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# Mining the Watkins collection of wheat landraces for novel sources of eyespot resistance

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Eyespot is an economically important stem base disease of wheat caused by the soilborne fungal pathogens *Oculimacula yallundae* and *Oculimacula acuformis*. The most effective method of controlling the disease is host resistance. However, there are only three genetically characterized resistances in wheat varieties and further sources of resistance are required. Previous studies have identified resistances in wild relatives, but use of these resistances has been limited by linkage drag with deleterious traits exacerbated by low rates of recombination. Therefore, the identification of novel resistances in hexaploid wheat germplasm is desirable. The Watkins collection currently consists of 1056 hexaploid wheat landraces that represent global wheat diversity at the time of its collection in the 1920s and 1930s. As such, it may contain beneficial agronomic traits such as eyespot resistance. The Watkins collection was screened for resistance to *O. yallundae* based on a glasshouse test of all 1056 accessions and a polytunnel test of 44 accessions selected from a previous field trial. Resistant lines identified in these tests were retested against both *O. yallundae* and *O. acuformis*. This identified 17 accessions with resistance to one or both of the pathogen species. From these, two accessions (1190094.1 and 1190736.3) provided a high level of resistance to both pathogen species. An $F_4$ population derived from accession 1190736.3 indicated that the resistance to *O. acuformis* in this accession is conferred by a single gene and therefore would be suitable for introgression into elite wheat varieties to provide an alternative source of eyespot resistance.

*Keywords:* genebank collections, *Oculimacula acuformis*, *Oculimacula yallundae*, *Triticum aestivum*

## Introduction

Eyespot is a fungal disease of the stem base of cereal crops including wheat, barley and rye, and is caused by two species of fungi, *Oculimacula acuformis* and *Oculimacula yallundae* (Crous *et al.*, 2003). Severe infection results in lodging and premature ripening of grain, leading to reduced crop yield, and is considered economically important in temperate areas, particularly northwest Europe and the Pacific North West of the USA (Fitt, 1992). Fungicides have been widely used to control the disease, but effective control can be problematic as it requires precise application timings (Burnett & Hughes, 2004) and because resistance has arisen to many of the widely used chemicals (Parnell *et al.*, 2008). Therefore, the use of disease resistant varieties is considered the most economical and effective strategy to control eyespot disease.

There are three main sources of genetic resistance to eyespot that are known to be present in commercial wheat varieties. These are *Pch1*, *Pch2*, and a QTL on chromosome 5A termed QPch.jic-5A. *Pch1* is the most potent of these and was introduced into wheat from the wild grass *Aegilops ventricosa* (Maia, 1967). However, *Pch1* has been associated with lower yield in the absence of the disease (Koen *et al.*, 2002) and this has limited the use of the resistance in elite varieties, particularly in Europe (Johnson, 1992). Both *Pch2* (Law *et al.*, 1975) and QPch.jic-5A (Burt *et al.*, 2011) originate from the partially eyespot resistant French cultivar Cappelle Desprez. However, both *Pch2* and QPch.jic-5A confer only a moderate level of resistance and are unlikely to prevent yield loss under high disease pressure. There is, therefore, a need to identify additional resistances due to the paucity of eyespot resistance genes available within modern wheat varieties, and the limitations associated with those resistances that are currently used.

Resistance to eyespot has been identified in wheat relatives including *Triticum monococcum* (Cadle *et al.*, 1997), *Triticum tauschii* (Assefa & Fehrmann, 1998; Yildirim *et al.*, 1998), *Aegilops longissima* (Sheng & Murray, 2013) and *Dasypyrum villosum* (Murray *et al.*, 1994). However, the use of wild relatives in wheat breeding is often limited by linkage between the traits of interest and deleterious traits on introgressed segments (Feuillet *et al.*, 2008). Reducing the size of alien introgressions and breaking adverse linkages is often difficult due to suppressed recombination between wheat chromosomes and wild relative homoeologues (Riley *et al.*, 1959). Therefore, the identification of novel eyespot resistance in collections of hexaploid wheat would be highly beneficial, as these resistances could be crossed into wheat varieties, genetically characterized, and deleterious traits back-crossed out more rapidly than is

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possible with alien introgressions. Despite these advantages, only a few studies have screened collections of hexaploid wheat for sources of eyespot resistance. For example, Börner *et al.* (2006) analysed eyespot resistance data from screenings of Gatersleben genebank accessions of wheat collected from 1933 to 1992. Data was available for seedling tests on 1881 accessions and field tests on 4885 accessions using a combination of natural and artificial infection. From these tests the majority of lines were deemed to be infected at a low or medium level, but 3% of accessions were determined to be resistant at the seedling stage and 10% of accessions were resistant at the adult plant stage. However, it was not shown if these resistant accessions contained known resistance genes or whether they contained novel resistance genes that are not present in commercial cultivars.

Both *O. yallundae* and *O. acuformis* are often present together in field populations (Fitt *et al.*, 2006); control of one leads to selection of the other and does not prevent wheat crops from becoming infected (Parnell *et al.*, 2008). Previous studies have identified differential levels of resistance against the two pathogens in accessions of *T. monococcum* (Burt *et al.*, 2010) and *D. villosum* (Uslu *et al.*, 1998). There is also evidence that the widely used *Pch2* resistance may be more effective against penetration by *O. acuformis* than by *O. yallundae* (Burt *et al.*, 2010), although this difference is not always reflected in altered fungal biomass accumulation (Esvelt Klos *et al.*, 2013). Although it may be technically possible to conduct marker-assisted selection of more than one eyespot resistance gene, in order to combine control of *O. yallundae* and *O. acuformis*, the use of single resistance genes that function against both pathogens would be more efficient. Therefore, it is important to investigate resistance to both *O. yallundae* and *O. acuformis* in order to develop cultivars that provide effective control against both pathogen species.

The Watkins collection of hexaploid wheat was assembled by the British botanist A. E. Watkins in the 1920s and 1930s from 32 countries across the world, and represents a sample of global genetic diversity in wheat landraces and early varieties at the time. The original collection consisted of 7400 lines, which was subsequently reduced by Watkins to 2750 maintained accessions (Miller *et al.*, 2001). The Watkins collection potentially contains useful variation for a number of agronomic traits, including resistance to a variety of diseases. This is evidenced by the identification of the leaf rust resistance gene *Lr52* (Dyck & Jedel, 1989; Hiebert *et al.*, 2005) and the stripe rust resistance gene *Yr47* (Bansal *et al.*, 2011) from Watkins accessions. As the collection consists of hexaploid wheat, any traits that are identified may be genetically characterized and introgressed into elite varieties more rapidly and with less linkage drag than traits identified in wild relatives.

Recently, 814 remaining accessions from the original collection were studied for their phenotypic diversity in field trials, and heterogeneous lines stabilized by single seed descent (SSD) as part of the UK Wheat Genetic Improvement Network (WGIN) (http://www.wgin.org.uk/wgin_2003-2008/index.php?area=Resources&page=results). This generated the current collection of 1056 stable lines held at the John Innes Centre (JIC), UK. As part of the WGIN project, Watkins accessions from the original non-stabilized collection were tested for their eyespot resistance during field trials carried out in 2007–2008 at Rothamsted Research, UK. These trials were conducted using natural infection rather than artificial inoculation and were scored for stem penetration by eyespot (R. Gutteridge, Rothamsted Research, Harpenden, UK, personal communication). This experiment revealed a range of susceptibility to eyespot within the collection (http://www.wgin.org.uk/resources/researchresults.php) and suggested that further investigations into eyespot resistance within the collection were worthwhile.

The specific objectives of this study were to (i) screen a subset of the Watkins collection identified as resistant in the 2007–2008 field trial for resistance to *O. yallundae*; (ii) screen all 1056 accessions from the stabilized collection to identify accessions with potentially high levels of resistance to *O. yallundae*; (iii) screen putative resistant accessions for resistance to both *O. yallundae* and *O. acuformis*; (iv) screen resistant accessions with SSR markers to infer the presence or absence of known resistances; and (v) characterize potential novel resistances in existing populations produced within the Wheat Institute Strategic Programme (WISP).

## Materials and methods

### Plant and fungal material

Seed of all Watkins accessions and populations derived from Watkins accessions were obtained from the stocks curated at JIC. All Watkins accessions are coded with the prefix '1190'. Hereafter in the present study, the prefix is not included in the accession code and only the three-digit unique code is used. The susceptible control cultivar Chinese Spring (CS), the moderately resistant cultivars Cappelle Desprez (CD) and Hobbit 'sib' (HS) both containing *Pch2* and QPch.jic-5A, and the highly resistant intervarietal single chromosome substitution line Hobbit 'sib'-VPM7D (HS/VPM7D) containing all three known resistances (*Pch1*, *Pch2* and QPch.jic-5A) were all obtained from the Germplasm Resources Unit (GRU) at the JIC.

All *O. yallundae* and *O. acuformis* isolates used in the JIC trials were from the JIC culture collection. For each species, a homogenized mixture of six different isolates, as detailed by Chapman *et al.* (2008), was used for inoculations.

### Seedling bioassays

In glasshouse and polytunnel trials, plants were grown over winter in unheated unlit conditions at JIC, Norwich, UK. In the polytunnel trial (experiment 1a), plants were inoculated as described by Chapman *et al.* (2008), harvested 8 weeks after inoculation, and visually scored for disease. Coleoptiles and leaf sheaths were peeled back successively and scored for disease symptoms on a scale reflecting the number of wheat leaf sheaths infected (slight lesion, not penetrating to adaxial surface) or completely penetrated (more severe lesion probably penetrated

to adaxial surface), where 0 = seedling uninfected, 1 = coleoptile infected, 2 = coleoptile heavily infected, 3 = first leaf sheath infected, 4 = first leaf sheath completely penetrated, 5 = second leaf sheath infected, 6 = second leaf sheath completely penetrated, etc. (Scott, 1971). In the glasshouse trial (experiment 2a), seeds were inoculated by applying a mixture of oat grains previously colonized with *O. yallundae* using the method of Bruehl & Nelson (1964), and peat and sand compost through which the seedlings emerged. Plants were harvested 10 weeks after sowing and scored as above.

In controlled environment (CER) trials (experiments 1b, 2b and 3), plants were grown at 10°C and 10 h day length. Plants were inoculated with macerated mycelium using the method of Chapman *et al.* (2008). Transparent PVC cylinders (3 cm long, 5 mm I.D.) were placed over emerging shoot tips. After 21 days, plants were inoculated by pipetting agar slurry (400 $\mu$L) into each cylinder. The inoculum was prepared by homogenizing the agar and associated fungal colonies with water (2:1). Plants were well watered and propagator lids used to increase the humidity. Plants were harvested 6–8 weeks after inoculation and scored for disease as above.

### *Experiment 1a: polytunnel trial to test putative resistant accessions identified from 2007 to 2008 field trial for resistance against* O. yallundae

Data from the 2007 to 2008 field trial conducted at Rothamsted Research was kindly provided by Dr Richard Gutteridge at Rothamsted Research. This data can be accessed online from WGIN (http://www.wgin.org.uk/resources/researchresults.php). Eyespot disease indices were calculated for each accession using the method of Scott & Hollins (1974). Thirty-nine putative resistant accessions with a disease index <40% and five susceptible accessions (018, 021, 173, 704, 775) with a disease index >75% were selected for retesting. Seed was obtained for these lines from the original non-stabilized collection.

Five seeds of each of the 44 selected accessions and three control lines (CS, CD and HS/VPM7D) were sown in five 7 × 7 cm pots in peat and sand compost, five seeds per pot. Pots were arranged as five complete randomized blocks, each block containing one pot of each Watkins accession and one pot of each control line, and were inoculated with *O. yallundae* using the macerated mycelium method of Chapman *et al.* (2008) as described above.

### *Experiment 1b: CER trial to assess levels of resistance to both* O. yallundae *and* O. acuformis *in accessions identified as resistant in experiment 1a*

Three Watkins accessions (166, 577 and 827) demonstrating evidence of resistance to *O. yallundae* in experiment 1a were selected for experiment 1b. Also included were the three control lines, and Watkins accession 011 as an additional susceptible control. Five seeds of each accession were sown in 7 × 7 cm pots in peat and sand compost, 50 seeds per accession. Pots were arranged as five complete randomized blocks, each block containing two pots of each line. In each block, one pot was inoculated with *O. yallundae* and the other with *O. acuformis* using the method of Chapman *et al.* (2008).

### *Experiment 2a: glasshouse trial to assess levels of resistance to* O. yallundae *in Watkins lines*

The 1056 stabilized Watkins accessions were screened for resistance against *O. yallundae* using oat grain inoculum in an unheated, unlit glasshouse. Nine seeds of each of the accessions were sown in 9 × 9 cm pots in peat and sand compost. These were arranged in propagation trays, each tray containing 19 Watkins accessions and one of the three control lines. All seeds were inoculated with *O. yallundae* using a colonized oat grain and peat and sand compost mixture as described above.

### *Experiment 2b: CER trial to assess levels of resistance to both* O. yallundae *and* O. acuformis *in accessions identified as resistant in experiment 2a*

Forty-four Watkins accessions demonstrating the highest levels of resistance to *O. yallundae* in experiment 2a were selected for experiment 2b. Also included were two Watkins accessions with high levels of susceptibility and the three control varieties. Five seeds of each accession were sown in ten 7 × 7 cm pots in peat and sand compost, 50 seeds per accession. Pots were arranged as 10 complete randomized blocks, each block containing one pot of each accession. Five blocks were inoculated with *O. acuformis* and five blocks were inoculated with *O. yallundae* using the method of Chapman *et al.* (2008).

### *Experiment 3: CER trial to phenotype the Paragon × Watkins accession 736 $F_4$ population for resistance to* O. acuformis

Watkins accessions × Paragon populations are under development within the Wheat Institute Strategic Programme (WISP) at JIC (http://wisplandracepillar.jic.ac.uk/). This includes a population derived from Paragon × Watkins accession 736. Paragon has previously been demonstrated to be susceptible to both *O. yallundae* and *O. acuformis* (data not shown) and Watkins accession 736 demonstrated resistance to both pathogen species in experiment 2b. To determine whether the resistance identified in Watkins accession 736.3 is inherited quantitatively or is controlled by few genes of large effect, 74 $F_4$ lines from the Watkins accession 736 × Paragon population were tested for eyespot resistance in a seedling bioassay. The experiment was conducted as four randomized complete blocks, with each block containing one pot (five plants) per line and three pots (15 plants) per parent line. All blocks were inoculated with *O. acuformis* because accession 736.3 had demonstrated a higher level of resistance to this species compared to *O. yallundae* in experiment 2b. Unfortunately, no populations developed from the other eyespot resistant accessions were available for inclusion in the present study.

## Statistical analysis

Analysis of variance was performed on visual disease scores from experiments 1a, 1b and 2b to assess the variation attributable to block and genotype using general linear modelling (GLM). In experiments 1b and 2b, the variation attributable to pathogen species and pathogen × genotype interaction was assessed. In each experiment, predicted mean disease scores were calculated for the accessions. Means for each accession were compared to the mean disease scores of the susceptible control CS or the moderately resistant control CD using *t*-values calculated within the GLM. A very high significance threshold ($P < 0{\cdot}001$) was used to limit the possibility of Type-I errors generated by multiple comparisons of genotypes.

For experiment 2a, a mixed level model was fitted to the data with tray included as a random effect and accession included as a fixed effect using a restricted maximum likelihood (REML) procedure. Best unbiased linear estimates (BLUEs) were generated for each accession, adjusting for the tray effect to account

for environmental variation across the glasshouse. BLUEs for each accession were compared to CD, using least significant differences generated from the mixed level model, to identify highly resistant accessions.

In experiment 3, GLM was used to predict mean disease scores for each $F_4$ line from the Paragon × Watkins accession 736 population. The mean disease score for each line was compared to the disease scores for the two parents using *t* probabilities calculated within the GLM. Lines with a disease score significantly lower ($P < 0{\cdot}001$) than Paragon were classified as resistant, whilst lines with a disease score significantly higher than accession 736 ($P < 0{\cdot}001$) were classified as susceptible. Lines with levels of resistance not significantly different from either parent were classified as intermediate. A chi-squared test was performed to compare the ratio of resistant: intermediate: susceptible lines to the expected segregation ratio for a single resistance gene of additive effect in an $F_4$ population (43·75% homozygous resistant, 12·5% heterozygous intermediate, 43·75% homozygous susceptible). All analyses were conducted in GenStat v. 14.

### Genotyping analysis

The selected Watkins accessions included in experiment 1b and the Watkins lines that demonstrated resistance to either or both of the pathogen species in experiment 2b were genotyped with the *Pch1*-linked STS marker Xorw1, as identified by Leonard *et al.* (2008); the *Pch2*-linked SSR markers Xwmc525 and Xcfa2040, as identified by Chapman *et al.* (2008); and the QPch.jic-5A QTL-linked markers Xgwm639 and Xbarc197 (Burt *et al.*, 2011), to determine the presence or absence of these known eyespot resistances. The lines CS (no resistance), CD (*Pch2* and QPch.jic-5A), HS (*Pch2* and QPch.jic-5A) and HS/VPM7D (*Pch1*, *Pch2* and QPch.jic-5A) were included as controls.

DNA was extracted from each wheat line using the CTAB method (Nicholson *et al.*, 1996), quantified using a PicoDrop spectrophotometer (Picodrop Ltd.), and diluted to 10 ng $\mu L^{-1}$ in sterile distilled water for use in PCRs. A 6·25 $\mu$L reaction volume consisted of 2·5 $\mu$L DNA, 3·125 $\mu$L Taq mastermix (QIAGEN) and 0·625 $\mu$L of the relevant primer pair (2 $\mu$M). The forward primer for each marker was labelled with 6-FAM, NED, PET or VIC fluorescent dyes (Applied Biosystems). PCR conditions were as described by Bryan *et al.* (1997). Samples were prepared by adding 1 $\mu$L of a 1:40 dilution of the PCR product to 10 $\mu$L formamide and 0·2 $\mu$L of LIZ 500 size standard (Applied Biosystems). Samples were run on an ABI 3700 capillary sequencer (Applied Biosystems) and the output data were analysed using peak scanner v. 1.0 (Applied Biosystems) to determine the product size of the amplicons. Lines were deduced to carry *Pch1* if they contained the VPM7D allele at Xorw1, *Pch2* if they contained the CD allele at Xwmc525 and Xcfa2040, and QPch.jic-5A if they contained the CD allele at Xgwm639 and Xbarc197.

## Results

### Experiment 1 seedling bioassays

Of the 44 Watkins accessions tested in experiment 1a, 34 demonstrated a moderate level of resistance against *O. yallundae*, with a disease score significantly lower ($P < 0{\cdot}001$) than the susceptible control CS (Table 1).

Table 1 Predicted mean disease scores for Watkins accessions when inoculated with *Oculimacula yallundae* in experiment 1a

| Line | Predicted mean disease score |
|---|---|
| HS/VPM7D | 3·0ab |
| 166 | 4·4a |
| 577 | 4·9a |
| 827 | 4·9a |
| 347 | 5·1a |
| 531 | 5·1a |
| Cappelle Desprez | 5·1a |
| 542 | 5·1a |
| 019 | 5·2a |
| 241 | 5·2a |
| 634 | 5·2a |
| 378 | 5·2a |
| 041 | 5·3a |
| 417 | 5·3a |
| 624 | 5·4a |
| 309 | 5·4a |
| 688 | 5·4a |
| 748 | 5·4a |
| 248 | 5·5a |
| 232 | 5·5a |
| 734 | 5·5a |
| 828 | 5·5a |
| 756 | 5·6a |
| 704 (S) | 5·6a |
| 021 (S) | 5·6a |
| 721 | 5·6a |
| 173 (S) | 5·7a |
| 108 | 5·7a |
| 482 | 5·7a |
| 799 | 5·7a |
| 595 | 5·7a |
| 743 | 5·8a |
| 018 (S) | 5·8a |
| 057 | 5·8a |
| 696 | 5·8a |
| 724 | 5·8a |
| 702 | 6·0 |
| 775 (S) | 6·0 |
| 823 | 6·1 |
| 255 | 6·1 |
| 638 | 6·2 |
| 794 | 6·2 |
| 653 | 6·3 |
| 011 | 6·4 |
| 718 | 6·4 |
| 322 | 6·6 |
| Chinese Spring | 6·8 |

(S), susceptible Watkins accession included as a control.
Statistically significant difference ($P < 0{\cdot}001$) between mean disease score of accession and control line, where a = significantly lower than Chinese Spring and b = significantly lower than Cappelle Desprez.

Only the control HS/VPM7D, containing *Pch1*, *Pch2* and QPch.jic-5A, had a disease score significantly lower than CD ($P < 0{\cdot}001$) with no Watkins accessions demonstrating a high level of resistance to *O. yallundae*. Lines 166, 577 and 827 had slightly lower, although not significantly lower, disease scores than CD.

Repeat testing of the resistant accessions 166, 577 and 827 was conducted to both *O. yallundae* and *O. acuformis* in experiment 1b. Accession 011 was also included as a further susceptible control, as although previously appearing to be resistant based on the single field trial data, it demonstrated a similar disease score to the susceptible control CS in experiment 1a. In experiment 1b, *O. yallundae* was significantly ($P < 0{\cdot}001$) more aggressive than *O. acuformis* across all lines (Tables 2 & 3). Therefore, it was not possible to compare the resistance in each line to the two pathogens directly and it was necessary to examine differences between accessions and control lines within each pathogen species. The moderately resistant control CD demonstrated significantly lower disease scores than CS when inoculated with *O. acuformis* but higher disease scores than CS when inoculated with *O. yallundae*. This suggests that the resistances contained in CD (*Pch2* and QPch.jic-5A) were ineffective in this experiment, presumably as a result of high disease pressure from aggressive *O. yallundae* isolates in a controlled environment that is more conducive to disease development than the more heterogeneous polytunnel environment.

Accession 166 demonstrated significantly lower disease scores than the susceptible CS when inoculated with either *O. yallundae* ($P < 0{\cdot}05$) or *O. acuformis* ($P < 0{\cdot}001$), suggesting that it contains resistance effective against both pathogens. However, the disease scores for accession 166 were significantly higher than that observed in the *Pch1*, *Pch2* and QPch.jic-5A control HS/VPM7D for both pathogens ($P < 0{\cdot}001$).

A significant interaction was detected between line and pathogen ($P < 0{\cdot}001$) in experiment 1b (Table 3) suggesting that lines may possess a differential level of resistance against the two pathogens. For example, accession 577 demonstrated disease scores significantly ($P < 0{\cdot}001$) lower than CS when inoculated with *O. acuformis*, but was more susceptible to *O. yallundae* than CS (Table 2). Despite demonstrating resistance to *O. yallundae* in experiment 1a, accession 827 provided no evidence of resistance to either pathogen in experiment 1b, again probably as a result of higher disease pressure as evidenced by the higher disease scores in all three control lines.

Table 2 Predicted mean disease scores for the Watkins accessions and controls when inoculated with *Oculimacula acuformis* and *Oculimacula yallundae* in experiment 1b

| Line | *O. acuformis* | *O. yallundae* |
|---|---|---|
| Chinese Spring | 6·4 | 7·4 |
| Cappelle Desprez | 3·9*** | 8·1 |
| HS/VPM7D | 2·6*** | 4·1*** |
| 011 | 6·1 | 8·5 |
| 166 | 4·3*** | 6·2* |
| 577 | 4·3*** | 8·2 |
| 827 | 5·8 | 7·7 |

*$P < 0{\cdot}05$; ***$P < 0{\cdot}001$ compared to Chinese Spring.

Table 3 Summary of variance components for eyespot disease scores using general linear modelling for seedling bioassays in experiment 1b and 2b

| | Experiment 1b | | Experiment 2b | |
|---|---|---|---|---|
| Source of variation | MS | *F*-value | MS | *F*-value |
| Line | 83·733 | 58·68*** | 28·059 | 9·52*** |
| Pathogen | 462·862 | 324·37*** | 1135·867 | 385·43*** |
| Line × Pathogen | 15·091 | 10·58*** | 7·227 | 2·45*** |
| Residual | 1·427 | | 2·947 | |

MS, mean squares.
***$P < 0{\cdot}001$.

## Experiment 2 seedling bioassays

BLUEs of disease scores for each accession in experiment 2a were approximately normally distributed and ranged from 2·3 to 7·7 (Fig. 1). High levels of resistance were rare in the collection; seven of the accessions (0·7%) had disease scores lower than the highly resistant HS/VPM7D and 163 of the accessions (15·4%) had disease scores lower than the moderately resistant CD. Two hundred and ninety-three (28%) of the accessions were highly susceptible, with disease scores higher than CS.

Forty-one lines (3·9%) with the lowest disease scores in experiment 2a were selected for retesting against both *O. yallundae* and *O. acuformis* in experiment 2b. Accession 166, which was identified as resistant in experiments 1a and 1b, was not among the most resistant lines in experiment 2a, and therefore was not included in the group of lines for retesting. Accessions 175.2 and 482.1

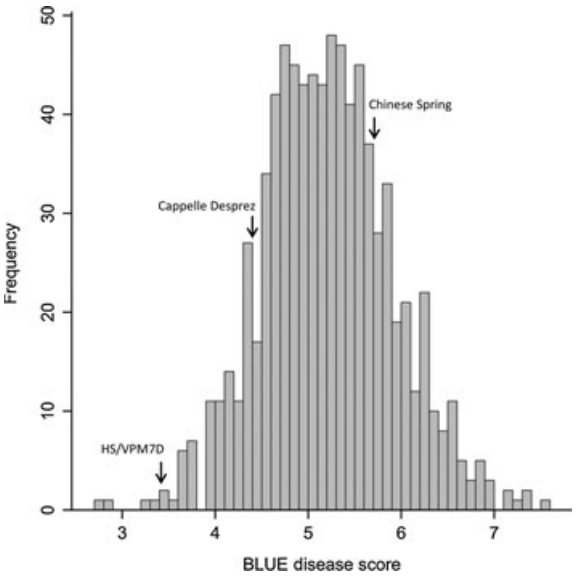


Figure 1 Histogram of best linear unbiased estimates (BLUEs) of disease scores generated from a mixed level model for the Watkins accessions when inoculated with *Oculimacula yallundae* in experiment 2a. BLUEs of disease scores for control lines HS/VPM7D, Cappelle Desprez and Chinese Spring are indicated by arrows.

were selected for inclusion as additional susceptible controls.

In experiment 2b, 11 lines demonstrated significantly ($P < 0{\cdot}001$) lower disease scores than Chinese Spring when inoculated with *O. yallundae* and seven lines demonstrated significantly ($P < 0{\cdot}001$) lower disease scores than Chinese Spring when inoculated with *O. acuformis* (Table 4). Of these lines, three lines (094.1, 736.3 and 674.1) demonstrated significant levels of resistance to both pathogens ($P < 0{\cdot}001$), although the level of resistance was higher in line 094.1 towards *O. yallundae* and higher in lines 736.3 and 674.1 towards *O. acuformis*.

As observed in experiment 1b, *O. yallundae* was significantly more aggressive than *O. acuformis* ($P < 0{\cdot}001$) across the experiment (Tables 3 & 4). However, it was also possible to detect a significant interaction between genotypes (Table 3), suggesting that some lines confer differential levels of resistance to the two pathogen species. For example, accessions 165.1, 704.1, 417.1 and 657.1 were significantly more resistant to *O. acuformis* than CS but not to *O. yallundae*, whereas accessions 049.1, 507.3, 488.1, 616.1, 411.1, 380.1, 399.2 and 741.1 were significantly more resistant than CS to *O. yallundae* but not to *O. acuformis* (Table 4).

Table 4 Predicted mean disease scores for the Watkins accessions and controls when inoculated with *Oculimacula acuformis* and *Oculimacula yallundae* in experiment 2b

| Line | *O. acuformis* | *O. yallundae* |
|---|---|---|
| 094.1 | 3·0a | 2·6a |
| HS/VPM7D | 2·3ab | 2·6ab |
| 049.1 | 4·1 | 4·4a |
| 507.3 | 3·6 | 4·5a |
| 736.3 | 2·3ab | 4·6a |
| 488.1 | 4·6 | 4·6a |
| 674.1 | 3·4a | 4·9a |
| 165.1 | 3·5a | 5·0 |
| Cappelle Desprez | 3·5a | 5·0a |
| 616.1 | 4·0 | 5·0a |
| 411.1 | 3·8 | 5·1a |
| 380.1 | 4·2 | 5·1a |
| 013.1 | 4·3 | 5·2 |
| 399.2 | 4·3 | 5·2a |
| 741.1 | 4·5 | 5·2a |
| 704.1 | 3·5a | 5·6 |
| 417.1 | 3·9a | 5·6 |
| 120.1 | 4·3 | 5·6 |
| 288.1 | 4·4 | 5·6 |
| 482.1 (S) | 6·4 | 5·8 |
| 657.1 | 3·3a | 5·9 |
| 605.2 | 4·9 | 5·9 |
| 408.2 | 4·0 | 6·0 |
| 399.1 | 4·8 | 6·0 |
| 286.1 | 5·2 | 6·0 |
| 013.2 | 4·3 | 6·1 |
| 522.1 | 5·1 | 6·3 |
| 077.1 | 3·7 | 6·4 |
| 328.4 | 4·3 | 6·4 |
| 503.1 | 5·2 | 6·4 |
| 588.1 | 5·3 | 6·4 |
| 376.2 | 4·4 | 6·5 |
| 639.1 | 3·9 | 6·6 |
| Chinese Spring | 5·1 | 6·6 |
| 730.4 | 3·9 | 6·8 |
| 730.1 | 4·1 | 6·9 |
| 175.2 (S) | 4·5 | 6·9 |
| 590.2 | 5·1 | 6·9 |
| 042.1 | 3·9 | 7·0 |
| 672.1 | 4·2 | 7·0 |
| 014.2 | 5·0 | 7·0 |
| 677.2 | 4·8 | 7·1 |
| 122.1 | 4·7 | 7·2 |
| 103.1 | 4·7 | 7·3 |
| 589.1 | 4·9 | 7·3 |
| 727.1 | 4·8 | 8·0 |

(S), susceptible Watkins accession included as a control.
Statistically significant difference ($P < 0{\cdot}001$) between mean disease score of accession and control line, where a = significantly lower than Chinese Spring and b = significantly lower than Cappelle Desprez.

## Genotyping analysis

Accessions in experiment 1b and 2b with significantly lower disease scores than CS ($P < 0{\cdot}001$) when inoculated with either *O. yallundae* or *O. acuformis* were selected for genotyping with markers linked to the known eyespot resistances *Pch1*, *Pch2* and QPch.jic-5A (Table 5). None of the Watkins lines demonstrated any evidence of containing *Pch1*. This result was expected because the *Pch1* resistance was first introduced into hexaploid wheat in 1967 (Maia, 1967), over 30 years after the Watkins collection was assembled. Accessions 166.1, 736.1, 507.3 and 704.1 possessed the CD-associated allele for one of the two SSR markers linked to *Pch2* suggesting that they may contain this resistance. However, none of the lines possessed a CD-associated allele at both *Pch2*-linked loci. Only two accessions (166.1 and 741.1) possessed the CD allele for the tightly linked QPch.jic-5A marker Xgwm639. Twelve accessions (577.1, 049.1, 488.1, 094.1, 736.1, 674.1, 507.3, 411.1, 380.1, 399.2, 741.1 and 417.1) possessed the CD allele at the more distant Xbarc197 locus. Only one accession (741.1) possessed a CD allele at both QPch.jic-5A loci.

## Seedling resistance against *O. acuformis* in Paragon × Watkins accession 736 $F_4$ population (experiment 3)

Following infection with *O. acuformis*, an approximately bimodal frequency distribution of disease scores was observed in the 74 $F_4$ lines of the Paragon × accession 736 population (Fig. 2). Out of the 74 lines, 29 lines were classified as resistant, 30 lines were classified as susceptible, and 15 lines were classified as intermediate by comparison to the parents using *t* probabilities. This segregation ratio does not differ significantly from that expected for a single gene of additive effect in an $F_4$

Table 5 Allele sizes (bp) for STS marker Xorw1 linked to *Pch1* (chromosome 7D), and SSR markers linked to *Pch2* (chromosome 7A) and the QPch.jic-5A (chromosome 5A), for control lines and for Watkins lines with resistance to *Oculimacula yallundae* or *Oculimacula acuformis* identified as resistant in experiments 1b or 2b

| Experiment | Line | Country of origin | *Pch1* | *Pch2* | | QPch.jic-5A | |
|---|---|---|---|---|---|---|---|
| | | | Xorw1 | Xwmc525 | Xcfa2040 | Xgwm639 | Xbarc197 |
| Control | CS | | 170 | 213 | 298 | 164 | 180 |
| Control | CS/CD7A | | 170 | 211[a] | 317[a] | 164 | 180 |
| Control | CS/CD5A | | 170 | 213 | 298 | 141[a] | 186[a] |
| Control | HS | | 170 | 211[a] | 317[a] | 141[a] | 186[a] |
| Control | HS/VPM7D | | 180[a] | 211[a] | 317[a] | 141[a] | 186[a] |
| 1b | 166.1 | India | 170 | 213 | 317[a] | 141[a] | 189 |
| 1b | 577.1 | Iran | 170 | 224 | 312 | 145 | 186[a] |
| 2b | 049.1 | Spain | 170 | 203 | 326 | Null | 186[a] |
| 2b | 488.1 | USSR | 170 | 213 | 294 | 160 | 186[a] |
| 2b | 094.1 | India | 170 | 220 | 308 | 146 | 186[a] |
| 2b | 736.3 | USSR | 170 | 211[a] | 319 | Null | 186[a] |
| 2b | 674.1 | Iraq | 170 | 213 | 322 | 148 | 186[a] |
| 2b | 657.1 | China | 170 | 217 | 326 | 172 | 183 |
| 2b | 165.1 | India | 170 | Null | Null | 151 | 189 |
| 2b | 507.3 | Australia | 170 | 211[a] | 304 | 170 | 186[a] |
| 2b | 616.1 | Yugoslavia | 170 | Null | 297 | 135 | 179 |
| 2b | 411.1 | India | 170 | Null | Null | 140 | 186[a] |
| 2b | 380.1 | Iran | 170 | 219 | 294 | 132 | 186[a] |
| 2b | 399.2 | China | 170 | 213 | Null | 164 | 186[a] |
| 2b | 741.1 | Afghanistan | 170 | 213 | 298 | 141[a] | 186[a] |
| 2b | 704.1 | Iran | 170 | 211[a] | 294 | 139 | 188 |
| 2b | 417.1 | India | 170 | 219 | 312 | 146 | 186[a] |

[a]Allele associated with resistance QTL.

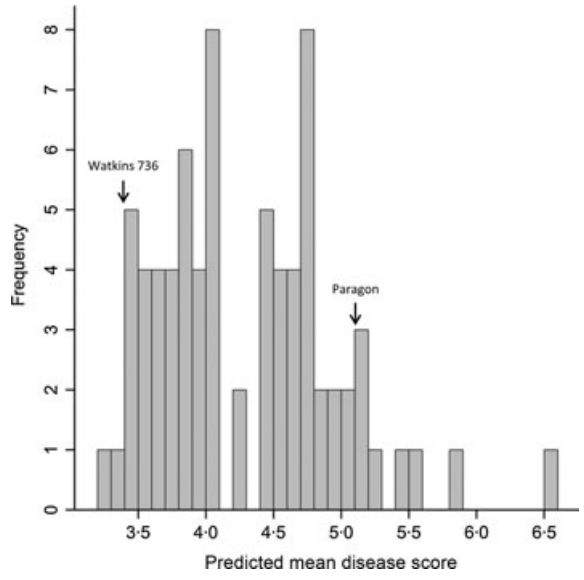


Figure 2 Frequency distribution of disease scores for 74 $F_4$ lines, derived from the Paragon × Watkins accession 736 population, when inoculated with *Oculimacula acuformis*. Predicted mean disease scores for parental genotypes are indicated by arrows.

population ($\chi^2$ = 4·1, d.f. = 2, $P$ = 0·124). This evidence suggests that a single resistance gene, originating from accession 736, is segregating in the population.

## Discussion

There is a paucity of sources of resistance to eyespot disease in wheat and there is therefore a requirement to identify new sources of resistance that can be introduced into elite varieties. In the current study, polytunnel and glasshouse screens for resistance followed by repeat testing in controlled environment rooms have identified accessions from the Watkins collection with resistance to either *O. yallundae* or *O. acuformis*. Within these accessions, two (094.1 and 736.3) were identified with levels of resistance similar to the resistant control HS/VPM7D. This control line possesses all three of the known resistances (*Pch1*, *Pch2* and QPch.jic-5A) and consistently confers a very high level of resistance to both *O. yallundae* and *O. acuformis*. Therefore, the identification of very few accessions with similar levels of resistance was expected, and any accessions that do exhibit such high levels of resistance are still considered to be worthwhile of further study.

Resistance was rare in the collection and the majority of accessions screened were found to be susceptible to eyespot disease. For example, 886 out of 1056 (84%) accessions tested with *O. yallundae* in the glasshouse screening test (experiment 2a) were more susceptible than the moderately resistant control CD. In contrast, Börner *et al.* (2006) observed a relatively low proportion of eyespot susceptible accessions in the Gatersleben collection of *Triticum* species accessions. However, that

study provided no controls for comparison and it is therefore not possible to compare these results with the eyespot resistance data from the Watkins collection. Although it is often possible to identify a higher proportion of resistant accessions in collections of wild relatives than was identified in the present study of hexaploid wheat accessions, the use of resistances from exotic germplasm may be limited by low rates of recombination with wheat and linkage drag with deleterious traits.

Differential resistance against *O. acuformis* and *O. yallundae* has been observed in wheat relatives, for example *A. longissima* (Sheng & Murray, 2013), *T. monococcum* (Burt *et al.*, 2010) and *D. villosum* (Uslu *et al.*, 1998). Data from the present study indicates that this also occurs in wheat. Of the resistant accessions identified in the present study, only four (24%) provided resistance to both *O. acuformis* and *O. yallundae*, whilst eight accessions (47%) provided resistance to *O. yallundae* and five accessions (29%) provided resistance to *O. acuformis*. The four accessions that demonstrated resistance against both *O. acuformis* and *O. yallundae* were 166 (experiment 1b), 094.1 (experiment 2b) 736.3 (experiment 2b) and 674.1 (experiment 2b). Accessions 166 and 674.1 conferred a level of resistance similar to the combined effects of *Pch2* and QPch.jic-5A (CD resistance) against both pathogens. However, accessions 094.1 and 736.3 also provided evidence of differential levels of resistance. The level of resistance identified in line 094.1 was similar to that conferred against *O. yallundae* by the combined effects of *Pch1*, *Pch2* and QPch.jic-5A (HS/VPM7D resistance) and similar that of CD against *O. acuformis*. In comparison, the level of resistance observed in 736.3 was similar to that of HS/VPM7D against *O. acuformis* and similar to CD resistance against *O. yallundae*.

High levels of variation are often associated with large-scale seedling bioassays of eyespot resistance (de la Peña *et al.*, 1996; Chapman *et al.*, 2008; Burt *et al.*, 2010). In the present study, a number of inconsistencies were observed between experiments. For example, accession 827 conferred a high level of resistance to *O. yallundae* in experiment 1a, which was conducted in a polytunnel, but in contrast there was no evidence of any resistance to either *O. yallundae* or *O. acuformis* from this accession in experiment 1b, which was conducted in a controlled environment room. This is probably because of the more homogenous environment providing suitable conditions for disease development, ensuring a higher disease pressure with a reduced possibility for disease escape. In addition, 11 accessions that were identified to provide resistance against *O. yallundae* in the glasshouse screening experiment (2a) had higher disease scores than the susceptible control CS in the controlled environment experiment (2b). It is probable that there was increased disease pressure and lower chance of disease escape in experiment 2b using the macerated mycelium technique than in experiment 2a in which a colonized oat grain technique was used. However, the use of different inoculation techniques and incubation environments in the present study should help to ensure that the resistances identified will be sufficiently potent and function consistently under high levels of disease pressure.

If the resistances identified in the Watkins accessions are simply inherited and can be mapped then these would be useful for introgression into modern wheat varieties as an alternative to the existing resistances. The resistances identified in accessions 166 and 674.1, although having effects against both *O. yallundae* and *O. acuformis*, were less potent than that observed in 094.1 and 736.3, not conferring a *Pch1* level of resistance to either pathogen. If any of the novel resistances identified in the present study are to be incorporated into elite varieties, then it is essential that their effects be maintained at the adult plant stage. Therefore, it is necessary for the resistant accessions to be tested in further field trials. This is particularly important as it has previously been demonstrated that seedling resistances may not be effective at the adult plant stage. For example, although the *Pch2* resistance from CD can be observed at the seedling stage, it confers little adult plant resistance (Muranty *et al.*, 2002). To replicate infection in the field, additional controlled environment experiments could also investigate the efficacy of the identified resistances against conidia applied in controlled concentrations (Sheng & Murray, 2013).

An $F_4$ population previously derived from the cross Paragon (susceptible UK spring wheat) × Watkins accession 736 was tested for resistance against *O. acuformis* to determine if this resistance was quantitative or probably due to few gene(s) of large effect. It was possible to categorize lines from this population as resistant (30), intermediate (15) or susceptible (29) by comparison to the disease scores of the parents. The proportions of these were consistent with the resistance being conferred by a single additive gene. A recombinant inbred line (RIL) population with a higher level of homozygosity will be developed from the Paragon × Watkins accession 736 $F_4$ population for genetic mapping, and for phenotyping with both *O. yallundae* and *O. acuformis*. It is important that the final RIL population is phenotyped with both pathogens to determine if the resistance is controlled by one locus with differential efficacy against the two eyespot species, or whether more than one locus is involved. A further biparental population is currently under development from a cross between Paragon and Watkins accession 094.1 to determine the genetic basis of the high level of resistance identified in this accession.

If the resistances from accessions 736.3 and/or 094.1 can be genetically located, and particularly if the same loci confer resistance to both pathogens, then these may be useful to plant breeders to complement or be used as alternatives to the existing eyespot resistances. Genetic mapping and identification of closely linked molecular markers will enable the backcrossing of this resistance into modern wheat varieties. This process will be assisted by the availability of high-throughput SNP marker systems in wheat, such as KASP™ (Allen *et al.*,

2013). Although backcrossing using hexaploid wheat lines as sources of resistance is likely to be quicker with less linkage drag and higher rates of recombination than when using wild relatives of wheat, the success and speed of the process will depend on the genetic location of the resistances. For example, if they are located in centromeric regions, rates of recombination will be lower (Akhunov *et al.*, 2003) and it may be difficult to break deleterious linkages. In addition, there are still fewer wheat SNP markers available on the D-genome compared to the A- and B-genomes (Allen *et al.*, 2013), which may hinder the mapping and marker-assisted selection of any resistances that are present on the D-genome.

Genotyping of the resistant accessions suggested that some of the resistances present in the Watkins collection may be novel. None of the resistant accessions had CD haplotypes at both SSR markers flanking *Pch2* and only one accession (741.1) had CD haplotypes at both markers flanking QPch.jic-5A, suggesting that the presence of these resistances is rare. However, the presence of these resistances cannot be entirely excluded as both resistances are QTL covering large genetic areas and the SSR markers are not diagnostic. Accession 094.1 might contain QPch.jic-5A (it has the CD allele at marker Xbarc197), but the level of resistance demonstrated is significantly higher than would be expected to be conferred by this QTL alone, indicating that there are additional novel resistances functioning in this accession that are conferring some, or all, of the observed resistance to the two eyespot pathogens. This suggests that the genetics controlling the resistance conferred by line 094.1 merits further investigation. There was also some evidence that accession 736 might contain *Pch2* and QPch.jic-5A, having the CD alleles for markers Xwmc525 and Xbarc197 respectively. However, the level of resistance observed in accession 736 was significantly higher than would be expected from either resistance individually or from the combination of the two resistances. In previous studies using the same methods, it was not possible to identify either *Pch2* or QPch.jic-5A as a single major effect with both resistances characterized as quantitative trait loci (QTL) (Chapman *et al.*, 2008; Burt *et al.*, 2011). This contrasts with the clear segregation apparent in the Paragon × Watkins accession 736 $F_4$ population. Taken together, this evidence suggests that the resistance identified in Watkins accession 736 has not previously been characterized and that it may be conferred by a single gene of major effect.

In conclusion, the Watkins collection of wheat germplasm contains some effective and potentially novel resistances to both causal pathogens of eyespot disease that could provide further options for plant breeders. The most potent of these from accessions 736.3 and 094.1 will be characterized in further studies. Accession 736 is of particular interest as evidence suggests that the resistance observed in this accession against *O. acuformis* may be controlled by a single locus, making it a suitable target for rapid introgression into wheat varieties.

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