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Using molecular methods to study eyespot disease in wheat

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Summary Report

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Contents

1 Abstract

Eyespot is a damaging stem-base disease of cereals caused by the fungi *Oculimacula acuformis* and *O. yallundae*. This project modelled the effects of agronomy (i.e. previous cropping, cultivation methods, soil texture, fungicide treatment, sowing date or place of winter wheat (WW)) in the rotation as 1st WW, 2nd WW or monoculture on DNA of *Oculimacula* spp. quantified in roots, 5 cm stem from the base and upper 10 cm stem sections. Fifty wheat fields were surveyed throughout England in 2012/13 season and in each field 36 randomly selected winter wheat plants were collected at growth stage (GS) 21-33, GS 39-44 and GS 66-77 over a one hectare area. DNA concentrations of *O. yallundae* and *O. acuformis* were quantified using Real-time PCR (qPCR) assays. PCR results indicated that *O. acuformis* was the predominant species causing the disease with the highest DNA concentrations of the pathogen measured at GS 65-77. Previous crop had a significant effect on DNA of *Oculimacula* spp. and WW rotational sequence contributed significantly to it. Seed treatment and fungicide application at GS 32 were found to significantly influence fungal biomass in plant tissues. In a separate study, a range of fungicide treatments were applied at GS 32 or GS 39 of the winter wheat crop and *Oculimacula* DNA was extracted from soil before fungicide application and at GS 51 and quantified using a qPCR assay. In addition, the impact of the disease on plant physiology was investigated by probing photosystem II efficiency. Chlorophyll fluorescence induction kinetics were used to calculate the performance index of plants as an estimate of photosynthetic efficiency following treatment for disease control. Boscalid application at GS 32 significantly increased performance index at GS 69, reduced disease severity, fungal biomass of *O. acuformis* in soil and DNA of *O. yallundae* in wheat stems. To quantify live, viable inoculum of individual *Oculimacula* spp in soil or in plants a two-step reverse transcriptase PCR assay was tested to synthesize and quantify cDNA from fungal mRNA obtained via RNA extraction. The assay was shown to efficiently quantify cDNA with traces of genomic DNA amplification beyond 35 cycles. Once validated in soil or in planta such an assay can potentially be utilized to increase our understanding of the epidemiology of eyespot disease and increase the accuracy of predicting disease risk associated with viable inoculum in field.

2 Introduction

Each year a significant percentage of crop yield is lost due to diseases caused by plant pathogens. Yield losses result in reduced income in the agricultural sector and decreased production for trades that require it, thus impacting on the economy. This study focuses on two fungal pathogens: *Oculimacula yallundae* and *O. acuformis*, the causal agents of eyespot disease of cereals. Research in eyespot disease increased in the 1980s when pathogen populations became resistant to the fungicides used to control them (Fitt, 1988) and now eyespot is considered to be the most important stem-base disease of cereals in temperate countries.

Eyespot is primarily a disease of cereals. Those affected include wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereale*) and oats (*Avena sativa*) (Scott et al., 1975; Cunningham, 1981). The main host of *O. yallundae* is wheat whilst *O. acuformis* is equally pathogenic to rye as well as wheat. This difference in the pathogenicity of *O. yallundae* and *O. acuformis* has led to the two species being classified as W- (wheat-) type and R- (rye-) type respectively. Some isolates of *Oculimacula* sp. C-type and S-type can infect grasses, *Agropyron repens* and *Aegilops squarrosa*, respectively as well as wheat and barley but were later shown to belong to *O. yallundae* due to their sexual reproduction compatibility (Cunningham, 1981; Nicholson et al., 1995). Eyespot is acknowledged to be agriculturally and economically most significant on wheat (Scott et al., 1975) and so the majority of published studies focus on investigating the disease in this host.

Yield loss and economic impact

Eyespot results in reduced tiller numbers, grain number per head, and 1000-grain weight (Scott and Hollins, 1974; Murray and Bruehl, 1986). Ray et al. (2006) showed that *O. yallundae* and *O. acuformis* reduced stem bending strength, ear weight and yield by 6 and 11% respectively. Yield reductions of up to 50% have been reported in commercial fields with severe disease (Murray, 2010). However as the last published figures are more than 15 years old current losses in the UK remain unknown. In a study spanning ten years (1989-1998), eyespot caused the highest percentage yield loss in five out of the ten years (Hardwick et al., 2001). Cook et al. (1991) estimated that between the years 1985-1989 eyespot disease caused an annual national loss of £26.3 million and Hardwick et al. (2001) estimated the loss in 1998 to be £23.6 million.

Survival of Oculimacula *spp.*

Oculimacula spp. survive and proliferate on infected host stubble from previous crops (Fitt et al., 1988; Daniels et al., 1991). Spores are produced from the infected debris in the cool, moist climate during autumn and spring and are dispersed onto neighboring crop by rain splash (Glynne, 1953; Fitt, 1988). The pathogens initially colonise the coleoptile and then progress through successive leaf sheaths (Bateman & Taylor, 1976), eventually causing severe lesions which block nutrient flow and weaken the stem resulting in lodging (Ray et al., 2006).

Infection and symptom development

By using electron microscopy Daniels et al. (1991) first showed that spores of each species penetrate differently upon contact with the plant surface. *O. yallundae* spores produce long single germ tubes which align with cell wall grooves and produce numerous appressoria along the length of the tube whilst spores of *O. acuformis* produce multiple, short, lateral hyphae with a single appressorium at their terminal end (Daniels et al., 1991). Further work showed that ascospores (produced during the sexual cycle) follow similar infection process despite their difference in generation and dispersal methods (Daniels et al., 1995).

Although *O. yallundae* and *O. acuformis* have different penetration mechanisms, visual symptoms of the disease are identical for both fungi. Several weeks after penetration events have occurred brown-bordered, eye-shaped lesions develop at the base of the stem. Slight lesions are not considered damaging, but a high incidence of moderate and severe lesions cause yield losses (Fitt et al., 1988). Further penetration of disease through successive leaf sheaths is influenced by temperature and weather conditions (Fitt et al., 1988). Higher temperatures can accelerate development of the pathogen however warm, dry weather can increase the rate of death of leaf sheaths and increase growth of new leaf sheaths (Wan et al., 2005). This means that in some conditions the plant can out-grow the disease; i.e. the rate of death of outer leaf sheaths is greater than fungal penetration of the leaf sheaths beneath (Fitt et al., 1988).

If, however, weather conditions are conducive to eyespot disease development the fungi will continue to grow through the stem and moderate or severe lesions will form. A direct effect of moderate and severe lesions is interference with transport of nutrients up the stem which affects grain production causing whiteheads whilst an indirect effect of severe lesions is softening of the stem bases, making wheat prone to lodging (Fitt et al., 1988; Ray et al., 2006).

Sporulation and dispersal

Large numbers of conidia of *Oculimacula* spp. are produced at the tips of short lateral hyphae (Daniels et al., 1991) on crop debris remaining in the field after harvest and can be rain-splash dispersed to wheat seedlings (Glynne, 1953; Fitt, 1988). This method of spore dispersal allows the rapid spread to nearby seedlings where multiple infections are initiated on the plant coleoptile. Secondary sporulation occurs after penetration of the host and when mycelial plates have developed within the coleoptile and later on stems. For many years it was believed that *Oculimacula* spp. only reproduced asexually but it has been shown that both species are capable of sexual reproduction. Discovery of a sexual stage in the life cycle of any pathogen is important because it means that genetic recombination can occur between individuals. This makes it possible for the fungi to respond to selection pressures or even, over time, to segregate, which can eventually generate new species (Dyer et al., 1993). Upon incubation of infected straw on moist sand, sexual reproductive structures (apothecia) were seen and a sexual stage was described for *O. yallundae (syn. Tapesia yallundae)* the teleomorph for *Helgardia yallundae (syn. Pseudocercosporella yallundae* Wallwork & Spooner, 1988). Further investigation revealed that *O. yallundae* possesses a heterothallic mating system. This means that isolates can be divided into two complementary mating groups (MAT1-1 and MAT1-2). Reproduction resulting in viable offspring is only possible when isolates of opposite mating types are crossed (Dyer et al., 1993).

Further work by Dyer et al. (1996) proved that *O. acuformis* (syn. *Tapesia acuformis* (teleomorph of *H. acuformis*) displayed the same heterothallic mating system and that mating did not occur between *Oculimacula* spp.

Ascospores (sexual spores released from apothecia) can be wind-dispersed, increasing the dispersal range of the pathogen much wider than that of the splash-borne conidia produced in the asexual cycle (Glynne, 1953; Dyer et al., 1993; Parnell et al., 2008).

Disease control

Burnett & Hughes (2004) and Burnett et al. (2012) identified a number of factors which influence the risk of disease; these include environmental factors such as soil type, temperature, humidity and rainfall. Others are agronomy factors which can be manipulated by the growers to reduce disease risk, such as sowing date, previous cropping and tillage. For example, sowing seed later in the season (i.e. November rather than September) is known to reduce the severity of eyespot lesions (Cook et al., 1991; Hardwick et al., 2001). There is some divergence in opinion regarding the influence of soil cultivation on eyespot disease incidence. Generally, it is thought that soil inversion can unearth crop debris which can act as a source of inoculum for fresh outbreaks of eyespot disease (Ray et al, 2004; Colbach & Saur, 1998); however Matusinsky et al. (2009) found no difference in disease incidence when soil was managed variably. It is important to note that Matusinsky et al. (2009) studied plants in soil which had been constantly used for cereal growth but the authors do not specify whether these cereals were hosts of *Oculimacula* spp and which species predominated. Sowing eyespot susceptible crops after at least a one year break from cereals can also lower risk of disease, with grass being suggested as an effective break crop (Cook et al, 1991; Hardwick et al., 2001). Due to the survival of *Oculimacula* spp., on debris from previous host crops, planting cereal monocultures year upon year means there is little opportunity for the levels of inoculum existing on the debris to decline (Matusinsky et al., 2009).

Performance of fungicides may vary depending on which *Oculimacula* species is dominant. A study by Bateman et al. (2000) found that cyprodinil was equally active against both pathogens but prochloraz was most effective against *O. yallundae*. This result was confirmed in a further study by Ray et al. (2004) in which both fungicides were tested on *O. acuformis*. More recently, fungicide performance against the species was evaluated by Burnett et al. (2012), the researchers reported that prothioconazole, boscalid and cyprodinil were equally effective in reducing disease index of eyespot.

Known genes conferring resistance to eyespot are few, making it difficult to rely on use of resistant cultivars for eyespot control (Chapman et al., 2009). At present the most effective resistance genes used in breeding programmes are Pch1, Pch2 and Pch3 available in the gene pool of wheat (*Triticum aestivum*).

Pch1 was integrated into hexaploid wheat from *Aegilops ventricosa*, (Kimber, 1967; Doussinault et al., 1983). The most popular breeding line, VPM-1 (VPM = Ventricosa x Persicum x Marne), has been used widely as a source of Pch1 in breeding programs (Doussinault et al., 1983). Introduction of the Pch1 gene into winter wheat was found to effectively slow the development of eyespot disease through the leaf sheaths (Lind, 1999; Thiele et al, 2002).The wheat variety Cappelle Desprez is

known to have a resistance to eyespot conferred by the presence of the Pch2 gene. Pch3 was a single dominant resistance gene, found in *D. villosum* shown to confer resistance to *O. yallundae*, but not to *O. acuformis* (Uslu et al. 1998), suggesting that the genetic basis of resistance to the two eyespot pathogens can differ. Cumulative effects of many resistance genes conferring minor effects can sometimes be less effective at pathogen control than the effect of one strong resistance gene, however multi-gene resistance is more difficult for pathogen populations to overcome, which makes this type of resistance more desirable (Hulbert et al., 2001).

Current diagnostic methods for disease and viability testing

Fungal isolations on selective media

Stem bases with suspected eyespot infection can be plated onto a suitable media and any fungal growth identified by visual or molecular methods. This method can be effective when plates are assessed by an experienced pathologist; however the similarity in culture and spore morphology can cause confusion during identification. One further drawback of plating infected material is that the method is very time consuming by nature and the fungi can take many weeks to grow, leaving plates highly susceptible to contamination from other fungi or bacteria (Breeuwer & Abee, 2000; Chitarra et al., 2006).

Plating is however one way of detecting the presence of viable inoculum. This method has the advantage of it being impossible to gain false-positive results because dead organisms simply will not grow on media; however the length of time required to grow and quantify the pathogen leaves less opportunity to apply preventative sprays before the pathogen takes hold. In addition, other more competitive organisms may grow faster on the media thus preventing the growth of the slower *Oculimacula* spp.

Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) works by immobilising an antigen from a sample to a plate and washing over a specific antibody-enzyme complex, which attaches to any complementary antigen present. Plates are treated with enzyme substrate to develop a visible signal which allows visualisation of the results (Clark, 1981). This method can be used to effectively diagnose and quantify fungal plant pathogens (Harrison et al., 1990) but is not an option for detection of viable cells. An ELISA assay using viable and non-viable plant pathogenic bacteria samples found no distinction between live and dead cells (Crother & Vruggink, 1980).

DNA-based methods

DNA-based methods, such as conventional and Real-time PCR, amplify any DNA present in the environment which is complementary to the primer template, regardless of whether the source organism is viable or not (Simpkins et al., 2000). Conventional PCR uses forward and reverse primers to amplify sequences of DNA when present in a sample. A sequence of specific temperature cycles a number of times after which samples can be subjected to agarose gel electrophoresis and

placed under a UV light source to visualise PCR product. Real-time PCR uses forward and reverse primers along with a fluorescent probe which emits light when DNA is amplified. By monitoring the emitted fluorescence the amplification can be monitored in real time and products can be detected and quantified without any further processing. This method is faster and has less risk of contamination than conventional PCR due to the reaction and visualisation being carried out in a sealed environment.

DNA can survive in the environment up to three weeks after cell death with one report successfully amplifying bacterial plasmid DNA from seven-month-old dried leaf lesions (Hartung et al., 1993) thus it is likely that false-positive results will occur during diagnoses (Josephson et al., 1993). In the case of *Oculimacula* spp., the use of conventional PCR primers, and especially Real-time PCR primers designed by Walsh et al. (2005), allow for fast and effective identification of each fungal species; however, even though it is possible to diagnose eyespot infection and to quantify sample DNA accurately using real-time PCR, it is not possible to amplify from only viable cells unless PCR is used in conjunction with intercalating agents (discussed below) or probes (Chitarra et al., 2006).

Intercalating agents are chemicals which bind between complex molecular structures, altering their shape. Two such agents, ethidium monoazide (EMA) and propidium monoazide (PMA) become cross-linked to DNA when exposed to light, disrupting its structure and making it incompatible with amplification primers (Nocker et al., 2007). The EMA/PMA technique was developed on bacteria; however Vesper et al. (2008) successfully used PMA to distinguish between live and dead *Aspergillus* spp. This provides evidence the method is not limited to bacteria; however it is limited in efficacy to cells with damaged membranes. It is known that cells can be non-viable but have intact membranes (Vesper et al., 2008; Nocker & Camper, 2009) such as those treated by UV light and to combat this Nocker & Camper (2009) suggested the novel concept of activity-labile compounds (ALCs). These theoretical compounds would enter the cell and either would be deactivated by cell metabolism or would bind to DNA when a cell is no longer active. This would allow for distinction between cells which may be dead with intact membranes and live, functioning cells. Even though there is no methodology for this approach the concept of ALCs remains interesting, especially as some recent studies have found the EMA/PMA method grossly overestimated the numbers of viable bacteria in a sample (Fittipaldi et al., 2011; Lovdal et al., 2011).

RNA based methods

Due to the time-consuming nature of plating methods and the unreliability of PMA-PCR, an RNA-based method for quantifying viable inoculum may be most suitable. In a study by Ingle & Kushner (1996) it was shown that two *E-coli* mRNA transcripts had very short half-lives which could be measured in minutes; illustrating that RNA is less stable than DNA and so degrades quickly after cell death (Nocker & Camper, 2009). It is noted that different mRNAs have different degradation times, suggesting that it is important to be aware of the half-lives of target sequences before an mRNA based assay is developed (Beelman & Parker, 1995; Ingle & Kushner, 1996).

Reverse-transcriptase (RT-) PCR is a diagnostic method which targets mRNA (Bleve et al., 2003; Guo et al, 2005). The method has been used frequently in plant virology but much less in fungal plant pathology. A study on *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola)*, the causal agent of septoria leaf blotch disease of wheat, by Guo et al (2005) showed it is possible to detect viable fungal inoculum using RT-PCR. Guo et al (2005) state that the technique was able to detect inoculum before visual symptoms were seen, however it is also affirmed that some spores used to initially inoculate the trial remained attached to leaf surfaces even after washing. It is possible that at least some of the pathogen mRNA detected whilst the disease was symptomless originated from the spores used to inoculate the plants. This study also does not investigate the possibility of a Real-time RT-PCR which would be useful in many ways. It is generally agreed that Real-time PCR is superior to conventional PCR in that it allows for easy quantification of material (Templeton et al., 2003) however the high sensitivity of PCR means that the presence of any genomic DNA in a RT-PCR reaction could result in false-positive signal (Simpkins et al., 2000). Another RNA-based method, nucleic-acid sequence based amplification (NASBA), has been used to detect many bacterial pathogens, including viable cells of *Ralstonia solanacearum*, and medically important *Candida* spp. but has not yet been validated for use on plant pathogenic fungi (Widjojoatmodjo et al., 1999; Simpkins et al., 2000; Bentsink et al., 2002). This method works by amplifying RNA below the melting temperature of DNA (Simpkins et al., 2000). It has, however, been suggested that NASBA is relevant only when used in comparing pre- and post- treatment scenarios which would limit its use in plant pathology (Birch et al., 2001).

Given the monocyclic nature of eyespot it is necessary to use molecular techniques which target mRNA to provide accurate predictions on the disease severity based on levels of viable inoculum, not just total inoculum present (Houseley & Tollervey 2009). The most economical and efficient way of quantifying RNA levels is likely to be via the two-step reverse transcriptase (RT-) PCR assay which uses reverse transcription enzymes to synthesize cDNA from fungal mRNA obtained via RNA extraction (Bleve et al. 2003). The same primers used for DNA analysis in quantitative PCR can then be used to quantify cDNA levels of the same target generated by the reverse transcriptase PCR.

Viability testing technology is frequently implemented in many sectors such as water and food safety, sterility testing, healthcare and agriculture (Nocker et al., 2007) but is less used in relation to plant health. Overestimation of the presence of pathogenic threat is common (Nocker & Camper, 2006) and can lead to unnecessary preventative measures being initiated. Accurate quantification of pathogen viability can potentially improve risk prediction and aid our understanding of pathogen survival and disease initiation and development.

3 Aim and Objectives

This project aimed to utilise existing, and develop new, molecular methods to study eyespot disease epidemiology.

The objectives were:

1. To determine the impact of agronomy factors on DNA of *Oculimacula* spp. quantified by Real – time PCR in roots and stems of wheat.
2. To quantify the effect of eyespot disease and fungicide treatment on the efficiency of photosystem (PS) II, estimated using chlorophyll fluorescence, and the yield of naturally infected winter wheat by *Oculimacula* spp.
3. To develop Real-time RT-PCR assay for the quantification of live inoculum of *O. yallundae* and *O. acuformis*.

IMPACT OF AGRONOMY FACTORS ON OCULIMACULA SPP. QUANTIFIED IN STEMS AND ROOTS OF WHEAT USING REAL-TIME PCR

A crop survey to investigate stem base diseases was carried out in 50 winter wheat (WW) fields distributed throughout England in 2011/12 season (Figure 1). Thirty six randomly selected winter wheat plants were collected from each field at growth stage (GS) 21-31, GS 39-44 and GS 65-77 across an area of 1 ha. Real-time PCR assays were used to identify and quantify both *O. acuformis* and *O. yallundae* (R-Type and W-Type eyespot, respectively) from the roots, 5 cm stem and 10 cm stem (Figure 2). This was to monitor changes in their populations from GS21-31 to GS 65-77. Agronomic information was collected, for example seed treatment, cultivation, previous crop, soil texture, fungicide treatment at GS 21-33 (T1), fungicide treatment at GS 33-39 (T2), sowing date and 1st WW, 2nd WW and CW (monoculture/continuous wheat).

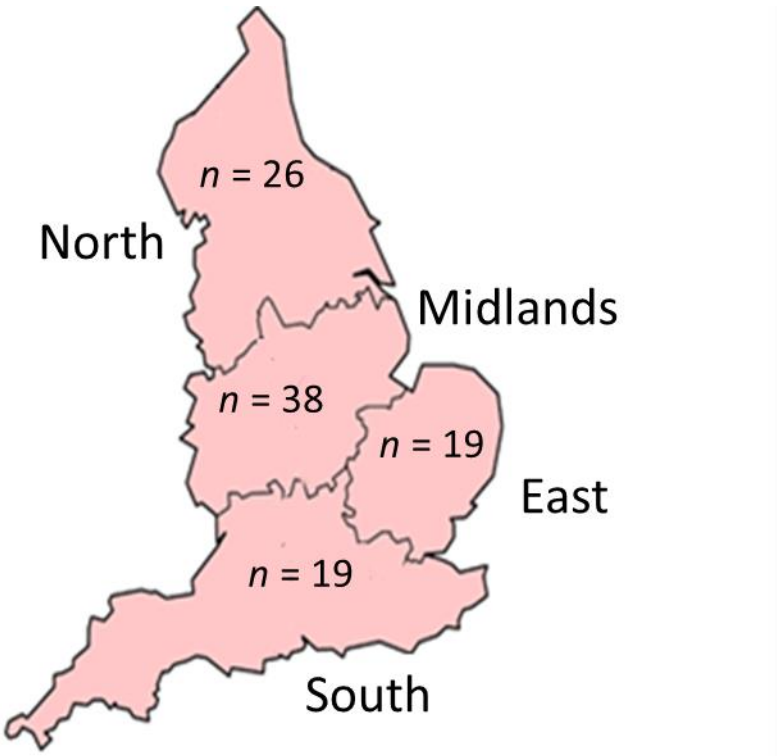

Figure 1. Map of England showing locations and numbers (*n)* of the fields sampled during the crop survey in 2011/2012.

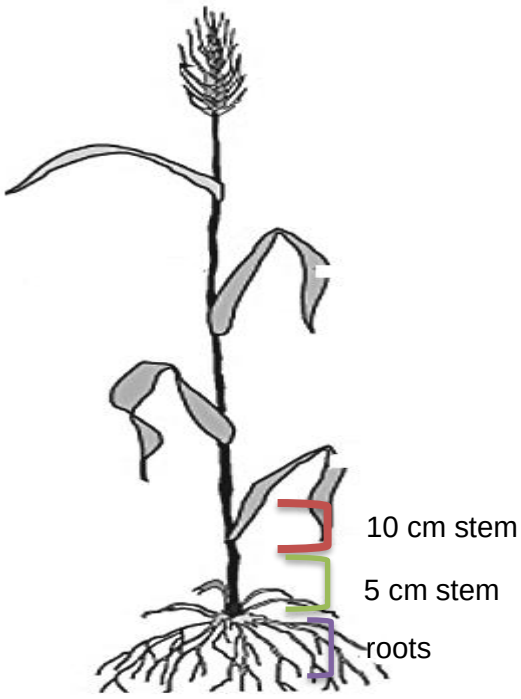

Figure 2. Diagram showing the size and type of wheat tissues sampled during the crop survey in 2011/2012.

Sampling and DNA extraction

Thirty six samples of crop material were removed from an area of one hectare in a gridline design from each sampled field. A gridline design was chosen to allow changes in populations of the pathogen to be monitored over time. The roots and stems were thoroughly washed to remove soil and debris then visually assessed for eyespot symptoms (Ray et al. (2004). The roots and stems were freeze-dried then stored for DNA extraction.

Stem bases used for disease assessments were prepared immediately afterwards for DNA extraction. The regions of each plant (Figure 2) were chopped finely, freeze-dried and milled to a powder in a ball mill before DNA extraction as described by Ray et al. (2004). Total DNA was measured by spectrophotometry (Beckman Instruments Inc.) and diluted to a concentration of 20 ng ml $^{-1}$ prior to PCR amplification.

Quantitative Real-time PCR assays for stem bases diseases

DNA samples of *O. acuformis* and *O. yallundae* were analysed by real-time Polymerase chain reaction (qPCR TaqMan®) in 96 well plates using the Biorad CFX 96. Primers, probe and qPCR conditions have been previously described in Walsh et al (2005).

Statistical analysis

Data were analysed using analysis of variance (ANOVA) or REML using Genstat (version 5 release 4.1). DNA quantities were analysed as pg fungal DNA ng^{-1} total DNA following log10 transformation. Relationships between disease score and pathogen DNA were determined by regression analyses.

RESULTS

Oculimacula species occurred in more than 76% of all tissues (roots, 5 cm and 10 cm stems) throughout the season (Table 1). The highest incidence of 98% was at GS 65-77 in roots and stem bases. DNA of both species was also quantified in roots with *O. yallundae* predominating (Table 2). However, on stems (5 cm and 10 cm) at GS 37-45 and 65-77 DNA of *O. acuformis* was 5-fold higher than *O. yallundae* (Table 2).

Table 1. Incidence of *O. yallundae* and *O. acuformis* DNA in the roots and stems of wheat at GS 21-31, GS 37-45 and GS 65-77.

	Incidence (%)					
	roots		5 cm stem		10 cm stem	
GS (n)	***O. y***	***O. a***	***O. y***	***O. a***	***O. y***	***O. a***
21-31(45)	93	96	96	96	-	-
37-45 (50)	88	90	96	96	67	80
65-77 (50)	98	98	98	98	98	76
Mean	93	95	97	97	83	78

O. y – Oculimacula yallundae, O.a – Oculimacula acuformis;

Factors influencing DNA in roots

Previous crop was the main factor influencing the quantity of pathogen DNA found in roots, with winter wheat roots accumulating significantly more DNA of *O. yallundae* (Figure 3A) and *O. acuformis* (Figure 3B) following wheat compared to other crops and legumes, respectively.

Table 2. Concentrations of *O. yallundae* and *O. acuformis* DNA in the roots and stems of wheat at growth stage (GS) 21-31, GS 37-45 and GS 65-77.

	Log 10 DNA (pg/ng $^{-1}$of Total DNA)					
	roots		5 cm stem		10 cm stem	
GS	***O. y***	***O. a***	***O. y***	***O. a***	***O. y***	***O. a***
21-31	-2.686	-2.173	2.411	-2.308	-	-
	(0.002)	(0.006)	(0.003)	(0.004)		
37-45	-2.136	-2.178	-2.04	-1.23	-3.26	-2.76

	(0.007)	(0.006)	(0.009)	(0.058)	(0.001)	(0.017)
65-77	-0.335	-1.285	0.627	0.74	-2.82	-1.54
	(0.462)	(0.052)	(0.236)	(5.495)	(0.001)	(0.028)
Mean	-1.719	-1.879	-1.693	0.933	-3.04	-2.15
	(0.019)	(0.013)	(0.02)	(0.116)	(0.001)	(0.007)

	SED	***P value***	**SED**	***P value***	**SED**	***P value***
GS	0.1756	<.001	0.2234	<.001	0.21	<.001
Species	0.1434	0.266	0.1824	<.001	0.21	<.001
GS x Species	0.2484	<.001	0.3159	0.019	0.297	0.066

Back-transformed means are shown in parenthesis. *O. y – Oculimacula yallundae, O.a – Oculimacula acuformis;*

O. acuformis DNA was also influenced by the rotational intensity of wheat with increased biomass of the pathogen in roots following continuous wheat (monoculture) compared to first wheat (Figure 4).

DNA of *O. yallundae* in wheat roots in clay/clay loam soils was significantly higher than in sandy/silty loam soils (Figure 5A).

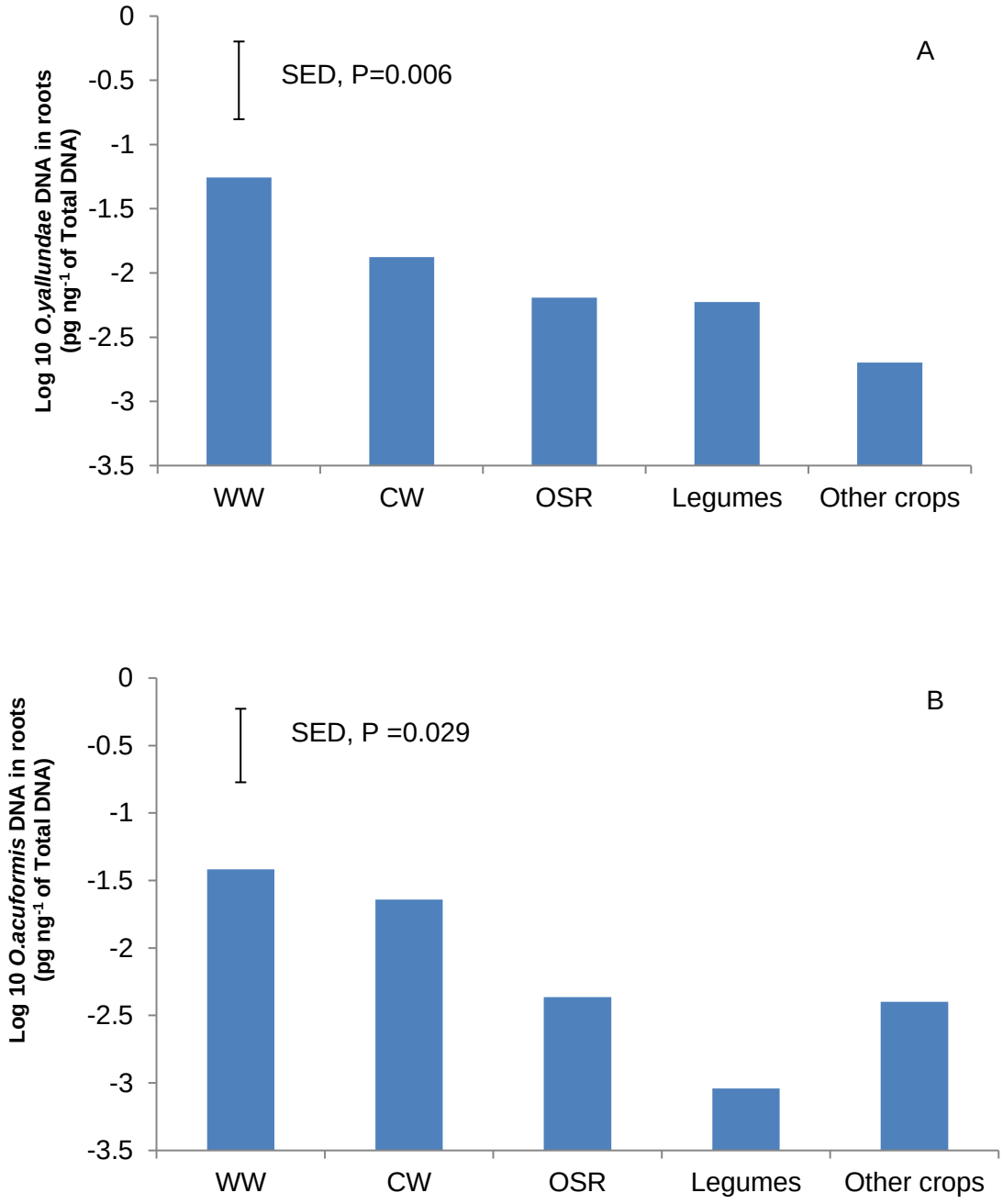

Figure 3. Effect of previous crop on DNA of *O. yallundae* (A) and *O. acuformis* (B) (pg ng^{-1} of Total DNA) in wheat roots.

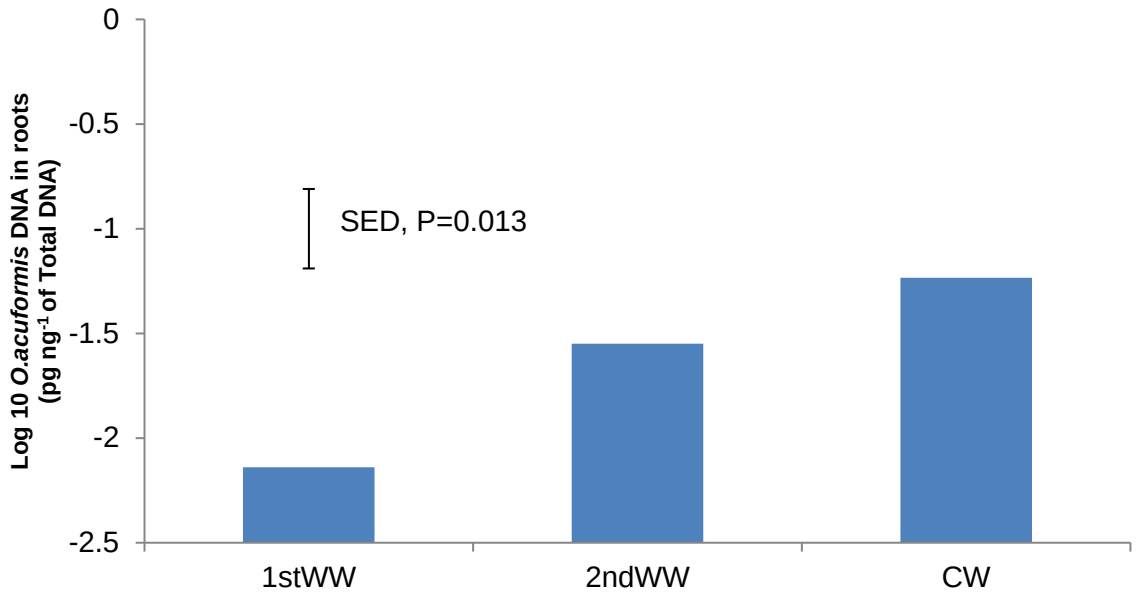

Figure 4. Effect of wheat rotational sequence on DNA of *O. acuformis* (pg ng^{-1} of Total DNA) in wheat roots.

The addition of chlorothalonil to triazoles in the fungicide spray at GS 31 also resulted in a significant increase of DNA of *O. yallundae* in roots by GS 65-77 (Figure 5B).

Factors influencing DNA in stem bases (5 cm)

The highest amounts of DNA of *Oculimacula* spp. was found following winter wheat (Figure 6). Stem-base DNA of *O. yallundae* was only reduced when following crops other than wheat, legumes or OSR (Figure 6A) whilst DNA of *O. acuformis* was significantly lower following OSR, legumes or other crops (Figure 6B) compared to following wheat.

DNA in 5 cm stem from the base of both *Oculimacula* spp. was influenced by the rotational intensity of wheat (Figure 7). However subtle differences between species were found, with DNA being significantly lower in first wheats than in second wheats for *O. yallundae* (Figure 7A), whilst for *O. acuformis* differences were only significant when DNA concentrations in first or second wheat were compared to continuous wheat (Figure 7B).

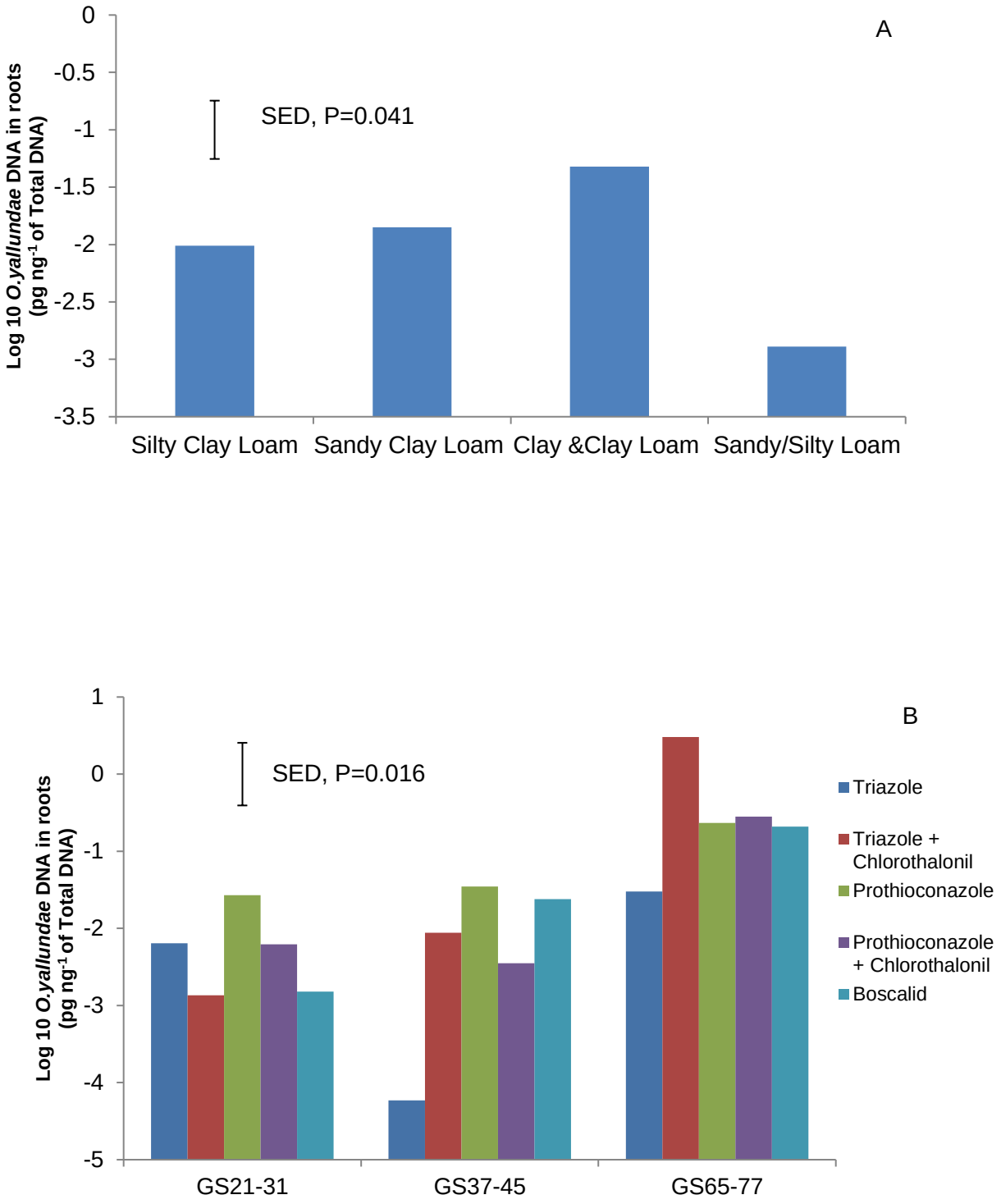

Figure 5. Effect of soil texture (A) and fungicide treatment at GS 31 (B) on DNA of *O. yallundae* (pg ng^{-1} of Total DNA) in wheat roots.

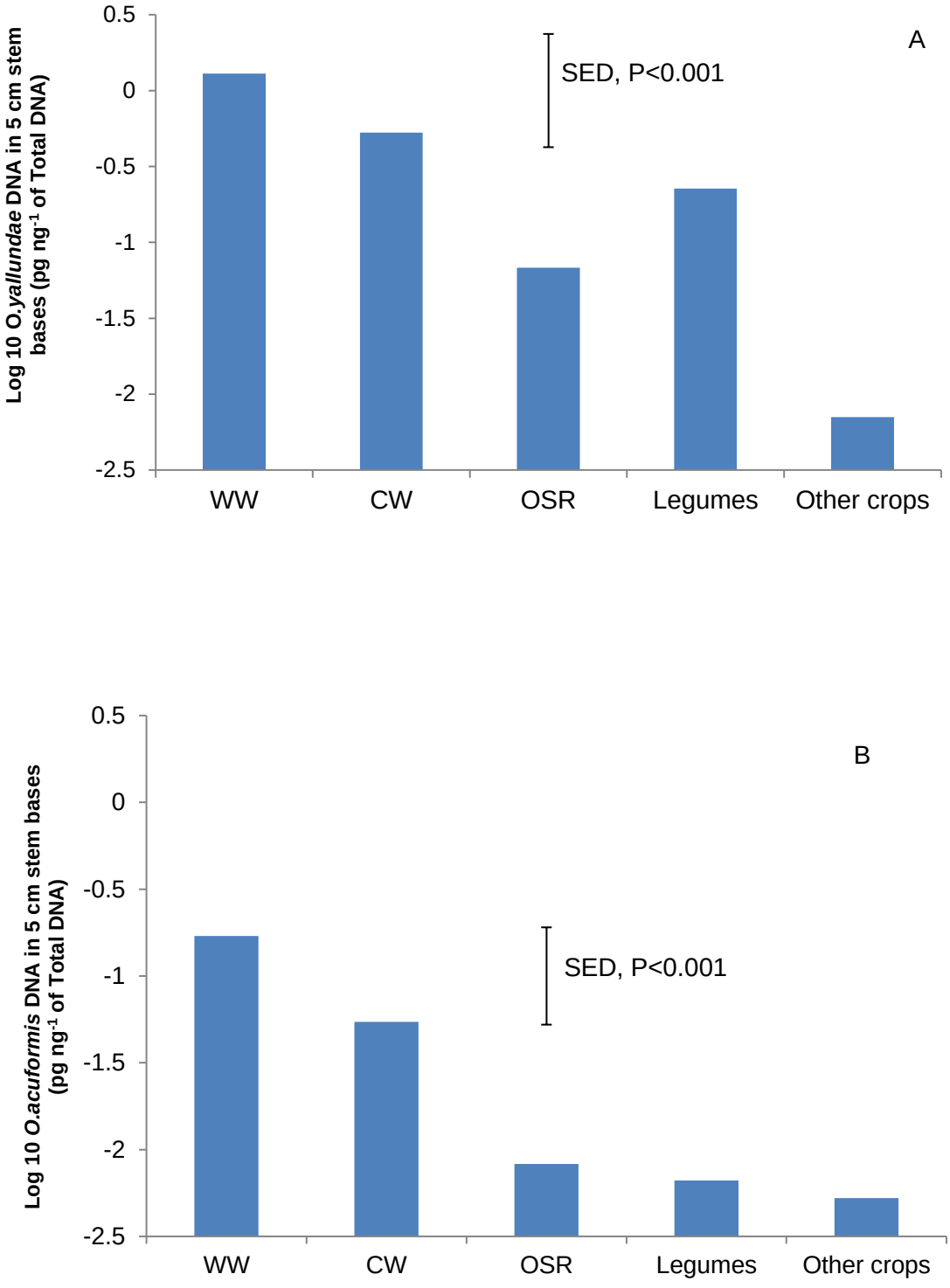

Figure 6. Effect of previous crop on DNA of *O. yallundae* (A, pg ng^{-1} of Total DNA) and *O. acuformis* (B) in wheat stem bases (5 cm).

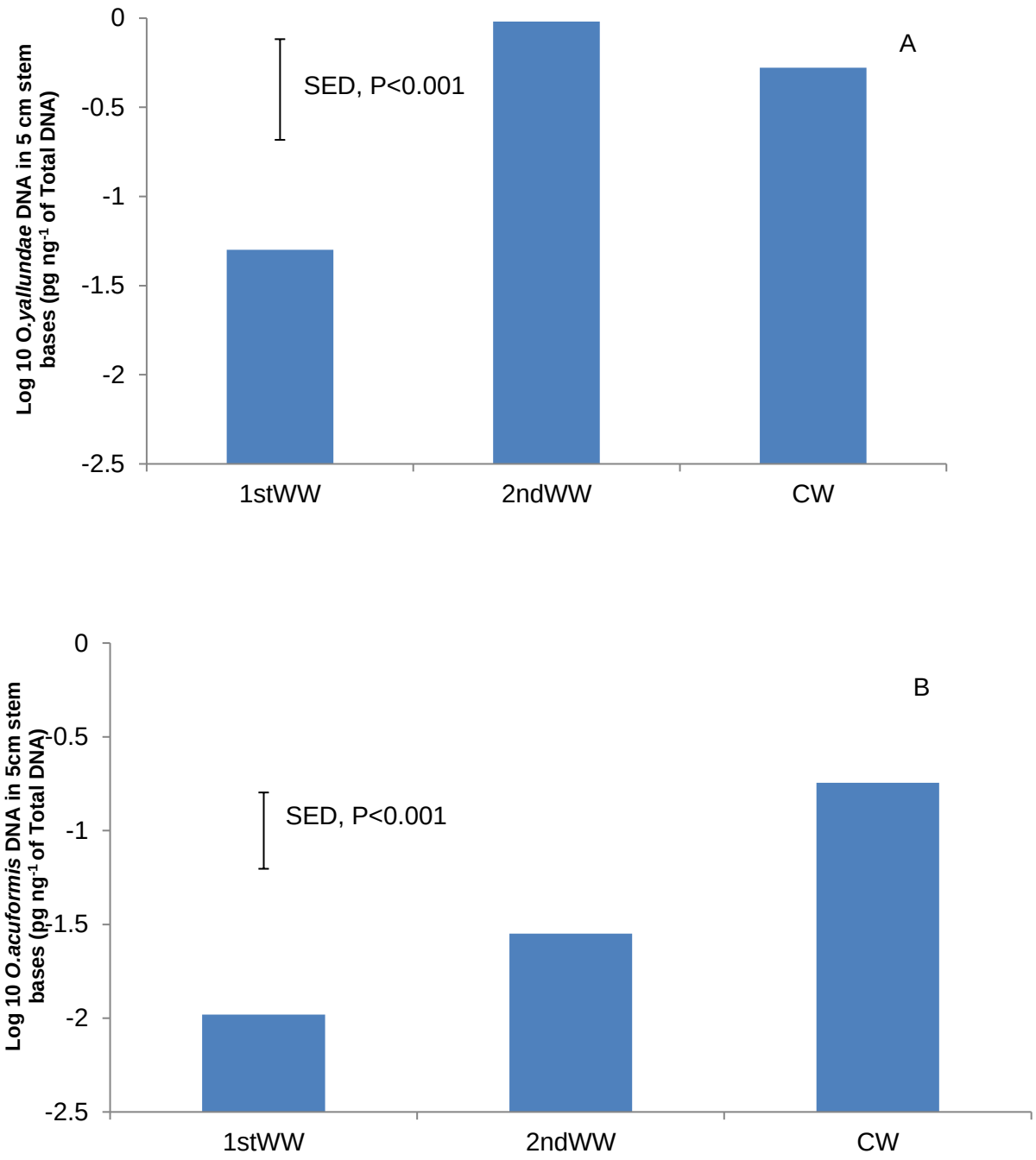

Figure 7. Effect of wheat rotational sequence on DNA of *O. yallundae* (A, pg ng^{-1} of Total DNA) and *O. acuformis* (B) in wheat stem bases (5 cm).

The following seed treatments were included in the model, Redigo Deter (Bayer CropScience, 50 g/L (4.3% w/w) prothioconazole and 250 g/L (21.4% w/w) clothianidin), Redigo (Bayer CropScience, prothioconazole 100 g/L), Latitude (Certis, 125 g/L (11.83 % w/w) of silthiofam) and a mixture of Redigo Deter and Latitude. Prothioconazole included in Redigo was found to significantly reduce the accumulation of DNA of *Oculimacula* spp. in stems up to 5 cm from the base (Figure 8). However, inclusion of clothianidin or silthiofam where prothioconazole dose was reduced resulted in greater

DNA accumulation of *O. yallundae* by GS 65-77 (Figure 8A), whilst for *O. acuformis* this was only observed when silthiofam was used alone as a seed treatment (Figure 8B).

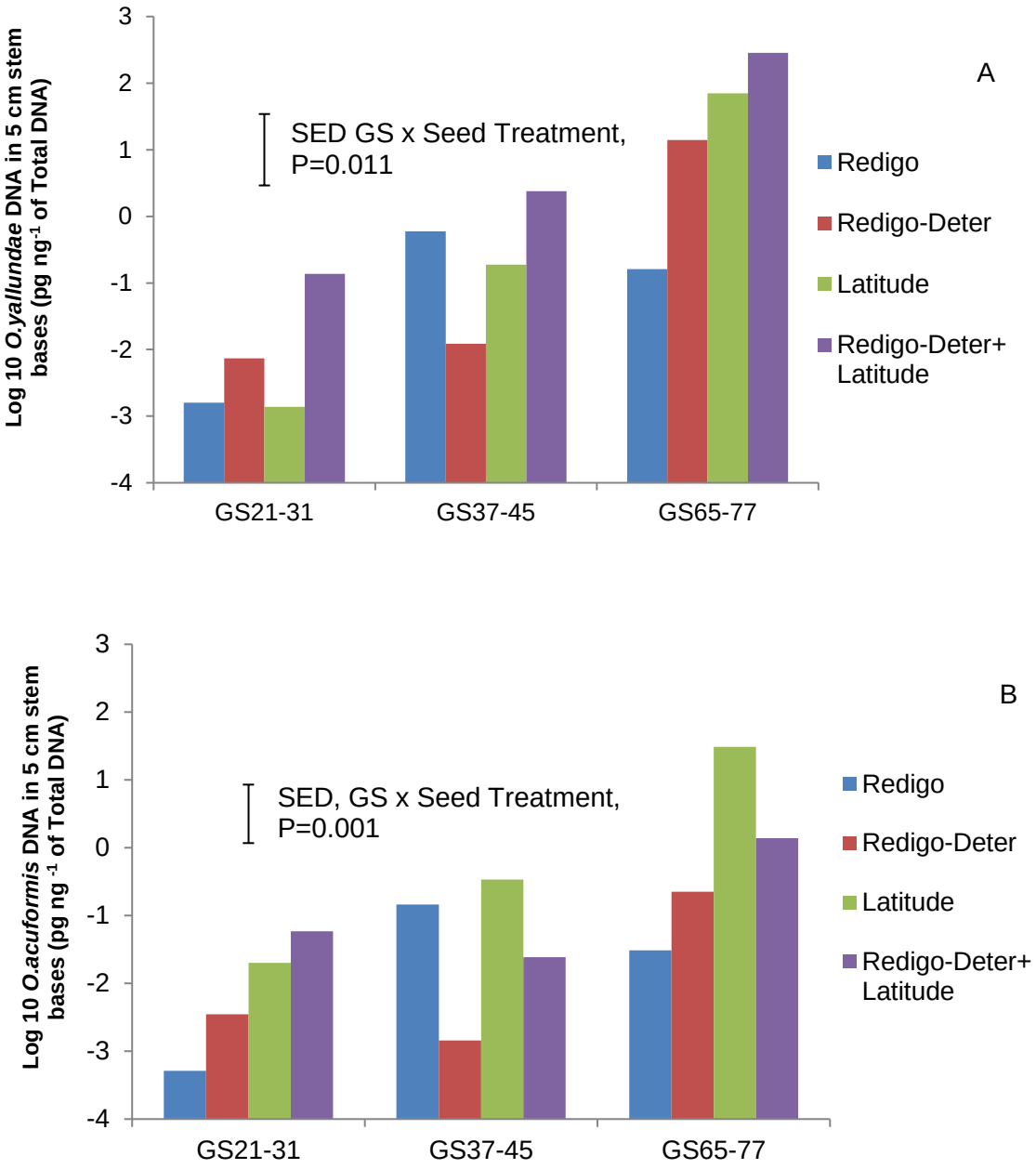

Figure 8. Effect of seed treatment on DNA of *O. yallundae* (A, pg ng^{-1} of Total DNA) and *O. acuformis* (B) in wheat stem bases (5 cm).

Factors influencing DNA in stem bases (10 cm)

The only agronomy factor influencing pathogen DNA in the upper 10 cm of the base of wheat stems was previous crop (Figure 9). OSR or other crops than legumes or wheat reduced DNA of *O. yallundae* (Figure 9A). In contrast, significantly less DNA of *O. acuformis* was quantified compared to wheat following legumes (Figure 9B).

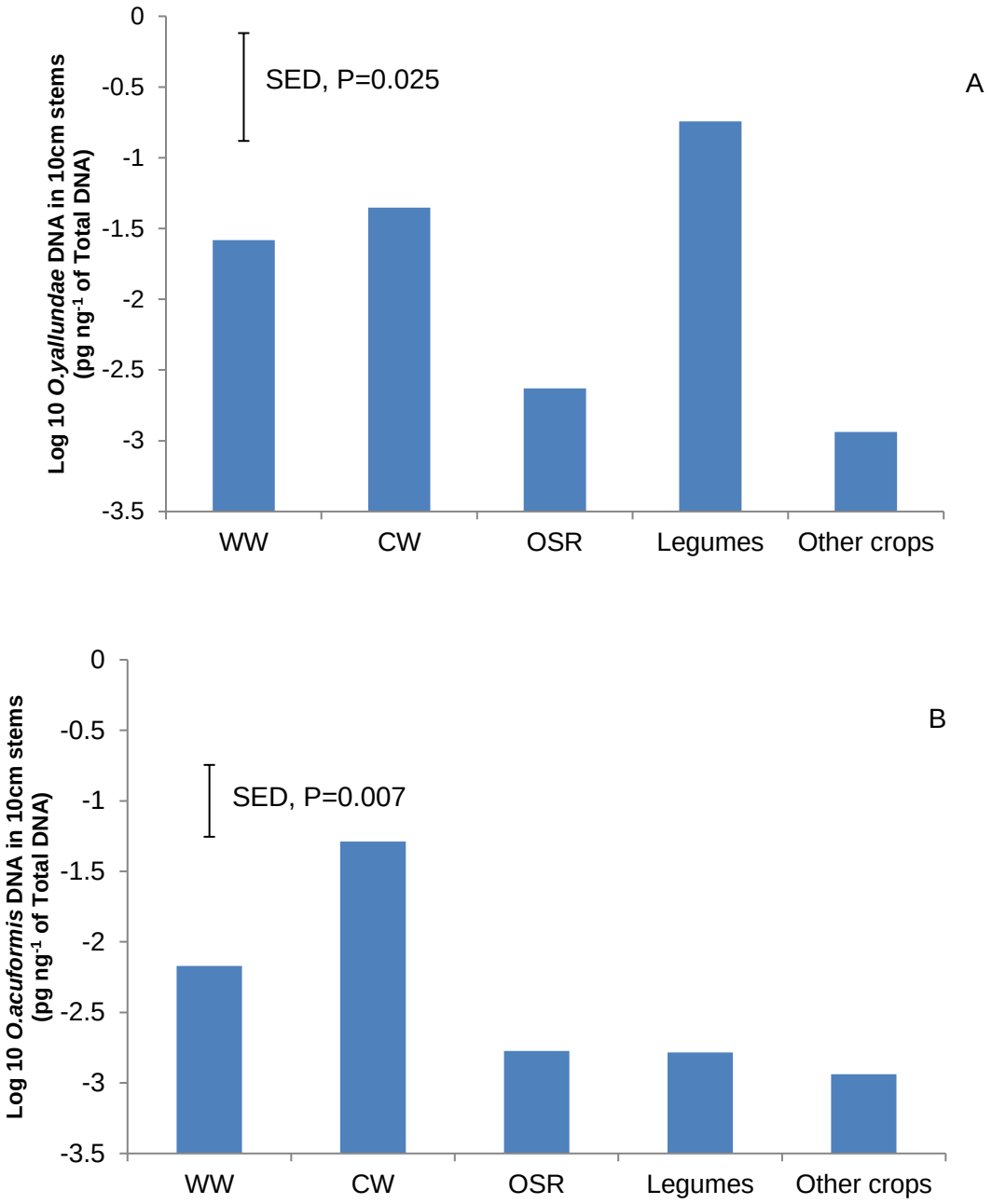

Figure 9. Effect of previous crop on DNA of *O. yallundae* (A, pg ng^{-1} of Total DNA) and *O. acuformis* (B) in wheat stems (10 cm).

EFFECT OF EYESPOT DISEASE AND FUNGICIDE TREATMENT ON THE EFFICIENCY OF PHOTOSYSTEM II AND YIELD OF NATURALLY INFECTED WINTER WHEAT

Experiments on winter wheat cv. Gallant, naturally infected by *Oculimacula* spp., were carried out at the University of Nottingham, Sutton Bonington Campus, UK in the 2010-2011 cropping season. Plots (2 x 12 m) were established as a second wheat following winter wheat. Fungicide treatments were applied at GS 32 or GS 39 with the aim of establishing a range of eyespot severity and to

evaluate the effect of timing of fungicide efficacy. The experimental design was factorial with two factors, fungicide application at GS 32 or at GS 39. The treatments included untreated control, Opus applied at 0.75 l ha^{-1} (epoxiconazole 93.75 g l^{-1}, BASF plc) or Opus 0.25 l ha^{-1} + Tracker 1 l ha^{-1} (epoxiconazole 98.25 g l^{-1}+ boscalid 233 g l^{-1}, BASF plc). Each treatment combination was replicated four times in randomised blocks.

One application of Amistar Opti (100 g l^{-1} azoxystrobin + 500 g l^{-1} chlorothalonil; Syngenta UK Ltd) was applied to all plots prior to GS 32 with the aim of controlling foliar pathogens. Other agronomy was as per standard farming practice.

Soil sampling

At GS 30, before fungicide application, soil was sampled over the trial site in a 'w' shaped sampling path with approximately 30 g soil taken at ten sampling points. Soil samples were allowed to dry at room temperature for 48 h in open bags and fragments of crop debris larger than 0.5 cm were removed by sieving. Soil (250 g) was used for DNA extraction. Soil was homogenised in Nalgene bottles containing ball bearings and cetyl-trimethyl ammonium bromide (CTAB) buffer and was further processed using a method developed at FERA (Sand Hutton, York, UK). DNA was eluted with a Kingfisher® mL (Thermo Electron) using MagneSil® paramagnetic particles (Promega UK Ltd.) and pathogen DNA quantification carried out per gram of soil. At GS 51 individual plots were sampled in the same manner.

Fluorescence measurements

Fluorescence was measured at GS 69 using the FluorPen FP 100-MAX (Photon Systems Instruments, Brno, Czech Republic). Twenty plants per plot were randomly selected and chlorophyll fluorescence induction kinetics measurements, including performance index measurements, were carried out on the flag leaf as per manufacturer's instructions. Performance index (Pi_Abs) combines three parameters:- density of PSII reaction centres, ability to feed electrons between PSII and PSI and trapping rate of PSII reaction centres, thus providing an overall estimate of crop photosynthetic efficiency.

Disease assessments and DNA quantification

At GS 87, whole plants were collected and secondary tillers discarded. Disease assessment was made on the main shoot using the ordinal rating scale of eyespot lesion severity, where 1 was slight, 2 was moderate and 3 was severe; according to the amount of stem girdling and softening as defined by Goulds & Polley (1990). Main shoots were then cut to 5 cm long stem bases, grouped according to their disease scores (slight, moderate or severe) and processed for DNA extraction using the method described by Ray et al. (2004). Total DNA in the extracts was measured using the Nanodrop

1000 (Thermo Fisher Scientific Inc., USA). Pathogen DNA was quantified using Real-time quantitative PCR assay using iQ supermix (Bio-Rad Laboratories Inc., UK). PCR conditions, probes and primers were used as described by Walsh et al. (2005).

RESULTS

At GS 30, DNA of *O. acuformis* and *O. yallundae* quantified in soil was 0.144 pg g^{-1} and 6.012 pg g^{-1}, respectively.

At GS 51, DNA of *O. acuformis* in soil was 86% lower in plots treated with epoxiconazole + boscalid at GS 32 than in untreated plots (Table 3). There was no significant effect of fungicide application on DNA of *O. yallundae* in soil (Table 3). Less DNA of both species was quantified in stems treated with epoxiconazole + boscalid at GS 32, however reductions were only significantly different from the untreated control for DNA of *O. yallundae* (Table 3).

ANOVA revealed that there were no significant interactions between timing of fungicide application for any of the collected data. Fungicide application at GS 39 failed to reduce disease score or raise the performance index (data not shown). Fungicide application at GS 32 was most effective in reducing disease severity and increasing performance index at GS 69 (Figure 10). Application of epoxiconazole + boscalid at GS 32 reduced disease score by 25% and increased performance index by 27% compared to untreated control (Figure 10). There were no significant differences between untreated and epoxiconazole only treatment for disease score or performance index.

There were no significant relationships between DNA quantity for individual *Oculimacula* spp. in soil at GS 51 and in stem at GS 87 (results not shown). However regression analysis showed a weak but significant relationship accounting for 14% of the variance between total *Oculimacula* DNA quantified in stems and visual disease score (Y= 0.65 (log10 total stem DNA) + 2.06, P = 0.029).

Regression analysis also revealed a significant relationship between disease score at GS 87 and yield, accounting for 24% of the variance (Y= 1.82 (DS at GS 87) + 14.8, P=0.005).

Table 3. Log10 transformed pathogen DNA quantified in soil and in stems, at GS 51 and at GS 87 respectively, and yield of cv. Gallant. Back-transformed means are shown in parentheses.

	Log10 Pathogen DNA (pg g^{-1})				
	Soil		Stems		Yield
Treatment a.i. (g a.i. ha^{-1})	***O.a***	***O.y***	***O.a***	***O.y***	**t ha^{-1}**
untreated	-0.54 (0.29)	-0.03 (0.93)	4.43 (27.04)	5.08 (120.23)	12.20
epoxiconazole (93.75)	-1.18 (0.07)	-0.40 (0.40)	4.54 (34.51)	4.95 (88.51)	12.61
epoxiconazole (98.25) + boscalid (233)	-1.32 (0.05)	-0.26 (0.55)	4.13 (13.37)	4.83 (67.45)	12.92
P	0.05	0.50	0.117	0.032	0.31
LSD	0.661	0.658	0.4088	0.1822	0.969
CV	11.8	13.6	9.4	3.7	7.7

O.a - O. acuformis, *O.y - O. yallundae*.

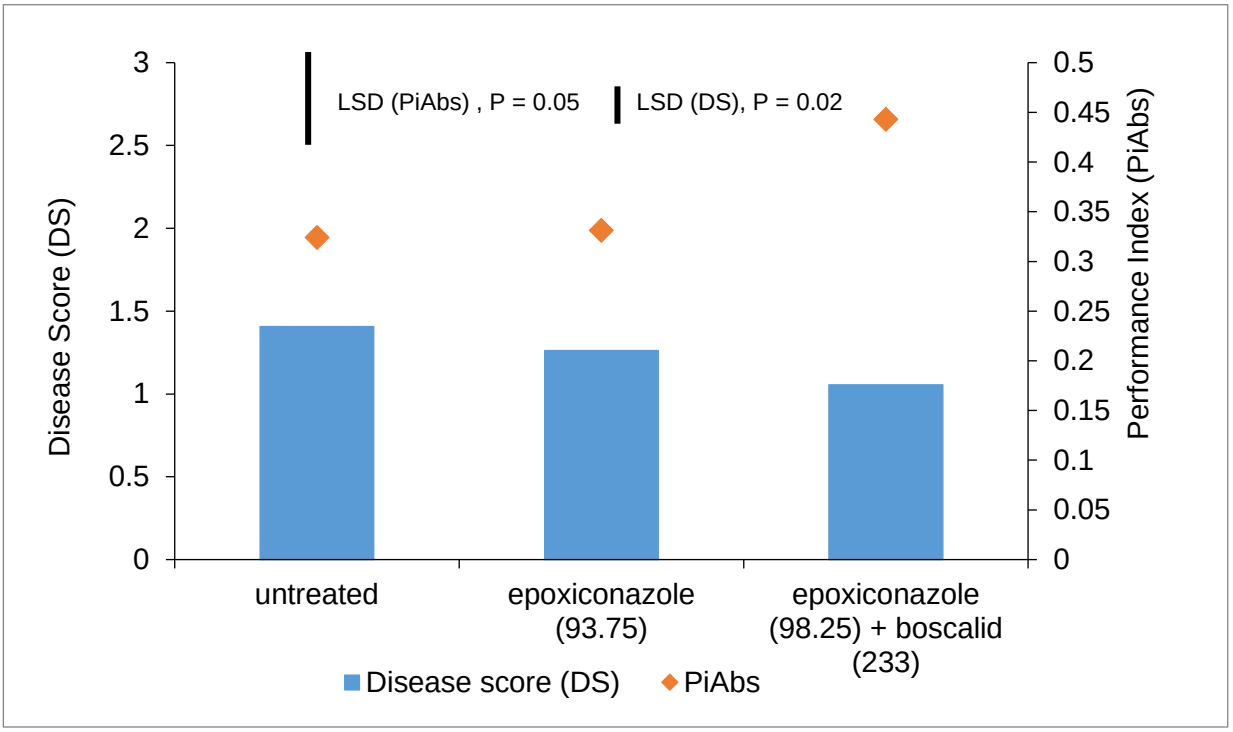

Figure 10. Eyespot disease score at GS 87 and performance index at GS 69 for winter wheat, cv. Gallant treated with different fungicides at GS32.

DEVELOPMENT OF REVERSE TRANSCRIPTASE (RT)-PCR ASSAY FOR THE QUANTIFICATION OF LIVE INOCULUM OF O. YALLUNDAE AND O. ACUFORMIS

Culturing Oculimacula yallundae *and* Oculimacula acuformis

Oculimacula yallundae and *O. acuformis* isolates (509 and 510, respectively from the culture collection at the University of Nottingham) were grown on potato dextrose (PD) agar for 7 days at room temperature. Conical flasks containing 100ml of liquid PD broth media were inoculated with ten plugs with one cm diameter from individual isolates. Cultures were subjected to agitation via an orbital shaker to ensure consistent, even hyphal growth and left to grow for two weeks until considerable hyphal growth was present. These cultures were used for RNA and DNA extraction.

RNA extraction

RNA was extracted using the RNease Plant Mini-kit (Qiagen) from fungal hyphae grown in liquid broth media. Before extraction, all equipment was sterilised and wiped with 70% ethanol to remove as much RNase as possible and therefore reduce the possibility of RNA degradation or contamination with RNase. Fungal hyphae ~100mg was taken from the broth and ground using a pestle and mortar in liquid nitrogen until a fine powder. This was then transferred using a sterile spatula directly into pre-cooled, 2ml Eppendorf tubes which contained the Qiagen RNeasy Plant Mini-kit lysis buffer and placed on ice. The rest of the extraction was carried out as per the manufacturer's instructions. This included an on-column DNA digestion step using Qiagen's RNase-free DNase Set, which contains DNase I. This step allows for the removal of most of the genomic DNA which is often also extracted using the RNeasy Plant Mini-kit as a contaminant and can be picked up by qPCR as positive amplification. RNA was quantified using a spectrophotometer and diluted to 20ng stock solutions. In the preparation of standards for quantification, 1ng solutions were made, followed by four subsequent 10-fold dilutions. RNA was stored at -80^{o}C.

DNA extraction

DNA was extracted from ~100mg fungal hyphae grown in liquid broth culture as described above to verify cDNA amplification by 2-step RT-PCR as well as generate standards for DNA quantification. DNA was extracted using the DNeasy Plant Mini-kit (Qiagen). The hyphae were ground in liquid nitrogen using a mortar and pestle until a fine powder. The rest of the extraction was carried out as per the manufacturer's instructions. DNA concentrations were determined using spectrophotometer and diluted to 20ng stock solutions. Standards were created from 1ng stock solution and four subsequent 10-fold dilutions. DNA was stored at -4^{o}C.

Reverse-Transcriptase (RT)-PCR

RT-PCR was carried out in 8-well PCR strips with a 20µl total reaction volume. The reaction mix consisted of 4µl iScript enzyme mastermix (Bio-rad), 1µl reverse-transcriptase, 2µl RNA template and 13µl Rnase-free water. The RT-PCR was carried out at the following conditions; 5 minutes at 25^{o}C, 30 minutes at 42^{o}C, 5 minutes at 85^{o}C with an optional indefinite hold temperature of 4^{o}C. The resulting cDNA was used immediately as the template for quantitative Real-time PCR analysis.

Real-time qPCR

Real-time qPCR was carried out in 96 well plates with a 25µl total reaction volume. The total reaction volume consisted of 12.5µl IQ supermix (Bio-rad), 7.175µl molecular grade water, 0.15µl of forward primer, 0.15µl of reverse primer and 0.025µl probe. PCR conditions, primers and probes were used as described by Walsh et al. (2005). Results were observed using CFX^{TM} Manager (Bio-rad). In each individual qPCR, DNA and/or cDNA standards were run in duplicate along with no template and positive controls for each step of the RT-PCR.

Viability validation

To check for the amplification of residual genomic DNA with extracted RNA, further *in vitro* studies were carried out by exposing live fungal hyphae in PD broth to temperatures of 20^{o}C, 40^{o}C or 60^{o}C for 2 hours and leaving them overnight to achieve fungal death and RNA degradation at least for the last two temperature treatments. Two replicates of isolate of each *Oculimacula* species were grown for two weeks in PD broth as described above for the RNA extraction. Flasks were then exposed to 20^{o}C, 40^{o}C or 60^{o}C and agitated for 2 hours and left to stand overnight prior to RNA extraction. Two step RT-PCR as described above was performed on each fungal extract from the different temperature treatment and quantitative PCR performed on the RNA to check for residual genomic DNA amplification.

RESULTS

cDNA Standard sensitivity and viability validation

Amplification of cDNA after 2-step RT-qPCR started at 19 cycles with expected intervals of 3-4 cycles generating a standard curve from 1 ng to 0.0001ng of cDNA for both *Oculimacula* species (Figure 11). However, residual genomic DNA in the RNA stocks also showed amplification without RT treatment. The time of amplification of genomic DNA and cDNA differed and showed a consistent 100-fold difference in amplification in favour of cDNA (Figure 11B, D).

Following temperature treatments, amplification of cDNA from 20 ng of RNA stocks of samples showed amplification starting at 16 cycles for treatment of 20^{o}C increasing to 23 cycles for treatment at 60^{o}C for 2 hours (Table 4). Late amplification >33 cycles was observed for the same samples of

RNA without RT treatment suggesting that there was traces of genomic DNA in turn indicating that the amplification of cDNA was the results of efficient RT of RNA (Table 4).

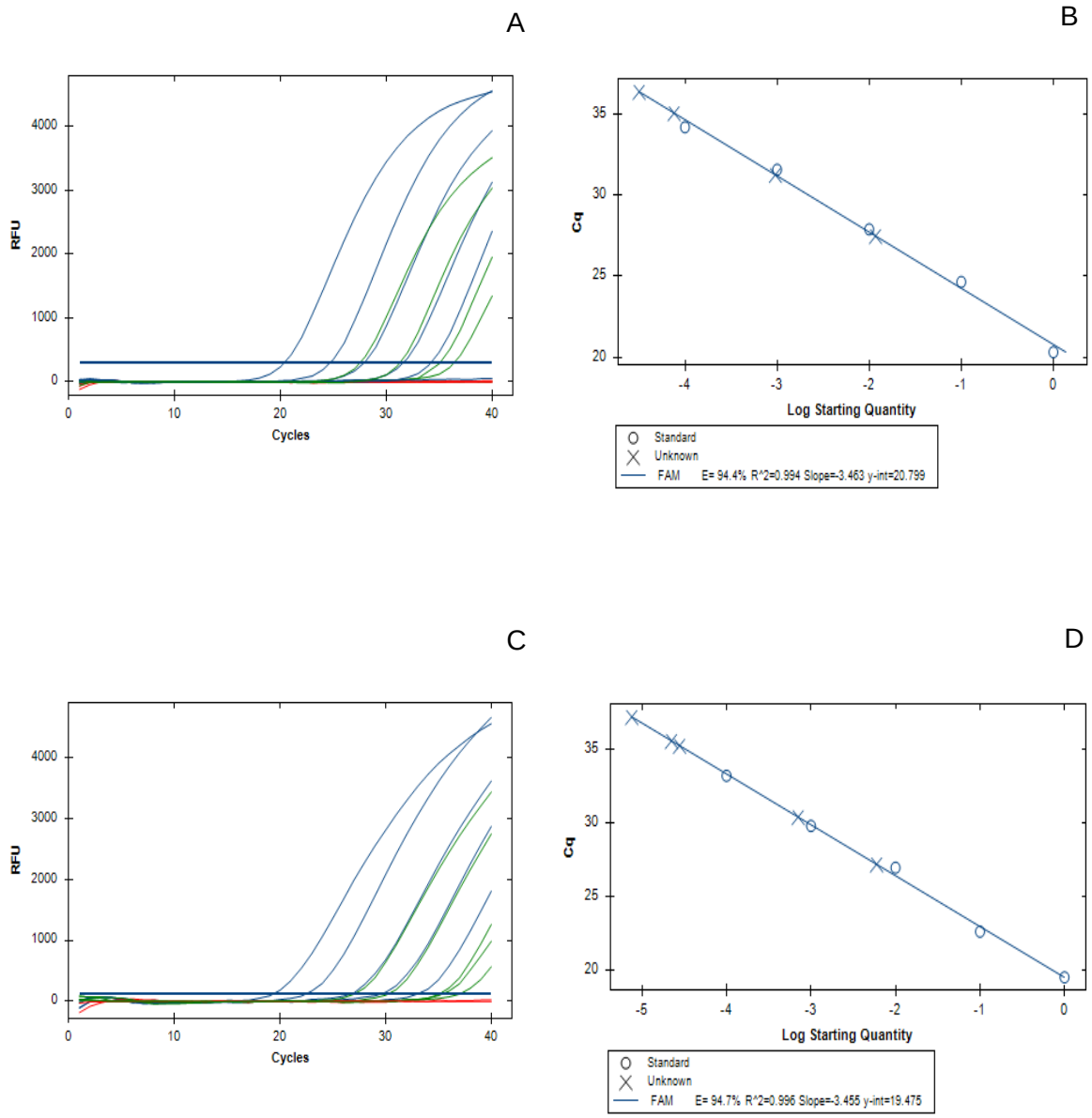

Figure 11. Relative fluorescence (RFU) and standard curves showing cycle of quantification (Cq) and in the box -PCR efficiency with regression parameters for quantification for *O. yallundae* (A-B) and *O. acuformis* (C-D). The unknowns in green are standards of pure RNA extractions from 1 ng diluted 10-fold showing amplification of genomic DNA, in red - water (-) control.

Table 4. Cycles of quantification (Cq) for cDNA fungal standards, cDNA and pure RNA from fungal hyphae of *O. yallundae* (*O.y*) and *O. acuformis* (*O.a*) treated with different temperatures for 2 hours.

Sample	Cq	
ng	*O. a*	*O. y*
Standards		
1	19.01	19.05
0.1	22.48	23.42
0.01	28.05	27.03
0.001	30.74	30.30
0.0001	34.19	34.08
cDNA (20)		
20ºC	16.02	15.05
20ºC	16.07	15.11
40ºC	18.70	17.73
40ºC	19.39	18.71
60ºC	21.54	21.39
60ºC	23.39	18.55
RNA (20)		
20ºC	34.79	33.20
20ºC	N/A	34.38
40ºC	N/A	33.98
40ºC	38.47	34.61
60ºC	35.29	35.32
60ºC	32.17	36.56
- **Ctrl**	N/A	N/A

4 Main Conclusions

These studies have shown that quantifiable DNA of both *Oculimacula* species is found in soil, roots and up to 10 cm from the base in the stems of winter wheat. It is therefore likely that the pathogens are capable of survival within the environment for longer periods of time than previously thought. Further studies using RT-PCR will be necessary to show how viable the inoculum is in different environments and type of soils. Although the number of sampled fields from the survey was adequate, the analysis was carried out using data collected in just one year and therefore conclusions must be made with caution. With this limitation in mind, results show that *O. acuformis* is generally more widespread as it was found in higher incidence in all tissues of sampled plants in wheat and remains the predominant species on stems up to 10cm from the base. However, *O. yallundae* was found in greater biomass in roots and soil.

The main agronomy factor influencing the quantity of pathogen DNA in all tissues was previous crop. DNA accumulation of both species was greatest following wheat although second wheat appear to select more for *O. yallundae* in stem bases whilst continuous wheat more for *O. acuformis*. In all tissues, other crops (maize or oats) were effective at reducing DNA of *O. yallundae*, whilst *O. acuformis* accumulated less also following OSR or legumes. Soil texture affected DNA of *O. yallundae* in roots, with sandy/silty loams accumulating less fungal biomass compared to heavier soils such as clay/clay loams. This may be related to the moisture and nutrient content of different soils and their ability to support fungal growth and favour infection. Seed treatment and fungicide application at GS 32 had significant effects on DNA of both species. Seed treatment where prothioconazole was used alone was most consistent in keeping a lower rate of accumulation of DNA of both species over the growing period. Silthiofam alone increased the DNA of *O. acuformis* at the end of the season, most likely indirectly, through removal of other competitors of *O. acuformis* as the fungicide is active against *Gaeumannomyces graminis* var. *tritici* causing take all on wheat that occupies the same space on stem bases and roots as *Oculimacula* species. Triazole application at GS 31 reduced DNA of *O. yallundae* in roots whilst boscalid + epoxiconazole reduced disease severity by 25%, DNA of *O. yallundae* in stems by 5% and DNA of *O. acuformis* in stems by 7% when compared to the untreated. Although there were no significant differences in final yield between treatments, boscalid + epoxiconazole treated plots yielded more than untreated or epoxiconazole only treated plots. Application of boscalid + epoxiconazole also increased the performance index of plants significantly compared to the untreated control. The observed increase in performance index in this case may be due to direct effects of the fungicides on PSII photochemistry, or due to indirect effects via reductions of disease and/or fungal biomass in stems. Boscalid is a succinate dehydrogenase inhibitor and fungicides of this class have been shown previously to positively act on PSII efficiency of wheat even in absence of disease.

The successful generation of cDNA standards and subsequent standard curves for both *O. yallundae* and *O. acuformis* allows for accurate quantification of viable spores or cells of the two causative agents of eyespot disease, *O. yallundae* and *O. acuformis.* Although genomic DNA was present in the standards as a contaminant from the RNA extraction process, cDNA was consistently and preferentially amplified by a factor of 100. This allowed for distinction between cDNA and genomic DNA. Furthermore, the validation studies showed that genomic contamination of DNA in the RNA stocks was low and efficiently removed by treatment with DNase I. However, due to the unstable nature of RNA and the susceptible nature of the RNA extraction process to RNase contamination, further degradation of RNA as well as RNA purity issues may affect the accuracy of quantification. For future studies relevant for field situations, RNA extraction will need to be modified and improved in speed and sample size prior to quantification. Potentially the developed assay here can be used to understand the survival of *Oculimacula* spp. in different soils/fields and how viable inoculum in soil and in planta contributes to disease development and severity.

5 Key Messages:

1. *Oculimacula* species have been found in quantifiable amounts in soil, roots and wheat stems up to 10cm from the base indicating greater survival potential and sources of inoculum.
2. *Oculimacula acuformis* remains the predominant pathogen in sampled fields and tissues.
3. Growing a second wheat in a rotation is likely to select for *O. yallundae*, whilst continuous wheat for *O. acuformis*.
4. Risk measured as DNA accumulation of *O. yallundae* and *O. acuformis* in stems up to 5 cm from the base is reduced by growing wheat following other crops (maize or oats) or OSR and legumes, respectively.
5. Prothioconazole applied as seed treatment reduced pathogen DNA accumulation across the season in stems up to 5 cm from the base.
6. Boscalid and epoxiconazole applied at GS 32 reduced pathogen DNA and disease severity and increased PSII efficiency of second wheat.
7. RNA –based quantitative molecular assay can potentially be utilized to further our understanding of the epidemiology of eyespot disease and increase the accuracy of predicting disease risk associated with viable inoculum in field.

6 References:

Bateman, G.L., Taylor, G.S. 1976. Significance of the coleoptile in establishment of seedling infection on wheat by *Pseudocercosporella herpotrichoides*. Transactions of the British Mycological Society, 67(3): 513-514.

Bateman, G.L., Edwards, S.G., Marshall, J., Morgan, L.W., Nicholson, P., Nuttall, M., Turner, A.S. 2000. Effects of cultivar and fungicides on stem‐base pathogens, determined by quantitative PCR, and on diseases and yield of wheat. Annals of Applied Biology, 137(3): 213-221.

Beelman, C.A., Parker, R. 1995. Degradation of mRNA in eukaryotes. Cell, 81: 179-183.

Bentsink, L., Leone, G.O.M., Van Beckhoven, J.R.C.M., Van Schijndel, H.B., Van Gemen, B., Van Der Wolf, J.M. 2002. Amplification of RNA by NASBA allows direct detection of viable cells of *Ralstonia solanacearum* in potato. Journal of Applied Microbiology, 93(4): 1365-2672.

Birch. K., Dawson, C.E., Cornett, J.H., Keer, J.T. 2001. A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. Letters in Applied Microbiology, 33: 296– 301.

Bleve, G., Rizzotti, L., Dellaglio, F., Torriani, S. 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable and molds contaminating yogurts and pasteurized food products. Applied and Environmental Microbiology, 69: 4116-4122.

Breeuwer, P., Abee, T. 2000. Assessment of the intracellular pH of immobilized and continuously perfused yeast cells employing fluorescence ratio imaging analysis Journal of Microbiological Methods, 39: 253-264.

Burnett, F.J., Hughes, G. 2004. The development of a risk assessment method to identify wheat crops at risk from eyespot. Project Report No. 347. Home Grown Cereals Authority, London.

Burnett F., Butler-Ellis C., Hughes G., Knight S., Ray, R. 2012. Forecasting eyespot development and yield losses in winter wheat. HGCA, Project Report No. 491.

Chapman, N.H., Burt, C., Nicholson, P. 2009. The identification of candidate genes associated with Pch2 eyespot resistance in wheat using cDNA-AFLP. Theoretical and Applied Genetics, 118: 1045–1057.

Chitarra, L.G., Breeuwer, P., Abee, T., Bulk, R.W. 2006.The use of fluorescent probes to assess viability of the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* by flow cytometry. Fitopatologia Brasileira 31: 349-356.

Clark, M.F. 1981. Immunosorbent assays in plant pathology. Annual Review of Phytopathology, 19: 83–106.

Colbach, N., Saur, L. 1998. Influence of crop management on eyespot development and infection cycles of winter wheat. European Journal of Plant Pathology, 104: 37–48.

Cook, R.J., Polley, R.W., Thomas, M.R. 1991. Disease-induced yield losses in winter wheat in England and Wales 1985–1989. Crop Protection, 10: 504–508.

Cother, E.J., Vruggink, H. 1980. Detection of viable and non-viable cells of *Erwinia carotovora* var.*atroseptica* in inoculated tubers of var. Bintje with enzyme-linked immunosorbent assay (ELISA). *Potato Research,* 23: 133–135.

Cunningham, P.C. 1981. Occurrence, role and pathogenic traits of a distinct pathotype of *Pseudocercosporella herpotrichoides*. Transactions of the British Mycological Society, 76: 3–15.

Daniels, A., Lucas, J.A., Peberdy, J.F. 1991. Morphology and ultrastructure of W and R pathotypes of *Pseudocercosporella herpotrichoides* on wheat seedlings. Mycological Research, 95: 385–97.

Daniels, A., Papaikonomou, M., Dyer, P.S., Lucas, J.A. 1995. Infection of wheat seedlings by ascospores *Tapesia yallundae*: Morphology of the infection process and evidence for recombination. Phytopathology, 85: 918-927.

Doussinault, G., Delibes, A., Sanchez-Monge, R., Garcia-Olmedo, F. 1983. Transfer of a dominant gene for resistance to eyespot disease from a wild grass to hexaploid wheat. Nature, 303: 698-700.

Dyer, P.S., Nicholson, P., Rezanoor, H.N., Lucas, J.A., and Peberdy, J.F. 1993. Two- allele heterothallism in *Tapesia yallundae*, the teleomorph of the cereal eyespot pathogen *Pseudocercosporella herpotrichoides*. Physiological and molecular plant Pathology, 43: 403-414.

Dyer, P.S., Nicholson, P., Lucas, J.A., Peberdy, J.F. 1996. *Tapesia acuformis* as a causal agent of eyespot disease of cereals and evidence for a heterothallic mating system using molecular markers. Mycological Research, 100: 1219-1226.

Fitt, B.D.L., Goulds, A., Polley, R.W. 1988. Eyespot (*Pseudocercosporella herpotrichoides*) Epidemiology in relation to prediction of disease severity and yieldloss in winter wheat - a Review. Plant Pathology, 37: 311-328.

Fittipaldi, M., Pino Rodriguez, N.J., Adrados, B., Agustí, G., Peñuela, G., Morató, J., Codony, F. 2011. Discrimination of viable *Acanthamoeba castellani* Trophozoites and cysts by propidium monoazide real-time polymerase chain reaction. The Journal of Eukaryotic Microbiology, 58: 359–364.

Glynne, M.D. 1953. Production of spores by *Cercosporella herpotrichoides*. Transactions of the British Mycological Society, 36: 46-51.

Goulds, A., Polley, R.W. 1990. Assessment of eyespot and other stem base diseases of winter wheat and winter barley. Mycological Research, 94: 819–22.

Guo, J.R., Schnieder, F., Beyer, M., Verreet, J.A. 2005. Rapid detection of *Mycosphaerella graminicola* in wheat using reverse transcription-PCR assay. Journal of Phytopathology, 153: 674–679.

Hardwick, N.V., Jones, D.R., Slough, J.E. 2001. Factors affecting diseases of winter wheat in England and Wales, 1989–1998. Plant Pathology 50: 453–462.

Harrison, J.G., Barker, H., Lowe, R., Rees, E.A. 1990. Estimation of amounts of *Phytophthora infestans* mycelium in leaf tissue by Enzyme linked immunosorbent assay. Plant Pathology, 39: 274-277.

Hartung, J.S., Daniel, J.F., Pruvost, O.P. 1993. Detection of *Xanthomonas campestris* pv. c*itri* by the polymerase chain reaction method Applied and Environmental Microbiology, 59(4): 1143-1148.

Houseley, J., Tollervey, D. 2009. The many pathways of RNA degradation. Cell, 136: 763-776.

Hulbert, S.H., Webb, C.A., Smith, S.M., Sun, Q. 2001. Resistance gene complexes: evolution and utilization. Annual Review of Phytopathology, 39: 285-312.

Ingle, C. A., Kushner, S.R. 1996. Development of an *in vitro* mRNA decay system for *Escherichia coli*: poly(A) polymerase I is necessary to trigger degradation. Proceedings of the National Academy of Sciences, 93**:** 12926-12931.

Josephson, K.L., Gerba, C.P., Pepper, I.L. 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Applied and Environmental Microbiology, 59(10): 3513–3515.

Kimber, G. 1967. The incorporation of the resistance of *Aegilops ventricosa* to *Cercosporella herpotrichoides* into *Triticum aestivum*. The Journal of Agricultural Science, 68: 373–376.

Lind, V. 1999. Variation of resistance to *Pseudocercosporella herpotrichoides* (Fron) Deighton in wheat genotypes carrying the gene *Pch1*. Plant Breeding, 118: 281–287.

Løvdal, T., Hovda, M.B., Björkblom, B., Møller, S.G. 2011. Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua*. Journal of Microbiological Methods, 85: 164–169.

Murray, T.D. 2010. Eyespot (Strawbreaker foot rot). In: Compendium of Wheat Diseases and Insects, W.W. Bockus, R.L. Bowden, R.M. Hunger, T.D. Murray, R.W. Smiley, and W. Morrill, eds., Third Edition. APS Press, Minneapolis, MN. 32-34.

Murray, T.D. Bruehl, G.W. 1986. Effects of host resistance to *Pseudocercosporella herpotrichoides* and foot rot severity on yield and yield components in winter wheat. Plant Disease 70: 851-856.

Nicholson, P., Dyer, P.S., Rezanoor, H.N., Lucas, J.A., Walkowiak-Cagara, I., and Hollins, T. W. 1995. Mating relationships between different pathotypes of *Tapesia yallundae*, the teleomorph of the eyespot pathogen *Pseudocercosporella herpotrichoides.* Mycological Research 99: 457–465.

Nocker A., Camper A.K. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Applied and Environronmental Microbiology, 72: 1997–2004.

Nocker, A., Sossa-Fernandez, P., Burr, M., Camper, A.K. 2007. Use propidium monoazide for live/dead distinction in microbial ecology. Applied and Environmental Microbiology, 73: 5111–5117.

Nocker, A., Camper, A.K. 2009. Novel approaches toward preferential detection of viable cells during nucleic acid amplification techniques. FEMS Microbiology Letters, 291: 137–142.

Parnell, S., Gilligan, C.A., Lucas, J.A., Bock, C.H., Van Den Bosch, F. 2008. Changes in fungicide sensitivity and relative species abundance in *Oculimacula yallundae* and *O. acuformis* populations (eyespot disease of cereals) in Western Europe. Plant Pathology 57, 509–517.

Ray R.V., Jenkinson P., Edwards S.G. 2004. Effects of fungicides on eyespot, caused predominantly by *Oculimacula acuformis*, and yield of early-drilled winter wheat. Crop Protection, 23(12): 1199–1207.

Ray, R.V., Crook, M.J., Jenkinson, P., Edwards, S.G. 2006. Effect of eyespot caused by *Oculimacula yallundae* and *O. acuformis*, assessed visually and by competitive PCR, on stem strength associated with lodging resistance and yield of winter wheat. Journal of Experimental Botany, 57: 2249–2257.

Scott, P.R., Hollins, T.W., and Muir, P. 1975. Pathogenicity of *Cercosporella herpotrichoides* to wheat, barley, oats and rye. Transactions of the British Mycological Society, 65: 529-539.

Simpkins, S.A., Chan, A.B., Hays, J.P., Pöpping, B., Cook, N. 2000. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. Letters in Applied Microbiology, 30: 75–79.

Templeton, K.E., Scheltinga, S.A., Van der Zee, A., Diederen, B.M.W., Kruijssen, A.M., Goossens, H., Kuijper, E., Class, E.C.J. 2003. **Evaluation of real-time PCR for detection of and discrimination between** *Bordetella pertussis***,** *Bordetella parapertussis***, and** *Bordetella holmesii* **for clinical diagnosis.** Journal of Clinical Microbiology, 41(9): 4121-4126.

Thiele, A., Schumann, E., Peil, A., Weber, W.E. 2002. Eyespot resistance in wheat x *Aegilops kotschyi* backcross lines. Plant Breeding, 121: 29–35.

Uslu, E., Miller, T.E., Rezanoor, N.H., Nicholson, P. 1998. Resistance of *Dasypyrum villosum* to the cereal eyespot pathogens, *Tapesia yallundae* and *Tapesia acuformis*. Euphytica, 103: 203–209.

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S., Vesper, A. 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). Journal of Microbiological Methods, 72: 180–184.

Wallwork, H., Spooner, B. 1988. *Tapesia yallundae* - the teleomorph of *Pseudocercosporella herpotrichoides*. Transactions of the British Mycological Society, 91: 703-705.

Walsh, K., Korimbocus, J., Boonham, N., Jennings, P., Hims, M. 2005. Using real-time PCR to discriminate and quantify the closely related wheat pathogens *Oculimacula yallundae* and *Oculimacula acuformis*. Journal of Phytopathology, 153: 715–721.

Wan, A.M., Bock, C.H., Fitt, B.D.L., Harvey, J.L., Jenkyn, J.F. 2005. Development of *Oculimacula yallundae* and *O. acuformis* eyespot on leaf sheaths of winter wheat in the UK in relation to thermal time. Plant Pathology, 54: 144–55.

Widjojoatmodjo, M.N., Borst, A., Schukkink, R.A.F., Box, A.T.A., Tacken, N.M.M., Van Gemen, B., Verhoef, J., Top, B., Fluit, A.C. 1999. Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. Journal of Microbiological Methods, 38: 81-90.