A real-time polymerase chain reaction protocol for quantifying growth of *Fusarium graminearum* during solid substrate cultivation on corn stover

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Solid substrate cultivation (SSC), in which microbes are grown on solid substrates in the absence of free water, is a traditional technology with the potential to produce novel chemicals and biomass products for use in the manufacture of biofuels. It is currently relatively difficult to accurately measure microbial growth on a solid substrate, and thus to optimize SSC production conditions. A quantitative real-time polymerase-chain reaction protocol (RT-PCR) was developed to measure growth of the corn stalk rot fungus *Fusarium graminearum* on corn stover. The RT-PCR assay gave results that were comparable to more traditional glucosamine assays, but it was more sensitive. Other advantages of the RT-PCR assay include its specificity and the potential for high-throughput automation. The RT-PCR approach will facilitate optimization of culture conditions in SSC systems, thus increasing the potential for using SSC in the future for chemical production and biomass processing.

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Abbreviations: SSC: Solid substrate cultivation; RT-PCR: Real time polymerase chain reaction; SmF: Submerged liquid fermentation; CRF: corn rot fungi.

Introduction

The conversion of waste agricultural and forestry biomass into value-added chemical products offers great promise for increasing industrial sustainability. Treatment with enzymes is an important part of most biomass conversion technologies, but in many cases this treatment is prohibitively expensive. For instance, ethanol fuel production from lignocellulose is currently not economically feasible primarily due to the cost of the necessary enzymes, and of the chemical pretreatment required to make the biomass accessible to the enzymes. The National Renewable Energy Laboratory Ethanol Project has stated that "enzyme technology offers the greatest opportunity for future cost reduction in biofuels production" and has identified enzymatic conversion technologies for lignocellulose, in particular, as a high priority for research and development [1-3].

Conversion of biomass by microbial cultures in solid substrate cultivation (SSC) is relatively simple and inexpensive, compared with direct enzymatic conversion [4-7]. An alternative to submerged liquid fermentation (SmF), SSC involves growth of microorganisms on solid materials in the absence of free liquid. In SSC, water is bound to the substrate particles, and aerobic microorganisms use this water to grow and to degrade the solid substrate for nutrients. Air and water vapor are circulated through the

particles, either by natural or forced convection. SSC can utilize readily available, inexpensive solid substrates (e.g., unrefined agricultural residues such as corn stover, wheat bran, wheat straw, cereal grains, rice hulls, and sugar beet pulp). SSC typically requires a lower capital investment because the equipment and process often do not need to be completely sterilized. Most SSC processes perform well under "semi-sterile" conditions and do not need the technologically sophisticated equipment that sterile cultivations (e.g. SmF) require. Another advantage of SSC is that the low moisture levels may favor the production of products that are not ordinarily produced, or that are produced in small quantities or have different properties, in SmF. For example, the β-glucosidase produced by *Aspergillus phoenicis* on sugar beet pulp in SSC was more thermostable than that produced in SmF [8]. SSC provides cultivation conditions that tend to favor the production of enzyme complexes more than SmF. The complex nature of SSC substrates requires a more diverse set of enzymes for degradation than do the more refined substrates typically used in SmF. Thus, a *Trichoderma reesei* mutant produces a cellulase system that is much more effective at hydrolyzing pretreated sawdust when grown in the presence of that substrate [5]. In addition, SSC generally results in higher volumetric productivity due to a high concentration of substrate per unit volume of the reactor, resulting in lower capital and operating costs. Downstream processing and waste disposal are often simplified in SSC compared to SmF. Often the whole product is used (i.e., no separation or purification), in which case no waste is generated. If drying is required, less water in the SSC process results in lower energy inputs.

Corn stover is the residue of corn production (stalks, leaves, cobs, and husks), and is one of the most abundant single sources of lignicellulosic biomass in the United States [9]. Its potential as a feedstock for ethanol production has led to efforts to evaluate and improve methods for harvest, storage, and transport, and to assess the economic and environmental impacts of stover utilization [9-11]. The theoretical yield of ethanol from one ton of stover is approximately 100 gallons [12]. It has been estimated that 80-100 million dry tons of stover per year could be sustainably harvested in the U.S. [10]. Bioconversion of this stover could provide up to 10 billion gallons of ethanol annually, about one sixth of the volume necessary to reach our national goal for domestic ethanol production by 2030 [13].

Filamentous fungal plant pathogens that cause corn stalk rot diseases (corn rot fungi, or CRF) secrete an evolutionarily optimized combination of plant cell-wall degrading enzymes that efficiently break down corn stalk tissues. Cultivating CRF on stover in SSC approximates the natural environment of these fungi, potentially maximizing the rate of saccharification while decreasing capital costs. Pretreatment of biomass with adapted fungi in SSC has already been shown to improve the efficacy of enzymes in standard industrial saccharification protocols [14].

In spite of the potential advantages of SSC, it is currently not widely utilized for biofuels applications, primarily due to a lack of relevant research to optimize the processes. A major challenge for SSC research is the difficulty of precisely measuring microbial growth, because the microorganisms are intimately associated with the solid substrate. Accurate measurements of fungal growth over time help to elucidate the relationship between growth conditions and metabolite production. Several

approaches are currently used, e.g. measurement of chitin (glucosamine), ergosterol, and fungal enzyme activities, but these are all indirect measures of fungal growth [15, 16]. These protocols lack specificity, a problem for cellulosic biomass that is typically contaminated with multiple fungi, or in co-cultures of different fungal organisms. Furthermore, the existing assays are relatively time-consuming and labor intensive, and not suitable for large numbers of samples. Here we describe a real-time polymerase chain reaction (RT-PCR) assay, based on quantification of fungal DNA, as a convenient alternate means for measurement of fungal biomass in corn stover SSC. *Fusarium graminearum* (*Gibberella zeae*) is an aggressive colonizer of senescent corn stalks, and it is also a genetic model fungus, easily cultivated on artificial and natural substrates, and highly amenable to molecular and Mendelian manipulation [17]. A RT-PCR assay previously developed for *F. graminearum* in live wheat plants was adapted for use with corn stover. The assay uses a portion of the *Tri5* gene, which is specific to trichothecene-producing *Fusarium* strains [18].

Materials and Methods

Fungal strain and culture

F. graminearum strain PH-1 (obtained from Dr. Robert Bowden, Kansas State University) was routinely cultured on mung bean agar (MBA) at 23°C under continuous fluorescent light. MBA was prepared by boiling 40 g of mung beans in 1 L of nanopure water for 30 min., filtering the resulting liquid through Miracloth (EMD Biosciences, LaJolla, California), adding 20 g of agar (Bacto®Agar, Becton Dickinson & Company) to the filtrate, and autoclaving at 121°C for 20 min. For production of fungal DNA or mycelial inoculum, an agar plug was removed from a 3- to 4-day-old MBA culture, transferred into potato dextrose broth (PDB) (Difco), and shaken at 150 rpm and 23°C for 4-5 days. Mycelial fragments for inoculations were prepared by filtering the shake culture through Miracloth, washing the mycelial mat thoroughly with modified Fries minimal medium (FMM) (1.0 g NH_4 tartrate, 1.0 g NH_4NO_3, 1.0 g KH_2PO_4, 0.48 g $MgSO_4$. $7H_2O$, 1.0 g NaCl and 0.13 g $CaCl_2 \cdot 2H_2O$ per L, pH adjusted to 5.0), and grinding in a Waring commercial blender. To quantify the mycelial fragments, 1 ml of the ground slurry was transferred to a pre-weighed Eppendorf tube and centrifuged for 10 min at 10,000 rpm. The supernatant was decanted, and the wet weight of the mycelium was calculated.

Solid substrate cultivation on corn stover

Corn stover (consisting of dried leaves and stalks from a variety of different dent corn hybrids) was collected from the University of Kentucky Woodford County experimental farm at grain maturity in 2006, 2007, and 2008. The stover was dried at 45°C for 7-10 days to 8-10 % moisture, and then ground through a 20 mm screen. For RT-PCR and glucosamine analyses, 25 g of the dried, ground stover was autoclaved in 4 L Fernbach flasks. After sterilization, 17.5 ml of FMM was added. Each flask was inoculated with 3 ml of a mycelial fragment suspension in FMM. Medium without mycelium was added to control flasks. The solutions were distributed throughout the moistened stover by shaking the flasks vigorously. The flasks were closed with cotton plugs and foil caps and incubated without shaking at 29°C in the dark. Samples of the stover were collected from the flasks by using an autoclaved stainless steel olive grabber on day 0, and 2, 4, 6, 8, 10, and 20 days after inoculation.

Glucosamine estimation

Inoculated or uninoculated corn stover samples (450 mg) were dried for 48 h at 60°C to facilitate grinding to a fine powder in a coffee grinder. The ground samples were treated with 5 ml of 72 % sulphuric acid and incubated for 30 min at 130 rpm on a shaker at room temperature, then diluted with 54 ml of water and autoclaved for 2 hr at 121°C to hydrolyze. After hydrolysis, samples were neutralized to pH-7.0 with NaOH. Glucosamine content was estimated by using a colorimetric method [19], and comparing to a glucosamine standard at 650 nm. Data were statistically analyzed by using the multivariate Waller-Duncan K-ratio t test analysis protocol that is part of the Statistical Analysis System (SAS) (SAS Institute Inc. 1997).

Isolation of fungal and plant DNA

Genomic DNA was isolated from inoculated or uninoculated corn stover by using a modification of the protocol of Singh et al. [20]. Polyvinylpoly-pyrrolidone (PVPP) 40,000 (Sigma) was added (2 %) to CTAB extraction buffer (2 % Cetyl trimethyl ammonium bromide, 200 mM Tris HCl; 20 mM EDTA; 1.4 M NaCl). Stover samples were first dried for 48 h at 60°C to facilitate grinding. One gram of each dried sample was powdered in a coffee grinder and used for DNA extraction. Genomic DNA was also isolated from samples of fungal mycelium cultured in PDB, and from fresh corn tissues (leaf blades and sheaths) collected from V4 corn seedlings (inbred Mp305) grown in the greenhouse. These latter samples were ground under liquid nitrogen for extraction using CTAB buffer, as above. DNA was quantified by using a fluorometer (DyNA Quant 200, Hoefer, Pharmacia Biotech).

PCR amplification

F. graminearum trichodiene synthase primers (Tri5F: 5'-AGCGACTACAGGCTTCCCTC-3'; Tri5R: 5'-AAACCATCCAGTTCTCCATCTG-3') [21] and corn actin primers (Act1: 5'-CGTTGCTGCATCGA ACCTGTTTCA-3'; Act2: 5'-ACCTCAGGGCACCTAA ACCTTTCT-3') were used to amplify DNA isolated from inoculated and control corn stover samples. Each 25 µl PCR reaction was carried out in a 0.2 ml tube containing 2.5 mM dNTPs mix (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 20 pmol of each primer (Integrated DNA Technologies), 300 ng BSA, 1.25 units of Taq DNA polymerase (Invitrogen), and the DNA template. Samples were amplified in a BioRad iCycler. For amplication with the Tri5 primers, the cycler was programmed for an initial denaturation at 94°C for 3 min; followed by 40 cycles consisting of 94°C for 30 sec; 60°C for 11 sec; and 72°C for 30 sec; followed by a final extension at 72°C for 7 min. For amplification with the Act primers, the cycler was programmed for an initial denaturation at 94°C for 3 min; followed by 40 cycles consisting of 94°C for 30 sec; 68°C for 15 sec; and 72°C for 30 sec; followed by a final extension at 72°C for 7 min. Amplification products were visualized on a 1.4 % agarose gel.

RT-PCR

Genomic DNA was isolated as described from corn or fungal samples. DNA was resuspended in 120 µl of 1X TE buffer (10 mM Tris-HCl, pH=8.0 and 1 mM EDTA, pH=8.0), then diluted 40X in autoclaved nanopure water and quantified by RT-PCR in the 7900HT Fast Real-Time PCR system (Applied Biosystems). Dilutions of genomic DNA samples prepared from cultured *F. graminearum* PH1 (0.00128-100 ng) or from green corn tissues ground under liquid nitrogen (0.00128-100 ng) were used as standards.

The RT-PCR protocol was optimized by testing the effect of varying parameters including hybridization temperature, cycling conditions, and primer and $MgCl_2$ concentrations, on amplification and detection of the DNA standards. For each experiment, all standards and unknown samples were run in triplicate in Fast Optical 96-well reaction plates (Applied Biosystems). Each amplification was carried out in a 25 µl reaction containing 12.5 µl of SYBR green qPCR Master mix (New England Biolabs); 2.5 µM of each HPLC-purified fungal primer (Tri5F and Tri5R), or 4.0 µM of each HPLC-purified corn primer (Act1 and Act2); 300 ng of BSA; and 4 µl of the DNA template. PCR reactions were amplified and detected after initial denaturation and enzyme activation at 95°C for 15 min; followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 68°C for 22 sec, and extension and data acquisition at 72°C for 1 min; followed by dissociation. Amplified products were also visualized on a 1.4 % agarose gel. Data were statistically analyzed by using the multivariate Waller-Duncan K-ratio t test analysis protocol of the Statistical Analysis System (SAS Institute Inc. 1997).

Results and Discussion

Optimization of the RT-PCR protocol

RT-PCR quantification of fungal DNA using *F. graminearum* Tri5 primers, and of green corn leaf DNA using Act primers, was optimized for maximum sensitivity and reproducibility in a series of experiments. Tested variables included amplicon length, concentration of primer, template, and $MgCl_2$, annealing temperature, and cycling conditions (data not shown). The optimized assay produced linear (quantitative) detections of *F. graminearum* Tri5 at concentrations of between 1.28 pg and 100 ng of fungal genomic DNA, and of the corn actin gene at concentrations of between 64 pg and 100 ng of genomic DNA isolated from green corn tissues. The RT-PCR assay under these conditions is thus highly sensitive, capable of detecting the equivalent of approximately 30 fungal nuclei (based on a fungal genome size of 46 Mb), and 23 plant nuclei (based on a corn genome size of 2500 Mb).

Isolation of genomic DNA from corn stover

The genomic DNA prepared from corn stover samples appeared to be very degraded (Figure 1A). To determine if this degradation was due to the oven-drying treatment or to some other aspect of the DNA preparation method, DNA was prepared from fungal mycelium, senescent and green corn leaves, and corn stover, all of which were frozen in liquid nitrogen before grinding. DNA was also prepared from the same tissues that were oven-dried at 60°C for 48 hours before extraction. The DNA was isolated from 100 mg of the fresh-frozen and oven-dried tissues using the protocol developed for DNA isolation from corn stover. There was no DNA degradation in either fungal mycelium sample, or in green corn samples that had been frozen but not oven-dried (Figure 1B, C). The green corn tissues that had been oven-dried produced degraded DNA (Figure 1C). However, both the frozen and oven-dried senescent leaf and stover tissues also appeared degraded (Figure 1C). The degradation of plant DNA in stover was thus presumably due to natural processes that occur during plant senescence. Total DNA isolated from samples of inoculated oven-dried stover over time appeared to contain progressively larger quantities of high-molecular weight DNA (Figure 1D). It seems likely that this high molecular weight DNA is fungal, and that the increase from days 0 to 9 after inoculation represents an increase in fungal biomass, since

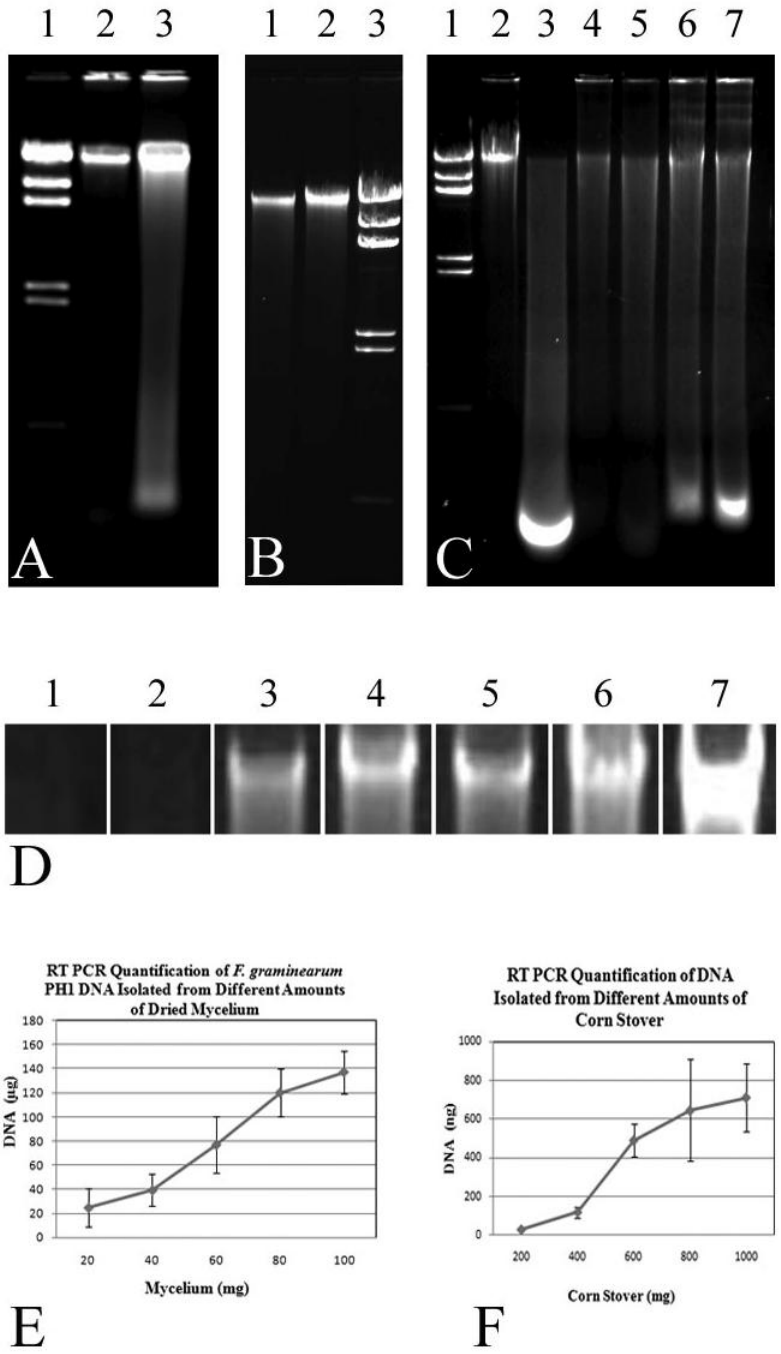

Figure 1: Genomic DNA isolated using different protocols from fungal and plant tissues. Representative results are shown. A) 1 = Hin*dIII*-digested λDNA marker. 2 = *Fusariumgraminearum* DNA prepared from fresh mycelium ground under liquid nitrogen. 3 = corn stover DNA, prepared from oven-dried stover. B) 1 = *F. graminearum* DNA prepared from fresh mycelium ground under liquid nitrogen. 2 = *F. graminearum* DNA prepared from oven-dried mycelium. 3 = Hin*dIII*-digested λDNA marker. C) 1 = Hin*dIII*-digested λDNA marker. 2 = Genomic DNA from green corn leaves ground under liquid nitrogen. 3 = Genomic DNA prepared from oven-dried green corn leaves. 4 = DNA prepared from senescent corn leaves ground under liquid nitrogen. 5 = DNA prepared from oven-dried senescent corn leaves. 6 = DNA prepared from corn stover ground under liquid nitrogen. 7 = DNA prepared from oven-dried corn stover. D) Genomic DNA prepared from oven-dried corn stover inoculated with *F. graminearum*. Only the DNA similar in size to the Hin*dIII*-digested λDNA marker is shown here. 1 = uninoculatedstover. 2 = 0 days post-inoculation.3=1 dpi.4 = 3 dpi.5 = 5 dpi.6 = 7 dpi.7 = 9 dpi. E) Real-time PCR quantification of DNA extracted from different amounts of mycelium (left) or corn stover (right). The DNA extracted from fungal mycelium was quantified using the *Tri5* primers and DNA extracted from stover was quantified by using the corn actin primers.

fungal DNA is not destroyed by the DNA preparation protocol. Amplification of plant and fungal DNA produced reasonably accurate relative measurements of plant and the fungal biomass, respectively, in spite of the degradation of the plant DNA (Figure 1E).

PCR inhibitors in corn stover

Plant tissues often contain unknown compounds that inhibit the PCR reaction. These inhibitors tend to reduce the sensitivity of the PCR assay. In order to test for inhibitors in corn stover, corn actin primers (Act1 and Act2) were used to amplify serial dilutions (1:20, 1:40, 1:60, and 1:80) of DNA samples isolated from uninoculated stover. PCR products were analyzed by gel electrophoresis. There was no amplification of the 1:20 DNA dilution, but the expected product was amplified from the 1:40, 1:60 and 1:80 dilutions (Figure 2A). Amplification of serial dilutions (1:20, 1:40, 1:60, and 1:80) of DNA samples from inoculated stover with Tri5 primers produced a similar result (Figure 2B). Again, the highest dilutions did not amplify. "Spiking" a dilution series (1:20, 1:40, 1:60, and 1:80) of uninoculated corn stover DNA with *F. graminearum* DNA (10 ng/ml), revealed a similar trend (Figure 2C). These data suggest that corn stover does contain PCR inhibitors. To avoid interference of the inhibitor with our RT-PCR analysis, all stover DNA samples were diluted at least 1:40.

PVPP has often been recommended for removal of inhibitory substances for PCR protocols [22-24]. Corn stover genomic DNA was isolated using CTAB buffer to which no PVPP had been added, or with the addition of 2 % PVPP. PCR amplification was performed with different dilutions (1:10, 1:20, 1:40) of corn stover DNA isolated with and without the use of PVPP. Addition of PVPP generally appeared to improve PCR amplification (Figure 2D). Thus, we adjusted our protocol to include 2 % PVPP in the CTAB buffer.

It has also been reported that the addition of BSA to the PCR reaction can be helpful for amplifying DNA in the presence of PCR inhibitors [25]. We added different amounts (0, 100, 200, 300, or 400 ng) of BSA to the PCR reactions. Dilutions of DNA samples isolated from inoculated stover were amplified in the presence or absence of BSA with Act primers, or with Tri5 primers. There was no amplification from the undiluted DNA in any of the BSA concentrations with the Act primers (Figure 2E). A 1:15 DNA dilution could be amplified only in the presence of BSA, and the amount of product generally seemed to increase as the BSA concentration increased, up to 300 ng of BSA (Figure 2E). Similar results were observed when samples were amplified with the Tri5 primers (data not shown). Thus, we added 300 ng of BSA to each of our PCR reactions. These measures (sample dilution and chemical additives) should help to minimize the effect of the PCR inhibitors on the sensitivity and performance of the RT-PCR assay.

Contamination of corn stover samples with trichothecene-producing fungi

Dilutions greater than 1:40 of genomic DNA isolated from uninoculated corn stover samples collected in 2007 or 2008 could be amplified with the *Fusarium* Tri5 primers (data not shown). RT-PCR data for the 2007 batch indicated the presence of 0.5 ng (±0.2) of fungal DNA per mg of oven-dried sample, and the 2008 batch averaged 0.9 ng (±0.3) per mg. This result suggests that these batches of stover were contaminated with endogenous trichothecene producing fungi. Because trichothecene producing *Fusarium* species, for

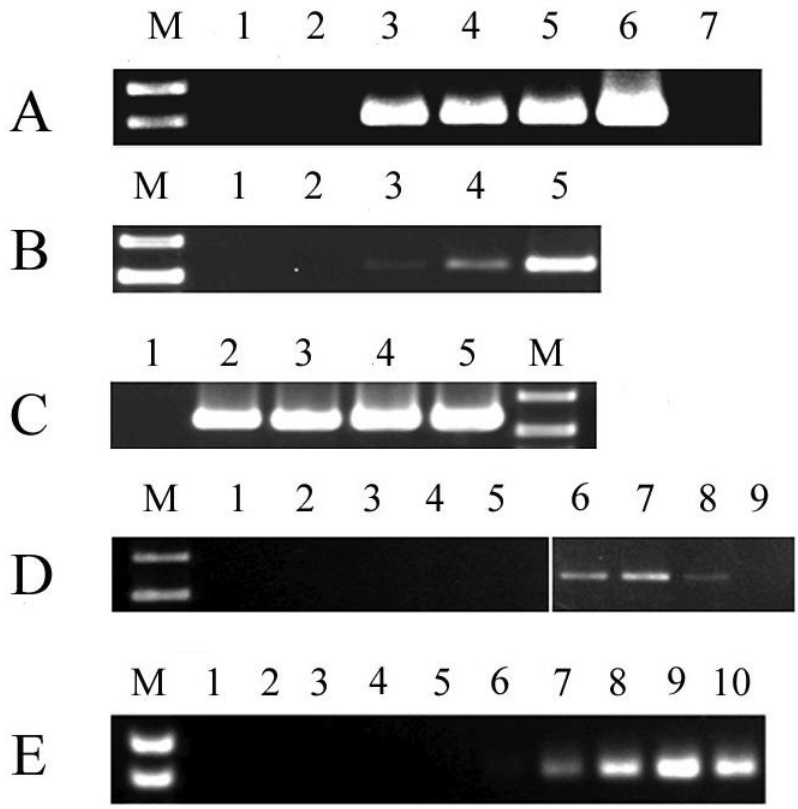

Figure 2: **A)** Dilutions of uninoculated corn stover DNA amplified with corn actin primers. Representative results are shown. M = MW markers, the upper marker is 650 bp and the lower is 500 bp from the1 kb ladder. 1 = blank (water control). 2 = 1:20 dilution stover DNA. 3 = 1:40 dilution. 4 = 1:60 dilution. 5 = 1:80 dilution. 6 = positive control (corn DNA). 7 = negative control (*F. graminearum* DNA). **B)** Dilutions of inoculated corn stover DNA amplified with *Tri5* primers. M = MW markers (650 and 500 bp). 1 = blank (water control). 2 = 1:20 dilution. 3 = 1:40 dilution. 4 = 1:60 dilution. 5 = 1:80 dilution. **C)** Dilutions of uninoculated corn stover DNA "spiked" with 10 ng*F. graminearum* DNA and amplified with *Tri5* primers. 1 = 1:20 dilution. 2 = 1:40 dilution. 3 = 1:60 dilution. 4 = 1:80 dilution. 5 = positive control (*F. graminearum* DNA). M = MW markers (650 and 500 bp). **D)** Dilutions of inoculated corn stover DNA, isolated with or without PVPP, amplified with *Tri5* primers. Representative results are shown. 1 = MW markers (650 and 500 bp). 2 = blank (water control). 2-5, DNA isolated without PVPP. 2 = undiluted DNA. 3 = 1:10 dilution. 4 = 1:20 dilution. 5 = 1:40 dilution. 6-9, DNA isolated from the same stover sample with PVPP. 6 = undiluted DNA. 7 = 1:10 dilution. 8 = 1:20 dilution. 9 = 1:40 dilution. **E)** Dilutions of inoculated corn stover DNA amplified with actin primers in the absence or presence of BSA. Representative results are shown. 1 = MW markers (650 and 500). 1 = undiluted corn stover DNA. 2 = undiluted DNA plus 100 ng BSA. 3 = undiluted DNA plus 200 ng BSA. 4 = undiluted DNA plus 300 ng BSA. 5 = undiluted DNA plus 400 ng BSA. 6 = 1:15 dilution DNA. 7 = 1:15 dilution plus 100 ng BSA. 8 = 1:15 dilution plus 200 ng BSA. 9 = 1:15 dilution plus 300 ng BSA. 10 = 1:15 dilution plus 400 ng BSA.

example *F. verticillioides*, are common inhabitants of corn stalks, this result is not surprising. In samples of corn stover collected in 2006, there was no detectable amplification of the Tri5 product in either non-diluted or diluted DNA (data not shown). Although 2007 and 2008 batches of corn stover contained background levels of Tri5 signal, this was not a problem for our experiments because we autoclaved the stover, killing any endogenous fungus. Thus, the signal did not increase over time in the stover controls. Furthermore, the background signal was never high enough to interfere with detection of fungal biomass in the stover cultures. However, one potential advantage of using evolutionarily-adapted microorganisms in SSC systems is increased competitiveness of the inoculated organism, possibly reducing the necessity for autoclaving the substrate. Primers with more specificity to *F. graminearum* could be used if the desire was to use non-autoclaved stover in SSC, or if levels of endogenous

infection in the stover were unusually high [26, 27].

Sampling effects

Corn stover is a heterogeneous substrate. This heterogeneity will tend to decrease reproducibility among samples. To evaluate the reproducibility of genomic DNA isolation from the stover, five replicate samples from the same uninoculated stover batch were extracted, and the DNA quantity recovered was evaluated both by fluorometry and by RT-PCR using the Act primers. The fluorometer produced an average reading from the 2007 batch of stover of 4.7 mg/g (±0.9) of DNA per gram of dried tissue, while RT-PCR for the same set of samples produced an average reading of 6.0 mg (±2.5). For the 2008 batch of stover, the fluorometer produced an average reading of 12.3 mg/g (± 2.3), while the RT-PCR for the same set of samples produced an average of 11.0 mg/g (± 6.7). The corn stover samples did not contain cob pieces, but they did contain coarse fragments of stalk rind as well as finer pith fragments. DNA yields from subsamples that were sorted to contain only rind fragments were much higher than from subsamples from the same batch of stover that contained only pith (RT-PCR results were an average of 21.8 mg/g (±7.2) for rind, versus 2.4 mg/g (±1.2) for pith). Furthermore, DNA isolated from rind tissues seemed to be less degraded (data not shown). Heterogeneity, both of the solid substrate and of the microbial growth on the substrate, is an inherent attribute of the SSC protocol, in contrast to SmF. Given this relatively high innate degree of variability, it is very important to sample extensively during SSC. This is where a high throughput assay capable of handling large numbers of samples, such as RT-PCR, would be particularly advantageous in comparison with other commonly used protocols for analysis of microbial growth.

Colonization of corn stover by *F. graminearum*

The most common protocol currently used to assess fungal growth in SSC measures glucosamine (chitin) content. Chitin is a major component of all fungal cell walls. We compared the performance of the RT-PCR assay with a standard glucosamine assay. Samples of corn stover inoculated with *F. graminearum* mycelial fragments were collected in triplicate immediately after inoculation (day 0) and on the 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 20^{th} days after inoculation. Half of each sample was subjected to the RT-PCR protocol, and the other half was analyzed for glucosamine content. The experiment was repeated twice with similar results. Results of one experiment are shown here. The RT-PCR results indicated a significant increase ($\alpha = 0.05$) in fungal DNA (microbial biomass) above the baseline for all three levels of inoculum after four days of growth. Fungal biomass generally appeared to increase over time, but between 4 and 10 days post inoculation (dpi) the difference was significant only for the lowest inoculum level, and there were no significant differences for any of the samples between 10 and 20 dpi (Figure 3A). Glucosamine content for all three inoculum levels did not rise significantly above the baseline until 6 dpi. There was a significant increase in glucosamine between 6 dpi and 10 dpi for the two highest amounts of inoculum, but between 10 and 20 dpi there was a significant difference only for the lowest level of inoculum (Figure 3B). The performance of the RT-PCR was generally comparable to the glucosamine assay for measuring fungal development on stover. Thus, results of both methods indicated that there was an increase in fungal growth and colonization of corn stover

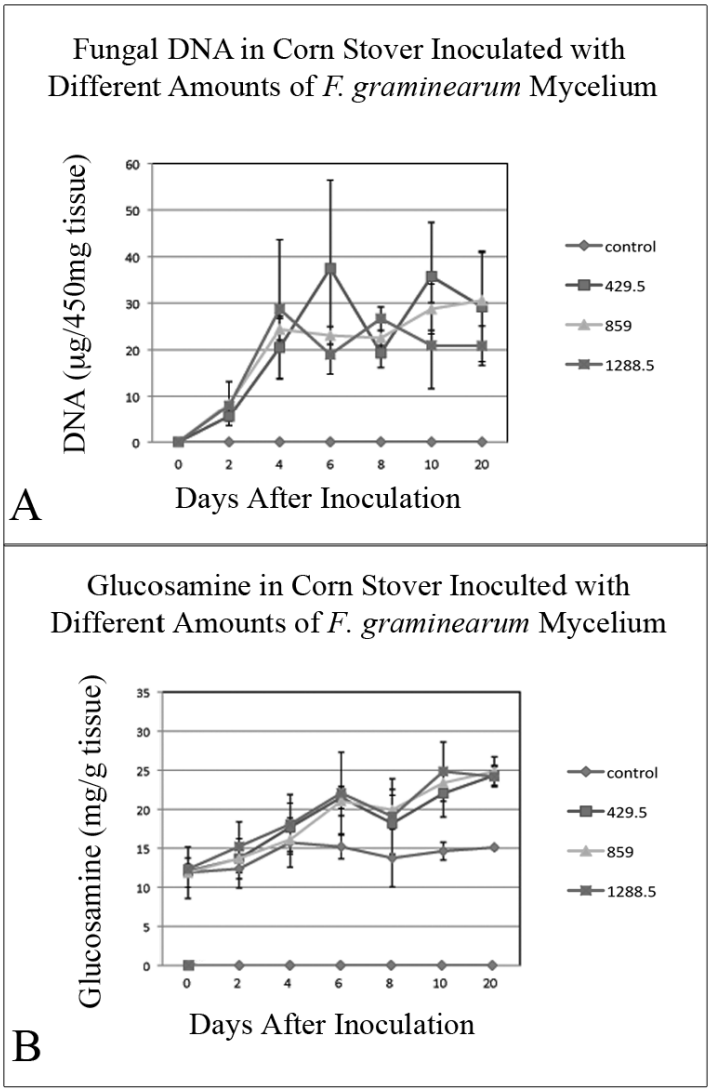

Figure 3: **A)** RT-PCR analysis and **B)** glucosamine analysis used to estimate fungal biomass in stover inoculated with different amounts of *F. graminearum* mycelium.

during the first week of cultivation, followed by a substantial reduction in the rate of fungal growth during the last two weeks of the experiments. The reason for this slowing of growth is unclear; however it is consistent with enzymatic saccharification of high-density solid substrate [28, 29].

The RT-PCR assay has two advantages over the standard glucosamine assay for measuring fungal development on stover. One is its specificity. All sources of agricultural biomass are typically contaminated with many types of fungi. Because the glucosamine assay lacks specificity, it will detect most of these contaminants. Thus, the baseline with the glucosamine assay for uninoculated stover was quite high, and partly as a consequence of this, the glucosamine assay was less sensitive than the RT-PCR assay. The RT-PCR assay showed a significant increase of fungal biomass above the baseline within 4 days, whereas the

glucosamine assay did not show a significant increase until 6 days after inoculation. In addition to increased sensitivity, the high degree of specificity makes it possible with RT-PCR to multiplex samples. Multiplexing would facilitate simultaneous analysis of the performance of microbial co-cultures, e.g. lignin-degrading combined with cellulose-degrading organisms.

Another advantage of the RT-PCR is the potential for semi-automated, high-throughput analyses. The ability to process large numbers of samples relatively quickly is important for optimization of SSC protocols, because between-sample variability tends to be relatively high due to heterogeneity of the solid substrate and of microbial growth. Increasing the number of samples in the RT-PCR would tend to decrease the standard error and improve the separation of treatments with similar means.

In conclusion, we developed a RT-PCR assay to measure growth of a CRF on corn stover by quantifying the fungal DNA. This method was very sensitive, detecting as few as 30 fungal nuclei, and its accuracy was comparable with the standard glucosamine assay. The assay also had a high degree of specificity and the potential for high-throughput automated analysis. This RT-PCR approach could make it easier to optimize and test the effect of various parameters on microbial growth in SSC, which should help to make SSC a more viable option in the future for biomass processing.

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