Plant Pathology (2014) **63**, 590–597 Doi: 10.1111/ppa.12127

Effects of relative humidity on infection, colonization and conidiation of *Magnaporthe orzyae* on perennial ryegrass

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Grey leaf spot, caused by *Magnaporthe oryzae*, causes severe damage on perennial ryegrass (*Lolium perenne*) turf. In this study, the effects of relative humidity (RH, 88 to 100% at 28°C) on infection, colonization and conidiation of *M. oryzae* on perennial ryegrass were investigated in controlled humidity chambers. Results showed that the RH threshold for successful *M. oryzae* infection was ≥92% at 28°C. The advancement of infection on the leaf tissue was further examined with a green fluorescent protein (GFP)-tagged *M. oryzae* strain. No appressorium formation was found when the inoculum was incubated at RH ≤ 88%. Additionally, the GFP-tagged staining provided a rapid method to quantitatively compare the fungal colonization from leaf tissue at different levels of RH. The fluorescence intensity data indicated that the fungal biomass was highest at 100% RH and there was no fluorescence intensity observed at 88% RH or below. Conidiation was only observed when RH was ≥96%, with the most abundant conidiation occurring 8 days after inoculation. Reduced conidiation was associated with decreasing RH, and no conidiation occurred at RH ≤ 92%. This study indicates that infection and conidiation of *M. oryzae* on perennial ryegrass required different thresholds: 92% and 96% RH for infection and conidiation, respectively. The quantitative data from this research will assist in prediction of grey leaf spot disease outbreaks and of secondary infection of perennial ryegrass.

Keywords: colonization, conidiation, infection, *Magnaporthe oryzae*, perennial ryegrass, relative humidity

Introduction

Grey leaf spot, caused by *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*), is a devastating foliar disease on perennial ryegrass (*Lolium perenne*) turf (Dernoeden, 1996; Uddin, 1999; Uddin *et al.*, 2003b). The disease has drawn great attention in the turfgrass industry as a result of severe outbreaks at golf courses in various regions of the USA during the past two decades (Landschoot & Hoyland, 1992; Schumann & Jackson, 1999; Uddin *et al.*, 1999, 2002; Harmon *et al.*, 2000; Pedersen *et al.*, 2000; Wong & de la Cerda, 2006). Grey leaf spot epidemics are particularly prevalent in the northeastern USA, usually reported on golf course fairways during the late summer, from late July to early September (Vincelli, 1999; Uddin *et al.*, 2003b). The pathogen overwinters as mycelia in plant debris or in live plant tissue (Suzuki, 1975). The host plant, perennial ryegrass, is widely cultivated for its desirable agronomic attributes, such as rapid germination, tolerance to close-mowing, rapid tillering, high density, upright growth, and dark green turf colour (Hannaway *et al.*, 1999). However, most perennial ryegrass cultivars available on the market are highly susceptible to *M. oryzae* (Bonos *et al.*, 2004). Therefore, grey leaf spot management is heavily dependent on expensive and extensive fungicide applications. However, strains with field-resistance to azoxystrobin have developed (Vincelli & Dixon, 2002; Harmon & Latin, 2003; Kim *et al.*, 2003).

Environmental conditions are important determinants of grey leaf spot epidemics in perennial ryegrass fairways and roughs. Grey leaf spot usually develops first in the canopy of high-cut perennial ryegrass where prolonged leaf wetness is present (Uddin *et al.*, 1999, 2003a). Leaf wetness and temperature have been identified as influential environmental factors for grey leaf spot development (Uddin *et al.*, 2003a). Periods of leaf wetness may be roughly estimated by the duration of high levels (≥90%) of relative humidity (RH; Sutton *et al.*, 1984). As a measurable and forecastable weather parameter, RH has been widely studied or applied as a predictor for disease development in various pathosystems at different stages, including fungal growth and sporulation of numerous plant pathogens (Hemmi & Imura, 1939; Mislivec & Tuite, 1970; Carisse & Kushalappa, 1992).

Critical cytological changes of *M. oryzae*, including germ tube development, appressorium formation and invasive hyphae, have been observed during its interaction with artificial (e.g. cellophane) and rice leaf surfaces (Howard & Valent, 1996; Koga & Nakayachi, 2004). A conidiation study of *M. oryzae* on rice seedlings indicated that scarce conidia production can occur at 90% RH, with more favourable production at 93% RH or higher at temperatures ranging from 16 to 34°C (Hemmi

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Published online 1 October 2013

Plant Pathology

& Imura, 1939). However, little information is available regarding the effects of RH on the development of grey leaf spot of perennial ryegrass and, to the authors' knowledge, no research has investigated the influence of various humidity levels on infection, colonization or conidiation by *M. oryzae* on perennial ryegrass.

Research on fungal–plant interactions has been facilitated by the use of green fluorescent protein (GFP) expressed in fungal cytoplasm (Howard, 2001; Lorang *et al.*, 2001). A rice plant strain of *M. oryzae* with high expression levels of GFP in the cytoplasm has been developed using *Agrobacterium tumefaciens*-mediated transformation (Rho *et al.*, 2002). The expressed GFP produces a strong and stable green fluorescence; photobleaching only occurs when the cells are illuminated with 340–390 nm or 395–440 nm light (Chalfie *et al.*, 1994). Additionally, GFP-labelling enables microscopic observation in real time: laser scanning confocal microscopy and conventional fluorescence microscopy are efficient methods to observe GFP-tagged fungi. Compared with conventional fluorescence microscopy, confocal laser scanning microscopy provides higher resolution and greater flexibility for image generation, processing and analysis (Conchello & Lichtman, 2005). As the intensity of GFP fluorescence is directly proportional to the amount of fungal colonization, which has been reported for *Cochliobolus heterostrophus* in maize leaves, this suggests a quick and accurate method to quantify the colonizing fungi (Maor *et al.*, 1998; Chen *et al.*, 2003).

The objectives of this study were to: (i) determine the humidity thresholds for critical events in the development of grey leaf spot on perennial ryegrass, such as infection, colonization and conidiation, and (ii) compare *M. oryzae* colonization and conidiation on perennial ryegrass at different RH levels. Results of this study may be useful for integrating RH into the current grey leaf spot forecasting programme, and assist turf managers in developing an integrated disease management strategy.

Materials and methods

Growth of perennial ryegrass and preparation of detached leaf blades

Perennial ryegrass cv. Legacy II seeds were surface-sterilized for 30 s in 0·5% NaOCl (Clorox), rinsed twice in distilled water and then grown in 4-cm diameter × 20-cm plastic conetainers (Stuewe & Sons) that were filled with a sterilized 50:50 (v/v) mixture of peat (Metro-Mix; Sun Gro) and sand. Two weeks after sowing, the plants were fertilized weekly with water-soluble 18-24-12 N-P-K fertilizer. Detached leaves were obtained from 6-week old plants. Leaf blades were cut into 4-cm sections, surface-sterilized with 0·5% NaOCl for 90 s then rinsed three times with sterile distilled water for 10 s.

Transformation of *M. oryzae* and preparation of conidial suspension

A monoconidial isolate of *M. oryzae*, collected from a perennial ryegrass fairway at Marple Township, Broomall, PA, USA, was used for all experiments. The fungus was maintained on nitrocellulose filter paper disks at −80°C for long-term storage. The filter paper disks were removed from storage, placed on potato dextrose agar (PDA) plates, and incubated at 28°C at 12 h:12 h light/dark cycles (177 μmol s^{-1} m^{-2}) for 5–7 days. Agar blocks (2 × 2 mm) containing actively growing mycelia were transferred to oatmeal agar (OMA) plates (Namai & Yamanaka, 1985) and incubated under the same conditions used for the PDA cultures. Conidia were harvested from the plates following 7–10 days' incubation at 28°C (Valent *et al.*, 1991).

Agrobacterium tumefaciens AGL-1 with pBMt2 (Mullins *et al.*, 2001; obtained from Dr Seogchan Kang, The Pennsylvania State University, Pennsylvania, USA) was grown at 28°C for 48 h in a minimal medium (MM; Hooykaas *et al.*, 1979) supplemented with kanamycin (Kan; 50 μg mL^{-1}). A 2 mL aliquot of the bacterial culture was harvested and incubated with induction medium (IM; Bundock *et al.*, 1995) amended with Kan (50 μg mL^{-1}) and acetosyringone (AS; 200 μM). Bacterial cells were grown for an additional 6 h before mixing them with an equal volume of *M. oryzae* conidial suspension (1×10^6 conidia mL^{-1}). This mixture (200 μL) was placed on nitrocellulose filters (0·45 μm pore size, 45 mm diameter; Whatman) on a co-cultivation medium (IM with 5 mM instead of 10 mM glucose) in the presence or absence of 200 μM AS. Following co-cultivation at 25°C for 24, 36 and 48 h, the fungal and bacterial cells on the filter paper were harvested with 2 mL MM with 200 μg mL^{-1} hygromycin B. Individual transformants were transferred into 24-well plates (Costar) that contained 1·5 mL OMA with hygromycin B (200 μg mL^{-1}) and incubated until conidiation occurred (Rho *et al.*, 2002). One conidium from each transformant was transferred to OMA to create monoconidial cultures. The transformed conidia were harvested by scraping the surface of the fungal colony on each plate with a sterile needle. Five millilitres of Tween 20 (0·1% v/v) was added to each plate and the suspension was then filtered through four layers of Veratec Graphic Arts cheesecloth (Fiberweb).

Preparation of humidity chambers

The influence of RH on development of grey leaf spot on perennial ryegrass was investigated using mini humidity control chambers. Each chamber consisted of a 90 mm diameter × 50 mm height glass dish, a 15 × 100 mm glass Petri dish lid, and a 75 mm diameter acrylic plastic stand as described by Dufault *et al.* (2006). Glass dishes were autoclaved for 45 min and plastic stands were sterilized using 0·5% NaOCl for 10 min, rinsed with sterile distilled water and then air dried. Filter papers (125 mm; VWR International) were cut to produce four wide tabs extending from different sides, and autoclaved for 45 min (Fig. 1). Glycerol–water solutions with different glycerol concentrations were prepared with a magnetic stirrer and 30 mL solution was added in each chamber (Dhingra & Sinclair, 1985; Table 1). The filter papers were placed on the plastic stand with the ends of the four tabs submerged in the glycerol–water solution (Fig. 1). Detached ryegrass leaf blades were placed on the soaked filter paper supported by the plastic stand. Humidity control chambers were sealed with Parafilm (Fisher Scientific) to maintain consistent RH levels. Temperature and RH in the chambers were monitored and recorded using iLog data loggers (Escort Data Loggers Inc.).

Effects of relative humidity on fungal infection

Detached leaf blades were inoculated by placing 100 µL conidial suspensions (8×10^4 conidia mL^{-1}) on each leaf blade

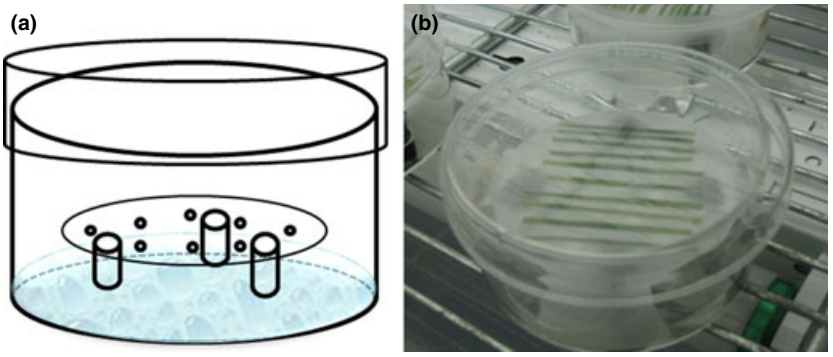

Figure 1 The mini humidity chamber containing a glycerol–water solution, devised for relative humidity experiment. (a) Diagram; (b) chamber containing 10 detached leaf blades.

Table 1 Relative humidity within humidity chambers, obtained with different glycerol solution concentrations at 28°C

Relative humidity[a] (%)	Glycerol solution concentration[b] (%)
100	0
97	7
96	9
94	15
92	22
91	24
88	30
85	36

[a]Relative humidity was determined using RH iLog data loggers (ESCORT Data Loggers Inc.).
[b]Glycerol–water solutions were prepared by adding respective quantity of glycerol to sterile water.

using a pipette. Inoculated leaf blades were incubated inside the humidity chambers with RH (85–100%, at 3% intervals). A completely randomized experimental design was used for each treatment. Each RH level was replicated in three separate humidity chambers and each chamber contained 10 detached leaf blades. All humidity chambers were placed in a Revco incubator (Thermo Fisher Scientific Inc.) at 28°C. Incandescent light was used to maintain a 12 h:12 h dark/light (177 μmol s^{-1} m^{-2}) cycle. Infection of *M. oryzae* was evaluated 10 days after inoculation using a compound bright field microscope (Nikon Eclipse E600; Nikon Instrument group). Histological examination with a bright field microscope at ×100 magnification was facilitated by staining tissues with chlorazol black E (Resendes *et al.*, 2001). The experiment was repeated following the same procedure. Observations with both wildtype and GFP-labelled isolates were conducted, with similar results.

Effects of relative humidity on development of disease

Plant tissues and humidity chamber preparation were prepared as previously described. Three replicate chambers were randomly used for each RH level (88–100% RH, at 4% intervals). Leaf tissues were inoculated with the GFP-tagged *M. oryzae* isolate (8×10^4 conidia mL^{-1}). Three pieces of detached leaf blades from each humidity chamber were observed every 24 h for the first 4 days after inoculation using an FV-1000 laser scanning confocal microscope (Olympus) with excitation and emission wavelengths of 488 nm and 508 nm, respectively. Observation of inoculum development was based on five developmental stages: intact conidia, germinated conidia with germ tubes only, germinated conidia with appressoria, invasive hyphae, and conidia with collapsed cells.

Effect of relative humidity on colonization of perennial ryegrass leaf tissue by *M. oryzae*

Fungal colonization was evaluated by measuring fluorescence intensity (FI) of GFP produced in the cytoplasm of transformed *M. oryzae*. Leaf tissues inoculated with GFP-tagged *M. oryzae* were incubated at RH levels ranging from 88 to 100% at 4% intervals. The FI of GFP produced from the colonized mycelia was measured daily for 14 days after inoculation. Soluble GFP from each replicate at each RH level was extracted by thoroughly homogenizing the colonized plant samples using a TissueLyser (QIAGEN) with 5-mm glass beads (Fisher Scientific) in 200 μL of prechilled extraction buffer (30 mM Tris–HCl, 10 mM EDTA pH 8, 10 mM NaCl, 5 mM DTT) (Chen *et al.*, 2003). TissueLyser parameters were set at 30 Hz for 5 min, and centrifugation steps were performed at 14 000 ***g*** for 15 min. Supernatant (800 μL) with soluble GFP was measured for green fluorescence using an SLM-Aminco 8100 spectrofluorometer (SLM Instruments Inc.) with an excitation wavelength of 485 nm and emission wavelength of 508 nm.

Effect of relative humidity on conidiation of *M. oryzae* on perennial ryegrass

Inoculated detached leaves were incubated in humidity chambers ranging from 88 to 100% RH, at 4% intervals at 28°C, as previously described. Three leaf blades from each humidity chamber were evaluated daily by quantifying the conidiation amount. Three replicate chambers were completely randomized for each RH level. For quantification of conidia, leaves were immersed in 500 μL Tween 20 (0·1%) solution in 1·5 mL centrifuge tubes (VWR International) and agitated for 30 s with a Vortex-Genie (Scientific Industries Inc.). The conidial density was then determined using a haemocytometer (Hausser Scientific). The daily quantification of conidial density continued every 24 h until conidia were no longer observed.

Data analysis

Daily fluorescence intensity measurement and conidiation at various RH levels were analysed by calculating the area under the curve (AUC). An analysis of variance (ANOVA) on the AUC data was performed to determine the effect of RH on the

development of infection over time. Moreover, the difference between the two experiments was determined by using a *t*-test. All statistical analyses were conducted using MINITAB v. 10 (Minitab Inc.)

Results

Infection of *M. oryzae* on perennial ryegrass

Microscopic examination of ryegrass leaf tissues revealed different extents of fungal colonization at RH levels from 85 to 100% at 10 days post-inoculation (dpi) (Fig. 2). No successful colonization of leaf tissue by the mycelia was observed at RH ≤ 88% (Fig. 2a,b). Colonization of tissue by *M. oryzae* was only observed at 92% RH and above (Fig. 2c–f). Visual assessment of inoculated leaf tissue samples indicated that increased colonization by mycelia was associated with increasing RH. Dense mycelial masses were observed at RH ≥ 96% (Fig. 2e,f).

Evaluation of advancement of inoculum on perennial ryegrass

Five developmental stages of *M. oryzae* were used to describe the progression of the pathogen (Fig. 3). All inocula remained as intact conidia for the first 3 days after inoculation at 88% RH (Fig. 4a). At 4 dpi, 23·3% of inocula still remained as intact conidia, 54·2% germinated and produced relatively short germ tubes (less than 10 μm) and 22·5% of the conidia had collapsed cells (Fig. 4a). At 92% RH, 82·8% of inocula germinated (53·9% with only germ tubes and 28·9% with appressoria) at 1 dpi (Fig. 4b). At 4 dpi, 82·9% developed into invasive hyphae (Fig. 4b). At 96% RH, more than 60% of inocula were observed as invasive hyphae at 1 dpi, which increased to almost 100% 4 dpi (Fig. 4c). At 100% RH, 87·4% of inocula were observed as invasive hyphae 1 dpi, and reached 100% by 2 dpi (Fig. 4d). Conidiophores and conidiation were observed at 4 dpi at 100% RH.

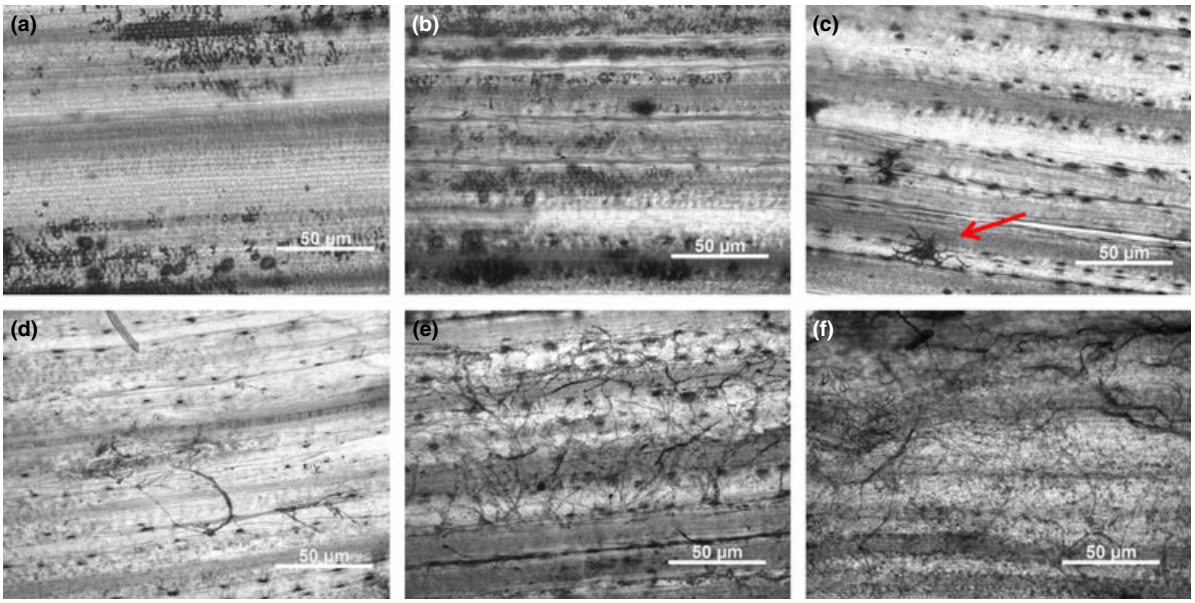

Figure 2 Effects of relative humidity (RH) on infection and colonization of perennial ryegrass tissue by *Magnaporthe oryzae*. Microscopic images were taken 10 days post-inoculation at RH from 85 to 100%, at 3% intervals. No mycelia observed in perennial ryegrass leaf tissues at 85% RH (a) and 88% RH (b); (c) colonizing mycelia (indicated by the arrow) observed at 91% RH (c) and 94% RH (d); intense mycelial colonization observed at 97% RH (e) and 100% RH (f).

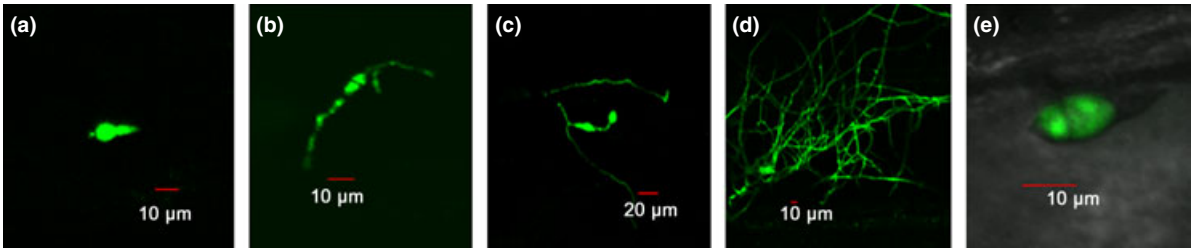

Figure 3 Confocal microscopic images showing the advancement of GFP-labelled *Magnaporthe oryzae* on perennial ryegrass leaf tissues. (a) An intact conidium; (b) a germinated conidium with germ tube only; (c) a germinated conidium with germ tube and appressorium; (d) invasive hyphae; (e) a conidium with degraded cell(s).

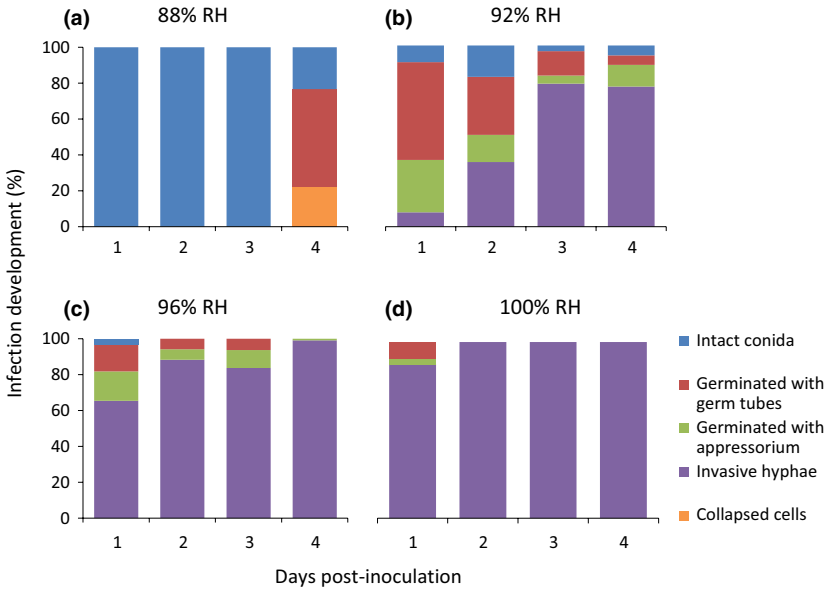

Figure 4 The effects of relative humidity (RH) on development and growth of *Magnaporthe oryzae* during the infection process on perennial ryegrass leaf tissue. Percentage of infection development into various stages was recorded daily for the first 4 days after inoculation. Stages comprised intact conidia, conidia germinated with germ tubes, germinated conidia with appressoria, invasive hyphae and collapsed cells. RH: (a) 88%; (b) 92%; (c) 96%; (d) 100%.

Results of both experiments followed a similar pattern; however, the percentage of collapsed cells was somewhat higher (28% in Experiment 2 compared to 17% in Experiment 1) 4 dpi at 88% RH. There were also some minor differences in percentage of intact conidia, germination of conidia with germ tube or appressorium, invasive hyphae, and collapsed cells at the various levels of humidity in Experiment 1 and Experiment 2.

Quantitative assessment of fungal colonization from infected leaf tissues

In Experiment 1, no fluorescence was detectable from the inoculated leaves when RH was 88% (Fig. 5). At 92% RH, FI was first detected at 10 dpi. The incubation period for first detectable fluorescence was 3 dpi at 100% RH. The highest FI value occurred at 100% RH at 11 dpi, but decreased sharply at 12 dpi. FI values increased at 96 and 92% RH until 14 dpi. The maximum FI value at 96% RH was comparable with the maximum FI value at 100% RH, although it was observed 3 days later at 96% RH.

The results in Experiment 2 followed a similar pattern. However, in Experiment 2, FI was first detected 11 dpi at 92% RH compared to 10 dpi in Experiment 1. At 96% RH, the level of detectable FI was found at 9 dpi compared to 6 dpi in Experiment 1 (data not shown).

Area under the curve (AUC) analysis indicated that there were significant effects ($P \leq 0{\cdot}05$) of RH on fungal colonization in both experiments (Table 2). A *t*-test was

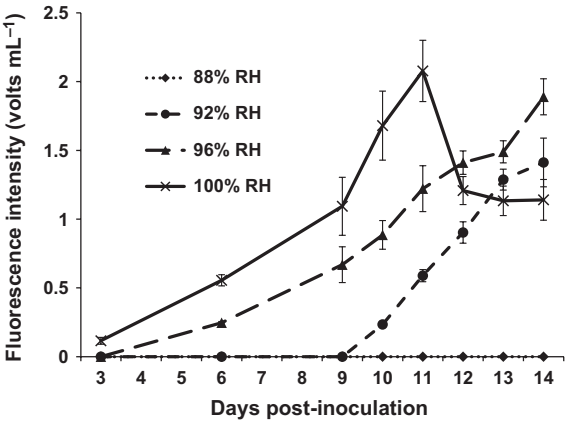

Figure 5 Effects of relative humidity (RH) on the extent of colonization of perennial ryegrass leaf tissue by GFP-labelled *Magnaporthe oryzae* using the measurement of fluorescence intensity. (Mean values of two experiments.)

Table 2 Analysis of variance for area under the curve (AUC) data of fluorescence intensity and conidiation under different relative humidity (RH) levels ($\alpha = 0{\cdot}05$)

Source	d.f.	SS	MS	*F*	*P*
Experiment 1					
Fluorescence intensity					
RH	2	98·05	49·02	34·82	0·000
Error	6	8·45	1·41		
Total	8	106·49			
Conidiation					
RH	1	77 940 104	77 940 104	313·48	0·000
Error	4	994 520	248 630		
Total	5	7 8934 624			
Experiment 2					
Fluorescence intensity					
RH	2	56·45	28·23	56·83	0·000
Error	6	2·98	0·50		
Total	8	59·43			
Conidiation					
RH	1	38 997 152	38 997 152	25·17	0·007
Error	4	6 197 401	1 549 350		
Total	5	45 194 553			

performed for pairwise comparison of the results of the two experiments. The test results showed there was no significant difference between the results of the two experiments ($P \geq 0{\cdot}528$).

Conidiation at various levels of relative humidity

No conidiation was observed when RH was ≤92% (Experiment 1; Fig. 6). Conidiation was first observed at 4 dpi at both 96 and 100% RH. The amount of conidiation was greatest at 8 dpi at both 96 and 100% RH. The amount of conidia increased from an initial 62 conidia μL^{-1} to a peak of 1700 conidia μL^{-1} at 100% RH, and from an initial 8 conidia μL^{-1} to a peak of 191 conidia μL^{-1} at 96% RH. Within 24 h after peak conidiation, conidia formation at 96% RH dropped to 10% of the peak level, but at 100% RH it only dropped to 50% of the peak amount. The conidiation quantity fluctuated over time but was never observed to reach the peak rates found at 8 dpi. By 21 dpi, no additional conidia were produced.

A similar pattern of conidiation was observed in the two experiments, although the peaks were lower in Experiment 2 with 134 conidia μL^{-1} and 1481 conidia μL^{-1} at 96 and 100% RH, respectively (data not shown). AUC analysis indicated that there were significant effects ($P \leq 0{\cdot}05$) of RH on fungal conidiation in both experiments (Table 2). Results of *t*-tests suggested there was no significant difference found between these two experiments ($P \geq 0{\cdot}071$).

Discussion

To the authors' knowledge, this is the first report of the effects of humidity levels on the major events during *M. oryzae*–perennial ryegrass interaction, including infection, colonization and conidiation. Additionally, this research has demonstrated the effective application of GFP-tagged *M. oryzae* and related procedures, such as confocal microscopy and fluorescence intensity measurement, to study the effect of RH on the interaction of *M. oryzae* and perennial ryegrass.

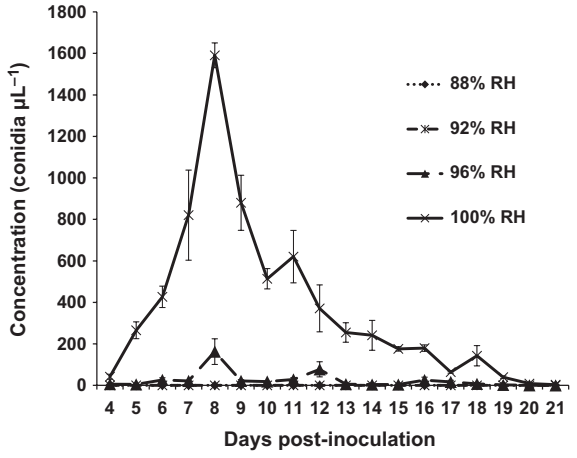

Figure 6 Effects of relative humidity (RH) on conidiation of *Magnaporthe oryzae* during the infection of perennial ryegrass. (Mean values of two experiments.)

The results of the current study indicate that RH has significant effects on both fungal colonization and conidiation. Also, specific RH ranges after inoculation are critical for these major events during grey leaf spot development. High humidity levels (RH ≥ 92% at 28°C) were required for successful infection by *M. oryzae* on perennial ryegrass, which was further illustrated by microscopic examinations. A high percentage of inocula developed into invasive hyphae, with shorter incubation periods at higher RH levels. For example, >80% of inocula developed into invasive hyphae within 24 h of inoculation at 100% RH, whereas it required at least 2 days to reach a similar infection rate at 96% RH or even longer at 92% RH. At low humidity levels (RH ≤ 88% at 28°C) conidia only germinated with short germ tubes (<10 μm) and without the formation of appressoria. Failure of appressorium formation resulted in the failure of *M. oryzae* infection on the perennial ryegrass. At the low RH levels, inocula also remained as intact condia or resulted in collapse of the cells. Therefore, an RH level of at least 92% is required for grey leaf spot development on perennial ryegrass if favourable temperatures for disease development are present.

The high moisture levels in the atmosphere may not only induce the formation of appressoria but also favour the building of turgor pressure in the appressoria of *M. oryzae*, which has been reported to be responsible for the penetration process (Howard & Valent, 1996). Once penetration is successful, invasive hyphae can be observed. A shorter inoculation time was required for germinated conidia to develop into invasive hyphae at higher RH levels. In a previous study on *M. oryzae* causing rice blast disease, free moisture on the surface was found to be required for *M. oryzae* development, such as germination and surface attachment (Hamer *et al.*, 1988). In the current study, the importance of moisture level (which is not only contributed from the surface moisture but also from the atmospheric moisture) for inoculum development was confirmed,.

Visual assessment from microscopic images indicates that heavy mycelial colonization on the leaf tissues was observed at near-saturated humidity levels (≥96% RH at 28°C). In the comparison of the FI of GFP from infected leaf tissues, FI levels increased faster with higher RH levels: although the FI peak at 100% RH was similar to that at 96% RH, the incubation period to reach the peak at 96% RH required three more days. This delay at 96% RH is evidently a direct effect of reduced RH level. Similarly, the increase in FI at 92% RH was slower than that at RH ≥ 96%.

The sudden decrease of FI after the peak amount may be explained by the cessation of fungal vegetative growth as a result of the deficiency of plant tissue nutrients and the collapse of colonized fungi. Studies have indicated the importance of nutrient availability to fungal growth;

the highest concentration of fungal biomass is found when the highest concentration of nutrients is present (Suberkropp, 1995). Although nutrient limitation restricts the maximum amount of fungal colonization, it may play a positive role in the induction of conidiation of *M. oryzae* on perennial ryegrass. Vegetative growth such as mycelial colonization requires nutrient absorption from infected plant tissues. At the same time, conidiation is induced as the colonization progresses. Other fungi, such as those within the genus *Alternaria* and *Aspergillus*, also follow similar sporulation and mycelia colonization trends (Broderick & Greenshields, 1981). Conidiation of *M. oryzae* on perennial ryegrass only occurred at near-saturated RH (≥96% at 28°C) levels, requiring higher RH levels than conidiation on rice seedlings (≥93% RH; Hemmi & Imura, 1939). Moreover, substrates with higher moisture content also favour the conidiation process (Henry & Andersen, 1948). High moisture levels may either occur continuously or intermittently to affect the fungal sporulation. For example, sporulation of *Alternaria porri* on potato and tomato plants is considerably higher during intermittent wet/dry periods than under continuously moist conditions (Rotem & Bashi, 1969).

In conclusion, the infection, colonization and conidiation of *M. oryzae* on perennial ryegrass were highly dependent on the atmospheric moisture levels. Near-saturated humidity conditions were most favourable for *M. oryzae* infection and conidiation. Results of this research could integrate RH into the current grey leaf spot forecasting system (Uddin *et al.*, 2003a), by providing critical infection and conidiation thresholds to assist the evaluation of disease outbreaks and the risk for secondary infections.

Acknowledgements

This research was funded by the Pennsylvania Turfgrass Council. The authors thank Dr Nicholas Dufault for assistance with the humidity chambers set-up; Dr Hye-Seon Kim, Dr Seogchan Kang and Alamgir Rahman for technical support with the transformation work, Missy Hazen for help with confocal microscopy at Penn State Microscopy and Cytometry Facility (University Park, PA) and Dr Marcus Ludwig and Fei Gan for technical assistance with the spectrofluorometer.

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