Occurrence and Survival of Apothecia of the Eyespot Pathogens *Oculimacula acuformis* and *O. yallundae* on Wheat Stubble in the U.S. Pacific Northwest

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Abstract

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Eyespot is a chronic disease of wheat caused by *Oculimacula yallundae* and *O. acuformis* that results in premature ripening of grain, lodging, and reduced grain yield. Discovery of the sexual stage of these *Oculimacula* spp. in the Pacific Northwest (PNW) of the United States is relatively recent and the role of apothecia in the epidemiology of eyespot is unclear. Our goals were to determine whether and when apothecia of these *Oculimacula* spp. are found in the PNW, and monitor their ability to survive over summer and over winter. Seventy-three harvested commercial wheat fields in Idaho, Oregon, and Washington were surveyed for apothecia during spring and fall 2012 and spring 2013. Apothecia of both species were found in both spring and fall in 19% of fields. Apothecia survived on straw placed on the soil surface over the summer but not the winter. This is the first report of *O. yallundae* apothecia in commercial wheat fields in the PNW. Occurrence of apothecia in spring and fall demonstrates that sexual reproduction of both species occurs regularly in the PNW and at a time when ascospores could serve as primary inoculum for infection of winter wheat. Results of this study are consistent with previous population genetic studies that found high genotypic diversity of both eyespot pathogens in winter wheat fields and provides a baseline for understanding the influence of sexual reproduction on population dynamics and genetics of both pathogens.

Eyespot disease, caused by the fungi *Oculimacula yallundae* (Wallwork & Spooner) Crous & W. Gams and *O. acuformis* (Boerma, R. Pieters & Hamers) Crous & W. Gams, is one of the most important diseases of winter wheat in the Pacific Northwest (PNW) region of the United States (Clarkson 1981; Scott and Hollins 1978). Both of these ascomycete fungi have similar life cycles and frequently coexist in the same field; however, the species differ in pathogenicity (Fitt et al. 1987; Scott et al. 1975), cultural characteristics (Hollins et al. 1985; Nicholson et al. 1991), sensitivity to fungicides (Bateman et al. 1995, 2000; Murray 1990, 1996; Parnell et al. 2008), isozymes (Julian and Lucas 1990; Priestley et al. 1992), and ribosomal internal transcribed spacer (ITS) sequence (Crous et al. 2003) and are reproductively incompatible (Dyer et al. 1996). Eyespot was found affecting cereals and grasses in the PNW more than a century ago (Sprague 1931), and its current distribution extends to the Great Plains and Midwestern and Northeastern United States. In the PNW, eyespot is found chronically in most winter wheat fields, where it can reduce yield by up to 50% (Murray 2006).

The asexual stage of the eyespot pathogens was first described by Fron in 1912 (cited by Chang and Tyler 1964). Seventy-five years later, Wallwork (1987) identified apothecia of *O. yallundae* in Australia. Since then, the sexual stages of both species have been reported in several places around the world (Hunter 1989; King 1990; Robbertse et al. 1994). In vitro crosses of *O. yallundae* have demonstrated a bipolar mating system governed by a single locus, *MAT1,* with two idiomorphs, MAT1-1 and MAT1-2 (Dyer et al. 1993; Moreau and Maraite 1996). Field observations have demonstrated that both MAT1-1 and MAT1-2 isolates often are found in the same field (Douhan et al. 2002a; Dyer et al. 1996).

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Apothecia of *O. acuformis* but not of *O. yallundae* were discovered in a survey of commercial wheat fields in the PNW (Douhan et al. 2002a). The absence of *O. yallundae* apothecia was surprising given that population genetic analyses of molecular markers suggested that sexual reproduction of *O. yallundae* isolates was likely occurring in the PNW (Douhan et al. 2002b). It is possible that apothecia of *O. yallundae* were not found because they are inconspicuous, occur at low frequency, or are not present in the PNW, though the latter would be contradictory to the data of Douhan et al. (2002b).

In Germany, apothecia of *O. yallundae* were produced in the beginning of the spring (early to mid-March) in commercial wheat fields and were present at a low frequency (King 1991). Other studies found similar results (Dyer and Lucas 1995; Dyer et al. 1996, 2001) and the low incidence of apothecia was attributed to the predominance of one mating type in a population, low sexual fertility, and unfavorable environmental requirements necessary to trigger sexual reproduction. Studies of sporulation of *Helgardia* spp., the asexual form of *Oculimacula*, have demonstrated that temperature, light, water, and nutrient requirements all influence sporulation (Chang and Tyler 1964; Glynne 1953; Higgins and Fitt 1985; Hollins and Scott 1980; Sprague and Fellows 1934; Ward and Friend 1979) and are assumed to influence production of apothecia; however, studies on the effect of specific environmental factors are absent.

Additional studies on the occurrence, distribution, and persistence of apothecia in commercial fields in the PNW are needed to determine the potential epidemiological role of ascospores in the disease cycle of the eyespot pathogens. The overall goals of this research were to develop a better understanding of the population biology of *Oculimacula* spp. and epidemiology of eyespot in PNW, which is essential to improve management of eyespot disease. The specific objectives of this study were to determine whether apothecia of *O. acuformis* and *O. yallundae* are present in commercial fields, their relative abundance and distribution, and their ability to persist over summer and winter.

Materials and Methods

Survey for apothecia in commercial wheat fields. Seventy-three harvested wheat fields in northern Idaho, northeastern Oregon, and eastern Washington were surveyed for the presence of *O. yallundae* and *O. acuformis* apothecia during May to June and September to October 2012 and June to July 2013. These time periods were

selected for sampling because they correspond to the times when temperature and precipitation are favorable for dispersal of ascospores (i.e., cool and moist). The number of fields surveyed was based on the availability of fields with standing winter wheat straw. Typical crop rotations vary across the region surveyed from wheat-summer fallow in the areas of lower rainfall (250 mm of annual precipitation) to 3-year rotations of winter wheat-spring wheat-legume in the areas of higher rainfall (500 mm of annual precipitation). The location of each field was identified by global positioning system (GPS) coordinates taken with a Garmin etrex handheld GPS device (Garmin Corp., Schaffhausen, Switzerland).

Fields were surveyed three or more months after harvest to allow time for apothecia to develop (Dyer et al. 2001), which was estimated based on coloration and appearance of the straw; therefore, fields with breakable yellowish-brown straw were preferred as samples. Approximately 100 to 150 straws per field were collected randomly along two 30-m transects, with a sample interval of approximately 5 m. Samples of straw were collected by digging standing straw, carefully removing loosely adhering soil from the crown and roots, and placing the straw in a paper bag. Samples were labeled and stored dry at 22°C to preserve them and avoid apothecia from discharging their ascospores before examination.

Apothecial assessment. Wheat stems with intact lower leaf sheaths were examined individually for the presence of apothecia using a dissecting microscope at ×10 magnification. When apothecia were found, morphological observations were used for identification of the genus *Oculimacula* (Hunter 1989; King 1991); specifically, dark-gray to brown apothecia approximately 1 mm in diameter with light-colored margins when open. The presence of one or more apothecia identified as *Oculimacula* spp. was designated as a positive occurrence for that location and sample. All observable apothecia were removed from straws with a dissecting needle and stored dry in 2-ml Eppendorf tubes at −20°C for DNA extraction and species determination.

DNA extraction. Apothecia were disrupted by agitation in 400 μl of 0.1% Nonidet P-40, with 0.2 g of acid-washed glass beads (425 to 600 μm in diameter; Sigma-Aldrich St. Louis). Agitation was performed for 3 min at 6 ms^{-1} in a Mini Beadbeater (Biospec Products, Inc., Bartlesville, OK), which resulted in disruption of approximately 99% of the apothecia and ascospores based on observation of samples under a microscope. DNA was extracted from apothecia and ascospore suspensions using a method adapted from Dellaporta et al. (1983), modified by the addition of 20 μg of glycogen (Thermo Scientific, Pittsburg) as a carrier for the DNA during isopropanol precipitation and placing the product in an incubator at −20°C for 30 min to improve DNA recovery (Williams et al. 2001).

Species determination. Quantitative polymerase chain reaction (PCR) was performed using a procedure modified from Walsh et al. (2005). All reactions were performed in an iCycler iQ Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA) using SYBR Green I fluorescent dye detection, rather than a TaqMan assay, in iCycler iQ PCR 96-well reaction plates. Species identification was based on primers designed from the ITS region of the ribosomal DNA (Walsh et al. 2005). Each reaction contained two species-specific forward primers (YALL F-H: 5′-GGG GGC TAC CCT ACT TGG CAG-3′ and Ac F-D: 5′-GCC ACC CTA CTT CGG TAA-3′) and one common reverse primer (Oculimacula-R: 5′-ATT CAA GGG TGG AGG TCT GRA C-3′).

The 20-μl reaction volume contained 1 μl of DNA extract, 10 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories), 0.5 μl each of purified desalted primers (20 nM; Sigma-Aldrich), and 8 μl of double-distilled sterile water. Thermocycling conditions consisted of an initial denaturation at 94°C for 4 min, followed by 40 cycles at 94°C for 15 s and annealing for 45 s at 62°C. After the final amplification cycle, a melting curve profile was obtained by heating to 95°C, cooling to 55°C, and incrementally heating to 95°C at the rate of 0.5°C per 10 s to detect nonspecific products or primer-dimers, as indicated by more than one peak in the profile. A cycle threshold line was scored positive between 14 and 35 and negative above 35, which was the limit of detection of the lowest concentration of ascospores tested (Vaerman et al. 2004). The reactions were performed using three replicates for each sample. Genomic DNA of either *O. acuformis* or *O. yallundae* isolates and distilled water were used as positive and negative controls, respectively.

Temporal occurrence of apothecia. The presence of *Oculimacula* spp. apothecia was monitored in two field plots at the Washington State University Plant Pathology Farm near Pullman during May to August 2012 (PPWest) and April to November 2013 (PPEast). To obtain alternating years of winter wheat stubble, PPWest and PPEast were seeded uniformly with a grain drill to 'Hill 81' wheat in September 2010 and 2011 and harvested in August 2011 and 2012, respectively. Plants in each plot were inoculated at Zadok's growth stage of 20 (tillering stage) (Zadoks et al. 1974) with conidia of compatible mating types of both species in November 2010 and 2011, as previously described (Douhan et al. 2002a). Following harvest, standing stubble was maintained with chemical weed control using glyphosate only and no mechanical tillage for 1 year after harvest. Prior to planting in September 2012 (PPWest) and 2013 (PPEast), the stubble was disked, harrowed, and then planted with Hill 81winter wheat using a grain drill. In 2013, four rows of stubble were left standing along one edge of PPEast to observe whether apothecia could form on second-year standing stubble.

Observations of apothecia were performed as described above for the field surveys, except that 100 straws were collected monthly using the random point sampling method (Beutler and Leneman 1966) to determine the incidence of apothecia.

Apothecial survival. Straws bearing open apothecia collected from inoculated field plots were photographed, labeled, and placed in a field containing newly harvested wheat stubble to determine the ability of apothecia to survive over summer. The experiment was conducted at the Plant Pathology Farm, Pullman, WA between June and September 2012. Straws were placed in one of three arrangements simulating how they are found after harvest under field conditions: laying on the soil surface; standing in rows of straw among other straws; or in a bundle of approximately 30 straws standing in a row, simulating intact harvested plants. Three replicates containing four straws each were distributed in a completely random design in the field. The labeled straws were collected after 3 months, immersed in water for 2 min to hydrate the apothecia, and photographed. Pre- and postincubation photos were compared and apothecia were counted. Only the rehydrated apothecia (open cup) were counted and compared with the photo taken at the beginning of the experiment. Two mature apothecia selected randomly from each straw were placed on slides, dissected, and observed with a compound microscope for the presence of asci and ascospores, which was interpreted as still being viable. The experiment was repeated from June to September 2013, and a similar experiment was conducted from November 2013 to March 2014 to determine whether apothecia were able to survive over the winter.

Data analysis. Survey data were summarized to represent the main wheat-growing areas of the PNW. Samples were pooled to determine the incidence and species distribution across the surveyed area. Incidence was expressed as the number of samples bearing apothecia out of the total samples collected. Treatments to test the effect of straw position on survival of apothecia were analyzed as a completely random design. Homogeneity of variance from individual experiments was conducted using Bartlett's test and data where combined to perform analysis of variance using PROC GLM of SAS software (SAS release 9.2; SAS Institute, Cary, NC). Tukey's multiple comparison test was applied to evaluate the statistical significance between mean values.

Results

Survey for apothecia in commercial wheat fields. In total, 7,825 wheat straws were collected from 73 harvested wheat fields in northern Idaho, northeastern Oregon, and eastern Washington. Apothecia of *O. yallundae* or *O. acuformis* were found in 19% of fields from the counties of Benewah and Latah, ID; Umatilla, OR; and Adams, Asotin, Columbia, Garfield, Walla Walla, and Whitman, WA. Apothecia occurred in 21% of Idaho and Washington fields and 8% of Oregon

fields (Table 1). Apothecia of both *O. yallundae* and *O. acuformis* occurred in 29% of the fields where apothecia were found, whereas apothecia of *O. yallundae* or *O. acuformis* only occurred in 36% of the fields with apothecia (Table 1). The incidence of apothecia within fields was low, ranging from 1 to 3% of the straws collected (data not shown).

A greater percentage of fields with apothecia were found in 2012 than in 2013 (22 and 16%, respectively; Table 2). In 2012, more fields with apothecia were found in spring (38%) than fall (10%). In spring 2013, 16% of surveyed fields had apothecia; unfortunately, very few harvested winter wheat fields with standing stubble were found in fall 2013 and, thus, no data are available (Table 2).

Temporal occurrence of apothecia. Mature apothecia of *Oculimacula* spp. were present in both PPWest and PPEast during all sampling periods. PPWest was sampled from May to August 2012; the greatest incidence of apothecia occurred in May (45%) and the least in July and August (17% each) (Fig. 1). PPEast was sampled from April to November 2013; apothecia were present on 22% of stems in April through June and decreased to less than 15% from July to September. In PPEast, stubble was allowed to remain standing into the second year after harvest in 2013 to determine whether apothecia could form on 2-year-old stubble. Incidence of apothecia in November was 52%, which was significantly greater than all other months (Fig. 1).

Apothecial survival. Survival of apothecia over summer was greater than 70% in all three arrangements of straw but greater on straw in bundles (85.1%) than standing in rows (77.4%) or laid on the soil surface (71.3%); however, survival did not differ statistically among these arrangements. In a similar experiment carried out over the winter of 2012–13, viable apothecia were not recovered from any of the configurations tested (results not shown).

Discussion

The presence of apothecia of *O. yallundae* and *O. acuformis* in wheat fields in three PNW states during spring and fall demonstrates that sexual reproduction is occurring in these fungi and that ascospores may have a more important role in the epidemiology of eyespot than previously believed. Our discovery of apothecia of *O. yallundae* in commercial fields in this study was not surprising because Douhan et al. (2003) concluded that recombination was occurring in PNW *O. yallundae* populations and apothecia of this fungus were found previously in an experimental plot inoculated with compatible mating types of *O. yallundae*. These results support and expand the findings of Douhan et al. (2002a), where apothecia of *O. acuformis* but not *O. yallundae* were found in commercial wheat fields in the PNW. They also explain why there is high genotypic diversity among isolates of the eyespot pathogens, given that ascospores are being produced during the time of year when primary infection of wheat by the eyespot pathogens is occurring.

The presence of apothecia in 19% of commercial fields surveyed in the PNW may be epidemiologically important; at the regional level, the presence of apothecia during fall, when winter wheat is planted, suggests that apothecia could provide ascospores that serve as primary inoculum initiating the disease cycle. The development of new infections via ascospores may also result from long-range

Table 2. Temporal occurrence of *Oculimacula yallundae* and *O. acuformis* apothecia in harvested winter wheat fields in northern Idaho, northeastern Oregon, and eastern Washington during spring and fall 2012 and 2013

		Fields with apothecia[a]			
Season	Samples[b]	OY	OA	OY + OA	Incidence (%)[c]
2012					
Spring	16	2	2	2	37.5
Fall	20	0	1	1	10.0
2013[d]					
Spring	37	3	2	1	16.2

[a] Number of fields in which apothecia of *O. yallundae* (OY), *O. acuformis* (OA), or both (OY + OA) were found.
[b] Number of fields surveyed.
[c] Percentage of fields with apothecia.
[d] No data are available in fall 2013 due to limited number of harvested winter wheat fields with standing stubble.

Table 1. Geographic occurrence of *Oculimacula yallundae* (OY) and *O. acuformis* (OA) apothecia in harvested winter wheat fields in Idaho, Oregon, and Washington from May 2012 to June 2013

			Species[a]			
State, county	*N*[b]	Fields with apothecia	OY	OA	OY + OA[c]	Incidence (%)[d]
Idaho						
Benewah	8	2	−	+	+	25.0
Clearwater	1	0	−	−	−	0
Idaho	1	0	−	−	−	0
Latah	3	1	−	−	+	33.3
Nez Perce	1	0	−	−	−	0
Subtotal	14	3	…	…	…	21.4
Oregon						
Umatilla	10	1	+	−	−	10.0
Union	1	0	−	−	−	0
Wallowa	2	0	−	−	−	0
Subtotal	13	1	…	…	…	7.7
Washington						
Adams	5	1	+	−	−	20
Asotin	5	1	+	−	−	20
Columbia	10	2	+	−	+	20
Franklin	1	0	−	−	−	0
Garfield	6	1	−	−	+	16.7
Walla Walla	5	2	−	++	−	40.0
Whitman	14	3	+	++	−	21.4
Subtotal	46	10	…	…	…	19.7
Total	73	14	5	5	4	…
Frequency	…	19.2	35.7	35.7	28.6	…

[a] Symbols: + = one field with apothecia, ++ = two fields with apothecia, and − = no apothecia observed.
[b] Number of fields surveyed.
[c] Fields with apothecia of both *O. yallundae* and *O. acuformis*.
[d] Incidence of apothecia by county and state.

dispersal, providing greater efficiency in the spatial distribution of the pathogen (Daniels et al. 1995). At the field level, recombination during sexual reproduction results in increases in genotypic diversity in populations of *O. yallundae* and *O. acuformis* (Dyer et al. 1996).

The incidence of apothecia was low within the 14 fields in which they were found, never exceeding 3% of stems; other studies in Europe and North America found similar results (Douhan et al. 2002a; Dyer et al. 1996, 2001). Dyer et al. (1996) hypothesized that the main reasons for limited sexual reproduction were the predominance of one mating type, low fertility of the pathogens, and absence of specific environmental triggers necessary for sexual reproduction. However, in a study carried out in the PNW, Douhan et al. (2002a) determined that both mating types of *O. yallundae* and *O. acuformis* were widely distributed in nearly equal proportion across all wheat fields sampled, suggesting that production of apothecia was not limited by a lack of compatible mating types. The fact that apothecia are forming, albeit in low percentages of infested stems, suggests that environmental conditions are not limiting and that isolate fertility or spatial distribution (or density) of mating types on a small scale within fields is a more likely explanation for the low incidence of apothecia.

This is the first report of apothecia of *O. yallundae* and *O. acuformis* occurring in commercial wheat fields during fall in the PNW and their presence, along with the production of viable ascospores, demonstrates the potential to incorporate genotypic variation resulting from recombination that was observed by Douhan et al. (2002b) into the disease cycle. The presence of apothecia in commercial wheat fields in the fall contrasts with previous studies in the United Kingdom and Germany (Hunter 1989; King 1991; Nicholson et al. 1997), where apothecia were found only during spring. However, Hunter (1989) and King (1991) considered the fall more suitable for occurrence of new eyespot infections because that is when winter wheat is planted.

Although the incidence of *Oculimacula* spp. apothecia was lower in fall than spring, environmental conditions might compensate and result in a greater chance of infection than for ascospores released in spring (Kirisits et al. 2012). A study in the United Kingdom came to a similar conclusion (Dyer et al. 1994); based on their findings, the authors suggested that ascospores may represent a potential source of inoculum for up to 9 months and that moisture and, to a lesser extent, temperature might influence the development and discharge of ascospores.

The occurrence of both *O. yallundae* and *O. acuformis* in the same fields is another aspect of the disease that has been discussed in previous studies (Bock et al. 2009; Hunter 1989). Bierman et al. (2002) reported that differences in competitive ability between these pathogens might favor *O. yallundae* infections during fall. Other studies in controlled environments support this conclusion, based on differences in mycelial growth rate between *O. yallundae* and *O. acuformis* (Hollins et al. 1985; Waldner-Zulauf and Gisi 1991), penetration of leaf sheaths (Bierman et al. 2002; Poupard et al. 1994), and ability for sexual reproduction (Moreau and Maraite 1995). Based on our results, apothecia of *O. yallundae* and *O. acuformis* occurred at similar times and frequencies during 2012 and 2013, suggesting that both species can coexist in the same field on the same host, despite having small niche differences (Fitt et al. 2006).

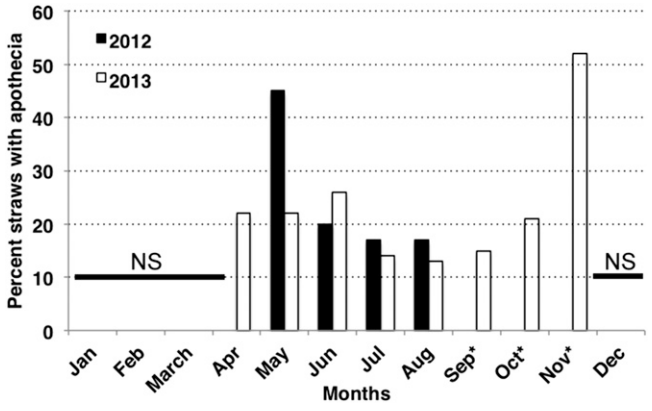

Fig. 1. Mean monthly occurrence of *Oculimacula* spp. apothecia in harvested field plots at the Washington State University Plant Pathology Farm previously inoculated with compatible mating-type isolates of *Oculimacula yallundae* and *O. acuformis*. Filled bars = percent straws with apothecia in PPWest during 2012. Open bars = percent straws with apothecia in PPEast during 2013. Asterisks indicate apothecia from second year of stubble in PPEast. NS (solid horizontal lines) = periods when no sampling occurred.

Based on survival of apothecia of *O. yallundae* on naturally colonized straws in field plots, apothecia are able to survive over summer but not over winter. Straws were arranged in three different configurations, simulating how they occur following wheat harvest, and survival over summer was greater than 70% in all treatments. This is the first report of apothecia surviving between seasons and demonstrates that apothecia produced in spring can survive over summer and produce or release ascospores during fall and, consequently, can serve as a source of primary inoculum (Bierman et al. 2002; Dyer et al. 2001). Survival over winter was tested in 1 year and further study is needed. However, we hypothesize that *Oculimacula* spp. survive the winter asexually as hyphae in colonized stem bases, and apothecia develop during spring and release ascospores, then survive over summer and release ascospores again during fall, when winter wheat seedlings are present. Our observations of apothecia survival over summer but not winter were done in one location over a 2-year period and are reflective of those conditions. Weather conditions during this study were typical for the region but weather, especially precipitation, varies greatly across the region, ranging from about 150 to over 500 mm annually. Consequently, further research is needed to confirm these observations over a broader range of wheat-production systems and environmental factors to better understand survival of apothecia over summer and winter. For example, the inability of apothecia to survive over winter may be due to the low temperatures or the prolonged wet conditions that predominate during winter.

The occurrence of apothecia during all months of evaluation in stubble of PPWest (2012) and PPEast (2013) (Fig. 1) suggests that *Oculimacula* spp. will reproduce sexually when compatible mating types are present. More studies are needed to determine when initial development of apothecia occurs and their ability to produce ascospores begins.

In summary, apothecia of *O. yallundae* and *O. acuformis* occur widely in commercial fields in the PNW during spring and fall on winter wheat stems infested while the plants were alive; ascospores are produced in both seasons and constitute a previously unrecognized source of primary inoculum in addition to conidia produced on colonized straw. As such, this research provides new insights into the role of the sexual stage of *Oculimacula* spp. in the epidemiology of eyespot disease that may help explain shifts in species composition (Douhan et al. 2002a) and contribute to the development of new disease management strategies.

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