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Germination of *Gibberella zeae* ascospores as affected by age of spores after discharge and environmental factors

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Abstract

The effects of age of ascospores (0–18 days after discharge), photon flux density (0–494 μmol m^{-2} s^{-1} PAR), temperature (4–30 °C), frost (−15 °C for 30 min), relative humidity (RH; 0–100%), pH (2.5–6.5) and dryness (0 and 53% RH for up to 40 min) on the germination of the ascospores of the mycotoxin-producing fungus *Gibberella zeae* (anamorph *Fusarium graminearum*) were studied. Freshly discharged ascospores germinated within 4 h at 20 °C and 100% RH. The rate of germination and the percentage of viable ascospores decreased over time after the spores were discharged from perithecia. The time course of ascospore germination was not significantly affected by photon flux density. The period of time required to obtain 50% germinated ascospores at 100% RH was 26.90 h at 4 °C, 10.40 h at 14 °C, 3.44 h at 20 °C and 3.31 h at 30 °C. There was no significant effect of frost on the percentage of viable ascospores. A small percentage (6.6 ± 3.8%) of the ascospores germinated at 53% RH. At RH ≥ 84% and 20 °C almost 100% of the freshly discharged ascospores germinated. The time course of ascospore germination was affected by pH. The maximum rate of ascospore germination was estimated to be at pH 3.76. Ascospores lost their ability to germinate following exposure to 0% RH almost instantaneously. No germinating spores were detected after an incubation period of 1 min at 0% RH. Incubating the ascospores at 53% RH decreased the percentage of viable spores from 93 to 6% within 10 min. The data demonstrate that age of spores, relative humidity, temperature and pH, but not photon flux density, are key factors in germination of *G. zeae* ascospores.

Introduction

Gibberella zeae (anamorph *Fusarium graminearum*) is the causal agent of head blight and of foot and crown rot of wheat and corn (Sutton, 1982). Losses due to the head blight epidemics in 1996 in the USA were estimated to be $100 million for Ohio, $38 million for Illinois and Indiana and $56 million for Michigan (McMullen et al., 1997). Other species of the genus *Fusarium* also contribute to epidemics, but *G. zeae* was reported to be the predominant species causing wheat head blight in the major wheat producing areas (Fernando et al., 2000; Markell and Francl, 2003). Infections result in yield loss from shrivelled grain and in contamination of the grain by mycotoxins (Bai et al., 2000; Bottalico and Perrone, 2002; Mesterházy, 2002). *Gibberella zeae* produces several mycotoxins, of which deoxynivalenol and zearalenone are the ones most frequently found in wheat flour (Schollenberger et al., 2002). Food and feed contaminated by mycotoxins represent health risks for both humans and animals (Sinha and Bhatnagar, 1998).

Gibberella zeae produces sexual spores (ascospores) and asexual spores (macroconidia) for inoculum dispersal. Several reports indicate that the ascospores may be more important for head blight

epidemics than the macroconidia because the inoculum for head blight requires aerial dispersal to the wheat heads (Sutton, 1982) and the ascospores can be forcibly discharged into the air from perithecia (Trail et al., 2002). First, wind dispersed ascospores were trapped at wheat anthesis (Paulitz, 1999), when the plants are most susceptible to infections. Second, Fernando et al. (1997) compared the spore dispersal gradients of airborne and splashborne fungi and concluded that disease foci of *G. zeae* in wheat probably arose from airborne ascospores rather than from splashborne macroconidia. Furthermore, Fernando et al. (2000) reported that the daily average densities of macroconidia sampled over wheat plots were an order of magnitude less than ascospore densities. Third, Brown et al. (2001) demonstrated the importance of the ascospores for disease severity and mycotoxin level by deleting the mating type locus that controls the sexual reproduction of *G. zeae* and concluded that control strategies should target the ascospores.

The environmental conditions favouring the formation of perithecia and the release of ascospores were studied in detail (Tschanz et al., 1976; Paulitz, 1999; Inch et al., 2000; Trail et al., 2002). In contrast, little information is available on the environmental conditions required for ascospore germination (Sung and Cook, 1981; Sutton, 1982) and survival (Jin et al., 2001). Detailed knowledge about ascospore germination and survival under various environmental conditions is expected to improve epidemiological models and that may lead to new approaches for controlling the disease. The objectives of this study were (i) to quantify the effects of environmental factors on ascospore germination and (ii) to identify factors that delay or inhibit ascospore germination.

Materials and methods

Production of macroconidia

A single spore isolate of *G. zeae* (anamorph *F. graminearum*) (Group II) was obtained from M. Goßmann (Humboldt University, Berlin, Germany). The identity of this isolate as *G. zeae* was confirmed by PCR using pathogen-specific primers (Ludewig, 2003) and morphological traits according to Nelson et al. (1983). *Gibberella zeae* was grown in Petri dishes on SNA medium (Nirenberg, 1976) at 23 °C in the dark. After 19–98 days, macroconidia were washed off using sterile deionized water. The macroconidia suspension was used as inoculum for the production of perithecia and ascospores.

Production of ascospores

Carrot agar (Klittich and Leslie, 1988) was prepared in 9-cm diameter Petri dishes. One droplet of a macroconidia suspension containing approximately 15,000 macroconidia per millilitre was applied to the centre of each carrot agar Petri dish. Cultures were grown at 20 °C and a 12-h photoperiod (photon flux density: 205 ± 14 μmol m^{-2} s^{-1} PAR; light-meter LI-250 equipped with quantum sensor LI-190SA; LI-COR Biosciences, Lincoln, USA). After 5 days, 1 ml of aqueous 2.5% Tween 80 (Carl Roth GmbH, Karlsruhe, Germany) was applied to the surface of the culture (Trail and Common, 2000) and spread across the mycelium using a sterile stainless steel rod. Perithecia developed 13–14 days after this induction with Tween 80 and could be easily detected with the bare eye. The lids of the carrot agar Petri dishes were checked daily for discharged ascospores. Ascospore discharge as indicated by the presence of ascospores in the droplets attached to the Petri dish lids was observed 67 ± 11 days after inoculation ($n = 35$ dishes). Since ascospores of *G. zeae* are discharged from asci along with epiplasmic fluid (Trail et al., 2002), the droplets attached to the inner side of Petri dish lids consisted of epiplasmic fluid diluted with condensed water. Droplets containing ascospores were marked using a waterproof pen and labelled with the date of discharge. Ascospores in droplets attached to Petri dish lids were washed off 0, 1, 8, 12 and 18 days after discharge using sterile deionized water and the resulting spore suspension was used in germination experiments. In some cases, air mycelium reached the Petri dish lids and produced macroconidia. To obtain a pure ascospore suspension free of mycelium and macroconidia, Petri dish lids with air mycelium were replaced by new lids prior to ascospore discharge. The number of replicates of the experiment on the effect of ascospore age on germination ranged from $n = 3$ to $n = 4$.

Germination tests

Spore germination tests were carried out following the procedure described by Beyer et al. (2004). Briefly, the inner side of Petri dish lids was coated with water agar (WA; 1.5%). One droplet of ascospore suspension was applied to the surface of each lid coated with WA. After the droplets disappeared due to evaporation and water uptake by WA, dry silica, constant humidity solutions or deionized water were filled into the Petri dishes, such that the bottom of the dishes was completely covered. Silica, saturated salt (constant humidity) solutions and water were used to adjust the humidity of the air within the dishes (Wexler, 1995). Petri dishes were closed and sealed with parafilm, such that the spores attached to the lid were incubated above the media which regulated the humidity within the dishes. Relative humidities tested were 0% (Silica), 29.8% ($CaCl_2$), 52.6% ($Ca(NO_3)_2$), 75.3% (NaCl), 84.7% (KCl), 92.7% (KNO_3) and 100% (deionized water). The number of replicates of the experiment on the effect of RH on ascospore germination was $n = 3$. Closed Petri dishes with ascospores attached to the lids were placed under the light microscope and the total number and the number of germinated spores per microscope field were counted periodically at 200 × using the same plates throughout a time course experiment. From these data, the percentage of germinated spores was calculated and used as dependent variable in graphs. An ascospore was referred to as "germinated" if at least one germ tube was clearly visible under the light microscope at 200 ×. Unless specified otherwise, germinating ascospores were incubated in darkness, because the ascospores were reported to be preferentially released at night (Paulitz, 1996).

Initially, the effect of ascospore density (10 ± 1, 13 ± 2, 18 ± 1, 30 ± 5 and 60 ± 10 ascospores mm^{-2}) on the time course of germination was tested. Droplets of ascospore suspension were applied to WA coatings as described above and the number of ascospores per squaremillimetre was determined for each spore density level in three microscope fields at 200 ×. The ascospore solution having a density of 48 ascospores per microlitre resulted in 60 ± 10 ascospores per squaremillimetre on WA and lower spore densities were obtained by dilution with sterile deionised water prior to the application of the suspension to WA coatings. The number of replicates was $n = 3$. The average spore density of the ascospore solutions used in the experiments reported in this paper was 47 ± 18 ascospores per microlitre.

To quantify the effect of temperature on ascospore germination, time courses of ascospore germination were monitored at 4, 14, 20 and 30 °C. RH was adjusted to 100% and spores were incubated on WA in darkness as described above. The number of replicates was $n = 3$.

The experiment on the effect of light on germination was carried out in a Heraphyt growth chamber (model HPS 500, Heraeus-Vötsch, Balingen, Germany). Irradiance within the growth chamber was provided by 14 Lumilux Plus Eco warm white lamps (Osram, München, Germany) and the photon flux density within the growth chamber decreased exponentially with the distance from the light sources. The regression equation for the relationship between photon flux density (μmol m^{-2} s^{-1} PAR) and the distance from the light sources (cm) was: Photon flux density $= 81.74 \cdot e^{(108.05/(\text{distance} + 60.09))}$, $r^2 = 0.97$***. Different photon flux densities (0, 218, 315, 430, 494 μmol m^{-2} s^{-1}) were achieved by adjusting the distance of the ascospores from the light sources. The number of replicates was $n = 3$.

The effect of pH on germination was tested by incubating ascospores at 20 °C in citrate buffer (10 mM citric acid adjusted to pH 2.5, 3.5, 4.5 and 6.5 using NaOH). Citrate buffer droplets (5 μl) containing ascospores were applied to the inner surface of Petri dish lids. Filter paper wetted with deionised water was inserted into the Petri dishes and Petri dishes were closed, such that the citrate buffer droplets attached to the lids faced the wet filter paper and, hence, citrate buffer droplets with ascospores were incubated at 100% RH. The percentage of germinated spores within the citrate buffer droplets attached to the Petri dish lids was evaluated by light microscopy after 0, 3, 5, 7, 9, 11 and 24 h as described above. The number of replicates was $n = 3$.

To establish whether ascospores can survive frost, six 50-μl droplets of ascospore suspension were frozen (−15 °C for 30 min) in Petri dishes. Complete freezing of the droplets was confirmed by visual assessment. The ascospore suspension was applied to WA after thawing and the percentage of germinated spores determined by light microscopy after an 18-h incubation period at

20 °C and 100% RH in the dark. Untreated spores served as control. The number of replicates was $n = 6$.

Ascospores were incubated at (53% RH, explained later, Figure 1(d)) or below (0% RH) the critical humidity threshold for germination for up to 40 min and subsequently transferred to 100% RH to test if the ascospores lose their ability to germinate or if germination is just suppressed by low humidity until spores are transferred to high humidity again. Droplets of ascospore suspension were applied to the surface of microscope glass slides. Droplet drying was monitored and the slides were transferred to 0% RH (incubation over dry silica gel) or 53% RH (incubation in growth chamber) immediately after droplet drying. Ascospores were washed off the slides after 1, 5, 10, 20, 30 and 40 min using deionised water and the resulting spore suspension was applied to Petri dishes coated with 1.5% WA. The emergence of germ tubes was used as indication for spore viability and the percentage of germinated ascospores was evaluated after an 18-h incubation period at 20 °C and 100% RH in darkness as described above. The number of replicates was $n = 4$.

Statistics

Data were subjected to the procedures GLM (analysis of variance), REG (linear regression, Figure 1(b)) or NLIN (non-linear regression) of the Statistical Analysis System software package (version 6.12; SAS Institute Inc., Cary, NC, USA). Time courses of ascospore germination were compared at $P = 0.05$ using the REPEATED option of the GLM procedure. Non-linear curve fitting was carried out using either exponential (Figures 1(f) and 2) or sigmoid models (Figure 1(a, c–e, g)). Time courses of ascospore germination (Figure 1(a, c, e, g)) were described using a sigmoid regression model [$y = a/(1 + (x/x_{50})^b)$], where y = percentage of germinated ascospores, x = time, a = maximum percentage of germinated ascospores, x_{50} = period of time, until $a/2$ percent of the ascospores germinated and b = slope parameter. Significance of coefficients of determination (r^2) at $P \leq 0.05$, 0.01 or 0.001 is indicated by *, ** or ***, respectively. Unless specified otherwise, data are presented as mean ± standard error of the mean.

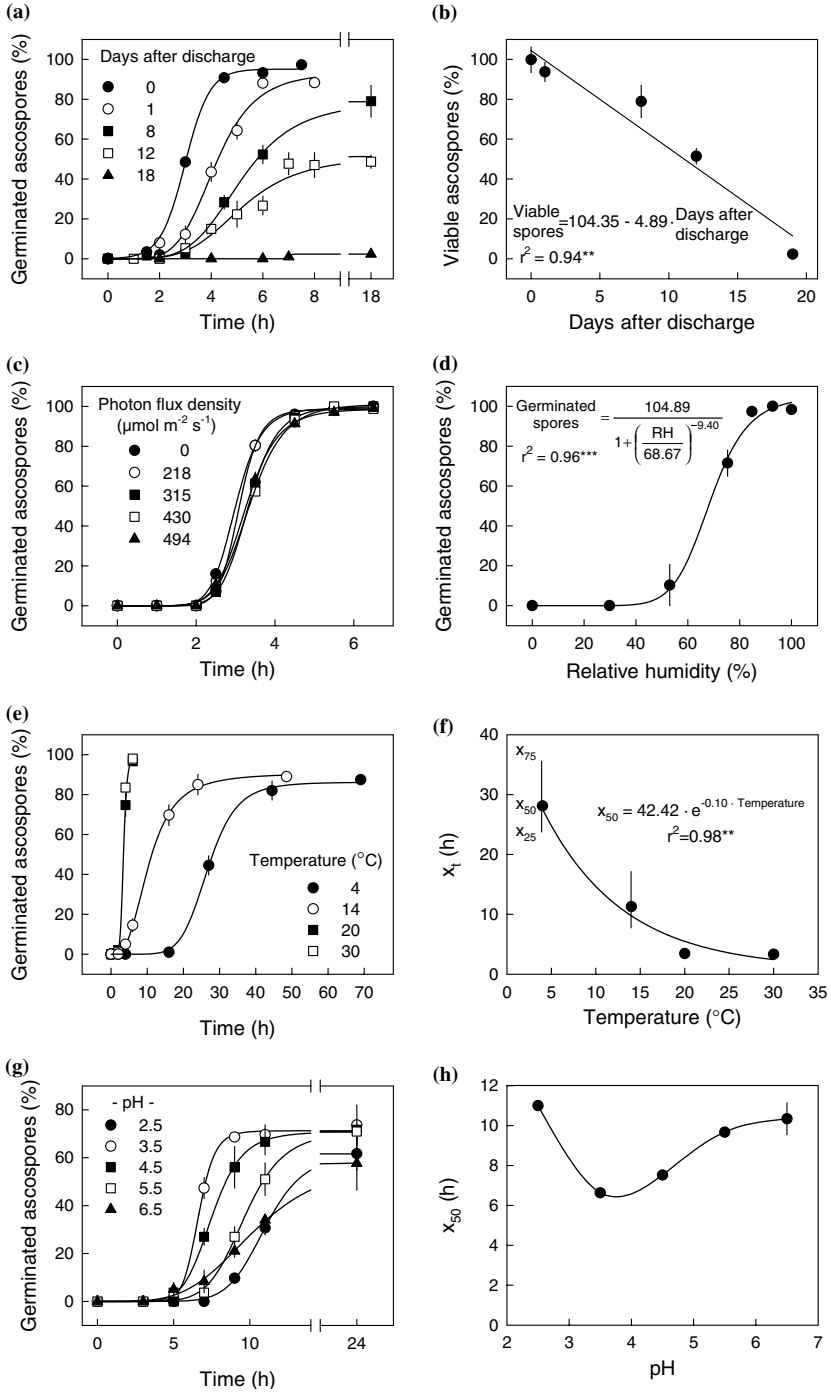
Figure 1. Ascospore germination of *Gibberella zeae*. Unless specified otherwise, spores were incubated at 20 °C and 100% relative humidity on a 1.5% WA coating in darkness. (a) Time courses of ascospore germination. Germination was monitored at 0, 1, 8, 12 and 18 days after ascospore discharge; (b) Relationship between the percentage of viable ascospores and ascospore age expressed as days after discharge. The percentage of viable ascospores and the respective standard errors were estimated by the regressions depicted in Figure 1(a); (c) Time courses of ascospore germination at different photon flux densities (see legend); (d) Percentage of germinated ascospores after an 18-h incubation period at 20 °C in darkness in relation to the relative humidity (RH); (e) Time courses of ascospore germination at 4, 14, 20 and 30 °C; (f) Relationship between the periods of time required to obtain 25 (x_{25}), 50 (x_{50}) or 75% (x_{75}) germinated ascospores and temperature. x_{25}, x_{50} and x_{75} were estimated from the regressions depicted in Figure 1(e); (g) Time courses of ascospore germination at different pH-levels. Ascospores were incubated in 10 mM citric acid buffer adjusted to the pH-levels given in the legend using NaOH; and (h) Periods of time required until 50% of the viable ascospores germinated in citic acid buffer as affected by pH. x_{50} was estimated from the regressions depicted in Figure 1(g) and the plot symbols were connected using a spline interpolation.

Results

Ascospore discharge as indicated by the presence of ascospores in the droplets attached to the Petri dish lids was observed at 67 ± 11 days after inoculation on carrot agar. The ascospores did not germinate in these droplets within the period of observation (up to 18 days after spore discharge).

The time courses of ascospore germination followed a sigmoid growth pattern (Figure 1(a)). There was no significant effect of ascospore density (range from 10 to 60 ascospores per square millimetre) on the time courses of germination on WA ($P = 0.17$, data not shown). The rate of germination (slope of the time courses in Figure 1(a)) as well as the maximum percentage of germinated spores decreased with time after the spores were discharged from perithecia (Figures 1(a) and (b)). Extrapolating the linear relationship between the percentage of viable ascospores and time expressed as days after spore discharge depicted in Figure 1(b) revealed that all ascospores lost their viability at 21.3 days after discharge. On WA, 26% of the freshly discharged ascospores formed one germ tube, 68% formed two germ tubes and 6% formed three germ tubes ($n = 50$).

The time courses of ascospore germination were not significantly affected by photon flux density (Figure 1(c), $P = 0.11$).



The percentage of germinated ascospores depended on relative humidity. No germination was observed at RH <50%. At humidities between 50 and 84%, the percentage of germinated ascospores increased with humidity and at humidities >84% almost 100% of the ascospores germinated (Figure 1(d)).

Temperature had a profound effect on the rate of ascospore germination (Figure 1(e)). The periods of time required to obtain 50% germinated

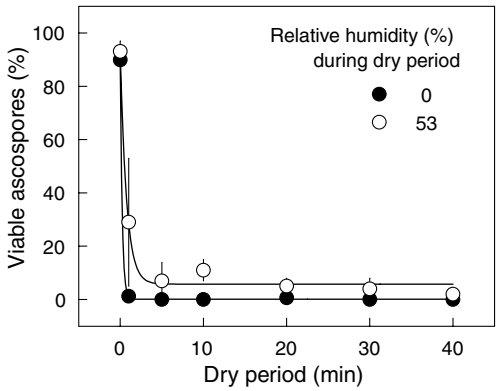


Figure 2. Relationship between the length of a dry period at 0 and 53% relative humidity and the percentage of viable ascospores of *Gibberella zeae*. Curve fitting was carried out using a regression model having the general equation [$y = y_0 + ae^{-bx}$], where y = percentage of germinated ascospores after incubation at 0 or 53% RH; y_0 = minimum percentage of viable spores; x = time; a, b = regression parameters.

ascospores were 26.90 h at 4 °C, 10.40 h at 14 °C, 3.44 h at 20 °C and 3.31 h at 30 °C (Figure 1(f)). The temperature sums required to obtain 25, 50 or 75% germinated ascospores were 3.62 ± 0.42, 4.39 ± 0.67 and 6.08 ± 1.52 degree days, respectively. There was no significant effect of frost (−15 °C for 30 min) on the percentage of viable ascospores (99.4 ± 1.5% vs. 97.3 ± 2.5% for control and frost treatment, respectively; $P = 0.14$).

Time courses of ascospore germination were affected by the pH of a citric acid buffer incubation solution (Figure 1(g)). The period of time required, until 50% of the viable ascospores germinated, was decreased when pH was increased from 2.5 to 3.5, and was increased when pH was further increased from 3.5 to 6.5 (Figure 1(h)). The spline interpolation depicted in Figure 1(h) revealed that the maximum rate of ascospore germination (which corresponds to the steepest point of the respective time course in Figure 1(g)), would be expected at pH 3.76.

Ascospores lost their ability to germinate due to an exposure to 0% RH almost instantaneously (Figure 2). No germinating spores were detected after an incubation period of 1 min at 0% RH (Figure 2). Incubation of the ascospores at 53% RH decreased the percentage of viable spores from 93 to 6% within 10 min (Figure 2).

Discussion

In a previous report (Sung and Cook, 1981), ascospores were obtained by crushing perithecia on glass microscope slides to extrude the spores. This method was not used here, because ascospores that were not yet discharged from perithecia may be immature and, hence, may germinate in a different manner than mature ascospores. Even though direct experimental evidence for differences between mature and immature ascospores is currently missing, only ascospores were used in this study that were discharged from perithecia to avoid the utilization of immature spores.

Tschanz et al. (1976) and Trail et al. (2002) studied the discharge of ascospores in *G. zeae* and found that ascospore discharge events started at 9 to 17 or approximately 15 days after inoculation, respectively. In the present study, ascospore discharge was observed at 67 ± 11 days after inoculation. This discrepancy may be related to the fact that the light regimes used by Tschanz et al. (1976) and Trail et al. (2002) could not be reproduced and light affects the formation of perithecia (Tschanz et al., 1976) and, hence, the period of time required for ascospore production. Moreover, variation has been reported between different isolates of *G. zeae*, in particular on the (molecular) genetic level (Miedaner et al., 2001) and with respect to aggressiveness and mycotoxin production (Gilbert et al., 2001), but the effect of isolate on the time course of macroconidial germination was rather small (Beyer et al., 2004). Recently, Zeller et al. (2004) studied the population structure of *G. zeae* in the USA using AFLP profiles and concluded that all populations were genetically similar and showed evidence of extensive interpopulation genetic exchange. However, since only one isolate was used in the present study, no direct data on the effect of isolate on germination of *G. zeae* ascospores are currently available and hence, the present study should be considered a baseline study.

The ascospores of *G. zeae* lost their viability after being discharged from perithecia (Figure 1(a)). This finding illustrates that ascospores need favourable conditions for germination immediately after discharge. In fact, ascospore discharge increases with relative humidity (Trail et al., 2002) and thus, ascospore discharge seems to occur preferentially when humidity conditions for

germination are also favourable. Currently, the reason for the loss of ascospore viability with time is unknown, but some factors may be excluded. First, the humidity in the carrot agar Petri dishes must have been approximately 100% as indicated by the permanent presence of droplets at the Petri dish lids due to condensation. Therefore, the loss of spore viability cannot be attributed to desiccation in this case. Second, the temperature was 20 °C which is usually favourable for fungal growth and survival. Third, only discharged ascospores were used such that immaturity is unlikely to be the reason for the limited spore viability.

Since significant portions of non-viable ascospores were detected only a few days after discharge, the pure presence of ascospores in wheat plots should not be interpreted as a sufficient prerequisite for inoculum potential. Additionally, the ability of the spores to germinate should be tested. This will particularly be important when long-distance dispersal of ascospores by wind is investigated. In this case, ascospores may have been discharged several hours or even days ago. Viable ascospores were swollen (both, before and during the formation of germ tube(s)) and internally homogenous, whereas non-viable spores were not swollen and internally granular (Figure 3(a)). Even if three out of four ascospore cells were not viable, the remaining viable cell still formed a germ tube and a macroconidium (Figure 3(b)). Hence, viable and non-viable ascospore cells can be easily distinguished by light microscopy.

A small portion of ascospores (8.2 ± 3.0%) germinated at humidities as low as 53% which is an unusual low humidity for fungal spore germination or fungal growth (Ayerst, 1969). A lower humidity requirement of air dispersed spores may be expected in comparison with spores spread by rain splash dispersal. However, the low humidity required for ascospore germination illustrates that *G. zeae* ascospores are a dangerous inoculum, because periods of time with less than 53% relative humidity are rare events in the temperate zones, where head blight epidemics were most severe in the past (McMullen et al., 1997; Bottalico and Peronne, 2002). Data obtained at our institute in northern Germany (latitude 54°18′, longitude 10° 06′) in wheat plots in the seasons 1997 and 1998 indicate that only 9.16% of the hours in June (around wheat anthesis) had average relative humidities lower than 50% (S. Röding, pers. comm.). In term, approximately 90% of the hours in June had suitable humidity levels for the germination of at least a fraction of ascospores. Hence, models predicting ascospore germination using weather data will have a very limited usefulness for disease management, because low humidities suitable for suppressing ascospore germination hardly occur, at least in northern Germany.

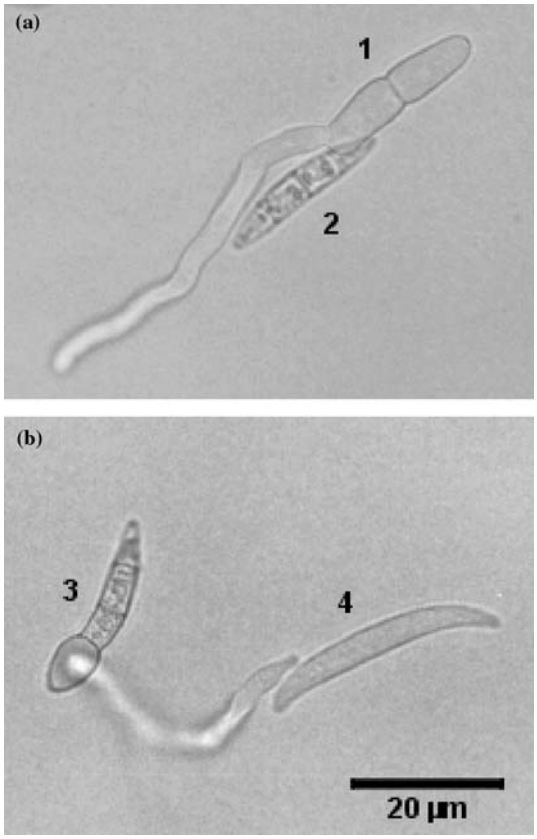


Figure 3. (a) Germinated (viable (1)) and non-germinated (non-viable (2)) ascospore of *Gibberella zeae* and (b) *Fusarium graminearum* macroconidium (4) produced by a partly collapsed ascospore (3).

The ascospores of *G. zeae* lost the ability to germinate due to an exposure to low relative humidity within only a few minutes (Figure 2). On the one hand, the ascospores may not have survived the desiccation but on the other hand, dryness may have induced constitutive dormancy in *G. zeae* ascospores according to the definition by Sussman and Douthit (1973). However, if dryness induced ascospore dormancy, then re-hydration of the spores failed to recover them from dormancy.

Moreover, long distance ascospore dispersal by wind will hardly be effective if the spores are subjected to relative humidities $< 50\%$ during their voyage, because they will lose their ability to germinate. Ascospores of *G. zeae* are discharged along with droplets of epiplasmic fluid (Trail et al., 2002). These droplets may protect ascospores from desiccation until they evaporate.

The rate of ascospore germination was at maximum at pH 3.7. Decreasing the pH from 3.7 to 2.5 or increasing the pH to 6.5 delayed ascospore germination by 66 or 56%, respectively. Effects of pH on spore germination in other fungal species were reported previously. For instance, the alkalinity of $Ca(OH)_2$ solutions is thought to be linked to the mechanism inhibiting spore germination and germ tube growth of the ascomycete *Venturia inaequalis* (Schulze and Schönherr, 2003). The pH dependence of germination may be interpreted as evidence for the involvement of enzyme activity in the process of ascospore germination. For example, Clausen and Green (1996) observed the maximum activity of fungal polygalacturonase between pH 3.4 and 3.9. However, avoiding pH 3.7 in foliar applied sprays at wheat anthesis, e.g. by adding pH-buffers, may help to reduce the infection efficacy of *G. zeae*.

Ascospores of *G. zeae* are preferentially released at night (Paulitz, 1996) and the formation of perithecia depends on light (Tschanz et al., 1976). The present results established that the time courses of germination were not significantly affected by photon flux densities up to 494 μmol m^{-2} s^{-1} PAR. Hence, darkness is neither a prerequisite for ascospore germination nor does it promote germination.

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