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Solvent comparison in the isolation, solubilization, and toxicity of *Stachybotrys chartarum* spore trichothecene mycotoxins in an established in vitro luminescence protein translation inhibition assay

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Abstract

It is well known that non-viable mold contaminants such as macrocyclic trichothecene mycotoxins of *Stachybotrys chartarum* are highly toxinigenic to humans. However, the method of recovering native mycotoxin has been without consensus. Inconsistencies occur in the methods of isolation, suspension, preparation, and quantitation of the mycotoxin from the spores. The purpose of this study was to provide quantitatively comparative data on three concurrent preparations of 10^6 *S. chartarum* spores. The experiments were designed to specifically evaluate a novel method of mycotoxin extraction, solubilization, and the subsequent inhibitory effect in an established in vitro luminescence protein translation assay from 30 day-old spores. The mycotoxin-containing spores swabbed from wallboard cultures were milled with and without glass beads in 100% methanol, 95% ethanol, or water. Milled spore lysates were cleared of cell debris by filter centrifugation followed by a second centrifugation through a 5000 MWCO filter to remove interfering proteins and RNases. Cleared lysate was concentrated by centrivap and suspended in either alcohol or water as described. The suspensions were used immediately in the in vitro luminescence protein translation assay with the trichothecene, T-2 toxin, as a control. Although, mycotoxin is reported to be alcohol soluble, the level of translation inhibition was not reliably satisfactory for either the methanol or ethanol preparations. In fact, the methanol and ethanol control reactions were not significantly different than the alcohol prepared spore samples. In addition, we observed that increasing amounts of either alcohol inhibited the reaction in a dose dependent manner. This suggests that although alcohol isolation of mycotoxin is desirable in terms of time and labor, the presence of alcohol in the luminescence protein translation reaction was not acceptable. Conversely, water extraction of mycotoxin demonstrated a dose dependent response, and there was significant difference between the water controls and the water extracted mycotoxin reactions. In our hands, water was the best extraction agent for mycotoxin when using this specific luminescence protein translation assay kit.

Keywords: *Stachybotrys chartarum*; Spore; Trichothecene mycotoxin; In vitro protein translation assay

1. Introduction

Fungi infestation of natural and fabricated materials can be a major source of respiratory disease in humans. Both the cellular components and the byproducts of

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fungi metabolism directly influence the adverse health effects that are associated with such exposures. Organic dust toxicity syndrome, first reported among workers in a variety of agricultural and industrial settings, is thought to involve inhalation exposure to a poorly defined mixture of substances that may include toxic fungi (Enaarson and Chan-Yeung, 1990; Weber et al., 1993). The severity of the disorder may by associated with the amount of mycotoxin present and essentially depends on the number of fungi spores inhaled within the airborne dust (Trout et al., 2001; Fischer and Dott, 2003). Pulmonary hemosiderosis among infants was reported in Cleveland, OH, in the early 1990s (Etzel et al., 1996), and although a connection was attempted, the findings were not sufficient to support an association between *Stachybotrys chartarum* and the disease (Montana et al., 1997; Etzel et al., 1998; CDC MMWR, 2000). Mycotoxicosis specifically refers to a reaction to the mycotoxins produced by fungi. In addition, during exponential growth, many fungi release approximately 500 low molecular weight volatile microbiological organic compounds (mVOCs) as byproducts of metabolism including ethanol (Miller, 1992). Indeed, the mVOCs comprise a great diversity of chemical structure ranging from ketones to aldehydes to alcohols as well as moderately to highly modified aromatics and aliphatics. Although VOCs may be involved in human disease, it is difficult to confirm. Alternatively, there are many more described mechanisms by which fungi cause disease (i.e., immunologic hypersensitivity to the fungus and fungal mycosis host-cell toxic reaction associated with mycotoxin). Collectively, the data support the hypothesis that mycotoxin toxicity has an important role as the agent of disease causation.

Numerous studies have attempted to delineate the mycotoxins produced by fungi. Typically the solubilization of mycotoxins is optimized depending on the downstream application. For instance, because mycotoxins are highly soluble in organic solvents and only slightly soluble in physiological fluids, researchers interested in the physical properties of mycotoxins use overnight extractions in ethyl acetate, petroleum ether, methanol, and dichloromethane for characterization by gas chromatography/mass spectroscopy (GC/MS), high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and ELISA (Harrach et al., 1981; Sorenson et al., 1987; Jimenez et al., 1997; Nielsen et al., 1998, 1999; Jarvis et al., 1998; Bo-Kwak and Dong-Hwa Shon, 2000; Andersen et al., 2002). Alternatively, researchers interested in conducting toxicity assays addressing the de novo consequences of fungi inhalation choose to purify mycotoxins in less toxic suspensions including methanol, ethanol, water, or saline suspension (Harrach et al., 1982; Pasanen et al., 1993; Jimenez et al., 1997; Vesper et al., 1999; Yike et al., 1999; Rao et al., 2000a,b). Although any of these extraction agents are acceptable, the reported toxicity of the suspended mycotoxin varies widely and is extraction specific.

It would be advantageous to develop a consensus method for the recovery of trichothecene mycotoxin from fungi spores with direct application in a standardized in vitro toxicity assay. The purpose of this study was to provide quantitatively comparative data on three concurrent preparations of 10^6 *S. chartarum* spores. The experiments were designed to specifically evaluate mycotoxin extraction, solubilization, and the subsequent inhibitory effect in an established in vitro luminescence protein translation assay from 30 day-old spores. In addition, we present a method of spore mycotoxin preparation that shortens the procedure from overnight extraction and purification to less than 1 h.

2. Methods and materials

2.1. Materials and reagents

Lyophilized T-2 trichothecene mycotoxin used as a positive control in the luciferase translation assay was purchased from Sigma, St. Louis, Mo. The Rabbit Reticulocyte Lysate Kit and Steady Bright-Glo Reagent were purchased from Promega, Madison, WI. Steady Bright-Glo Reagent was divided into 0.5 mL sizes, and all reagents were stored in the dark at −70 °C until needed.

2.2. Preparation and storage of T-2 toxin

Lyophilized stock of T-2 toxin was suspended in 95% ethanol to ensure complete solubility and subsequently diluted in cold deionized sterile water at specific concentrations for easy use in the translation assay. The final concentration of ethanol in the T-2 preparations was below 0.01% in the translation reaction mixture. T-2 toxin suspensions stored at −70 °C for 30 days exhibited no loss of toxicity.

2.3. Fungi growth conditions

Conidia of toxinigenic *S. chartarum*, strain 63-07, were inoculated onto the paper of a wetted 3″ by 3″ piece of autoclaved sterile, 3/8″ thick wallboard in a petri dish. The wallboard was incubated in a clear acrylic-walled static chamber desiccator (32×39×51 cm)

designed to provide controlled environments for the fungal growth (Foarde et al., 1992, 1994). The chambers were placed in a temperature-controlled (21±3 °C), HEPA (High Efficiency Particulate Absolute) filtered room. A 97% RH (relative humidity) was maintained by insertion of a bottom tray containing a saturated potassium sulfate salt solution (Greenspan, 1977), and RH was checked daily with a chamber-mounted hygrometer.

2.4. Preparation of S. chartarum mycotoxins

Spores were collected by gently rolling a wetted sterile cotton swab over the *S. chartarum* growth and immersing the spore covered swab in cold deionized sterile 18-ohm water. This preparation was immediately washed once with 200 μL of cold deionized sterile 18-ohm water and pelleted at 1800 *g* for 1 min. The pellet was suspended in cold deionized sterile 18-ohm water, and spore integrity and purity was assessed by light microscopy and quantitated by hemacytometer. A 1×10^6 spore suspension was pipetted into a thin-walled PCR tube, pelleted as before, and gently suspended in the appropriate diluent to approximately 100 μL. The tubes were filled with approximately 100 μL of 0.5 mm glass beads or an additional 100 μL appropriate diluent. The tubes were capped and subjected to milling in a bead beater (Biospec Products, Bartlesville, OK) on high for 30 seconds, paused for 30 seconds, and milled for an additional 30 seconds. The cellular debris was pelleted, and the supernatant was passed through Ultrafree-MC 5000 NMWL microcentrifuge filter (Millipore Corp, Bedford, MA) at 1800 g for 25 min to remove small interfering proteins and RNases (Yike et al., 1999). The filtrate was evaporated to near dryness at 65 °C in a Speed Vac Plus centrifuge (Savant Instrument, Farmingdale, NY). At no time did the sample dry completely. Methanol extracts were evaporated for 8 min, ethanol extracts for 11 min, and water extracts for 25 min. Extracts were reconstituted in 10 μL of 100% methanol, 95% ethanol, or sterile deionized water and placed on ice until needed.

2.5. In vitro luminescence protein translation

The translation of firefly luciferase mRNA was carried out as previously described (Yike et al., 1999) with some modification. A master mix of the translation reaction was made by combining 8.75 μL Flexi Rabbit Reticulocyte Lysate, 0.25 μL Complete Amino Acid Mixture (1 mM), 0.35 μL Potassium Chloride (2.5 M), 0.25 μL RNA substrate (omitted for negative control), and Nuclease-Free Water to a final volume of 12.5 μL for each reaction. The luciferase protein translation assay was performed by combining 0.5, 1.0, or 2.0 μL of the extracts to master mix with RNA substrate to a final volume of 12.5 μL in a PCR tube. The reaction tubes were placed in a thermocycler (BioRad, Hercules, CA) programmed for a single cycle of 90 min at 30 °C. The samples were removed from the thermocycler within 5 min of completion and either used immediately or stored at −70 °C to be used within 24 h.

2.6. Luciferase activity assay

An aliquot of Steady Bright-Glo Reagent was thawed slowly to room temperature by placing the tube in a small beaker of water in the dark 30 min before the start of the activity assay. The Steady Bright-Glo Reagent (25 μL) was pipetted into test wells of a Costar 96 well ½ volume white polystyrene plate (Corning Inc., Corning, NY). Each mycotoxin sample (2.5 μL) was added by pipette and mixed in by swirling the pipette tip in a circular motion 3–5 times. Pipette mixing of the mixture was not done to reduce the generation of interfering foaming. The plate was covered by a foil-lined lid and was either read within 10 min or stored for up to 2 h in the dark without loss of activity. The plate was read in a Tecan GENios (Tecan US, RTP, NC) using luminescence mode and XFLUOR4 software. The activity of all samples was expressed as percent control (water added in place of toxin or extract). Control samples with luciferase mRNA and no toxin added consistently yielded about 400,000 RLU for 100% luciferase mRNA translation.

3. Results

3.1. Alcohol extracted S. chartarum spores

There was no discernible dose dependent linear trend in luciferase protein translation inhibition by increasing either methanol or ethanol extracted spore mycotoxin concentration (Figs. 1 and 2) because there is virtually no difference in results using either alcohol alone at or alcohol extracted mycotoxin at 0.5–2.0 μL. In fact, there was no significant difference between the spores milled in alcohol and the alcohol controls regardless of whether milled with glass (+G) or without glass (−G). Within these data sets, any alcohol in the reaction was inhibitory. However, as alcohol increased in the final reaction volume (v/v) from 0.5–2.0 μL, the translation of luciferase mRNA decreased in a linear fashion.

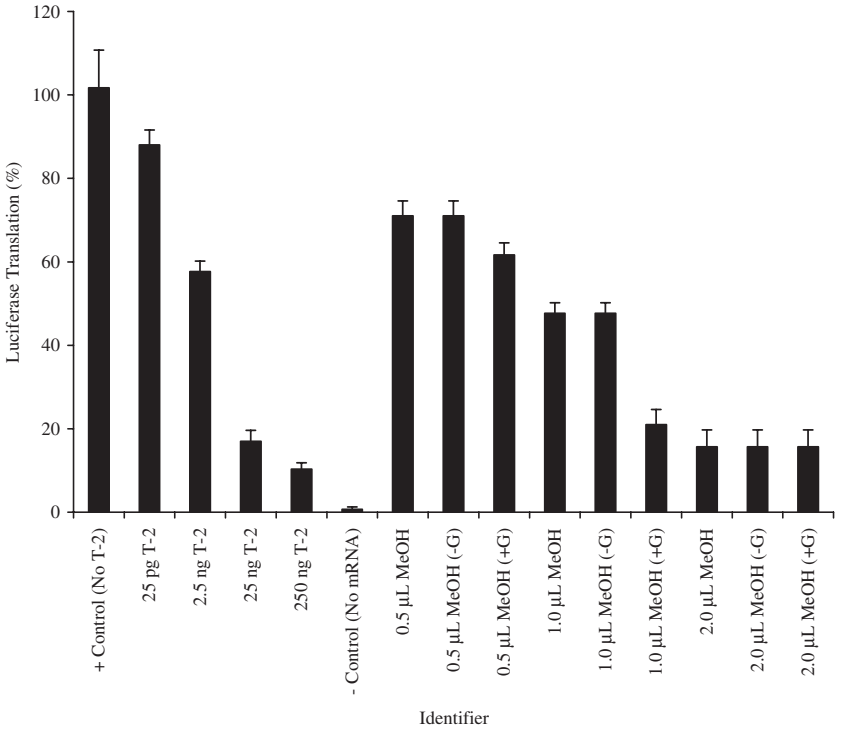

Fig. 1. Mean effects of T-2 toxin and methanol (MeOH) prepared *S. chartarum* spores milled with glass beads (+G) and without glass beads (−G) on luciferase protein translation. Data shown are the means±standard error of the means of three independent experiments.

3.2. Water extracted S. chartarum spores

There was a discernible dose dependent linear trend in luciferase protein translation inhibition by increasing water extracted spore mycotoxin with similar translation inhibition regardless of whether the spores were milled with (+G) or without glass (−G) (Fig. 3). In fact, there was significant difference between the milled spores and the water control. Within the data set, water did not inhibit luciferase protein translation as the water increased in the final reaction volume (v/v) from 0.5–2.0 µL.

3.3. Water re-suspended milled spores

The alcohol and water milled spore isolates were evaporated to near dryness and suspended in water only. Each water suspended isolate was submitted to the protein translation assay as before (Fig. 4). Neither the methanol nor the ethanol isolates suspended in water inhibited the reaction at all. In fact, they display a similar pattern of inhibition as shown for water alone. However, the evaporated water isolate resuspended in water inhibited the luciferase protein assay in a dose dependent linear response.

4. Discussion

Trichothecenes are very stable small molecular weight compounds that are resistant to UV light, heat (>100 °C), and acids (Kuhn and Ghannoum, 2003). *S. chartarum* mycotoxins may have developed to serve as a chemical defense system against insects, microorganisms, nematodes, grazing animals, and humans. Their potential harmful effects are well documented and reviewed in the literature (Etzel, 2002; Kuhn and Ghannoum, 2003). Historically,mycotoxins were extracted by a variety of methods depending on the downstream application. However, not all solvents are amendable for all molecular toxicity and cytotoxicity applications. Therefore, the optimization of mycotoxin extraction is preferable so as to obtain a better understanding of physiological mycotoxin cytotoxicity.

Water extraction of *S. chartarum* spore mycotoxins produced a discernible dose dependent linear trend in luciferase protein translation inhibition and is only slightly enhanced by glass milling. Conversely, methanol and ethanol completely inhibited the luciferase protein translation assay even when the solvent concentration was less than 5% (v/v). The situation

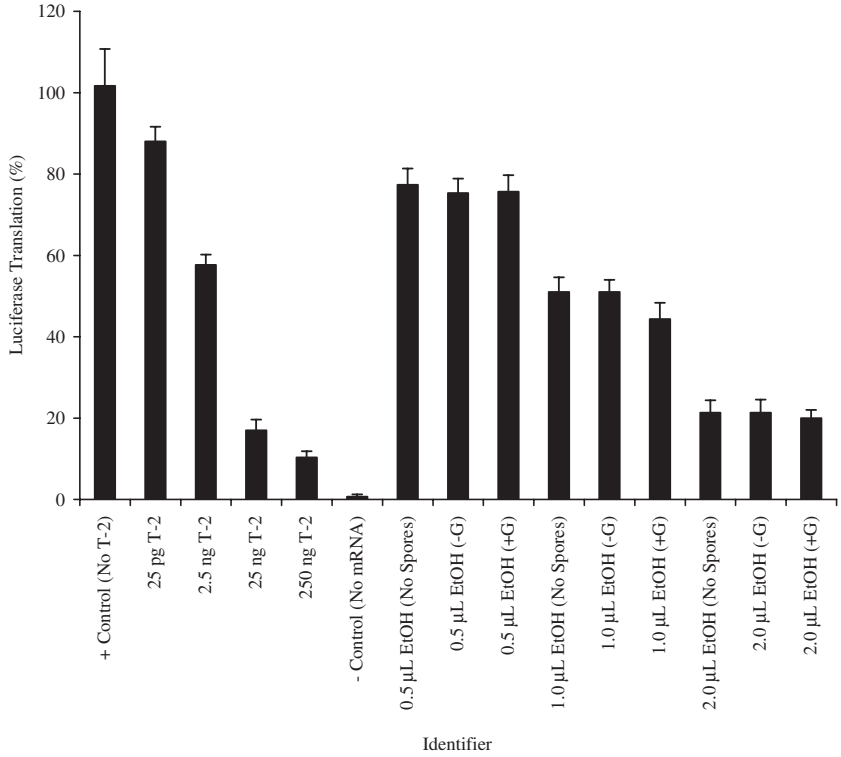

Fig. 2. Mean effects of T-2 toxin and ethanol (EtOH) prepared *S. chartarum* spores milled with glass beads (+G) and without glass beads (−G) on luciferase protein translation. Data shown are the means ± standard error of the means of three independent experiments.

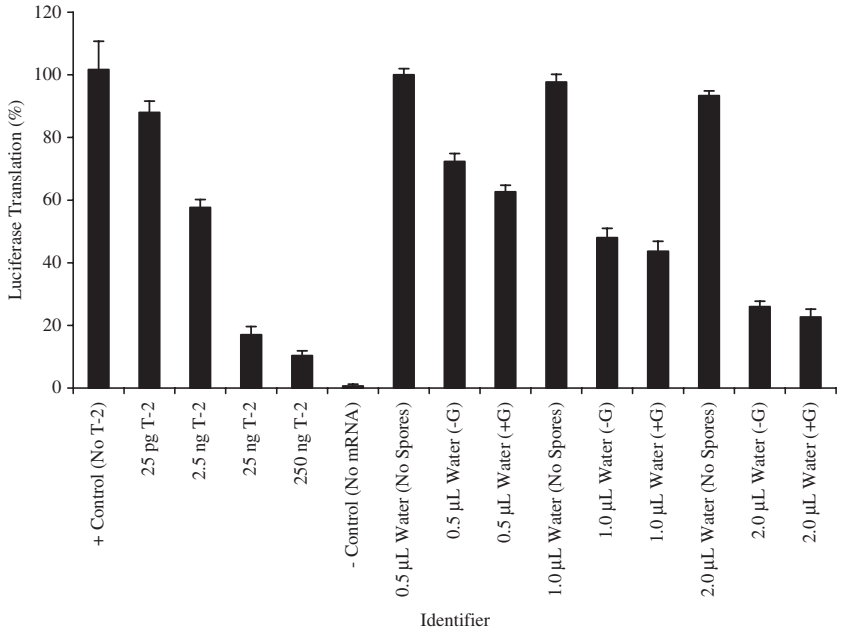

Fig. 3. Mean effects of T-2 toxin and water prepared *S. chartarum* spores milled with glass beads (+G) and without glass beads (−G) on luciferase protein translation. Data shown are the means ± standard error of the means of three independent experiments.

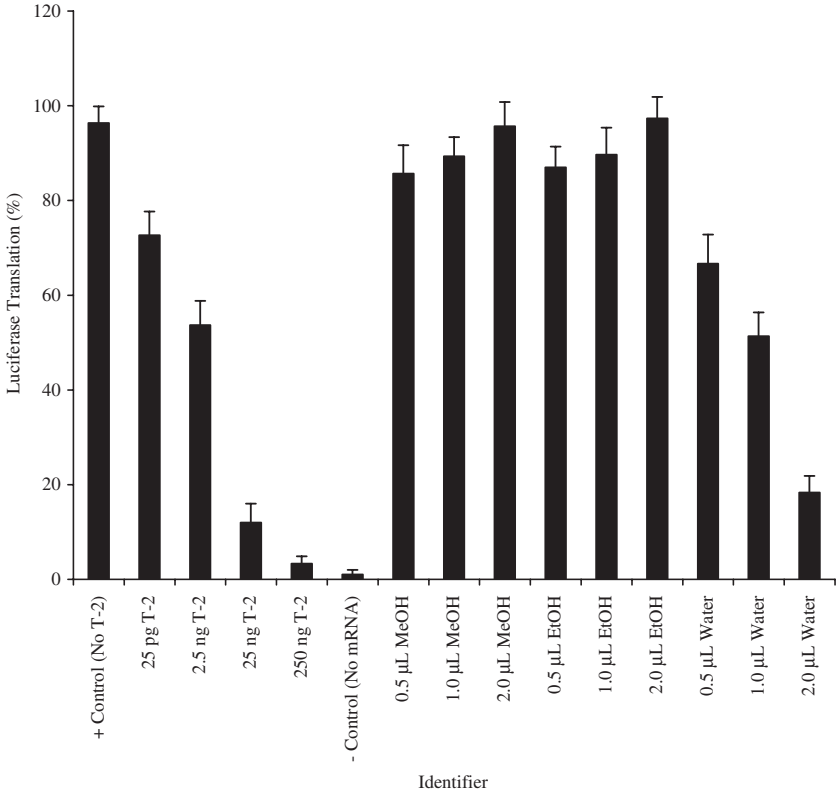

Fig. 4. Mean effects of T-2 toxin and methanol (MeOH), ethanol (EtOH), and water prepared *S. chartarum* spores milled with glass beads. Each sample was centrivap dried and suspended in 10 µL water only. Data shown are the means ± standard error of the means of three independent experiments.

did not improve with milling either in the presence of or absence of glass beads.

It has been suggested that methanol extraction of mycotoxin from *S. chartarum* spores decreased the toxigenicity of the spore in the mouse model (Rao et al., 2000b). However, the effects of the extracted spores could not be attributed to mycotoxin alone. The study did not conclusively demonstrate that the methanol extracted any mycotoxin at all or whether the reduction in toxicity was attributed to either methanol induced spore sterilization or methanol extraction of cellular components, although a limited bronchoalveolar lavage response occurred the animals were sacrificed after only 24 h. Since no mycotoxin induced antigen follow up was done (Park et al., 1994) and no assessment of the extraction was provided, factors other than mycotoxin mediated toxicity cannot be dismissed entirely as the agents responsible for the limited response. Although it is not our contention that the organic solvents are incapable of isolating mycotoxin, we are suggesting that the amounts and relative strength of mycotoxin may be enhanced by isolation in physiological fluid. Simply extracting the spores in water may have given more relevant information. This was first shown in the late 1970s and early 1980s by isolating macrocyclic trichothecenes from swine stomach and intestinal fluid (Nummi and Niku-Paavola, 1977) and isolating water soluble mycotoxins from *S. atra* (Harrach et al., 1982).

Suspending alcohol extractions (evaporated to near dryness) in water did not result in luciferase protein translation inhibition. In fact, neither the methanol nor the ethanol isolates suspended in water inhibited the reaction at all, and each displayed a similar pattern of non-inhibition as shown for water alone. However, the evaporated water isolate re-suspended in water inhibited the luciferase protein assay in a dose dependent linear response.

It is difficult to reconcile the results we obtained in this study with others. To be sure, methanol and ethanol will inhibit this in vitro protein translation assay when 5% or greater in the final reaction (v/v). Interestingly, ethanol is the most common mVOC released by fungi (Miller, 1992). Additionally, lyophilized T-2 mycotoxin is only readily soluble after first adding 95% ethanol.

However a lyophilized sample of mycotoxin is not in its physiological state. Alcohol preparation of *S. chartarum* spores resulted in an unreliable response in this in vitro luminescence protein translation assay and the alcohol was non-permissible in the reaction. It is not unexpected that methanol is toxic in the luciferase protein translation assay. Methanol is generated from fossil fuels and is extremely toxic in very small amounts, causing blindness and permanent injury including death. It is considered five times more toxic than most volatile organic solvent vapors.

It was not the purpose of this study to identify the trichothecene mycotoxins released by milling *S. chartarum* spores. However, it is evident that in this study *S. chartarum* trichothecene mycotoxins that inhibit protein synthesis are extractable by water. This is not surprising since the effects of mycotoxins are optimized in nature in physiological fluids. Extraction of spore mycotoxins by milling reduces the time typically required to isolate trichothecene mycotoxins from overnight to 1 h. This is important because the spores of most fungi will germinate over time in a variety of media. It would be interesting to evaluate water extractions of other types of fungi contaminated surfaces with particular interest placed on the strength of mycotoxins isolated from different genus of fungi and relevance between strains.

5. Conclusion

Trichothecenes are very stable small molecular weight compounds with potentially harmful effects, and there have been numerous studies attempting their delineation. Due to the inherent difficulties and inaccuracies reported in distinguishing and isolating mycotoxins, our laboratory set out to optimize the extraction and analysis of spore bound mycotoxins in a well-established luciferase protein translation assay.

We discovered that methanol and ethanol did not effectively extract mycotoxins from spores as there was no discernible dose dependent linear trend in luciferase protein translation inhibition. In fact, there was virtually no difference in the results using either alcohol alone or alcohol extracted mycotoxin. Milling and alcohol extraction did not influence this finding. However, alcohol to 5% or more final volume in the translation assay was inhibitory in a dose dependent linear fashion.

Water was clearly best at extracting spores mycotoxin. The results demonstrated that luciferase protein translation inhibition followed a discernible dose dependent linear trend. Milling was beneficial, and water did not inhibit the reaction as demonstrated by alcohol.

Re-suspending dried alcohol milled spore extractions did not inhibit luciferase protein translation at all. In fact, the inhibition was similar to water alone. However, the evaporated water isolate re-suspended in water inhibited the luciferase protein assay in a dose dependent linear response.

This study suggests that although alcohol isolation of mycotoxin is desirable in terms of time and labor, the presence of alcohol in the luminescence protein translation reaction was not acceptable. Conversely, water extraction of mycotoxin demonstrated a dose dependent response and there was significant difference between the water controls and the water extracted mycotoxin reactions. In our hands, water was the best extraction agent for mycotoxin when using this specific luminescence protein translation assay kit.

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