Germination of *Fusarium graminearum* Ascospores and Wheat Infection are Affected by Dry Periods and by Temperature and Humidity During Dry Periods

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ABSTRACT

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The effects of temperature and relative humidity (RH) on germination of *Fusarium graminearum* ascospores, and of dry periods (DP) of different lengths and of temperature and RH during DP on ascospore survival were studied both in vitro and in planta. Optimal temperatures for ascospore germination at 100% RH were 20 and 25°C; germination was ≤5% when ascospores were incubated at 20°C and RH ≤ 93.5%. Viable ascospores were found at all tested combinations of DP duration (0 to 48 h) × temperature (5 to 40°C) or RH (32.5 to 100% RH). Germination

declined as DP duration and temperature increased. Germination was lower for ascospores kept at 65.5% RH during the DP than at 76.0, 32.5, or 93.5% RH. Equations were developed describing the relationships between ascospore germination, DP duration and temperature or RH during DP. Durum wheat spikes were inoculated with ascospores and kept dry for 0 to 48 h at approximately 15°C and 65% RH; plants were then kept in saturated atmosphere for 48 h to favor infection. Fungal biomass, measured as *F. graminearum* DNA by quantitative polymerase chain reaction, declined as DP increased to 24 and 48 h at 3 and 9 days postinfection but not in spikes at maturity.

Additional keywords: *Fusarium* head blight, *Gibberella zeae*, modeling, spore survival.

Fusarium graminearum Schwabe produces sexual spores (ascospores) and asexual spores (macroconidia). The sexual form of *F. graminearum* is *Gibberella zeae* (Schwein.) Petch. In this work, we refer to *F. graminearum* following the “one fungus one name” provision (Geiser et al. 2013; Hawksworth 2011). Several reports indicated that ascospores may be more important than macroconidia for *Fusarium* head blight (FHB) epidemics (Fernando et al. 1997; Osborne and Stein 2007; Sutton 1982). Ascospores are produced on previous crop debris in the field, such as maize residues (Khong and Sutton 1988; Pereyra et al. 2004), and are then forcibly discharged into the air from perithecia (Trail et al. 2002). They can initiate infection after traveling short distances (de Luna et al. 2002; Fernando et al. 2000; Keller et al. 2010; Markell and Francl 2003; Paulitz 1996) and also long distances (Del Ponte et al. 2003; Francl et al. 1999; Maldonado-Ramirez et al. 2005; Prussin et al. 2014a, b; Schmale et al. 2012).

The environmental conditions favoring the formation of perithecia and the discharge of ascospores (Inch et al. 2000; Manstretta and Rossi 2015a, b; Paulitz 1999a, b; Trail et al. 2002; Tschanz et al. 1976) as well as their germination (Beyer and Verreet 2005; Gilbert et al. 2008; Sung and Cook 1981) have been well studied. These processes are greatly affected by relative humidity (RH) (Jennings and Turner 1996; Xu 2003). Studies on ascospore germination have consistently indicated that ascospore germination is maximal at high RH (from about 84 to 100% depending on the report) and that the germination rate rapidly diminishes as RH declines below these levels; according to the literature, no germination occurs below 50 to

55% RH (Beyer et al. 2005; Beyer and Verreet 2005; Sung and Cook 1981; Ye 1980).

Processes leading to ascospore infection (development and maturation of perithecia, ascospore ejection, and ascospore germination) are then all strictly dependent upon high environmental humidity. Ascospores are usually ejected from perithecia under wet conditions provided by rainfall or atmospheric humidity (Manstretta and Rossi 2015b). After becoming airborne, ascospores eventually settle on the surface of a spike and germinate within a few hours if humidity is high (Beyer and Verreet 2005). Therefore, ascospore discharge seems to preferentially occur when humidity conditions for germination are favorable. Nevertheless, ascospores may be deposited on wheat spikes when humidity is unfavorable for germination if any of the following occur: (i) ascospores are ejected from perithecia significantly after the ejection has been triggered by moisture (Manstretta and Rossi 2015b) so that some of the ascospores become airborne under dryer conditions; (ii) a portion of the seasonal ascospores are discharged and dispersed under dry conditions (Manstretta and Rossi 2015b); or (iii) there is a time lag between ascospore ejection and deposition (Schmale et al. 2006).

As a consequence of these three possibilities, *F. graminearum* ascospores can be recovered from spikes on every day regardless of moisture conditions (Francl et al. 1999). Ascospores present on the spikes under unfavorable humidity conditions for germination may survive until the next period of high humidity favors germination. Little and ambiguous information is available on the time ascospores remain viable once they have been discharged from the perithecium. Jin et al. (2001) found that the ascospore germination rate declined rapidly within 12 days. Beyer and Verreet (2005) observed that ascospores lost their viability after being discharged from perithecia and detected significant portions of nonviable ascospores within a few days after discharge. Schmale and Bergstrom (2004) found

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significant viability of ascospores on both artificial (plastic) and real wheat spikes after 3 days of exposure in natural environments. Similarly, Francl (1998) observed that ascospores remained viable on spikes for about 48 h. Some ascospores were found to retain their viability for a considerable length of time after discharge from the perithecium (Beyer and Verreet 2005; Schmale et al. 2006).

Effect of relative humidity on ascospore survival is unclear. Beyer and Verreet (2005) found that ascospores lost their ability to germinate when exposed to 0% RH for 1 min, and incubation of ascospores at 53% RH decreased the percentage of viable spores from 93 to 6% within 10 min. In contrast with this drastic effect of RH, Gilbert et al. (2008) reported that ascospores exposed at 30, 60, and 90% RH for 4 to 48 h were still able to germinate on water agar, even though germination rates fell when temperature increased between 15 and 30°C; the lowest rate of germination, 13%, occurred after 48 h of incubation at 30°C and 60% RH.

As indicated in the previous paragraph, information on how environmental conditions affect ascospore viability is ambiguous and incomplete. Relevant questions remain unanswered. What is the viability at temperatures lower or higher than those tested by Gilbert et al. (2008)? What are the dynamics of viability when ascospores are exposed to dryness (i.e., periods with low RH) for durations between 4 and 48 h (which were the only two durations considered by Gilbert et al. [2008]), i.e., for those dry periods (DP) that usually occur in the normal daily pattern of RH (Peixoto and Oort 1996)? Does the reduction in ascospore viability *in vitro* reflect the reduction in infection severity in planta? Answering these questions should improve epidemiological models and lead to new approaches for controlling the disease.

The objectives of this study were to (i) quantify the survival of *F. graminearum* ascospores as affected by temperature, RH, and exposure duration by considering a broader range of conditions than in the previous published works; and (ii) assess the effect of DP on ascospores after their deposition on wheat spikes by means of *in planta* trials.

MATERIALS AND METHODS

Preparation of ascospore suspensions. Two strains of *F. graminearum* were used; one was isolated from wheat kernels in Italy (Emilia-Romagna) (Università Cattolica del Sacro Cuore, Piacenza, Italy), and the other was isolated from oat kernels in Germany (Institut für landwirtschaftliche Kulturen, Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Sanitz, Germany). These strains were maintained on 1.5% water agar at 4°C. Small plugs of agar were transferred to the center of 60-mm-diameter plates containing carrot agar (Klittich and Leslie 1988) and incubated at 20°C in the dark until the fungi had completely colonized the agar surface (in 5 to 7 days). After the aerial mycelium was gently removed with a sterile metal rod, 1 ml of an aqueous solution (2.5%) of Tween 20 (Sigma-Aldrich, St. Louis, MO) was uniformly spread on the agar surface. The plates were then covered with the lid but not sealed (to allow air circulation) and incubated at 20°C with a 12-h light/12-h dark photoperiod; a mixture of white and UV light was used to induce perithecia.

After 14 days, the perithecia were mature and were individually transferred with a needle to a tube containing 1.5 ml of water. The suspension of perithecia was mixed with a vortex apparatus to allow mature ascospores to exit from perithecia and become suspended in the water. The suspension was then passed through a sterile, single layer of cheesecloth to remove the perithecia. The concentration of ascospores in the suspension was determined with a Bürker hemocytometer and was adjusted to 2×10^4 ascospores/ml. Separate ascospore suspensions were prepared for each fungal strain for the *in vitro* experiments, while equal amounts of the two single-isolate suspensions were mixed for the *in planta* experiments. Microscopic examination confirmed that ascospores in the suspensions had not germinated at the initiation of these experiments.

***In vitro* experiments.** Two types of experiments were performed to study (i) the effect of temperature (temp) and RH on the germination of *F. graminearum* ascospores, and (ii) the effect of ascospore exposure to DP that differed in length, temperature, and RH on ascospore survival. Both experiment types were run three times. In all experiments and replicate runs, germination of ascospores was observed on microscope slides as previously done by Gilbert et al. (2008).

Effect of temperature. Drops (20 µl each) of the ascospore suspension of each of the two fungal strains were placed on microscope slides (three drops per slide) and dried in a laminar flow cabinet. The slides were kept in the cabinet just long enough to allow the drops to dry. The ascospores on the slides were examined at the end of this drying phase, and none had germinated. The microscope slides were then moved from the laminar flow cabinet and arranged in Petri dishes (94 mm diameter, one slide per dish) over a wet blotting paper.

To study the effect of temperature on ascospore germination in a saturated atmosphere, the dishes were placed in incubators at 100% RH and at 5, 10, 15, 20, 25, 30, 35, or 40°C for 24 h. To study the effect of time on ascospores germination, the dishes were incubated at 100% RH and 20°C for 3, 6, 12, and 24 h (dishes to be checked at these different times were arranged in the incubator according to a complete randomized design). Germination was assessed using a light microscope (200× magnification) for 600 ascospores per treatment, resulting from two slides × three drops per slide × 100 randomly chosen ascospores per drop; a treatment is defined as the combination of one incubation temperature, or one incubation time, × two fungal strains. An ascospore was scored as germinated if at least one germ tube was clearly visible.

To study the effects of temperature on germination during DP of varying lengths, microscope slides were arranged in Petri dishes over a dry blotting paper and incubated at 60% RH and 5, 10, 15, 20, 25, 30, 35, or 40°C for DP of 0, 3, 6, 9, 12, 24, or 48 h (dishes to be checked at these different times were arranged in the incubator according to a complete randomized design); 60% RH was chosen as the baseline for DP and temperature evaluation because no or few germination occurs at 60% RH (Beyer et al. 2005; Sung and Cook 1981). Temperature and RH inside incubators were monitored for the duration of the experiments using data loggers (Tinytag Plus 2 TGP-4500, Gemini Data Loggers, Chichester, UK). No condensation was observed on slides or in Petri dishes for the duration of the experiment. At the end of each DP, the blotting paper was wetted with 1.5 ml of sterile-deionized water, and dishes were sealed with Parafilm (Neehan, WI) to obtain a saturated atmosphere (100% RH). These dishes were then incubated at 20°C for 24 h before germination was assessed as described earlier. The percentage of germinated ascospores was recorded as a measure of ascospore viability.

Effect of RH. For assessment of RH effects on ascospore germination, microscope slides were prepared as described earlier (three drops of ascospore suspension per slide, two slides per fungal strain). Once the drops had dried, the slides were placed on a metal grid in plastic boxes (19 × 13 × 4 cm) and incubated at 20°C and with 32.5, 65.5, 76.0, 93.5, or 100% RH for 24 h before germination was assessed as described earlier. Different RH levels were obtained by placing saturated aqueous salt solutions (CaCl₂, NH₄NO₃, NaCl, and KNO₃ for 32.5, 65.5, 76.0, and 93.5% RH, respectively; Dhingra and Sinclair 1995) or distilled water (for 100% RH) in the boxes. The boxes were sealed in transparent plastic bags, and the real value of RH inside the boxes was measured with data loggers (Tinytag Plus 2 TGP-4500, Gemini Data Loggers) for the duration of the experiment. Boxes with the different RH levels were arranged in the incubator according to a complete randomized design.

To study the effects of RH on ascospore germination during DP of varying lengths, an experiment was set up as described earlier and microscope slides were incubated for 0, 3, 6, 12, or 24 h (boxes to be assessed at these different times were arranged in the incubator according to a complete randomized design). At the end of each DP,

microscope slides were transferred to another box in which salt solutions were replaced with distilled water to obtain 100% RH. After 24 h at 100% RH, ascospore germination was assessed as described earlier.

In planta experiments. Durum wheat plants (cultivar Saragolla, which is susceptible to FHB) were grown in pots ($14 \times 14 \times 20$ cm³) in a greenhouse; there were 10 spikes per pot at heading. On 20 May 2013 and 9 May 2014, when plants were at full anthesis (stage 65 of the BBCH scale, Meier 2001), spikes were inoculated with an ascospore suspension (2×10^4 ascospores/ml, Rossi et al. 2007) of the two fungal strains, prepared as described earlier; 1 ml of ascospore suspension was nebulized per spike. After inoculation, plants were subjected to DP that lasted for 0, 6, 12, 24, or 48 h; air temperature during the DP was $15.2 \pm 2^\circ\text{C}$ in 2013 and $15.5 \pm 2^\circ\text{C}$ in 2014; RH was $65.5 \pm 5\%$ in 2013 and $65.1 \pm 5\%$ in 2014 (these RH levels were considered similar to dry conditions tested in the laboratory experiments). For the uninoculated control, spikes were nebulized with sterile distilled water instead of an ascospore suspension. Pots were assigned to the different DP and to the uninoculated control (treatments) according to a randomized block design, with four replicate pots per treatment (10 spikes per pot).

At the end of each DP, plants were sealed in a transparent plastic bag for 48 h. To guarantee high humidity, the internal side of the plastic bags was sprayed with water. After 48 h at near 100% RH, the plastic bags were removed, and plants were kept outdoors. In 2013, average temperature for the whole period outdoors was 19.5°C , with a total of 66 h with RH $\geq 90\%$, and 78.8 mm of rain in 6 rainy days. In 2014, average temperature was 16.2°C , with a total of 179 h with RH $\geq 90\%$, and 91.8 mm of rain in 6 rainy days. Symptoms first appeared 7 to 9 days after infection.

Spike samples (five spikes per sample) were collected 3 and 9 days after the plants had been sealed in plastic bags and at maturity on 24 June 2013 and on 22 June 2014. Spikes were carefully washed under tap water, brushed to remove epiphytically mycelia, and washed again under tap water for 30 min. Spikes were then dried with blotting paper, frozen in liquid nitrogen, and stored at -80°C until they were processed for DNA content.

In 2014, FHB index was calculated assessing 20 random spikes per DP (five spikes for each replicate pot per treatment) at 20 days postinfection (dpi). FHB severity was assessed on individual spikes as the percentage of spikelets with bleaching per spike, sometimes with pink/orange mycelia at the edge of the glumes. FHB index was then calculated per each replicate pot as average disease severity.

***Fusarium* DNA analysis.** *F. graminearum* mycelium was grown and lyophilized as described by Terzi et al. (2007). DNA was extracted from the lyophilized mycelium as described by Al-Samarrai and Schmid (2000). A 10-fold dilution series of the DNA was used to prepare a standard curve for *F. graminearum* DNA as described in the next section.

DNA extraction from wheat samples. After spike tissue was finely ground in liquid nitrogen and with a mortar and pestle, DNA was extracted according to Terzi et al. (2007). The primer pair 22F (AATATGGAAACGGAGTTCATCTACA)/122R (ATTGCCGGTGCTGAAAGT), designed from the *Tri5-Tri6* intergenic sequence (AY134892 accession number), was used for the real-time quantitative polymerase chain reaction (qPCR) analysis of the samples (Terzi et al. 2007). qPCR was carried out in a 20- μl reaction volume containing 10 μl of KAPA SYBR FAST ABI Prism qPCR kit (Kapa Biosystems, Wilmington, MA), 0.4 μl of both the F and the R primer (10 μM), 2 μl of template DNA solution (10 ng/ μl), and 7.2 μl of water. The amplification was conducted with a 7300 Real-time PCR System (Applied Biosystems, Foster City, CA) and with the following cycling protocol: 95°C for 3 min and 40 cycles of 95°C for 3 s and 60°C for 30 s. A melting curve analysis step (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) was included in each run to confirm the amplification of a single gene product and to control for false-positive results caused by primer-dimer hybridization and nonspecific amplifications. For the quantification of *Fusarium*

DNA, a standard curve was generated by using the 10-fold dilution series of pure *F. graminearum* DNA that was described in the previous section; for this standard curve, cycle threshold (Ct) values of the diluted DNA were plotted against the $\log_{10}$ amount of DNA. The quantity of fungal DNA in the wheat samples was estimated based on the comparison with the standard curve (which was run in parallel reactions), and the results were expressed as picogram of fungal DNA per nanogram of total DNA (of plant and fungal origin) extracted from a sample.

As reported by Terzi et al. (2007), the lower detection limit of the assay was 4 pg of fungal DNA, corresponding to about 100 *F. graminearum* haploid genomes. The specificity of the assay was previously evaluated on a panel of different *Fusarium* species (Terzi et al. 2007).

Data analysis. The effects of DP duration, temperature, and RH on ascospore viability in the in vitro experiments were assessed with a factorial analysis of variance (ANOVA) for a complete randomized design, with four main factors: DP duration and temperature or RH were considered fixed factors while replicate run and fungal strain were considered random factors, with three and two levels, respectively. Before analysis, percentages were arcsine-transformed to make variances homogeneous.

Regression analysis was used to study how ascospore germination was affected by DP duration $\times$ temperature and DP duration $\times$ RH. The data were rescaled by dividing the percentage of germinated ascospores at any combination of DP $\times$ temperature or DP $\times$ RH, depending on the experiment, by the percentage obtained with no exposure to dryness (DP = 0 h). Different linear and nonlinear regression models were compared based on the Akaike's information criterion (AIC), and the model with the lowest AIC value was selected as the best (Burnham and Anderson 2002).

The effects of DP duration on the *F. graminearum* DNA content in the in planta experiments were assessed with ANOVA for a factorial randomized block design, with two main, fixed factors: DP duration and year. ANOVA was performed for data at 3 dpi, 9 dpi, and maturity separately. The effects of DP duration on the FHB index in 2014 were assessed by a one-way ANOVA for a randomized block design. Before analyses, picogram of fungal DNA per nanogram of total DNA were transformed using the natural logarithm function and FHB index values were arcsine-transformed to make variances homogeneous.

SPSS (SPSS ver. 21, IBM SPSS Statistics, IBM Corp., New York) was used for all statistical analyses.

RESULTS

Ascospore germination as affected by temperature and RH but without prior exposure to dryness. At 100% RH, irrespective of the fungal strain, no ascospores germinated after 24 h of incubation at either 5 or 40°C , germination reached the maximum at 20 and 25°C (Fig. 1A). At 20°C and 100% RH, almost all ascospores had germinated by 12 h (Fig. 1B). When ascospores were kept at 20°C with 32.5, 65.5, 76.0, or 93.5% RH for 24 h, germination ranged from 3 to 5% (data not shown).

Ascospore germination after exposure to dryness. In all treatments some ascospores remained viable, i.e., in all combinations of DP $\times$ temperature and DP $\times$ RH. When ascospores were not exposed to dryness (DP = 0 h), $86.5 \pm 1.9\%$ germinated (average of the three experiments and of the two fungal strains). DP duration ($P < 0.001$) and the interaction DP $\times$ temperature during the DP ($P < 0.001$) significantly affected ascospore viability (measured as germinated ascospores) and globally accounted for approximately 60% of the total variance of the experiment. The percentage of ascospores that germinated declined as the DP duration increased, and the reduction in germination increased as temperature increased during the DP (Fig. 2A). After a 48-h DP, germination was reduced by $<50\%$ at 5 and 10°C compared with ascospores not exposed to dryness, by 59.5 and 68.2% at 15 to 30°C , respectively, and by $>90\%$ at 35 and 40°C , respectively (Fig. 2A).

Irrespective of temperature ($P = 0.721$ for the interaction of strain $\times$ temperature $\times$ DP), the German strain was less sensitive to dryness than the Italian one ($P = 0.013$ for interaction strain $\times$ DP). When ascospores were not exposed to dryness, germination for the two strains was similar (87.4 and 85.6%, respectively) (Fig. 2B). For ascospores exposed to DP of any duration (>0 h), germination was higher for the German strain than for the Italian strain; after 48 h of dryness, germination was 38.4% for the German strain and 22.0% for the Italian strain (Fig. 2B).

Equation 1 provides a good fit to the rescaled data for ascospore germination as a function of DP duration and temperature during the DP:

$$Y = a + b \times DP + c \times DP \times \text{temp}^2 \quad (1)$$

where Y is the rescaled ascospore germination (i.e., ascospore viability; 0 to 1); DP is the duration of the dry period (in hours); temperature is the temperature during the DP ($^{\circ}\text{C}$); and a , b , and c are equation parameters: $a = 0.98 \pm 0.01$, $b = -0.009 \pm 0.001$, and $c = -7.73 \times 10^{-6} \pm 0.90 \times 10^{-6}$. This equation accounted for the curvilinear decrease of ascospore germination over time when ascospores were kept in a dry atmosphere and at increasing temperature (Fig. 3A). Standard errors of equation parameter estimates were low, and the R^2 was 0.95. A plot of observed versus predicted values did not show systematic deviations (Fig. 3B), and the regression line between the two series of data had an intercept (a) of 0.02 and a slope (b) of 0.97, which were not significantly different from 0 ($P = 0.345$) and 1 ($P = 0.122$), respectively.

DP duration and RH during the DP significantly affected ($P = 0.022$ and 0.015 , respectively) ascospore viability, but the interaction DP $\times$ RH did not ($P = 0.454$). Neither experiment nor fungal strain significantly affected viability ($P = 0.073$ and 0.323 , respectively). Germination decreased as DP duration increased (Fig. 4). Germination of the ascospores not exposed to dryness was $81.5 \pm 2.9\%$; irrespective of DP duration, germination was lower for ascospores kept at 65.5% RH than at 76.0 or 32.5% RH, and at 93.5% RH. For instance, at DP = 6 h, germination was reduced by 63.4, 48.6, 46.0, and 30.9% at 65.5, 76.0, 32.5, and 93.5% RH, respectively, compared with the ascospores not exposed to dryness (Fig. 4).

Fig. 1. Effect of **A**, temperature and **B**, incubation time on germination of *Fusarium graminearum* ascospores incubated at 100% relative humidity (RH) and without a prior dry period. For quantification of germination, drops (20 μl each) of an ascospore suspension (2×10^4 ascospores/ml) were placed on microscope slides, briefly dried in a laminar flow hood, and then incubated with 100% RH and at different temperatures for 24 h (**A**) or at 20°C for different times (**B**). The percentage of germination was assessed by examining 100 random ascospores per drop. Dots are means and error bars are standard errors of 36 measurements (three experiments, two fungal strains, and six replicate drops).

Fig. 2. Effect of dry periods (DP) on germination of *Fusarium graminearum* ascospores. **A**, Dots represent different temperature regimes during the DP: $\blacklozenge = 5$, $\diamond = 10$, $\blacktriangle = 15$, $\triangle = 20$, $\blacksquare = 25$, $\square = 30$, $\bullet = 35$, and $\circ = 40^{\circ}\text{C}$. **B**, Dots represent one fungal strain originating from Italy ($\bullet$ IT) and one from Germany ($\circ$ DE). For quantification of germination, drops (20 μl each) of an ascospore suspension (2×10^4 ascospores/ml) were placed on microscope slides, briefly dried in a laminar flow hood, and then incubated at 60% relative humidity at different temperatures and for different times. At the end of each incubation period, ascospores were transferred to a saturated atmosphere at 20°C for 24 h before the percentage of germination was assessed based on examination of 100 random ascospores per drop. **A**, Dots are means of 36 measurements (three experiments, two fungal strains, and six replicate drops) and error bars are the standard errors averaged over each DP duration. **B**, Dots are means and error bars are standard errors of 144 measurements (three experiments, six replicate drops, and eight temperatures).

Fig. 3. **A**, Fit of the effect of dry period (DP) duration and temperature during the DP on relative germination of *Fusarium graminearum* ascospores using equation 1 (see text), and **B**, plot of predicted versus observed data. **B**, The line is the linear regression between the two series of data, with $a = 0.02$, $b = 0.97$, and $R^2 = 0.95$. Rescaled germination was calculated by dividing germination of the ascospores exposed to dryness by germination of ascospores that were not exposed to dryness.

Equation 2 provides a good fit to the rescaled data for ascospore germination as a function of DP duration and RH during the DP:

$$Y = (a + b \times RH + c \times RH^2 + d \times RH^3) - (e + f \times RH + g \times RH^2) \times \ln(DP) \quad (2)$$

where Y is the rescaled ascospore germination (i.e., ascospore viability; 0 to 1); DP is the duration of the dry period (in hours); RH is the RH during the DP (%); a to g are equation parameters: $a = 3.29 \pm 1.01$, $b = -0.126 \pm 0.055$, $c = 0.002 \pm 0.001$, $d = -9.85 \times 10^{-6} \pm 0.80 \times 10^{-6}$, $e = -0.038 \pm 0.107$, $f = 0.009 \pm 0.004$, and $g = -7.84 \times 10^{-5} \pm 0.90 \times 10^{-5}$. This equation accounted for the decrease in ascospore germination during a DP and the curvilinear effect of RH during such a DP (Fig. 5A). Standard errors of equation parameter estimates were low, and the R^2 was 0.95. A plot of observed versus predicted values did not show systematic deviations (Fig. 5B), and the regression line between the two series of data had an intercept (a) of 0.04 and a slope (b) of 0.95, which were not significantly different from 0 ($P = 0.343$) and 1 ($P = 0.148$), respectively.

Spike infection after exposure to dryness. The qPCR reaction generated satisfactory standard curve when Ct values were plotted on the $\log_{10}$ amount of pure DNA of *F. graminearum* in a 10-fold dilution series. The slope of the standard curve (from the most concentrated to the least concentrated sample) was -3.3 in 2013 and -3.6 in 2014, both in the optimal range, and the values obtained from three replicates had a high uniformity. The melting curve showed a single peak, indicating that primer dimers were absent and that other targets had not been amplified.

Results for fungal biomass at 3 dpi were different in 2013 versus 2014 (the interaction between dpi $\times$ year was significant: $P < 0.001$). At 3 dpi in 2013, *F. graminearum* DNA was detected only in spikes not exposed to dryness (Fig. 6, left). At 3 dpi in 2014, *F. graminearum* DNA was detected regardless of DP duration but the quantity detected tended to decrease as DP duration increased (Fig. 6, right).

At 9 dpi, fungal biomass was affected by DP duration ($P = 0.019$), irrespective of the year ($P = 0.870$ for the interaction dpi $\times$ year). In both years, the fungal DNA content did not significantly differ with DP of 0, 6, and 12 h but was lower with DP of 24 and 48 h than with shorter DP (Fig. 6).

Fig. 4. Effect of dry period (DP) duration and relative humidity during the DP on germination of *Fusarium graminearum* ascospores. Dots represent length of DP: $\blacklozenge$ = 3, $\square$ = 6, $\blacktriangle$ = 12, and $\circ$ = 24 h; the symbol $-$ represents germination of ascospores not exposed to dryness. For quantification of germination, drops (20 μ l each) of an ascospore suspension (2×10^4 ascospores/ml) were placed on microscope slides, briefly dried in a laminar flow hood, and then incubated at different humidity levels and for different times at 20°C. At the end of each incubation period, ascospores were transferred to a saturated atmosphere at 20°C for 24 h, and the percentage of germination was assessed based on examination of 100 random ascospores per drop. Dots are means and error bars are standard errors of 36 values (three experiments, two fungal strains, and six replicate drops).

At harvest (35 dpi in 2013 and 43 dpi in 2014), fungal biomass was greater than at 3 and 9 dpi but was not affected by year ($P = 0.184$) or DP duration after inoculation ($P = 0.654$); the overall average for *F. graminearum* DNA content at harvest was 268.8 ± 37.6 pg/ng of total DNA (Fig. 6).

At 20 dpi in 2014, FHB index was 32.9 ± 7.5 , 22.3 ± 5.3 , 27.9 ± 3.5 , 23.6 ± 7.4 , and 17.5 ± 5.7 for spikes exposed to 0, 6, 12, 24, or

Fig. 5. A, Fit of the effect of dry periods (DP) and relative humidity during the DP on relative germination of *Fusarium graminearum* ascospores using equation 2 (see text), and B, plot of predicted versus observed data. B, The line is the linear regression between the two series of data, with $a = 0.04$, $b = 0.95$, and $R^2 = 0.95$. Rescaled germination was calculated by dividing germination of the ascospores exposed to dryness by germination of ascospores that were not exposed to dryness.

Fig. 6. Biomass of *Fusarium graminearum* (expressed as the natural logarithm of picogram [pg] of fungal DNA/nanogram [ng] of total DNA) in durum wheat spikes inoculated with ascospores and then exposed to dry periods (DP) for 0 to 48 h. Spikes were inoculated (1 ml of ascospore suspension of the two fungal strains, 2×10^4 ascospores/ml) at full anthesis and kept outdoors (but protected from rain) at 15°C and 65% relative humidity (RH) for 0 to 48 h (the DP). At the end of each DP, plants were sealed in a transparent plastic for 48 h at 100% RH to allow infection. Dots are means and error bars standard errors of 4 replicated samples (20 spikes per sample) at 3 days after infection (lined bars), 9 days after infection (white bars), and at maturity (black bars).

48 h of dryness, respectively, after ascospore inoculation. These differences were not significant ($P = 0.448$).

DISCUSSION

In this paper, most of the *F. graminearum* ascospores discharged from mature perithecia of the two fungal isolates germinated within 12 h at 100% RH and 20°C, but only few germinated within a 24-h period at lower RH levels (between 93.5 and 32.5%). Beyer et al. (2005) and Beyer and Verreet (2005) also reported that a high percentage of ascospore germination occurred only with a very high RH, even though the germination percentages at RH > 70% were higher in these earlier studies than in our experiments. Ascospores were supported by microscope slides in our study and by 1.5% water agar in the two other studies, i.e., the ascospores may have had access to more water in the two other studies than in our study.

This paper has provided new information about the viability of *F. graminearum* ascospores after exposure to a DP and on the effect of temperature and RH during that DP. None of the tested combinations of DP duration, temperature, and RH resulted in the death of all ascospores. In other words, viable ascospores were found at all temperatures, relative humidities, and DP tested, confirming that some *F. graminearum* ascospores are able to remain viable under conditions that are unfavorable for germination (Beyer and Verreet 2005; Franci 1998; Gilbert et al. 2008; Jin et al. 2001; Schmale and Bergstrom 2004; Schmale et al. 2006). Nevertheless, the proportion of viable ascospores sharply decreased with an increase in the duration of the DP, and the rates of decrease depended on temperature and RH. As previously observed by Gilbert et al. (2008) for a narrower temperature interval (15, 20, and 30°C), viability decreased with increasing temperature, and the decrease was the highest at 35 and 40°C in the current study. Consistent with Gilbert et al. (2008), viability was lower at 65.5% RH than at either higher or lower RH levels, with germination dropping from 82% at 100% RH to 17% after a 24-h exposure at 65.5% RH at 20°C. Similar response to RH was also found in other fungi (Akusie and Clerk 1981; Leong et al. 2006; Nicholson and Moraes 1980; Tisserat and Kuntz 1983).

Beyer and Verreet (2005) found that incubation of ascospores at 53% RH (20°C) decreased the percentage of viable spores from 93 to 6% within 10 min. Such a low viability of ascospores in the study of Beyer and Verreet (2005) is difficult to explain based on the results in Gilbert et al. (2008) and in the current study. Given that the ascospores of the two *F. graminearum* strains used in this study differed somewhat in survival ability, the discrepancy in ascospore survival among studies may be due to the difference among the *F. graminearum* isolates. The effect of *F. graminearum* isolates on ascospore survival should be further investigated. Genetic variability of *F. graminearum* has been reported (Leslie and Summerell 2006), but few and contradicting studies were conducted on the effect of this variability on fungal response to temperature (Brennan et al. 2003; Cook and Christen 1976; Ramirez et al. 2006; Rossi et al. 2001, 2002) or water activity (Jiménez et al. 1996).

Beyer and Verreet (2005) reported that viable ascospores are swollen (both before and during the formation of germ tubes) and internally homogeneous, and that nonviable spores are not swollen and are internally granular. These authors concluded that viable and nonviable ascospore cells can be easily distinguished by light microscopy. We also found that internally granular ascospores failed to germinate, but we also found swollen and internally homogeneous ascospores that failed to form germ tubes even after ≥24 h under optimal conditions.

To our knowledge, this is the first published report concerning the effect of ascospore viability on infection severity of wheat plants. Infection severity after ascospore-inoculated spikes were exposed to dry conditions was evaluated by quantifying (via qPCR) the fungal biomass in the spike tissue as picograms of *F. graminearum* DNA per nanograms of total DNA. qPCR has been extensively used

to detect, identify, and quantify *F. graminearum* (reviewed in Morcia et al. 2013) and to study the genes involved in mycotoxin synthesis (Doohan et al. 1999; Lysøe et al. 2009; Marín et al. 2010; Yoruk and Albayrak 2014). Correlation between qPCR results and FHB severity and/or mycotoxin contamination has been frequently reported (Burlakoti et al. 2007; Demeke et al. 2010; Horeváj et al. 2011; Rossi et al. 2007; Schnerr et al. 2001; Terzi et al. 2007; Zhang et al. 2009).

Consistent with the effects of DP on in vitro ascospore viability, exposure of wheat spikes inoculated with ascospores to DP before infection reduced fungal biomass at both 3 and 9 days after inoculation. This reduction in fungal biomass in the early stages of spike invasion did not result in differences in the ultimate disease level, as demonstrated by FHB index and fungal biomass. This result was not unexpected. Wheat spikes were inoculated at full flowering with a high inoculum dose (2×10^4 ascospores per spike), and although exposure to DP reduced ascospore viability and thus the initial infection severity, this did not reduce fungal spread within the spike tissue during kernel ripening. Severe FHB is due to the infection of several spikelets and to the spread of the fungus through the rachis (Bai et al. 2002; Brown et al. 2010; Del Ponte et al. 2007; Franci et al. 1999; Schaafsma et al. 2005).

The relationship between inoculum density and disease observed by Stack (1989) and Stein et al. (2009) may explain the lack of differences in disease severity at kernel maturity in our experiments. In one inoculation experiment with 1, 10, 100, and 1,000 ascospores per wheat spike, Stack (1989) found approximately 5, 10, 20, and 70% disease severity; in another experiment, grain yield per spike was approximately 2.0, 1.3, 1.1, and 0.4 g at the four inoculum doses, respectively, with a grain yield of 2.1 g in the uninoculated spikes. Therefore, each additional ascospore in the inoculum accounted for 5.0, 0.5, 0.15, and 0.055% severity, and 0.1, 0.08, 0.01, and 0.0017 g of yield loss at the four inoculum doses, respectively. It follows that the loss of ascospores at high inoculum doses (as in this work) may have little or no effect on the final disease. Based on the results of Stein et al. (2009); Fig. 1B in that study), reducing the final disease severity in our experiments would have required that our inoculum dose be reduced by approximately 75% (from 2×10^4 to 5×10^3 spores per spike). Such a reduction in inoculum dose, however, would have probably caused fungal biomass to be undetectable a few days after inoculation, and detecting differences in fungal biomass a few days after inoculation was an important objective in this work.

In the field, loss of ascospores due to reduced viability during DP may have a different effect on the final disease than in the conditions of this study because fewer ascospores are deposited on spikes in the field than in artificial inoculations. Panisson et al. (2002) trapped from 0.66 to 1.35 ascospores/cm² of trapping surface per day during wheat anthesis in Brazil. Manstretta et al. (2015) trapped an average of 2.67 ascospores/cm² per day in passive spore traps that were placed in the wheat canopy to mimic wheat spikes. Paul et al. (2007) found that the mean daily number of propagules of *F. graminearum* (both ascospores and conidia) recovered from wheat spikes, quantified as colony forming units (CFUs) per spike, varied among locations and among years at one location. For any given location and year, there was substantial day-to-day variation ranging from 0 to >5,000 CFUs per spike. Franci et al. (1999) observed multiple inoculation events consisting of ≥50 CFUs of *F. graminearum* per spike per day in areas with moderate and severe epidemics, and low average inoculum levels and isolated cases of deposition >50 CFUs in nonepidemic areas.

Overall, our results indicate that DP reduces the viability of the *F. graminearum* ascospores and the biomass of the fungus in the wheat spikes after infection. However, a percentage of the ascospores remain viable for an extended period and may serve as a source of inoculum for infecting hosts. This percentage depends upon length of the DP and on the temperature and RH during that period. Equations were developed that describe these relationships

and that estimate the proportion of ascospores that lose the ability to germinate when they experience unfavorable conditions after discharge from perithecia and when deposited on the wheat head. This information can be integrated in predictive models that consider the relative quantity of inoculum, such as the Brazilian model GIBSIM (Del Ponte et al. 2005) or the prototype model for *F. graminearum* ascospore risk of Gourdain and Rossi (2011).

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