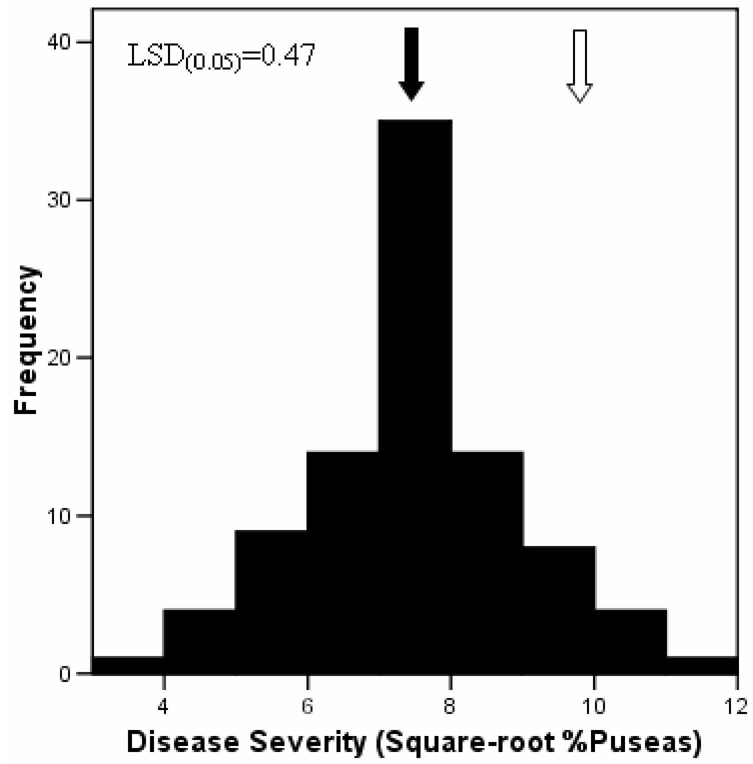


FIGURE 2.

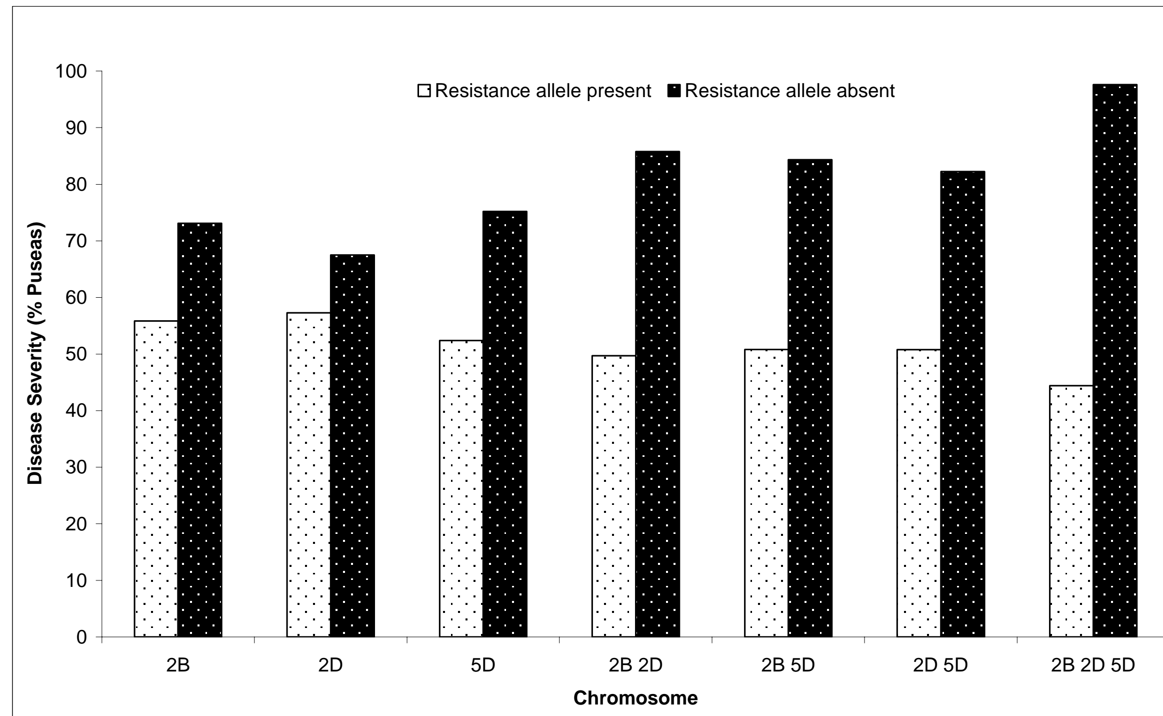
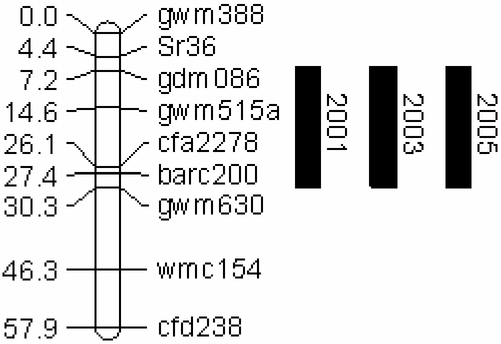
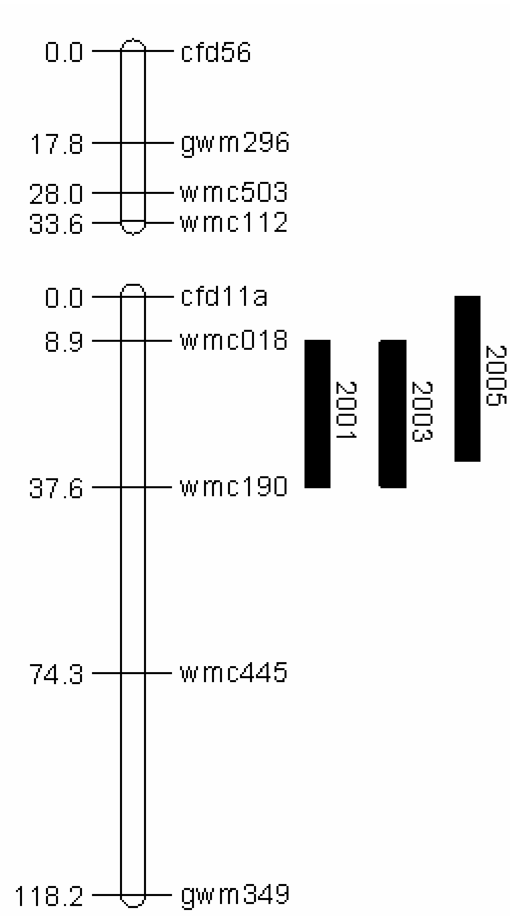


FIGURE 3.

a) Chromosome 2B



b) Chromosome 2D



c) Chromosome 5D