

The Eurotiomycete *Apinisia graminicola* as the causal agent of a leaf spot disease on the energy crop *Miscanthus x giganteus* in Northern Germany

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Abstract *Miscanthus x giganteus* is a fast growing, perennial energy crop for temperate climates. Because of its high annual biomass production rates and its characteristics as a low-input crop, an expansion of field cultivation can be anticipated to cover increasing demands for sustainable biomass production. However, knowledge about pathogens that could have an impact on biomass production is still limited for *M. giganteus*. Here, we report about the isolation of the filamentous fungus *Apinisia graminicola* from necrotic leaf lesions of *M. giganteus* grown on a field trial plot in Northern Germany. Inoculation assays with the isolated *A. graminicola* strain confirmed its capacity to cause a leaf spot disease on *M. giganteus*. Additional inoculation assays revealed that

A. graminicola also caused necrotic lesions on leaves of the model grass *Brachypodium distachyon*. Generally, symptoms of *A. graminicola*-caused leaf spot disease were stronger on *B. distachyon* compared to *M. giganteus*. Incubation temperatures above 22 °C during *A. graminicola* infection resulted in stronger disease symptoms on both, *M. giganteus* and *B. distachyon* leaves. Microscopic analysis of cross sectioned, infected leaf tissue revealed an epiphytic mycelium formation on the surface and an endophytic colonization of the mesophyll leaf tissue, especially in *M. giganteus*. Our results revealed that the isolated *A. graminicola* strain is a causal agent of a leaf spot disease on grass leaves. Its potential on endophytic growth in *M. giganteus* might open new possibilities in studying this type of plant-fungal interaction on a cellular and molecular level in an energy crop.

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Introduction

To supply the increasing demand for energy and natural products deriving from renewable resources, the production of biomass has to be extended because the utilization of agricultural and forestry waste would not produce sufficient biomass if considered as sole lignocellulosic feedstocks. Therefore, cultivating energy crops can be considered as an alternative approach (Somerville et al. 2010), especially to fulfill requirements on sustainable agricultural production systems. These

would include a high biomass production, low level fertilization, low water consumption, and not being invasive (Dale et al. 2011). Adapted to temperate climates, the fast growing C₄ perennial grass *Miscanthus x giganteus* would fulfill most of these criteria (Lewandowski et al. 2000; van der Weijde et al. 2013). In addition, *M. giganteus* is one of the most productive energy crops in temperate climate zones (Dohleman and Long 2009; Heaton et al. 2008; Hulle et al. 2012). Its perennial nature is associated with an extended and permanent root system, the so-called rhizome, which can reduce soil erosion and store atmospheric CO₂ (Hughes et al. 2010). Its favorable agronomic traits describe *M. giganteus* as a low-input energy crop (Lewandowski et al. 2003). Several studies have revealed the potential of *M. giganteus* biomass production in temperate climates (Lovett et al. 2009; Mishra et al. 2013; Schorling et al. 2015; Stampfl et al. 2007; Thomas et al. 2013). Hence, an expansion of *M. giganteus* cultivation can be anticipated. Despite its potential for extended sustainable biomass production, the knowledge about emerging pathogens that can infect *M. giganteus* is still limited. Mainly two reasons support the importance of identifying *M. giganteus* pathogens: on the one hand, the possible impact on biomass yield losses due to infection (Stewart, and M. C. 2011); and on the other hand, to host pathogens of other crops (Glynn et al. 2015).

The growing interest in *M. giganteus* cultivation has promoted studies on identifying pathogens of this energy crop where fungal species represent the majority. Pusz and Płaskowska (2010) identified *Stagonospora tainanensis* as a *M. giganteus* leaf pathogen, which could reduce biomass yields; and *Pithomyces chartarum* is the causal agent of *M. giganteus* leaf blight with a biomass-reducing potential that might exceed that of *S. tainanensis* (Ahonsi et al. 2010; Ahonsi et al. 2013). In our recent study (Falter and Voigt 2014), we were able to show the biomass-reducing potential of *Bipolaris sorokiniana*, *Cochliobolus heterotrophus*, *Stagonospora macropycnidia*, and *Stagonospora nodorum*, which we identified as *M. giganteus* leaf pathogens and which have been well described pathogens of crops (Bakonyi et al. 1998; Oliver et al. 2012; Ullstrup 1972). The list of fungal species was recently extended by the study of Glynn et al. (2015), who identified *Rhizoctonia solani*, *Fusarium poae*, *Fusarium culmorum*, and *Sordaria fimicola* as putative, emerging *M. giganteus* leaf pathogens.

Our study was aimed at identifying and isolating fungal species from field-grown *M. giganteus* leaves that revealed necrotic lesions due to infection. We wanted to focus on those fungal species that have not been reported as *M. giganteus* pathogens before. The inclusion of *Brachypodium distachyon* as an additional host for newly identified and isolated fungal species from *M. giganteus* leaves would give a first idea about host specificity. Moreover, based on its characteristics as a model plant, *B. distachyon* would facilitate the establishment of model pathosystems with newly identified fungal species, which would provide relatively easy genetic access for molecular studies on the host side (Alves et al. 2009; Draper et al. 2001; International Brachypodium, I 2010). From necrotic leaf lesions of *M. giganteus*, we isolated the ascomycete *Apinisia graminicola*, a fungus that was first reported as a saprophyte on grass cuttings (La Touche 1968) and has not been described as a plant pathogen yet. The genus *Apinisia* is a member of the Onygenaceae, which is one out of four families within the order of Onygenales (Currah 1985; Kirk et al. 2008). Because many Onygenales reveal a keratinolytic, cellulolytic or chitinolytic ability, these enzymatic capabilities have been suggested for phylogenetic characterization of Onygenales together with molecular analyses (Currah 1985; Scott et al. 1993; Sugiyama et al. 2002). In addition, morphological features are used to separate families within the Onygenales and genera within a family. Genera assigned to the family of Onygenaceae can be separated based on ascocoma configuration, peridium morphology, conidia position and size, or ascospore shape (Currah 1985). In this regard, a globose ascospore shape is characteristic for the genus *Apinisia* as shown for *A. graminicola* (La Touche 1968; Sigler et al. 1998). Because of divergent ascospore shape and substrate specificity, *A. queenslandica*, considered to represent a second species of the genus *Apinisia* (Apinis and Rees 1976), has been suggested to be redispersed in the genus *Uncinocarpus* (Sigler et al. 1998). Hence, *A. graminicola* remains the only undisputed species of the genus *Apinisia*.

Materials and methods

Isolation and cultivation of fungal material from *M. giganteus* leaves

M. giganteus leaves with spot-like necrotic lesions were harvested from a small-scale field trial plot in Wedel,

Northern Germany (53°34'51.4"N, 9°42'41.5"E). Single necrotic lesions were cut out from leaves, surface sterilized with 70% ethanol, and transferred on plates containing CzV8CS medium (modified after Solomon et al. (2004): 33,6 g l⁻¹ Czapek Dox liquid medium (Oxoid), 3,0 g l⁻¹ CaCO₃, 200 ml l⁻¹ Campbell's V8 juice, 20,0 g l⁻¹ casamino acids, 20 g l⁻¹ peptone, 20 g l⁻¹ yeast extract, 3 g l⁻¹ adenine, 0,02 g l⁻¹ biotin, 0,02 g l⁻¹ nicotinic acid, 0,02 g l⁻¹ thiamine, 15 g l⁻¹ agar agar). Cultivation of plates with necrotic *M. giganteus* leaf tissue was performed at 18 °C under near-UV light and white light (TL-D 36 W/08, TL-D 36 W/840, Philips, Netherlands) in a 12-h photoperiod until occurrence of fungal growth out of the tissue. To obtain pure fungal cultures, growing fungal hyphae were separated and cultivated on new CzV8CS plates. Purity of fungal culture was confirmed by sequencing.

Determination of fungal material from *M. giganteus* leaves

The CTAB protocol was used to isolate genomic DNA of the isolated fungus (Leslie and Summerell 2006). An approximately 900 bp DNA fragment of the 28S ribosomal gene was amplified via PCR by using LROR (5'-ACCC GCTGAACTTAAGC-3') and LR5 (5'-TCCT GAGGGAACTTCG-3') primers as described by Vilgalys and Hester (1990). For the purification of the PCR product, the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) was used according to manufacturer's instruction. To identify the fungal species, the PCR product was sequenced using primers LROR, LR5, LR3R (5'-GTCTTGAAACACGGACC-3') and LR3 (5'-CCGTGTTTCAAGACGGG-3') (all primers adapted from Vilgalys and Hester (1990)). The resulting partial sequence of the 28S ribosomal RNA was blasted against the nucleotide collection database at the National Center for Biotechnology Information (NCBI, <http://blast.ncbi.nlm.nih.gov/blast.cgi>) and stored in GenBank with the Sequence ID: KF647896. The fungal strain isolated from *M. giganteus* leaves was identified as *Apinisia graminicola*.

Plant material for leaf inoculation assays

B. distachyon (inbred line Bd21 (Huo et al. 2006)) was grown in a growth chamber at 22 °C and a 16 h photoperiod (HPI-T PLUS 400 W and SON-T PIA AGRO

400 W, Philips, Netherlands). *M. giganteus* was grown in a greenhouse with an additional light supply to provide 16 h light if required.

Droplet inoculation of detached leaves

Detached leaves of 4–5 week old *B. distachyon* and *M. giganteus* were used in the inoculation assays. Droplet inoculation followed the description in Falter and Voigt (2014). In brief, detached leaves were surface sterilized and transferred on water agar supplemented with kinetin (1 mg/ml). 10 µl droplets of gelatine-tween20-solution containing mycelium of the newly isolated fungal strain were used for detached leaf inoculations. Incubation of inoculated leaves was performed at 22 °C, 26 °C or 30 °C in a photoperiod of 16 h light and 8 h darkness for 7 days. Control leaves were treated with droplets of the gelatine-tween20-solution.

The relative necrotic leaf area as a scale for disease severity was determined with the ImageJ tools (Rasband, W.S., US National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997–2011) “freehand selection” and “measure” as described in Falter and Voigt (2014).

Statistical analysis

Descriptive statistics including the mean and the standard error of the mean (±SE) along with the Tukey range test for multiple comparison procedure in conjunction with an ANOVA were used to determine significant differences. $P < 0.05$ was considered significant.

Microscopy of inoculated leaves

The microscopy of inoculated leaves with the confocal laser-scanning microscope LSM 780 (Zeiss, Germany) was performed according to the description in Falter and Voigt (2014) using calcofluor white to visualize fungal mycelium on the leaf surface (Herth and Schnepf 1980), wheat germ agglutinin coupled with the green fluorescent dye CF488A to visualize fungal mycelium inside the infected leaf tissue in cross sections (Mirelman et al. 1975), and aniline blue staining to visualize plant cell walls in cross sections.

Results

Identification of a phytopathogenic fungus isolated from *M. giganteus* leaves

We isolated a fungus from *M. giganteus* leaves showing disease symptoms from a small field trial plot located in Wedel, Northern Germany. Spot-like disease symptoms were restricted to single leaf sections without indication of an epidemic outbreak or severe, large-scale necrosis. For the isolation of putative phytopathogenic fungi from infected tissue, spot-like necrotic lesions were cut out from *M. giganteus* leaves (Fig. 1a) and placed on CzV8CS medium. The cultivation of a filamentous fungus growing out of the necrotic leaf tissue was maintained until reaching a pure culture with only one fungal strain. Sequencing of a 28S ribosomal RNA gene fragment from the purified fungal strain revealed a 99% sequence identity to the Ascomycete *Apinisia graminicola* (La Touche 1968) (Supplementary Data Fig. 1). Cultivation of the isolated *A. graminicola* strain on nutrition-rich CzV8CS medium revealed the same morphological characteristic of a dense, white mycelium growth (Fig. 1b) that was previously described for this fungal species (La Touche 1968). Inoculation of intact *M. giganteus* leaves under greenhouse conditions with the isolated *A. graminicola* induced disease symptoms of spot-like necrotic lesions, which were comparable to those observed on field-grown *M. giganteus* plants (Fig. 1a). We found similar spot-like necrotic lesions also on detached *M. giganteus* leaves after

A. graminicola inoculation (Fig. 2a) indicating the suitability of the detached leaf assay in surveying infection of this plant pathogenic fungus.

Temperature-dependent disease severity on *B. distachyon* and *M. giganteus* leaves

Similar to the induction of disease symptoms on detached *M. giganteus* leaves, the isolated *A. graminicola* strain caused spot-like necrotic lesions on detached *B. distachyon* leaves (Fig. 2a). Comparing necrotic lesion formation as a scale for disease severity (Falter and Voigt 2014) on both plant species, *B. distachyon* revealed a higher susceptibility to *A. graminicola* than *M. giganteus*, where only a relatively small leaf area showed necrotic symptoms (Fig. 2). At 22 °C, necrotic lesions covered 2.5% of the leaf area in *B. distachyon* and only 1.5% in *M. giganteus*. In previous studies, global warming has been identified as a potential driver of altered pathogen growth and pathogenicity, and higher temperatures might also impact plant development and defense responses (Chakraborty 2013; Siebold and von Tiedemann 2013). To test possible scenarios of global warming, we also tested disease severity of the isolated *A. graminicola* strain on *B. distachyon* and *M. giganteus* leaves at 26 °C and 30 °C. Interestingly, higher incubation temperatures during *A. graminicola* infection resulted in stronger disease symptoms on both, *B. distachyon* and *M. giganteus* leaves (Fig. 2). On *M. giganteus* leaves, the area of necrotic lesions increased to 3% at 26 °C and further rose to 4% at 30 °C (Fig. 2b), which was almost three times higher

Fig. 1 Disease symptoms on *M. giganteus* plants infected with *A. graminicola*. **a** Necrotic lesions on *M. giganteus* leaves from which the *A. graminicola* strain was isolated, scale bar =1 cm. **b** Growth morphology of isolated *A. graminicola* strain on CzV8CS medium

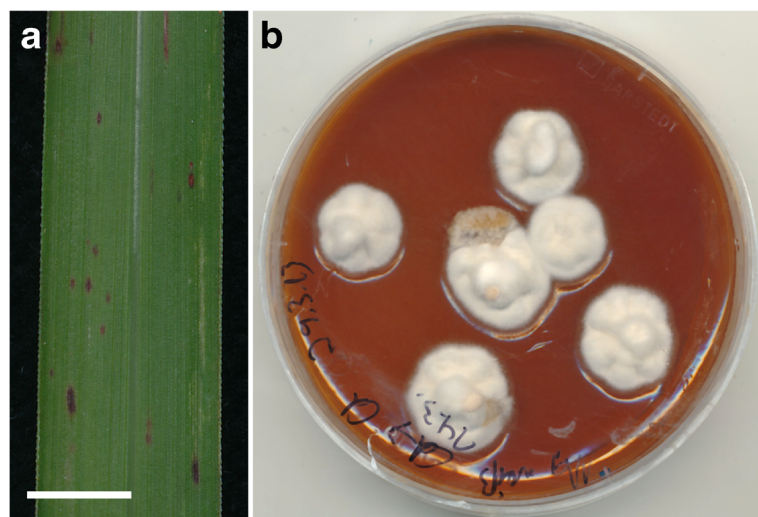
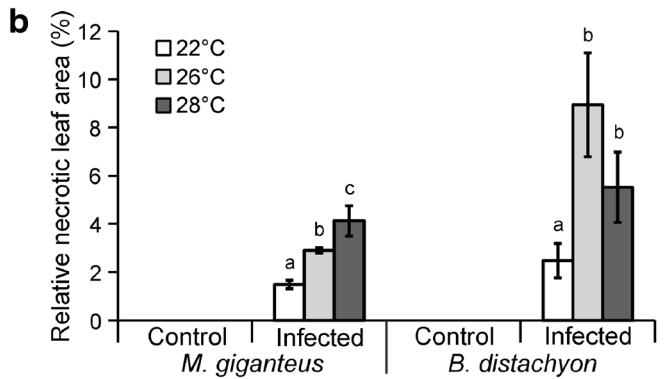
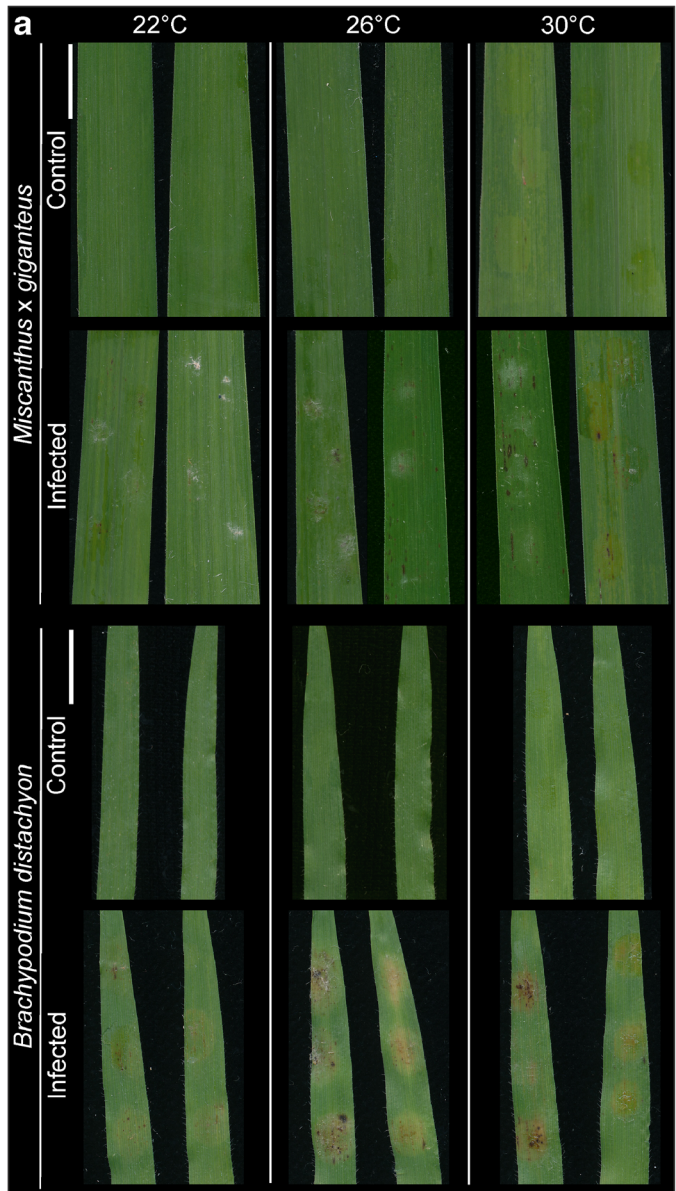


Fig. 2 Temperature-dependent disease phenotypes on *M. giganteus* and *B. distachyon* leaves caused by *A. graminicola*. Inoculation of detached leaves was performed with 10 μ l droplets containing mycelium, control leaves with gelatine-tween20 solution only. Leaves were incubated at 22 °C, 26 °C, and 30 °C. **a** Photos represent disease phenotypes 7 days post-inoculation (dpi), scale bar =1 cm. **b** Determination of the relative necrotic leaf area formation 7 dpi as an indicator for disease severity at 22 °C, 26 °C, and 30 °C; letters a, b, and c indicate groups with $P < 0.05$ by Tukey's test, error bars represent SE, and $n = 4$



than at 22 °C. On *B. distachyon* leaves, the temperature dependent increase in disease severity of *A. graminicola* was higher compared to *M. giganteus* leaves. Disease symptoms were almost four times stronger at 26 °C compared to 22 °C; and necrotic lesions covered 9% of the leaves at the higher temperature (Fig. 2b). This relatively high level of disease severity remained stable at 30 °C (Fig. 2b).

Cellular analysis of infected *B. distachyon* and *M. giganteus* leaves

To examine fungal colonization on leaf surfaces, *A. graminicola* hyphae were stained with calcofluor white to facilitate high-resolution imaging using a confocal laser-scanning microscope. On both, *M. giganteus* and *B. distachyon* leaf surfaces, *A. graminicola* revealed dense hyphal network formation that exceeded the area of necrotic lesion formation, especially on *M. giganteus* leaves (Figs. 2a and 3), which would be characteristic for epiphytic fungal growth. The occurrence of appressoria-like hyphal structures above developing necrotic lesion formation (Fig. 3) supported the plant pathogenic capacity of the isolated *A. graminicola* strain. Moreover, aggregation of hyphae occurred in areas of necrotic lesion development, which could have ring-like shapes (Fig. 3). The production of conidiophores on both, *M. giganteus* and *B. distachyon* leaves (Fig. 3) showed that *A. graminicola* was able to complete its life cycle on this leaf tissue. Microscopic

analysis of cross sections in the area of necrotic lesion formation confirmed that the isolated *A. graminicola* strain was able to penetrate and colonize *M. giganteus* and *B. distachyon* leaf tissue. Interestingly, not only epiphytic growth on the leaf surface but also internal hyphal growth exceeded necrotic lesion formation in *M. giganteus* leaf tissue. Lateral hyphal growth underneath the penetration area and outside the area of lesion formation was apoplastic (Fig. 3), which would resemble an endophytic growth capacity. Both, epiphytic and endophytic growth of *A. graminicola* was less pronounced in *B. distachyon* (Fig. 3).

Discussion

Combining the relatively limited genetic variety and an expanding cultivation area of *M. giganteus* because of an anticipated increasing demand of sustainable biomass production, this energy crop might be challenged by emerging pathogens. Among pathogens, fungal species would have a high potential to lower biomass yields after infection and successful colonization. In our study, we were able to identify and isolate an *A. graminicola* strain from field-grown *M. giganteus* that can cause a leaf spot disease. Interestingly, *A. graminicola* was first described as a saprophyte on decomposing grass cuttings in the UK where the same visible white aggregates of cleistothecia were formed as on V8 juice agar (La Touche 1968). Until now, this fungal species has not

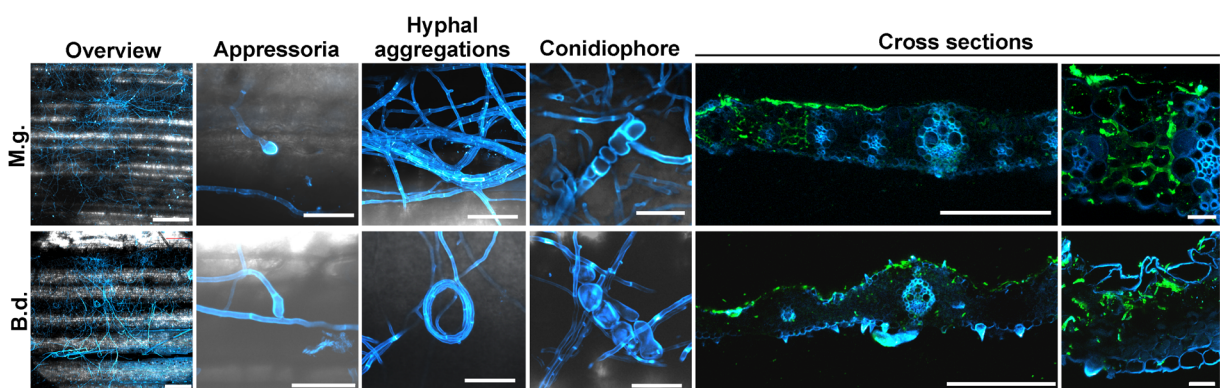


Fig. 3 Microscopic analysis of *M. giganteus* (M.g.) and *B. distachyon* (B.d.) leaves infected with *A. graminicola*. Inoculation of detached leaves was performed with 10 μ l droplets containing mycelium. Micrographs showing superficial hyphal growth, appressoria, and hyphal aggregations were taken 3 days post-inoculation (dpi). Calcofluor white (*blue fluorescence*) was

used to stain mycelium. Micrographs from leaf cross sections represent infection progress 7 dpi, mycelium was stained with wheat germ agglutinin CF488 conjugate (*green fluorescence*) and the leaf tissue was visualized with aniline blue (*blue fluorescence*). Scale bars =20 μ m for appressoria and magnified cross sections, scale bars =100 μ m for overviews and cross sections

been reported as a plant pathogen. Because the isolated *A. graminicola* strain not only infected and colonized *M. giganteus* but also *B. distachyon* leaf tissue, it might not be considered as a *forma specialis* of *M. giganteus*. *A. graminicola* belongs to the Onygenales (Sugiyama et al. 1999), which is an order of Ascomycota. Recent phylogenetic studies placed *A. graminicola* in the subcluster of Onygenaceae (Johnston et al. 2015) confirming previous classification of this fungus (Currah 1985; Scott and Untereiner 2004). In this subcluster, *A. graminicola* showed highest phylogenetic relation to *Arachnotheca glomerata*, which can be found in soils (Uchiyama and Udagawa 1995). The most related subcluster contained members of keratinophilic fungal species belonging to the family of Arthrodermataceae (Johnston et al. 2015), including the pathogenic fungus *Microsporium audouinii* that can cause mycetoma, a chronic subcutaneous infection of humans (Burton et al. 1980). Even though this might reveal a relative relation to a pathogenic fungus, *A. graminicola* could not have been anticipated from previous reports of its occurrence or phylogenetic analysis to be a causal agent of a leaf spot disease on *M. giganteus*. This is in contrast to already known fungal pathogens of *M. giganteus* with previous reports about infection of other plant species. *Pithomyces chartarum*, causing leaf blight of *M. giganteus* (Ahonsi et al. 2010), has also been reported to cause leaf spot of smooth brome (*Bromus inermis*) (Eken et al. 2006) and leaf blight of wheat (*Triticum aestivum*) (Tóth et al. 2007); *Stagnospora tainanensis*, infecting especially young *M. giganteus* leaves (Pusz and Płaskowska 2010), was previously described as the causal agent of sugarcane (*Saccharum officinarum*) leaf blight (Hsieh 1979); and several *Fusarium* species, including *F. culmorum* and *F. graminearum* as causal agents of *Fusarium* head blight in wheat (Osborne and Stein 2007), caused rhizome rot in *M. giganteus* (Scauflaire et al. 2013). Also in our recent study, where we screened for *M. giganteus* leaf pathogens, all four identified filamentous fungi causing necrotic lesions have previously been reported as pathogens of Poaceae including wheat and maize (Falter and Voigt 2014). A reason why *A. graminicola* has not been previously identified as a causal agent of a leaf spot disease could be related to its disease severity. On field-grown and greenhouse-grown *M. giganteus* leaves, *A. graminearum* caused relatively small necrotic lesions. Disease symptoms of a leaf spot disease were similar to those caused by *S. tainanensis* (Pusz and

Płaskowska 2010), but covered a smaller relative leaf area. Compared to other fungal pathogens with an observed or potential capacity of an epidemic outbreak on *M. giganteus* leaves, associated with biomass reduction, like *P. chartarum* (Ahonsi et al. 2013) and *C. heterostrophus* (Falter and Voigt 2014), respectively, we would not expect severe epidemic outbreaks and significant biomass losses caused by *A. graminicola* infection based on our current knowledge. However, our infection assay data clearly indicated that higher temperature would support severity of *A. graminicola* infection of *M. giganteus* leaves, which we have previously reported also for the plant pathogenic fungi *S. nodorum* and *S. tainanensis* (Falter and Voigt 2014). Hence, increasing temperatures due to climate change could support the emergence of new plant diseases caused by fungi that have not been reported as *M. giganteus* pathogens in temperate climates yet.

Our microscopic studies of infected leaf tissue suggest that *A. graminicola* penetrates directly through the cell wall of epidermal cells. This was supported by the observation that we did not find invasions through stomata. Moreover, *A. graminicola* hyphae formed appressoria-like structures on leaf surfaces in areas of necrotic lesion formation and tissue colonization. The extent to which the observed hyphal aggregations, associated with ring-like shapes, in areas of lesion formation would represent specialized infection structures is not clear. The morphology of *A. graminicola* hyphal aggregations differed from so-called infection cushions. These specialized infection structures have been described as a type of compound appressoria. A direct association of these specialized structures with infection was shown for the genera *Rhizoctonia*, *Sclerotinia*, and *Fusarium* (Boenisch and Schäfer 2011; Emmett and Parbery 1975). We also found cushion-like hyphal aggregations on *M. giganteus* and *B. distachyon* leaves infected by *B. sorokiniana*, *C. heterostrophus*, *S. macropyrenidia*, and *S. nodorum* in our recent study (Falter and Voigt 2014). In general, formation of appressoria, invasion, and initial fungal colonization of leaf tissue was not different between *M. giganteus* and *B. distachyon*. Therefore, *B. distachyon* would qualify as a host plant in a model pathosystem to study *A. graminicola* infection and tissue invasion. In this regard, the model grass *B. distachyon* would have the benefit of a high genetic diversity (Gordon et al. 2014), accessibility to a relatively small, sequenced genome, efficient transformation protocols, and easy cultivation (Alves et al. 2009; Draper et al.

2001; International Brachypodium, I 2010). However, endophytic growth of *A. graminicola* would not be fully covered by the *B. distachyon* – *A. graminicola* pathosystem because we observed extended apoplastic growth of *A. graminicola* in the mesophyll tissue without causing disease symptoms, which is typical for endophytes (Stone et al. 2000), only in *M. giganteus* leaves. Fungal endophytes can modify plant disease severity (Busby et al. 2016). For example in maize, which is phylogenetically closely related to *M. giganteus* (Lawrence and Walbot 2007), the fungal endophyte *Acremonium zeae* can support resistance to *Aspergillus flavus* and *Fusarium verticillioides*, which are associated with kernel rotting and mycotoxin production (Wicklow et al. 2005). Recently, Chirino-Valle et al. (2016) showed in their study that the application of specific mixtures of *Trichoderma* species, which are known as fungal root endophytes (Harman 2006), was beneficial for *M. giganteus* biomass production. This effect of *Trichoderma* species has been associated with an inhibition of plant pathogens through mycoparasitism (Druzhinina et al. 2011), antimicrobial compound production (Reino et al. 2008), and induction of host plant resistance (Harman 2006). In addition, the production of secondary metabolites by *Trichoderma* species can promote plant growth (Hermosa et al. 2014; Vinale et al. 2008). Therefore, direct application assays with *A. graminicola* on *M. giganteus* could reveal whether this fungus with endophytic growth characteristics would be beneficial for *M. giganteus* growth or provide increased pathogen resistance.

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