Fumonisin B_1 and beauvericin accumulation in wheat kernels after seed-borne infection with *Fusarium proliferatum*

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Fusarium proliferatum is a fungal pathogen causing ear rot of maize. The fungus infects a range of other plants but the economic impact of these diseases has not been established. Recently, *F. proliferatum* and its mycotoxin fumonisin were found in wheat grains. Here we report that seed-borne infection of wheat with *F. proliferatum* resulted in systemic colonization of wheat plants and contamination of wheat grains with fumonisins and beauvericin. *F. proliferatum* strains originating from different hosts were able to infect wheat via seeds. Colonization of wheat plants with the fungus was highest in the stems, followed by leaves; one third of the strains reached kernels, causing accumulation of fumonisins and beauvericin to 15–55 μg kg^{-1}. The results show that seed-borne infection of wheat with *F. proliferatum* can lead to contamination of wheat kernels with mycotoxins fumonisins and beauvericin.

Key words: systemic colonization, mycotoxins quantification, fungal DNA, mycotoxins contamination

Introduction

Fusarium proliferatum is a member of *Gibberella fujikuroi* species complex which consists of eight mating populations (MPs) and a number of asexual lineages (Leslie et al. 2006), described as morphological species (Nirenberg and O'Donnell 1998). Most members of *G. fujikuroi* species complex are economically important plant pathogens. Decreased yield as well as diminished quality of plant products due to *Fusarium* infection cause significant economic losses worldwide (Placinta et al. 1999). *F. proliferatum* is a member of the complex with a wide host range. The pathogen was predominantly found in maize (*Zea mays* L.) but it also infects rice (Abbas et al. 1999) and sorghum (Bacon and Nelson 1994) and causes crown, spear and root rot of asparagus (von Bargen et al. 2009), bulb rot in garlic and onion (Stankovic et al. 2007), and fruit rot in date palms (Abdalla et al. 2000).

Grains infected with *F. proliferatum* accumulate mycotoxins. *F. proliferatum* and *F. verticillioides* are the main source contamination with fumonisins, which are toxic and carcinogenic. Fumonisin B_1 is the most abundant fumonisin found in maize. Fumonisins also can be detected in symptomless infected maize kernels (Bacon and Hinton 1996). These mycotoxins inhibit sphinganine/sphingosine N-acyltransferase (ceramide synthase), a key enzyme in the sphingolipid metabolism and triggers apoptosis (Riley et al. 1996). High levels of fumonisins in maize were associated with outbreaks of equine leukoencephalomalacia (Wilson et al. 1990) and swine pulmonary edema (Osweiler et al. 1992). Association between fumonisin exposure and esophageal cancer was reported in South Africa (Rheeder et al. 1992). Besides fumonisins, *F. proliferatum* was shown to synthesize moniliformin (Marasas et al. 1986), beauvericin (Moretti et al. 1994) and fusaproliferin in maize kernel cultures (Ritieni et al. 1995), and fusarins in mineral medium consisting of ultrapure water (1 l), $(NH_4)_2HPO_4$ (1 g), KH_2PO_4 (3 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (5 g), sucrose (40 g) and glycerol (10 g; final pH 6.2), which was also satisfactory for moniliformin production (Miller et al. 1995). Besides *F. proliferatum,* other causal agent pathogens of *Fusarium* head blight synthesize moniliformin, beauvericin, fusarins, and fusaproliferin in grains. Moniliformin is produced by *F. culmorum* in rice culture (Scott et al. 1987), by *F. avenaceum* and *F. oxysporum* in parboiled rice culture (Abbas et al. 1989). Beauvericin is produced by *F. avenaceum* in wheat (Logrieco et al. 2002) and by *F. poae*, *F. oxysporum* (Logrieco et al. 1998) and *F. verticillioides* (Leslie et al. 2004) in maize kernels. Fusarin C is produced by major causal agents of *Fusarium* head blight in maize grains, and synthesized by *F. avenaceum* in parboiled rice culture (Abbas et al. 1989). *F. verticillioides* can produce trace levels of fusaproliferin in autoclaved yellow maize kernels (Leslie et al. 2004). Fusaproliferin is not produced by any *Fusarium* species that has been found in wheat except for *F. proliferatum*.

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Systemic infection of host plants has been reported for several *Fusarium* species. The entire maize plant can be systemically colonized by *F. verticillioides* without symptoms. In this way, *F. verticillioides* can be transmitted via seeds (Munkvold et al. 1997). Bacon and Hinton (1996) reported that maize kernels infected with *F. verticillioides* (*F. moniliforme* in their article) served the pathogen as a dissemination vehicle. The fungus grew intercellularly without visual signs of diseases through the whole plant, whereas both intercellular and intracellular hyphae were found in plants with disease symptoms. Plants other than maize can be systemically colonized with *F. verticillioides*, too (Dastjerdi and Karlovsky 2015).

Infection of wheat kernels with *F. proliferatum* was reported for the first time from Canada, where the pathogen was found as a rare contaminant of tombstone kernels (Clear and Patrick 1990). In 1992, *F. proliferatum* was isolated from infected wheat seeds in Mazandaran (Zamani-Zadeh and Forutan 1992). The following study from Canada identified *F. proliferatum* as the causal agent of black point symptom in wheat (Conner et al. 1996). Spontaneous infection of wheat plants grown in a space shuttle with *F. proliferatum* (Bishop et al. 1997) motivated a series of physiological studies with little relevance for field pathology but the same laboratory identified *F. proliferatum* as an opportunistic pathogen of wheat under stress conditions (Kwon et al. 2001). A survey of fumonisins determination in cereals in Spain reported fumonisins in 8 of 17 samples and *F. proliferatum* infecting 2 of 17 samples naturally (Castella et al. 1999). Field pathologists became interested in *F. proliferatum* in wheat after contamination of wheat kernels with fumonisins was reported from Italy (Cirillo et al. 2003). In spite of a growing number of studies of *F. proliferatum* in wheat in recent years (Desjardins et al. 2007, Palacios et al. 2011, Busman et al. 2012, Amato et al. 2015), our understanding of this pathosystem is still limited.

The objective of present study was to assess the ability of *F. proliferatum* to colonize wheat plants systemically and to compare aggressiveness of strains isolated from different hosts towards wheat.

Material and methods

Fungal strains and wheat plants

Sixteen single spore strains of *F. proliferatum* isolated from garlic, onion, asparagus, maize, silver grass and a dead larva of *Zeuzera pyrina* in Germany, France, Syria and Austria were used (Table 1). The strains were taxonomically assigned to *F. proliferatum* based on micro-morphological features on carnation leaf agar (CLA) (Leslie et al. 2006) and the taxonomical identification was confirmed by species-specific PCR with primers PRO1 and PRO2 (Mulè et al. 2004).

Table 1. *F. proliferatum* strains

Strains[aT]	Host	Year	Origin
F. pro 2-G	Garlic	2000	France
F. pro 67-S	Silver grass	1993	Brandenburg, Germany
F. pro 78-S	Silver grass	1993	Brandenburg, Germany
F. pro 86-S	Silver grass	1993	Brandenburg, Germany
F. pro 2-O	Onion	2008	Baden-Wuerttemberg, Germany
F. pro 5-O	Onion	2008	Rheinland-Pfalz, Germany
F. pro 29-M	Maize	2005	Brandenburg, Germany
F. pro 76-M	Maize	2008	Baden-W. Germany
F. pro 219-A	Asparagus	2000	Rheinland-Pfalz, Germany
F. pro 223-A	Asparagus	1997	Brandenburg, Germany
F. pro 227-A	Asparagus	2003	Goldgeben, Lower Austria
F. pro 231-A	Asparagus	2003	Goldgeben, Lower Austria
F. pro 241-A	Asparagus	2003	Upper Austria
F. pro 245-A	Asparagus	2003	Burgenland, Austria
F. pro 259-A	Asparagus	2003	Lower Austria
F. pro 3-L	Insect Larva	2005	Syria

[a] Original host is specified by the last character of strain label; G = Garlic; S = Silver grass; O = Onion; M = Maize; A = Asparagus; L = insect larva (dead)

Bread wheat (*Triticum aestivum* L.) cultivar "Taifun" (KWS, Germany) was grown under greenhouse conditions. The cultivar is rated as susceptible to *Fusarium* infection (score 6 on German scale from 1 [resistant] to 9 [susceptible], Anonymous 2014).

Preparation of inocula

Mung bean medium according to Bai and Shaner (1996) in 50 ml portions in 100 ml Erlenmeyer flasks was autoclaved at 121 °C for 20 min. Three discs of 5 mm diameter from potato dextrose agar (PDA) plates overgrown with *F. proliferatum* were inoculated into each flask and incubated at 25 °C without light for 4 days with shaking at 150 rpm. Using four-layer cheese cloth for filtering the mycelia, conidia were collected, enumerated in a haemocytometer cell and stored in − 80 °C.

Inoculation of wheat plants

Wheat seeds were surface-disinfested for 3 min in 1.2% sodium hypochlorite and rinsed three times with sterile water. 50 wheat kernels were incubated in 10 ml of a conidial suspension (10^4 spores ml^{-1}) of each *F. proliferatum* strain for 48 h on a shaker. Inoculated seeds were sowed in twice-autoclaved soil in pots (10 kernels per pot, five replicates per strain). Un-inoculated controls were treated with sterile water. The experiment was carried out twice.

In a greenhouse, the temperature cycled between 26 °C (from 0600 to 2200) and 22 °C (from 2200 to 0600); light of intensity 4000 to 5000 lux was maintaining for 16 h; relative humidity was 50%. Plants in pots were watered every other day till the kernels were ripe. After two more weeks without watering, ears, first flag leaves, and the internodes between last node and ear were collected.

Re-isolation of fungi from wheat plants

The stems (between the ear and the last node of the inoculated plants which were fully asymptomatic) were cut into 2–3 cm sections, surface sterilized with 1.2% sodium hypochlorite for 3 min and rinsed three times in autoclaved distilled water. Stem sections were placed on Czapek-Dox-Iprodione-Dichloran (CZID) medium (Abildgren et al. 1987) and incubated at 25 °C. Un-inoculated controls were handled in the same way. Fungal growth was observed after 3–4 days. Taxonomic identity of the isolates was confirmed by PCR with primers PRO1/PRO2 specific for *F. proliferatum* (Mulè et al. 2004).

Quantification of fungal DNA in wheat stems, flag leaves and kernels

Wheat kernels, flag leaves and stems were dried for 48 h at 45 °C and ground. Total DNA was extracted from 50 mg using a CTAB method (Brandfass and Karlovsky 2008). The pellet was dissolved in 50 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). DNA solution was diluted fifty fold prior to PCR analysis. Inhibition assay was carried out to confirm the absence of matrix effects on PCR at this dilution. *F. proliferatum* DNA in plant tissue was quantified as described previously (Nutz et al. 2011). The lowest standard set as a limit of quantification corresponded to DNA content of 0.935 ng g^{-1}.

Mycotoxin analysis in kernels

100 mg of ground kernels were extracted with acetonitrile/water (84/16, v/v) as described (Adejumo et al. 2007); samples for beauvericin analysis were not defatted. HPLC separation and MS/MS detection was performed essentially as described (Adejumo et al. 2007) but ion trap 500-MS (Varian, Darmstadt, Germany) was used as a detector. Beauvericin was quantified in a positive ionization mode as a sodium adduct with mass transitions *m/z* 806 > 645 (quantification), *m/z* 806 > 545 and *m/z* 806 > 384 (confirmation). Calibration curves were constructed using analytical standards dissolved in methanol/water (1:1, v/v) with a correction for recovery and matrix effects. The limit of quantification for both beauvericin and fumonisin B_1 was 25 ng g^{-1}.

Statistical Analyses

Statistical analyses were performed with SPSS 22.0 (IBM SPSS statistics, USA). The Pearson correlation coefficient (2-tailed) between mycotoxins (fumonisin B_1 and beauvericin) production and fungal DNA was calculated.

Results

F. proliferatum strains originating from different plants were capable of infecting wheat systemically

We inoculated wheat seeds with conidia of *F. proliferatum* and sowed them in soil. The germination rate dropped from 94% for un-inoculated seeds to 84–92% for seeds inoculated with *F. proliferatum*. No disease symptoms were observed in the inoculated plants. To determine whether plants inoculated with *F. proliferatum* were colonized systemically, we attempted to re-isolate the pathogen and detect its DNA in stems, flag leaves and harvested kernels. Mycelia growing out from stems of infected wheat plants (Fig. 1a) were confirmed to belong to *F. proliferatum* by species-specific PCR (Fig. 1b). No mycelia grew out from surface-sterilized stems of un-inoculated plants.

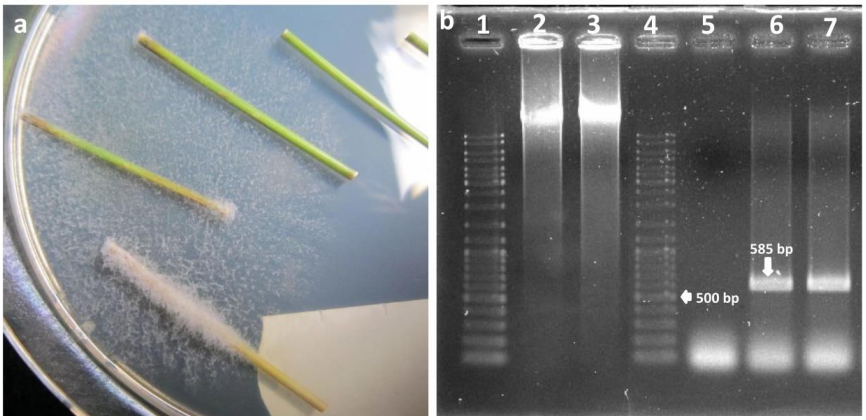

Fig. 1. Re-isolation of fungi from colonized wheat stems. (a) Fungal mycelia growing out from surface-sterilized wheat stem from seed-inoculated plants. (b) Two fungal colonies were randomly selected and fungal DNA was extracted (lanes 2 and 3). Assignment to *F. proliferatum* was confirmed by PCR (lanes 6 and 7). Lanes 1 and 4: DNA size standards; lane 5: negative control

The presence of *F. proliferatum* DNA in stems, flag leaves and harvested kernels corroborated systemic colonization (Fig. 2). *F. proliferatum* strains originating from different hosts successfully colonized wheat plants; the extent of colonization among strains from the same host plant differed. Strain *F. proliferatum* 29-M isolated from maize accumulated the highest amount of fungal DNA both in wheat stems and kernels.

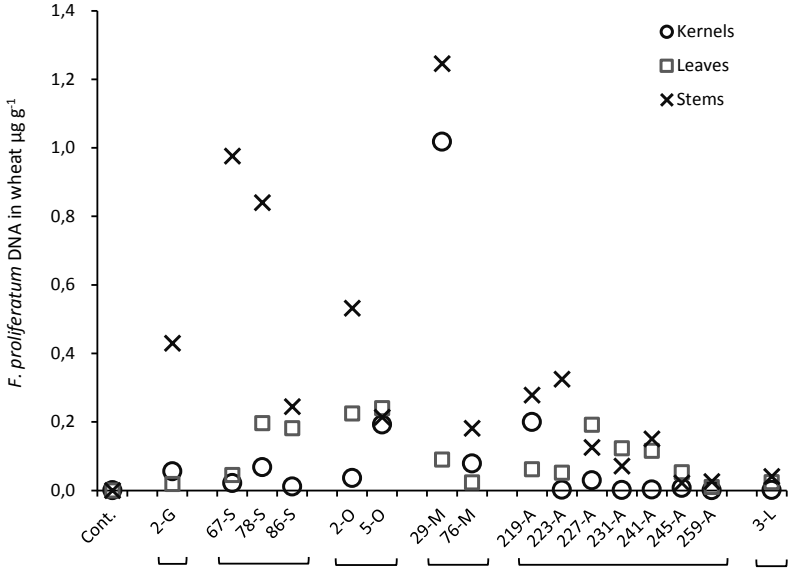

Fig. 2. Fungal DNA of *F. proliferatum* strains in wheat stems (the internode between the last node and ear), the first flag leaves, and kernels was quantified by real time PCR

The amount of fungal DNA in kernels was lower than in stems and leaves (Fig. 2). The experiment was carried out for the second time with similar results (Fig. 3).

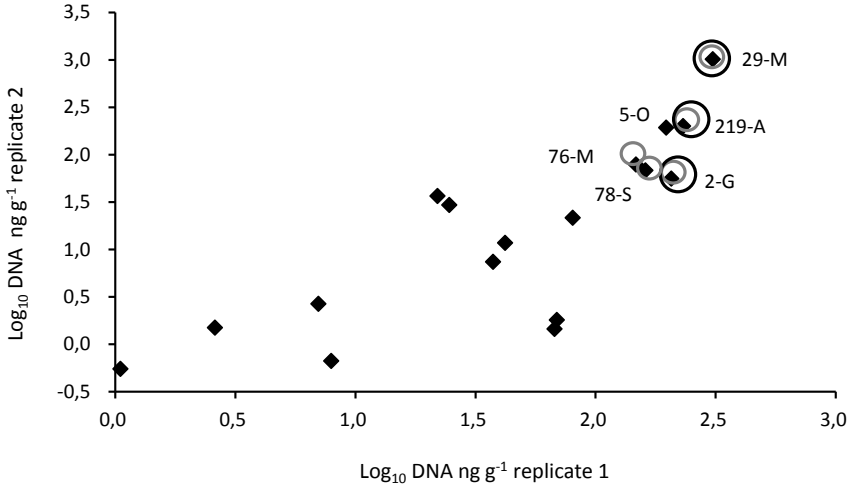

Fig. 3. Fungal DNA in kernels of wheat inoculated with *F. proliferatum* in two independent experiments. Samples encircled in gray contained beauvericin; samples encircled in black contained fumonisin B_1

Fumonisin B_1 and beauvericin accumulation

Mycotoxins fumonisin B_1 and beauvericin were found in 1/3 of infected wheat kernels (Fig. 4). The strains causing mycotoxin accumulation originated from different host plants.

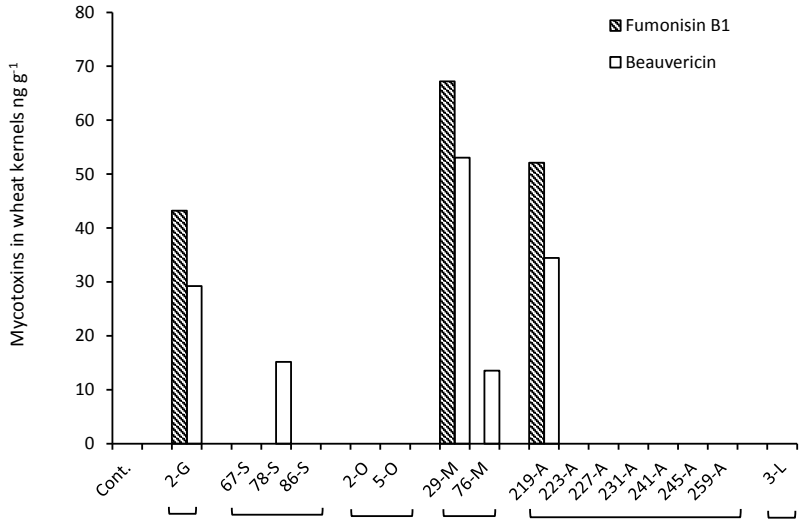

Fig. 4. Fumonisin B_1 and beauvericin quantification in colonized wheat kernels infected by different tested *F. proliferatum* strains with soak-inoculation mature seeds method

We found a strong positive correlation (r = 0.817) between DNA amount and fumonisin B_1 concentration in wheat kernels. A strong correlation (r = 0.871) was also found between *F. proliferatum* DNA and beauvericin content (Fig. 5).

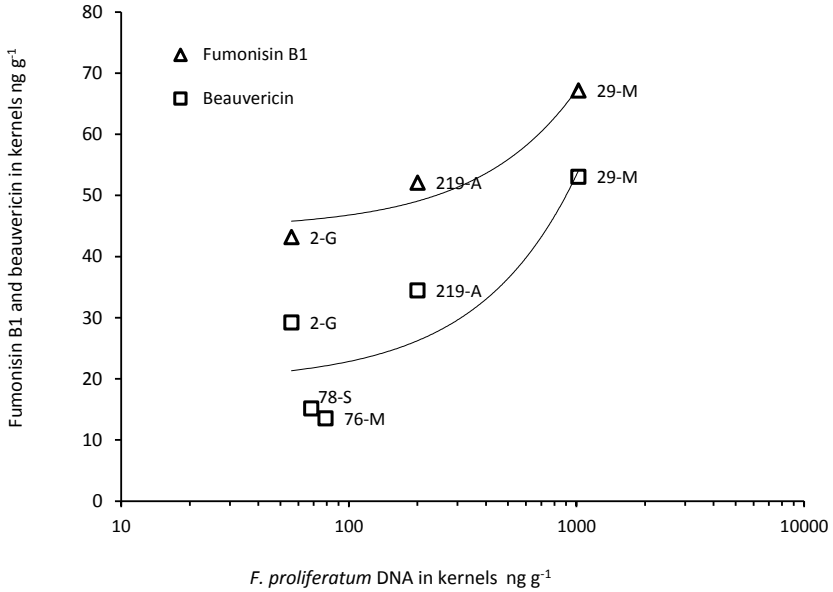

Fig. 5. Correlation of mycotoxin accumulation and amount of *F. proliferatum* DNA in wheat kernels

Discussion

F. proliferatum strains isolated from all host plants tested (garlic, onions, asparagus, maize, silver grass and dead larvae) were able to systemically infect wheat. The aggressiveness of the strains differed to a large extent. The limited number of strains used did not allow us to conclusively attribute these differences to the origin of the strains but low amount of biomass accumulated by all seven strains isolated from asparagus indicated that the specialization of *F. proliferatum* to asparagus may be accompanied by a decline of aggressiveness towards wheat. This might also be the case with onion and silver grass; more strains have to be tested to corroborate this hypothesis.

Only *F. proliferatum* strains that accumulated the largest amounts of DNA in kernels caused detectable contamination of kernels with mycotoxins (Fig. 3). Fumonisin B_1 was only detectable in kernels infected with three *F. proliferatum* strains out of sixteen strains tested. Interestingly, these three strains originated from different host plants: maize, asparagus, and garlic. Infection of wheat with two other strains resulted in the accumulation of beauvericin but not fumonisin B_1. The amounts of fumonisin B_1 in infected kernels were much lower than the levels commonly found in maize, supporting the hypothesis of Busman et al. (2012) that wheat kernels are a less favorable substrate for the production of fumonisins than maize. Fumonisin levels in naturally infected wheat were low, too (Desjardins et al. 2007, Palacios et al. 2011, Amato et al. 2015).

F. proliferatum DNA was found in largest amounts in stems, less in leaves and the lowest amounts in kernels. We speculate that *F. proliferatum* spreads within pith parenchyma (including the vascular system), which offers abundant nutrients and humidity, as shown for *F. graminearum* and *F. culmorum* (Guenther and Trail 2005, Mudge et al. 2006). Tight correlation between the amount of *F. proliferatum* DNA and mycotoxin concentration in kernels (Fig. 5) indicated that mycotoxins were produced in kernels rather than being transported to kernels from the stem with the transpiration stream. This speculation is in line with the results of Winter et al. (2013) on deoxynivalenol, which was produced in large amounts in stems of wheat plants infected with *F. culmorum* but did not reach kernels due to an anatomical barrier between grain and rachilla designated as "xylem discontinuity". Analysis of fumonisin and beauvericin content in stems of wheat plants infected with *F. proliferatum* via seeds will be needed to test this hypothesis. We have not observed any symptoms on the inoculated plants except that the leaves near the first node were more chlorotic as compared to control plants. Desjardins et al. (2007) reported kernel black points in wheat inoculated with *F. proliferatum* but they injected wheat spikelets at early anthesis while we studied seed-borne infection.

Accumulation of mycotoxins in symptomless wheat grain used for human and animal consumption poses a risk to human and animal health. Furthermore, residues of infected wheat plants can serve as source of inoculum of *F. proliferatum* for susceptible crops in the next season. Systemic infection of wheat with *F. proliferatum* therefore deserves attention, particularly in crop production systems in which seed coating with fungicides is not practiced.

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