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The effect of different carbon sources on phenotypic expression by *Fusarium graminearum* strains

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Abstract Two *Fusarium graminearum* strains were cultured in glucose yeast extract peptone broth or minimal medium broth to measure the production of mycelial biomass, pH, mycotoxins, and aurofusarin pigment, when limited to single carbon sources (at 1%), including xylan, cellulose, starch, or glucose. A random complete block design with factorial arrangement and analysis of variance at a significance level of 0.01 were employed to test for treatment differences. Overall, the *F. graminearum* strains produced significantly more biomass, deoxynivalenol, and aurofusarin with xylan than with cellulose. No significant differences were found in terms of 15–acetyldeoxynivalenol production from the four carbon sources. The presence of significant interactions between the strains, carbon sources, and media led to the following specific differences. In yeast extract peptone broth, R-9828 strain yielded significantly more deoxynivalenol production with xylan than cellulose and R-9832 produced significantly more mycelium (biomass) with xylan than cellulose. R-9828 strain yielded significantly more deoxynivalenol production than the R-9832 strain. Also in yeast extract peptone broth, cellulose led to significantly higher pH values than other carbons, which might be due to the limited ability of the *Fusarium* strains to utilize cellulose as an energy source. Aurofusarin was the only expressed analyte to show a significant difference in minimal medium broth, and R-9832 produced significantly more aurofusarin with xylan than with cellulose in the broth. These results suggest that xylan may induce *Fusarium* growth and deoxynivalenol production to assist the infection process and may support the theory that *F. graminearum* invades through xylan in the cell walls of cereals.

Keywords Trichothecene · Aurofusarin · Xylan · Cellulose · pH · Biomass

Abbreviations

ANOVA	Analysis of Variance
AU	Absorbance Unit
3-ADON	3-acetyldexynivalenol
15-ADON	5-acetyldeoxynivalenol
CMC	carboxymethycellulose
CLA	carnation leaf agar
DON	deoxynivalenol
FHB	Fusarium head blight
GYEP	glucose yeast extract peptone broth
HPLC	high performance liquid chromatography
MM	Minimal medium broth

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NIV	nivalenol
PDA	photodiode array detector
RCBD	random complete block design
ZEN	zeralenone

Introduction

Fusarium head blight (FHB) or head scab is a destructive disease of small grains including wheat, barley, and maize (Goswami and Kistler 2004). *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*) is generally regarded as the most important species causing FHB, which not only reduces grain yield and quality, but also contaminates grain with toxic metabolites (mycotoxins) such as trichothecene.

The fungus produces two main types of secondary metabolites: polyketides such as zeralenone (ZEN) and aurofusarin pigment and B-trichothecenes (possess carbonyl functionality at the C-8 position) such as deoxynivalenol (DON) and 15–acetyldeoxynivalenol (15-ADON) (Medentsev et al. 1993; O'Donnell et al. 2000).

Medentsev et al. (1993) found that feeding the mycelia of 20 *F. graminearum* isolates to laying hens led to egg deterioration, the egg deterioration syndrome was not associated with the presence of trichothecenes, but rather a yellow-orange-coloured metabolite, which was identified as aurofusarin. They further found that aurofusarin inhibited the growth of bacteria, yeast, and fungi. The biological roles of aurofusarin on cereals have not been thoroughly investigated. Lysoe et al. (2006) found the mutant with the deletion of aurofusarin gene cluster did not show decreased pathogenicity in barley seedling. Voigt et al. (2007) proposed that *F. graminearum* produced polyketides including aurofusarin to antagonize competing organisms during saprophytic growth phase.

Trichothecenes are thought to be important virulence factors in infection of both maize and wheat caused by *F. graminearum* because knockout gene clusters that control trichothecene production makes the fungus less virulent (Proctor et al. 2002). Trichothecenes were found in the host cells at the early stage of infection by immunocytochemical location in infected wheat spikes by *F. culmorum* (Kang and Buchenauer 1999). The toxins were thought to help with the invasion process by inhibiting protein synthesis, causing electrolyte loss and impairing host defense reactions (Bushnell et al. 2003).

Jansen et al. (2005) studied the infection patterns in barley and wheat spikes inoculated with wild-type and a knockout trichodiene synthase gene mutant of *F. graminearum*. They found that the hyphae of germinating fungal spores of both the wild type and knockout mutant invaded along the epicarp in the space between lemma and palea, damaging the fruit coat layers first and the endosperm (consisting of accumulating starch and protein) subsequently. The observation suggested that trichthecenes were not a virulence factor during the initial infection. However, the non-trichothecene–producing *F. graminearum* mutants that were able to initiate infection could not spread throughout the head, and their growth was limited to the rachis node between the rachis and floret. Without the aid of trichothecene, fungus penetration was inhibited by the development of heavy cell wall thickening in the rachis node; eventually, infection of adjacent florets through the phloem and along the surface of the rachis were restricted. Buchenauer and Kang (2004) also found that DON levels in resistant wheat varieties were considerably lower than those in susceptible ones as determined by labeling densities. Thick layered appositions and large papillae were more noticeable in host tissues of resistant wheat varieties than in susceptible varieties, so the reactions were seen as plant structural defences. Cytological studies confirmed that those *Fusarium* species invaded spike tissues of resistant wheat varieties more slowly than tissues of susceptible ones. They suggested that the production of DON and the structural reaction of resistant wheat varieties were active defence reactions against the infection and spreading of *Fusarium* species in the spiking tissue.

Other studies investigating the host–pathogen interaction also recognized plant cell walls as major barriers to the pathogen's infection and colonization of plant tissue (Bushnell et al. 2003). Studies of the *Fusarium* infection process (*F. culmorum, F. graminearum,* and *F. avenaceum*) (Buchenauer and Kang 2004; Kang and Buchenauer 2000; Kang et al. 2005) indicated that the pathogens secreted cell wall degrading enzymes, including cellulase, xylanase,

and pectinase in wheat spikes at the early stage of infection (6–24 h). Measurable change (less dense) of the host cell walls was observed as a result of the action of cell wall degrading enzymes. The pathogens penetrated through the interior surface of the lemma and palea and inside the ovary by infection peg.

Other study with barley illustrated similar results. When barley seeds were inoculated with *F. graminearum* and *F. poae* at the late-milk to early-dough stages in a greenhouse (Schwarz et al. 2002), these fungi produced considerable amounts of xylanase, proteinase, and beta-glucanase in inoculated barley samples. These results suggested that the fungi secreted the enzymes to help with infection on the grain. Additionally, starch granule pitting and the absence of protein matrices in the endosperm of infected barley were observed (Schwarz et al. 2001), and the highly expressed alpha-amylase levels resulting from the fungal infection were suggested as the cause.

Cereal cell walls consist mainly of cellulose (60% of mass) and hemicelluloses (Carpita et al. 2001). In cereals, the most important hemicelluloses are arabinoxylans and β-glucan (Izydorczyk and Biliaderis 1995). Cellulose microfibrils are embedded in a matrix consisting of pectin and arabinoxylans in both endosperm cell walls and non-endospermic tissues of wheat, barley, corn, and rice. The structural features of arabinoxylans allow the formation of non-covalent interactions of arabinoxylan molecules with other polysaccharides such as β-glucan, cellulose, and pectin in the matrix of cell walls (Hellweg 2003; Izydorczyk and Biliaderis 1995, Izydorczyk and MacGregor 2000).

Many questions regarding the biochemical mechanism of the invasion process and host-pathogen interaction remain unanswered. This study aimed to imitate the *in planta* environment and to assess the role carbon sources play in the infection process with respect to the host-pathogen interaction mechanism. This is the first study to directly investigate the cell wall compound (xylan and cellulose) as carbon sources in *in vitro* conditions. The experiment was designed to determine how a single carbon source in two media consisting of different nitrogen sources would affect fungal growth and the production of the secondary metabolites including mycotoxins and aurofusarin pigment. It tested the hypothesis that carbon sources would affect phenotypic expression by *F. graminearum* differently.

Materials and methods

Experimental design and statistical analysis

A random complete block design (RCBD) with factorial arrangement was used (Giesbrecht and Gumpertz 2004). The total experimental units were 64 (2 media×4 carbon sources×2 strains×2 duplicates×2 replicates). Each treatment was done in duplicate and the replication was completed on two successive days (n=4). Non-inoculated broth samples corresponding to each treatment were used as controls. Factorial analysis of variance, specifically, general linear model and multivariate tests with least square mean multiple comparison at a significance level of 0.01, was employed to test for treatment differences.

Interaction means that the effect of one independent variable on the dependent variables (factors) depends on the value (level) of some other independent variable(s) in a study (Giesbrecht and Gumpertz 2004). Interaction effect may be easily identified by being graphically represented in an interaction graph/plot. Lack of interactions between variables is shown by parallel lines even if the lines are not horizontal in an interaction graph (Hinkelmann 2004). Non-parallel lines show interaction, however, they do not automatically indicate that the interaction is significant. Whether or not the interaction is significant depends on the degree to which the lines are not parallel and consequently is determined by statistical analysis results. If interaction is significant (indicated by significant F-test), we need to determine the nature of its significance. There are two types of interactions. One is called anti-directional interaction: the two lines on the interaction graph cross over each other. This is also called "true" or cross-over interaction, meaning the factors are not responding the same at different levels. Another is co-directional interaction: the unparallel properties of the two lines are caused by variation in magnitude, but not in opposite direction of treatments. In other words, the interaction is significant because of differences in magnitude between the levels of a factor.

Two approaches could be used as a follow-up to the significant interaction effect of factorial ANOVA:

(i) tests of simple main effects and (ii) statistical comparison of cell means (Oshima and McCarty 2006). The approach of tests of simple main effects was employed in the liquid culture experiment. It included two steps: (i) examining what (magnitude variation, cross-over, or both) contributed to the interaction effect graphically and then (ii) conducting the test of simple main effects by factorial ANOVA.

Fusarium graminearum strains used

Two *F. graminearum* strains (R-9828 and R-9832) were selected for the study because they demonstrated virulence to wheat in greenhouse trials and were known DON producers in solid substrates. R-9828 was a strain from South Africa belonging to lineage 3 of *F. graminearum,* while R-9832 was a strain from the United States belonging to lineage 7 (O'Donnell et al. 2000). Both strains are available in the Fusarium Research Center Collection at Pennsylvania State University. The stock cultures were maintained on carnation leaf agar (CLA) slants or plates.

Inoculum preparation

Carboxymethycellulose (CMC, Sigma, St Louis, MO) broth (Booth 1971) was used to prepare inoculum. One piece of mycelium grown from a CLA slant was put into 500 ml of CMC broth in an Erlenmeyer flask. The inoculated CMC flasks were incubated in a shaker incubator (Series 25, New Brunswick Scientific Co., Edison, New Jersey) at a rate of 200 rotations per minute with ambient lighting and at room temperature (~25°C). After incubation for seven days, the CMC broth was aseptically filtered through four layers of sterile cheese cloth to remove mycelia, and combined into a roughly 1000 ml macroconidia suspension. The final macroconidia suspension was adjusted to the concentration of 10,000 macroconidia/ml with sterile distilled water; the level of inoculum was pretested and proven to be efficient in inoculation for the study. The macroconidia were counted by hemocytometer.

Media inoculation and incubation

Two broth media were tested for mycotoxin production in the experiment: minimal medium (MM) and glucose yeast extract peptone (GYEP). The GYEP broth, a complex medium, contained yeast extract (DIFCO, Detroit, MI) and peptone (DIFCO, Detroit, MI) respectively at a concentration of 1 g/l (Miller and Greenhalgh 1985). Minimal medium broth, a synthetic medium, consisted of 0.5 g sodium sulfate (EM Science, Gibbstown, NJ), 6.75 g K_2HP0_4 (Sigma, St. Loius, MO), 2.35 g KH_2P0_4 (Mallinckrodt, Paris, KY), 1.25 g NaCl (EM Science, Gibbstown, NJ), 0.275 g ammonium chloride (Baker, Phillipsburg, NJ), and trace $MgSO_4$ (Baker, Phillipsburg, NJ) in 1 l distilled water. The MM was modified from the compositions of Czapek-Dox medium and Modified Fries (Jiao et al. 2008; Miller and Greenhalgh 1985).

For both GYEP and MM media, the primary carbon source was limited to single carbon including xylan, cellulose, starch or glucose, at a concentration of 10 g/l. Aliquots of 6 ml of broth were put into 10 ml test tubes with screw caps and autoclaved at 121°C for 15 min. One ml of the macroconidia suspension (10^4 spores/ml) was aseptically inoculated into each of the tubes. The tubes were capped loosely and incubated in a shaker incubator at 200 rotations per minute, at 28°C, with ambient lighting for 20 days, and stored on a bench at room temperature for one day before refrigeration (4°C) until analysis.

Biomass and pH determination

The mycelia of each sample were washed with distilled water and vacuum filtered through pre-dried and weighed filter paper (Whatman No. 4). The mycelia on the filter papers were dried at 60°C to a constant weight, and the biomass was indicated as the dry mycelium weight after subtracting the filter paper weight. The pH value of the broth of each sample was measured with a pH meter (Model 520A, Orion Research Inc., Boston, MA).

Mycotoxin analysis

One milliliter of the cultured broth was filtered through a 0.2 μm syringe filter into a vial, and 100 μl was injected for analysis. A Waters HPLC (2690 separation module, Waters Corporation, Milford, MA) with a photodiode array detector (PDA, model 996) at an output wavelength of 220 nm was used to assay the sample, and the data acquisition and processing were done by Millennium software (3.20 version, Waters, 2001).

A gradient elution with acetronitrile and water as mobile phase solvents was used to detect and quantify the five *Fusarium* mycotoxins including nivalenol (NIV), zeralenone (ZEN), DON, 15-ADON and 3-ADON. The analytical column was Phenomenex Synergi Hydro-RP 80A (4u, 4.0 × 150 mm), and the flow rate was set at 1 ml/min. The HPLC program had three stages : (i) running a 15% aqueous acetonitrile solution (15% water, 85% acetonitrile) for 6 min to allow NIV and DON to elute, (ii) immediately increasing to a 25% aqueous acetonitrile solution and remaining for 5 min for 15- and 3-ADON elution, and (iii) switching to a 80% aqueous acetonitrile solution for ZEN elution. The total run time for separation of the five mycotoxins, flushing, and re-equilibration was 40 min. The retention times for NIV, DON, 3-ADON, 15-ADON, and ZEN in a mixed standard solution were 2.6, 4.3, 8.8, 9.0, and 15.9 min respectively.

Ultrasound-assisted extraction and HPLC analysis of aurofusarin

After the dried mycelia of each sample were weighed for biomass determination, they were placed inside pre-labeled test tubes (12.5 × 1.5 cm) stationed in a test tube rack. The mycelia were suspended in 15 ml methylene chloride and extracted in an ultrasonic bath (Branson 3200, Bransonic Scientific, Danbury) at 40°C for 30 minutes. The methylene chloride was evaporated to dryness at 45°C under a stream of nitrogen gas and the mycelia were removed. The residue was re-dissolved in acetonitrile (0.7 ml), vortexed, and transferred to vials prior to HPLC analysis.

The pigment was also analyzed with a Waters HPLC (2690 separation module, Waters Corporation, Milford, MA). Waters uBondpack^{tm} C18 column (3.9x300mm) was used for separation, and a Waters photodiode array (PDA, model 996) was used for the detection with 381 nm as the UV output absorbance wavelength. Isocratic separation was achieved by a mobile phase of acetonitrile, water, and KH_2PO_4 in the ratio of 55:45:0.14 (v/v/wt) adjusted to pH 3.0 with phosphoric acid. The flow rate was 1 ml per minute at a temperature of 35°C. The identification of the aurofusarin compound was achieved by both retention time and UV spectrum. The retention time for aurofusarin was approximately 11.5 min. There was no purified aurofusarin available for a standard; so, it was not possible to make a calibration curve to quantify the pigment. As a result, peak area in absorbance unit (AU) was used as the unit to present data in the current study

Results and discussion

The HPLC method used could quantify the five common *Fusarium* mycotoxins mentioned in the section of Materials and methods, however, only DON and 15-ADON were detected in the two broths. Hence, the other three mycotoxins were not included in this study. The results also show that the two strains used in this study belong to 15-ADON chemotype.

The effects of carbon source, strain, and medium on biomass production

There was a significant two-way interaction between medium and strain. Figure 1a is the interaction graph in terms of mycelial mass production. All the interaction graphs in this study were plotted to illustrate the complex interaction effects from the three factors (carbon source, strain, and medium). The interaction profile shown in Fig. 1a indicated that *F. graminearum* strain R-9828 in GYEP media exhibited a different pattern from the rest of combinations and that the cross-over caused the interaction. Further ANOVA analysis pointed out that there were significant differences among the four carbon sources.

Overall, there also were significant differences between the four carbon sources on biomass production; xylan and glucose produced the most, followed by starch, and cellulose produced the least. Figure 1b shows the multiple comparison results for biomass in terms of strain and media. Strain R-9832 grown on xylan produced significantly ($P<0.01$) more biomass than on cellulose in GYEP broth. Strain R-9832 produced more biomass than R-9828 when glucose was used as the carbon source in GYEP medium. No significant differences in terms of biomass were found between the two *F. graminearum* strains or the four carbon sources in MM broth.

The effects of carbon source, strain, and medium on pH

In terms of pH values, Fig. 2a exhibits that there was no interaction between the carbon sources and strain

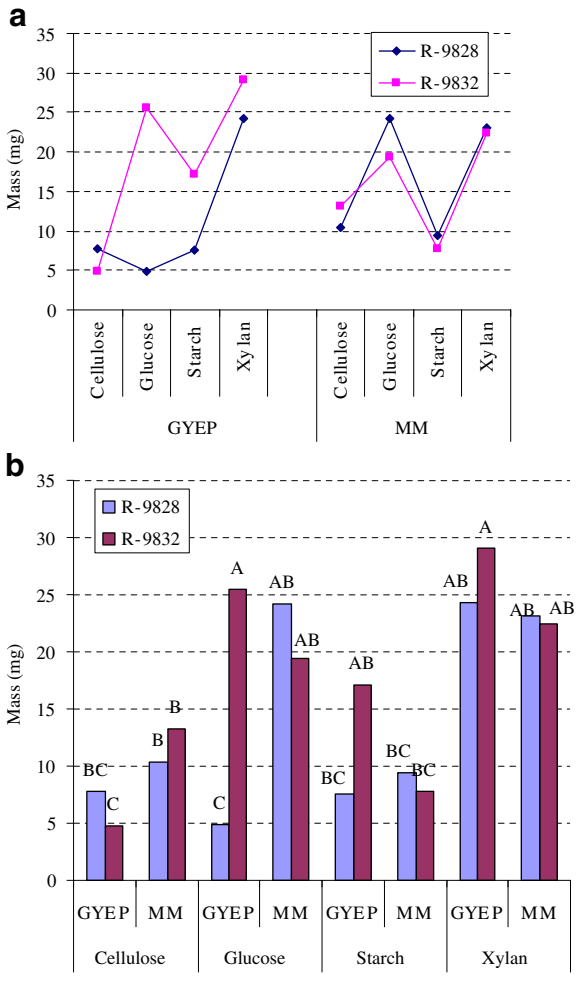

Fig. 1 **a**. The interaction profile of the average mycelial mass (mg) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. **b**. Average mycelial mass (mg) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. Different letters indicate significant differences: $P<0.01$, $n=4$. *LSD* stands for Least Significant Difference

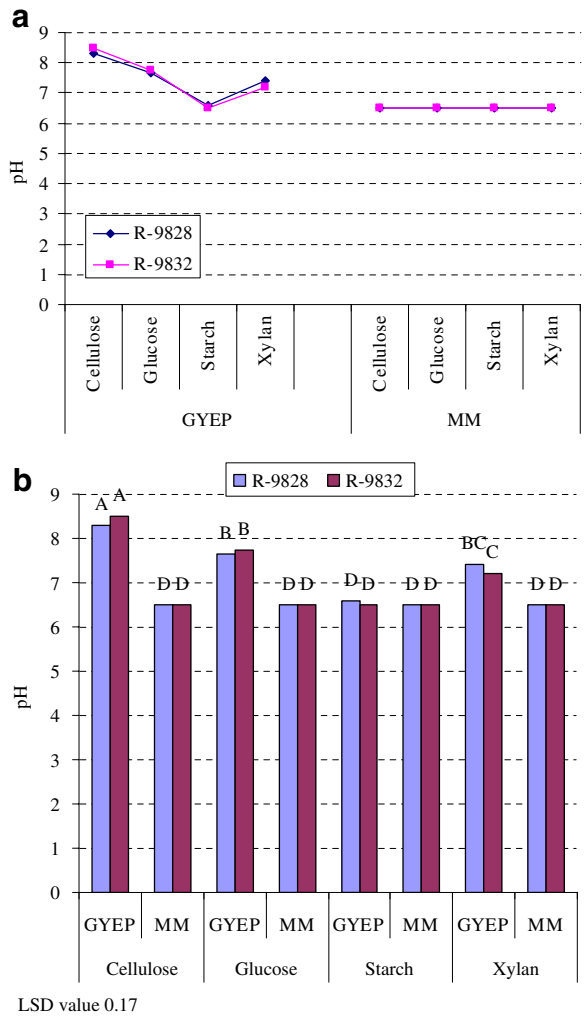

Fig. 2 **a**. Interaction profile of average pH values produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. **b**. Average pH values produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source (initial pH=6.5). Different letters indicate significant differences: $P<0.01$, $n=4$. *LSD* stands for Least Significant Difference

because the two lines were parallel in both GYEP and MM media. However, it shows that highly significant interaction ($P<0.01$) occurred between media and carbon source because the two parallel lines from GYEP would cross over the two parallel lines from the MM.

The initial pH values of the two liquid media in the study were both 6.5. After incubation for 20 days, the pH values of the GYEP are shown in Fig. 2b. Highly significant differences ($P<0.01$) in pH were found between the two media and among the carbon sources. Cellulose led to significantly higher pH values than other carbon sources (glucose, xylan, and starch) in the GYEP media. The two strains were not significantly different from each other for GYEP or MM broth in terms of pH values. Pestka et al. (1985) found that in GYEP media, pH increased initially, then decreased. The increase in pH seen in GYEP in the study was likely due to proteolysis of the peptone or proteins from yeast extract. Proteolysis

led to the formation of amine acids and ammonia that increased the pH. The subsequent decrease in pH may be because that *F. graminearum* utilizes the carbon as an energy source and produces metabolites such as carbon dioxide that lead to pH reduction. The significantly higher pH value of GYEP with cellulose as the carbon source when compared to other carbon sources might be due to the limited ability of the *F. graminearum* to utilize cellulose as an energy source.

Figure 2b also indicates that the pH values of all the four carbon sources in the MM broth remained at roughly its initial pH value (6.5), and there were no statistical differences in pH value among the four carbon sources and two strains. The no-pH-change phenomenon observed might be that MM broth only contains inorganic nitrogen (ammonium chloride) and does not contain proteinaceous nitrogen, therefore, the *F. graminearum* fungus does not have substrate for proteolysis activity to initiate pH increase in the first place.

The effects of medium, strain, and carbon source on DON production

In terms of DON production, the three-way interaction (involving medium, carbon, and strain) and all two–way interactions (medium x carbon, medium x strain, strain x carbon) were highly significant ($P<0.01$). Figure 3a displays the interaction profile of DON production. Within the same medium, either GYEP or MM, differences in magnitude may account for the two-way interaction between carbon and strain. Across the two media, cross-over interactions were observed because the two lines from GYEP would cross over the two lines from the MM. Hence, a cross-over effect may contribute to the three-way interaction and also two-way interactions involved medium (medium × carbon and medium × strain). The presence of significant interactions, two-ways and three-way, explained the different patterns in terms of DON productions.

Medium and nitrogen source Overall, the current study found that *F. graminearum* grown in GYEP medium produced significantly ($P<0.01$) more DON than in MM did. Other studies have investigated the effect of nitrogen sources on trichothecene production by *Fusarium* using Modified Fries broth and GYEP broth and gave similar results (Miller and Greenhalgh

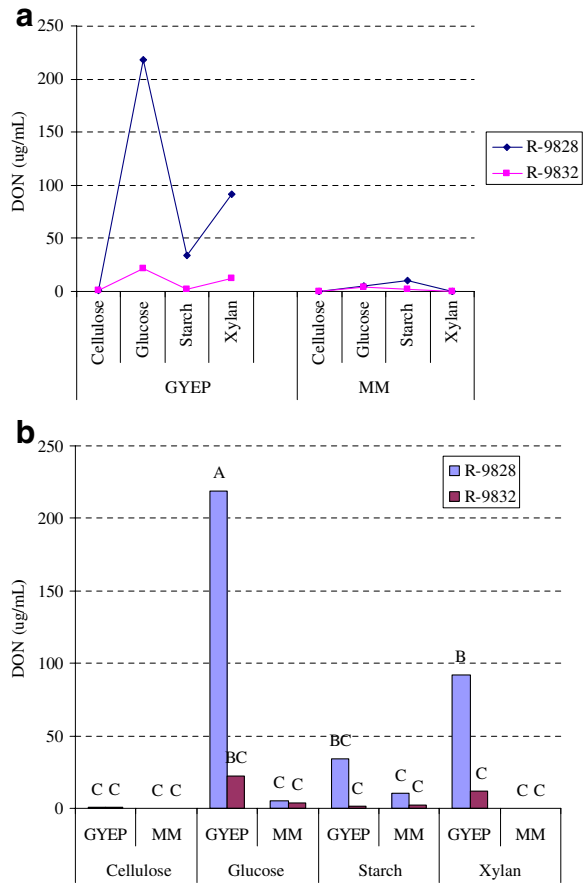

Fig. 3 **a**. Interaction profile of DON (µg/mL) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. **b**. DON (µg/mL) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. Different letters indicate significant differences: $P<0.01$, $n=4$. *LSD* stands for Least Significant Difference

1985; Pestka et al. 1985). The Modified Fries broth consists of 5.0 g NH_4NO_3, 0.5 g $MgSO_4$, 1.0 g K_2HPO_4, 0.1 g NaCl, 0.13 g $CaCl_2$, 0.02 g $FeSO_4$, and 30.0 g glucose, so, it had a similar composition as the MM broth in this current study. The two earlier studies indicated that the *F. graminearum* grown in Modified Fries broth produced lower amounts of trichothecenes (including DON, 15-ADON, NIV, and fusarenon X) than GYEP. The difference in mycotoxin production was attributed to nitrogen sources. They concluded that all other inorganic nitrogen sources such as nitrate and ammonia produced trace or non-

detectable amounts of DON and 15-ADON, proteinaceous nitrogen and possibly other unidentified factors in the yeast peptone extract played an essential role in trichothecene production.

The recent study by Gardiner et al. (2009) further confirmed and provided explanation for the different responses of *F. graminearum* to the nitrogen sources in triggering DON production. The expression of TR15 was used as a measurement of trichothecene production because it encodes the trichodiene synthase that catalyses the first committed step in the trichothecene biosynthesis pathway and its expression is correlated with the production of trichothecene both *in planta* and *in vitro* conditions. Their results indicated that amines such as arginine and agmatine and amino acids such as glutamine and methionine possessed strong inducing effects on TR15 expression and DON production in culture, while nitrate and ammonia was identified as a repressor of TR15 induction and DON production.

Strain Overall, strain R-9828 produced significantly more DON than strain R-9832 did. Specifically, strain R-9828 produced significantly more DON in GYEP medium with glucose and xylan compared to *F. graminearum* strain R-9832 (Fig. 3b). Miller and Greenhalgh (1985) also found that the two *F. graminearum* strains in their study had different response to various carbon sources in terms of trichothcene production. The different phenotypic expression of the two strains demonstrated in the current study could trace back to their genetic difference. Wolf-Hall and Bullerman (1998) characterized these two strains using random amplification of polymorphic DNA and found that they belonged to two distinctly different types. Strain R-9828 produced seven reproducible bands, while strain R-9832 produced only one very distinct and highly reproducible band. In addition, the Fusarium Research Center at Pennsylvania State University (personal correspondence, 2005) determined that R-9828 strain belonged to lineage 3 of *F. graminearum,* while R-9832 strain belonging to lineage 7 according to the phylogenetic analysis methodology of O'Donnell et al. (2000).

Carbon source Overall, the *F. graminearum* strains produced significantly more DON with xylan than cellulose. In GYEP medium, strain R-9828 yielded significantly more DON production with glucose, followed by xylan and starch, with the least amount by cellulose (Fig. 3b). Miller and Greenhalgh (1985) investigated the effects of carbon sources on trichothecene production in Modified Fries broth and semi-defined GYEP broth by two *F. graminearum* strains that did not produce the same monoacetate derivative of DON. One strain belonged to 15-ADON chemotype, and the other was a 3-ADON producer. They included glucose, D-fructose, D-galactose, sucrose, maltose, mannose, D-arabinose, D-xylose, raffinose, cellobiose, D-ribose, trehalose, sodium succinate, sodium acetate, sodium citrate, sodium proprionate, mannitol, myo-inositol, sorbitol, pectin, and starch in their study. The results indicated that the production of trichothecenes was carbon source dependent. The 15-ADON chemotype *F. graminearum* strain produced the most DON with xylose, mannitol, and proprionate, followed by glucose, arabinose, starch, pectin, trehalose, succinate, acetate, citrate, cellobiose, raffinose, and the rest of the carbon sources (fructose, mannose, galactose, sucrose, and maltose) resulted in trace or non-detectable amounts of DON. The R-9828 strain produced intermediate amounts of DON on starch in the current study and this agrees with the finding by Miller and Greenhalgh (1985).

The microbial degradation products of xylan consist of mainly xylose and arabinose (Izydorczyk and Biliaderis 1995; Izydorczyk and MacGregor 2000), while cellulose produced primarily cellobiose (Leschine 1995). Whether or not the degradation sugar differences (xylose and arabinose from xylan and cellobiose from cellulose) contribute to the different DON producing capacities of xylan and cellulose is a question that deserves further investigation.

To analyze the trichothecene induction mechanism, Jiao et al. (2008) examined the effects of 12 carbon sources on the production of DON and 3-ADON in modified Czapek liquid medium incubated with nine *F. graminearum* strains of 3-ADON chemotype. The modified Czapek liquid medium (pH 7.7) consists of 1 g K_2HPO_4,, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 10 mg Fe-EDTA, 2 gL-glutamic acid, and 10 g of carbon source per liter. The carbon sources include glucose, fructose, sucrose, 1-kestose, nystose, fructan, maltose, amylase, amylopectin, cellobiose, xylose or galactose. All of the nine strains tested produced

significantly higher concentrations of trichothecenes in the liquid media that were supplemented with sucrose, 1-kestose or nystose. Nystose and 1-kestose are fructo-oligosacharides and have structures very similar to sucrose. The trichothecene production by the rest of carbon sources was strain-specific.

Miller and Greenhalgh (1985) compared trichothecene production between a 3-ADON chemotype strain and a 15-ADON chemotyp strain in liquid media containing various carbon sources. The 3-ADON strain produced the most DON on acetate, proprionate, mannitol, and raffinose, followed by arabinose, mannonse, glucose, sucrose, xylose, and cellobiose, pectin and starch, fructose and galactose led to trace or not-detectable amounts of DON. Sucrose facilitated DON production by 3-ADON chemotype strain, as found by Miller and Greenhalgh (1985), is in agreement with the finding by Jiao et al. (2008). However, in the same study, Miller and Greenhalgh (1985) found that the *F. graminearum* strain of 15-ADON chemotype led to non-detectable DON on sucrose. It is evident that the carbon sources resulting in the highest yield of trichothecene by the 3-ADON chemotype were not consistent to those by the 15-ADON chemotype due to their different biosynthesis pathways.

The effects of carbon source, strain, and media on 15-ADON production

Figures 4a and b show the interaction profile and multiple comparison results of 15-ADON production as affected by carbon source and strain in GYEP and MM broth. Overall, no significant interactions existed in terms of 15-ADON production. In addition, there were no significant differences in terms of 15-ADON production between the two media, two strains, and four carbon sources. The very low 15-ADON production in the current study was likely to contribute to this insignificant result. Low 15-ADON contents (0.25 mg/l to 14.0 mg/l) were found in the study by Miller and Greenhalgh (1985) and Pestka et al. (1985). In contrast, Bily et al. (2004) found higher amounts of 15-ADON (600 to 1100 µg/g) in a two-step liquid media after incubating 20 days. The differences in 15-ADON production were likely caused by different media, strain, and incubation conditions.

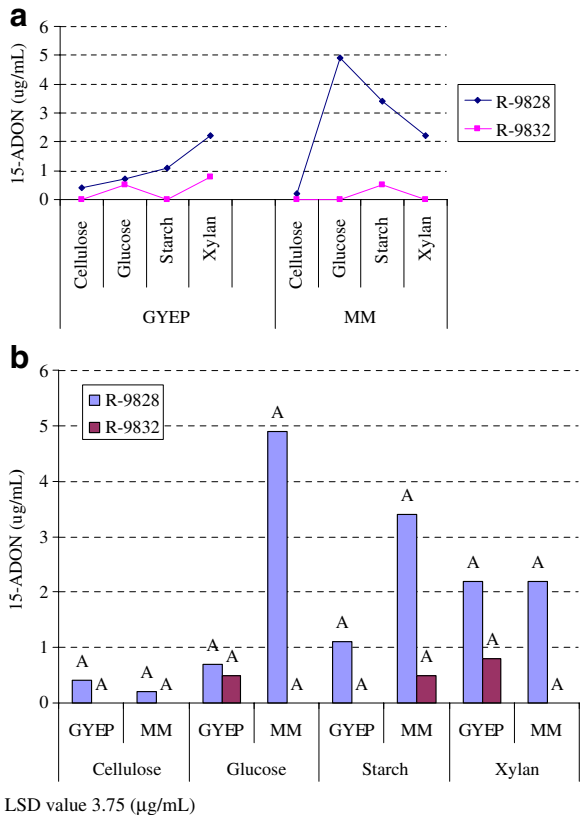

Fig. 4 **a**. Interaction profile of 15-ADON (µg/mL) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. **b**. 15-ADON (ug/mL) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. Different letters indicate significant differences: $P<0.01$, $n=4$. *LSD* stands for Least Significant Difference

The effects of carbon source, strain, and medium on aurofusarin production

There was a significant two-way interaction between media and strain in terms of aurofusarin peak area (Fig. 5a). Figures 5a exhibits that the variations in magnitude from the two strains and cross-over between the two media accounted for the interaction of aurofusarin peak area, and the area divided by biomass exhibited the similar result.

In MM broth, R-9832 produced significantly more aurofusarin on xylan than on cellulose (Fig. 5b). Overall, xylan led to significantly more aurofusarin production in MM broth for both strains than cellulose. Aurofusarin was the only expressed analyte to show a significant difference in MM broth in this

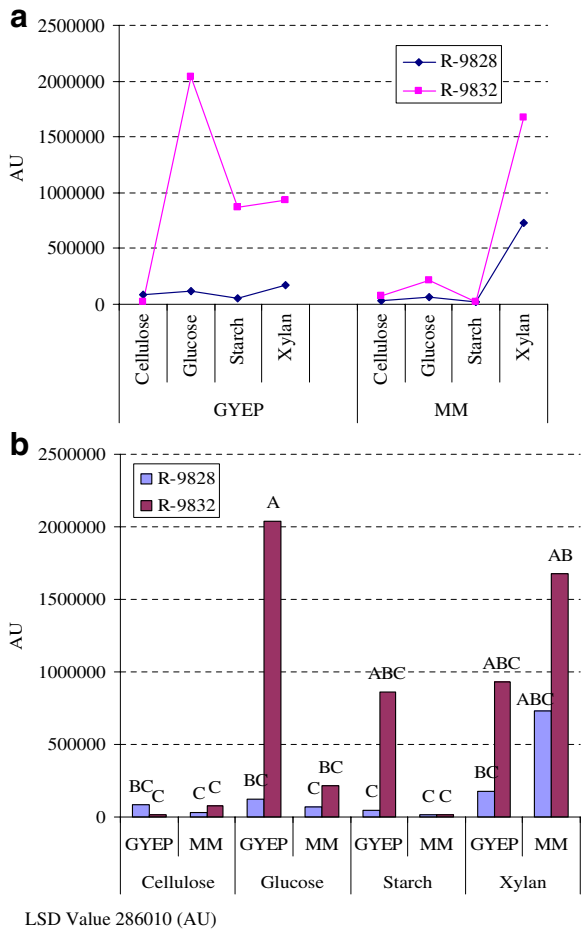

Fig. 5 a. Interaction profile of aurofusarin peak area (AU) DON (μg/mL) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. **b**. Aurofusarin peak area (AU) DON (μg/mL) produced by two *F. graminearum* strains (R-9828 and R-9832) grown in different media (GYEP and MM) containing a single carbon source. Different letters indicate significant differences: $P<0.01$, $n=4$. *LSD* stands for Least Significant Difference

study. The inorganic nitrogen source may be less influential in the case of aurofusarin production from xylan than for trichothecene production. The study by Bell et al. (2003) found that inorganic nitrogen such as nitrate increased the synthesis of polyketide in *Fusarium oxysporum* and may explain the result.

This is the first study to qualitatively analyze aurofusarin production from the mycelia of *F. graminearum* samples grown in liquid media. Aurofusarin pigment was found to be insoluble in water and was obtained by repeated extraction with $CHCl_3$ from the dried mycelia of *F. graminearum* and *F. culmorum,* which were cultivated on Raulin-Thom broth (Shibata et al. 1968). Burkhead (1990) used methylene chloride to extract aurofusarin from the dry mycelia of *F. graminearum* grown on soybean meal-glucose liquid broth. Unlike aurofusarin pigment, neither DON nor 15-ADON was extracted from the mycelia of the fungi in the study (data not presented). McCormick (2003) also reported that DON was only found in culture filtrates, but not in the mycelia of *Fusarium*. This was regarded as a strategy for mycotoxin-producing fungi to protect themselves from their own toxins (Stergiopoulos et al. 2002). These observations may suggest that aurofusarin pigment, unlike DON, is a constitutive part of the fungus, possessing no toxic effect to the fungus itself. It could be that *F. graminearum* produced aurofusarin to inhibit the growth of other microorganisms (Medentsev et al. 1993) and to antagonize competing organisms during saprophytic growth phase (Voigt et al. 2007).

In conclusion, *F. graminearum* produced considerable amounts of DON in GYEP medium with xylan and produced trace amounts of DON with cellulose in the current study, and the differences were significant. Cellulose yielded significantly higher pH values than xylan in GYEP media. The fungus also led to significantly more aurofusarin production on xylan in MM medium broth for both strains than on cellulose. These results may have implications for determining the type of carbon sources required for the fungal growth and secondary metabolite biosynthesis during the invasion process. It may support the theory that *F. graminearum* invades through xylan in the cell walls of cereals and suggests that xylan may induce *Fusarium* growth and DON and aurofusarin production to assist the infection process. *F. graminearum* attacked xylan, which interacts with and link together other cell-wall components (Izydorczyk and Biliaderis 1995; Izydorczyk and MacGregor 2000). As a result, the fungus penetrates through the surface to enter the cells in susceptible tissues such as the interior surface of the lemma and palea at an early invasion stage. On the other hand, cellulose provides scaffolding or supporting structure for cell walls and may be a more resistant structure that restricts the invasion of the pathogen.

It is well-documented by histological investigations that the thick-walled exterior epidermal cells of the lemma and palea in wheat and barley resist the

penetration of the *F. graminearum* more than the thin-walled epidermal cells during the initiation of head infection (Bushnell et al. 2003). However, histochemical knowledge is still very limited concerning the cell wall compositions of the differences between thick-walled and thin-walled cells. Hence, chemical composition differences in nitrogen-containing compounds and carbohydrates of the cell walls in thick- and thin-walled exterior epidermal cells deserve to be thoroughly investigated in the future.

Other composition differences such as lignin and phenolic compounds (Grabber et al. 2004; Siranidou et al. 2002) may also contribute to the variation in fungal growth and secondary metabolite production. Thickened lignified cell-walls with a "silico-subereux" nature were found in the exterior epidermal cells of the lemma and palea that are not quickly penetrated by head blight fungi (Bushnell et al. 2003). Defined liquid media including other compounds found in the cell walls such as β-glucans, pectins, lignin, and phenolic substances also deserve to be investigated in future study.

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