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RESEARCH ARTICLE

Effect of strain and cultural conditions on the production of cytochalasin B by the potential mycoherbicide *Pyrenophora semeniperda* (Pleosporaceae, Pleosporales)

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The seed pathogen *Pyrenophora semeniperda* has demonstrated potential as a mycoherbicidal biocontrol for eliminating persistent seed banks of annual bromes on western North American rangelands. This pathogen exhibits variation in virulence that is related to mycelial growth rate, but direct laboratory tests of virulence on seeds often have low repeatability. We developed a rapid and sensitive high pressure liquid chromatography method for quantification of the phytotoxin cytochalasin B in complex mixtures in order to evaluate its use in virulence screening. All 10 strains tested produced large quantities of this metabolite in solid wheat seed culture, with production varying over a fourfold range (535–2256 mg kg⁻¹). No cytochalasin B was produced in liquid potato dextrose broth culture, showing that its synthesis is strongly dependent on cultural conditions. In a *Bromus tectorum* coleoptile bioassay, solid culture extracts showed mild toxicity similar to the cytochalasin B standard at a concentration equivalent to 10⁻⁴ M cytochalasin B (72–95% of control), whereas at 10⁻³ M equivalent, the extracts exhibited significantly higher toxicity (8–18% of control) than the cytochalasin B standard (34% of control). This suggests the possible presence of other phytotoxic metabolites. Cytochalasin B production in solid wheat seed culture exhibited the predicted significant negative correlation with mycelial growth rate on potato dextrose agar, but the correlation was not very strong, possibly because cytochalasin B production and growth rate were measured under different cultural conditions.

Keywords: annual brome biocontrol; *Bromus tectorum*; HPLC (high pressure liquid chromatography); phytotoxins; seed pathogen; virulence screening

1. Introduction

Annual brome species, including cheatgrass or downy brome (*Bromus tectorum* L.) and red brome (*Bromus rubens* L.), are major invaders of semi-arid ecosystems in western North America, where tens of millions of hectares have been converted through cycles of repeated burning to near-monocultures of these grasses (Brooks et al., 2004). Restoration of annual brome-infested rangelands through direct seeding of more desirable species is not possible without some form of control, and even

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herbicides are of limited usefulness because they have no impact on the residual seed bank that persists following herbicide application.

We have determined that the cosmopolitan generalist seed bank pathogen *Pyrenophora semeniperda* (Brittlebank and Adams) Shoemaker has potential as a biocontrol agent for removing the annual brome residual seed bank (Meyer, Clement, & Beckstead, 2013). This pathogen is ubiquitous in the seed banks of annual bromes and under some conditions may destroy up to at least half of the current-year seed production (Meyer, Quinney, Nelson, & Weaver, 2007). Our intent is to develop this organism for augmentative or inundative biocontrol of the annual brome persistent seed bank in combination with other forms of control to increase the success of restoration seedings. This pathogen could also be useful as a biocontrol for persistent seed banks of many species of annual grass weeds problematic in intensive agriculture (Medd, Murray, & Pickering, 2003).

Our previous research indicates that the main target of this pathogen is seeds that are dormant in the soil seed bank (Beckstead, Meyer, Molder, & Smith, 2007). Rapidly germinating seeds may be infected, but usually escape mortality and develop into normal seedlings (Campbell & Medd, 2003). However, strains of this pathogen vary in their ability to cause mortality of non-dormant *B. tectorum* seeds. Our original hypothesis was that strains with faster growth rates would be more likely to kill non-dormant seeds, but experimental work revealed the opposite pattern, namely, that mycelial growth rate and ability to cause mortality of non-dormant seeds were negatively correlated (Meyer, Stewart, & Clement, 2010). We then proposed the alternative hypothesis that this apparent trade-off between virulence and growth was a consequence of the need to quickly produce large quantities of metabolically expensive phytotoxic metabolites that could slow or prevent germination of non-dormant seeds.

It was already known that an Australian strain of *P. semeniperda* produced large quantities of phytotoxic cytochalasin B (Figure 1) in wheat seed culture (Evidente, Andolfi, Vurro, Zonno, & Motta, 2002). This well-known fungal secondary metabolite exhibits cytotoxicity by interfering with actin filament development during cytokinesis (Möbius & Hertweck, 2009), making it likely to be the phytotoxin that can disable germinating seeds. The Australian *P. semeniperda* strain also produced smaller quantities of several additional cytochalasins, including cytochalasin A, cytochalasin F, deoxaphomin and three novel cytochalasins, Z1, Z2 and Z3

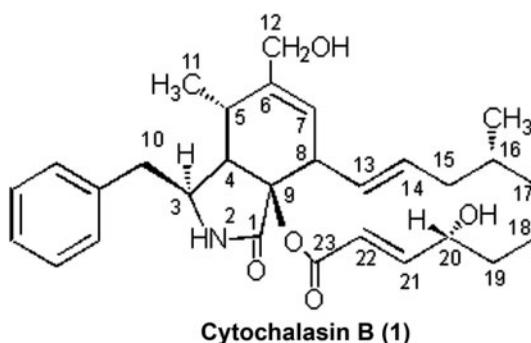


Figure 1. Chemical structure of cytochalasin B.

(Evidente et al., 2002). Cytochalasins are a large group of fungal metabolites produced by several genera of fungi. More than 60 different cytochalasins have been purified, identified and grouped in different sub-groups. The structure of cytochalasins, their biosynthesis and relationships between structure and activity were recently reviewed (Scherlach, Boettger, Remme, & Hertweck, 2010).

The first objective of the current project was to develop a rapid, sensitive method for quantifying cytochalasin B in complex mixtures, including the organic extracts of fungal cultures. We then used this quantification method to examine whether cytochalasin B production in *P. semeniperda* varied as a function of cultural conditions and whether among-strain variation in cytochalasin B production was negatively correlated with mycelial growth rate as predicted by the trade-off hypothesis. The practical goal was to improve our ability to screen large numbers of strains for potential virulence on non-dormant seeds. Our hope was that the repeatability of a chemical method would be greater than the repeatability of virulence tests on living seeds, which often yield highly variable results depending on largely unquantifiable variation in seed status and environmental conditions.

2. Materials and methods

2.1. Chemical methods

¹H nuclear magnetic resonance (NMR) spectra were recorded at 600 MHz in CDCl₃ on a Bruker (Kalsruhe, Germany) spectrometer. Solvent was used as an internal standard. Electrospray ionisation mass spectra (MS) were recorded on Agilent Technologies (Milan, Italy) 6120 Quadrupole LC/MS spectrometer. Analytical thin layer chromatography (TLC) was performed on silica gel (Kieselgel 60, F₂₅₄, 0.25 and 0.5 mm, respectively, Merck, Darmstadt, Germany) plates. The spots were visualised by exposure to UV radiation (254 nm), or by spraying first with 10% H₂SO₄ in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min. Analytical and high pressure liquid chromatography (HPLC) grade solvents for chromatographic use were purchased from Carlo Erba (Milan, Italy). Water was of HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). Anapop disposable syringe filters (10–0.2 µm) were purchased from Whatman (Maidstone UK).

2.2. HPLC apparatus

The HPLC system (Shimadzu, Tokyo, Japan) consisted of a Series LC-10Advp pump, FCV-10Alvp valves, SPD-10AVvp spectrophotometric detector and DGU-14A degasser. The HPLC separations were performed using a Macherey-Nagel (Duren, Germany) high-density reversed-phase Nucleosil 100–5 C₁₈ HD column (250 × 4.6 mm i.d.; 5 µm) provided with an in-line guard column from Alltech (Sedriano, Italy).

2.3. Strain selection, isolation and culture

We selected 10 strains to test for cytochalasin B production from a much larger set of isolates so that the selected strains represented a range of mycelial growth rates. Five of the strains were obtained from *B. tectorum* seed bank samples from the Ten Mile Creek population in Box Elder County, UT (TMC; –113.136 long, 41.8649 lat, 1453 m

elevation), two were from the Whiterocks enclosure in northern Skull Valley, Tooele County, UT (WRK; -112.7780 long, 40.3282 lat, 1446 m elevation) and three were from a population a few miles west of the Whiterocks enclosure on the Whiterocks Road (WRR; -112.8891 long, 40.3289 lat, 1567 m elevation). All field collections were made in November 2010.

The anamorph (*Drechslera campanulata*) of the pathogen *P. semeniperda* forms macroscopically visible fruiting structures (stromata) that protrude from the surface of killed seeds in the seed bank. To obtain pure strains from field-collected killed seeds, individual stromata were surface-sterilised, wounded by breaking off the tip and incubated in sterile water. New conidia produced at the wounded tip were then transferred to a small volume of sterile water using a needle, and the conidial suspension was poured over water agar. Excess water was decanted, and the plates were incubated for 8 h at room temperature. Single germinated conidia free of apparent contamination were then transferred using a needle under a dissecting microscope directly to modified alphacel medium plates for conidial production. We used the procedure of Campbell, Medd, and Brown (2003) to stimulate maximum conidial production. Conidia were then harvested, tested for germinability and stored dry at laboratory temperature in snap-cap vials (see Meyer et al., 2010 for detailed protocols). Mycelial growth rate was determined for each strain on quarter strength potato dextrose agar (PDA) at 20°C by placing a single germinated conidium at the centre of each of four plates, incubating for 14 days, and measuring colony diameter along four radial axes in each plate (Meyer et al., 2010).

We produced each of the 10 strains in solid wheat seed culture (Evidente et al., 2002). This cultural condition was selected because it induced cytochalasin B production in the earlier study. For each strain, we added 6.6 mg of conidia suspended in sterile water to 200 g soaked, autoclaved wheat seeds and placed the mixture in a sterile 1 L Erlenmeyer flask with an aluminium foil cap at laboratory temperature (ca. 22°C, considered near the optimum for the fungus; Campbell, Medd, & Brown, 1996). The flask was hand-shaken periodically during the 4-week incubation period to prevent caking together of the grains. The cultures were then spread in pans and air-dried for 6–10 weeks. Cultures were stored in the air dry condition for up to 6 months prior to extraction.

We also produced each of the 10 strains in potato dextrose broth (PDB) culture at laboratory temperature by inoculating 500 mL of broth in 1 L Erlenmeyer flasks with four 3 mm diameter plugs of mycelium taken from the edge of a 5-day-old culture grown on V-8 agar and incubating in shaker culture at laboratory temperature for 14 days. Mycelium was then removed from the medium by centrifugation and filtering, and the resulting filtrate was frozen at -20°C until extraction and analysis. The aqueous remainder solution from each extraction was also frozen for use in seedling bioassays to determine whether hydrophilic toxins remained in solution.

2.4. Extraction and purification of cytochalasin B

In order to obtain cytochalasin B in a pure form to be used as standard material, wheat cultures of *P. semeniperda* strain WRK1022 were dried and finely minced; 200 g of dried material was extracted with a MeOH–H₂O (1% NaCl) mixture (55:45 v/v), and the resulting extract was defatted with *n*-hexane and then re-extracted with

CH₂Cl₂. The organic extracts were dried (Na₂SO₄) and evaporated under reduced pressure yielding a solid mixed with a brown oil (456.2 mg) showing significant phytotoxic activity as measured using the method described below. The mixture was washed with small aliquots (5 × 1 mL) of MeOH. The solid residue was soluble in CHCl₃–MeOH (1:1 v/v) and essentially contained cytochalasin B as shown by TLC analysis carried out in comparison with an authentic sample of the toxin [*R*_f 0.55 and 0.48, using the solvent systems CHCl₃-*iso*-PrOH (9:1 v/v) and EtOAc-*n*-hexane (7:3 v/v), respectively]. The solid was crystallised twice from EtOAc-*n*-hexane (1:5 v/v) giving white needles (292.2 mg).

2.5. Extraction of *P. semeniperda* cultures

Solid wheat seed cultures for all 10 strains of *P. semeniperda* were extracted according to the protocol described earlier for WRK1022. In addition, filtrates from PDB culture for each strain were extracted with EtOAc. The organic extracts were dried (Na₂SO₄) and evaporated under reduced pressure. Extracts were kept frozen at –80°C until bioassay and chemical analysis.

2.6. HPLC analysis

The mobile phase used to elute the samples was MeCN–H₂O (6:4 v/v) at a flow rate of 1 mL/min. Detection was performed at 226 nm, corresponding to the maximum UV absorption of cytochalasin B (Cole & Schweikert, 2003). Samples were injected using a 20-μL loop and monitored for 15 min.

Construction of the HPLC calibration curve (Table 1) for quantitative cytochalasin B determination was performed with absolute amounts of the toxin standard (obtained as described above) dissolved in MeOH in the range between 0.01 and 20 μg/mL, in triplicate for each concentration. An HPLC linear regression curve (absolute amount against chromatographic peak area) for cytochalasin B was obtained based on weighted values calculated from eleven concentrations of the standard in the above range.

For each strain, 1 mg of the organic extract from solid culture was accurately weighed (±0.001 mg) and dissolved in 1 mL of MeOH. These suspensions were filtered using Anaport 10–0.2 μm filters, and aliquots of the samples (20 μL) were injected for the analysis into the HPLC instrument as described earlier. Each sample was assayed in triplicate. The quantitative determination of the metabolite was calculated by interpolating the mean area of the chromatographic peak using the equation from the calibration curve (Table 1).

Recovery studies were performed using a strain with low production of cytochalasin B (TMC1034). Pure cytochalasin B was added to 200 g of wheat

Table 1. Analytical characteristics of the calibration curve^a for cytochalasin B quantification.

<i>R</i> _t (min)	Range (μg)	<i>r</i> ²	Number of data points	Detection limit (μg)
4.44	0.01–20	0.999	33	0.001

^aCalculated in the form $y = a + bx$, where y is the chromatographic peak area and x is the μg of toxin. The calibration curve for this analysis run was: $y = 453.7 + 10387x$. In subsequent use of this method, it is best to generate a new calibration curve each time HPLC analysis is performed, due to possible differences in instrumentation and experimental conditions.

culture in the range of 4–20 mg. The samples were prepared as described earlier and the organic extracts analysed by HPLC to determine recovery. Three replicate injections were performed for each concentration.

2.7. Seedling coleoptile elongation bioassays

As a measure of the phytotoxicity of cytochalasin B and of the culture extracts, we performed seedling coleoptile elongation bioassays with host (*B. tectorum*) seeds. Cytochalasin B standards and organic extracts were first dissolved in MeOH, then a suitable aliquot was diluted quantitatively with deionised water to 1% MeOH so that the resulting 7 mL of solution contained a quantity of cytochalasin B standard that yielded a concentration of 10^{-3} or 10^{-4} M, or contained an equivalent quantity of culture organic extract. The solution for each strain and concentration was pipetted into three 10-cm plastic petri dishes (2.33 mL per petri dish) onto the surface of one Whatman no.1 filter paper. Cytochalasin B standards served as the positive controls for each concentration, while 1% MeOH served as the negative control.

Twelve host seeds were arranged onto the surface of each filter paper in a pattern that made it possible to track individual seeds. Petri dishes were sealed with parafilm to retard moisture loss, stacked in plastic bags and incubated at 20°C with a 12:12 hour photoperiod. Germination was scored each day, and germination day was tracked individually for each seed. Five days after germination, the coleoptile length of each seedling was measured and recorded using electronic calipers. Most seeds germinated within three days. Seeds that did not germinate (<5%) were excluded from analysis, while seeds that produced a radicle but no coleoptile were scored with a coleoptile length of zero. Coleoptile length data were log-transformed to improve homogeneity of variance prior to analysis of variance for a completely randomised design.

3. Results

3.1. Cytochalasin B quantification

A simple, rapid and sensitive HPLC method was successfully applied for the quantitative analysis of cytochalasin B in solid wheat seed culture for 10 *P. semeniperda* strains. The characteristics of the calibration curve, the absolute range and the detection limits of cytochalasin B are summarised in Table 1. In these experiments, we used a standard sample of cytochalasin B obtained by purification of the solid culture of strain WRK1022. Cytochalasin B was identified by comparison of its spectroscopic data (^1H NMR and ESI-MS) with those reported in literature (Capasso et al., 1987). The purity of this sample was ascertained by HPLC analysis.

Preliminary tests using various elution conditions with standard cytochalasin B on reverse phase C_{18} and C_8 columns produced unresolved asymmetric peaks. Satisfactory peak shape was obtained, in respect to those previously reported (Capasso, Evidente, Randazzo, Vurro, & Bottalico, 1991), using a C_{18} column eluted with an isocratic mixture of MeCN– H_2O (6:4 v/v) at a flow rate of 1 mL/min over 15 min (Figure 2a).

These latter conditions were used to quantify the cytochalasin B content in the organic extracts of different *P. semeniperda* strains. A representative HPLC chromatogram of the organic extract of *P. semeniperda* (strain TMC1026) is

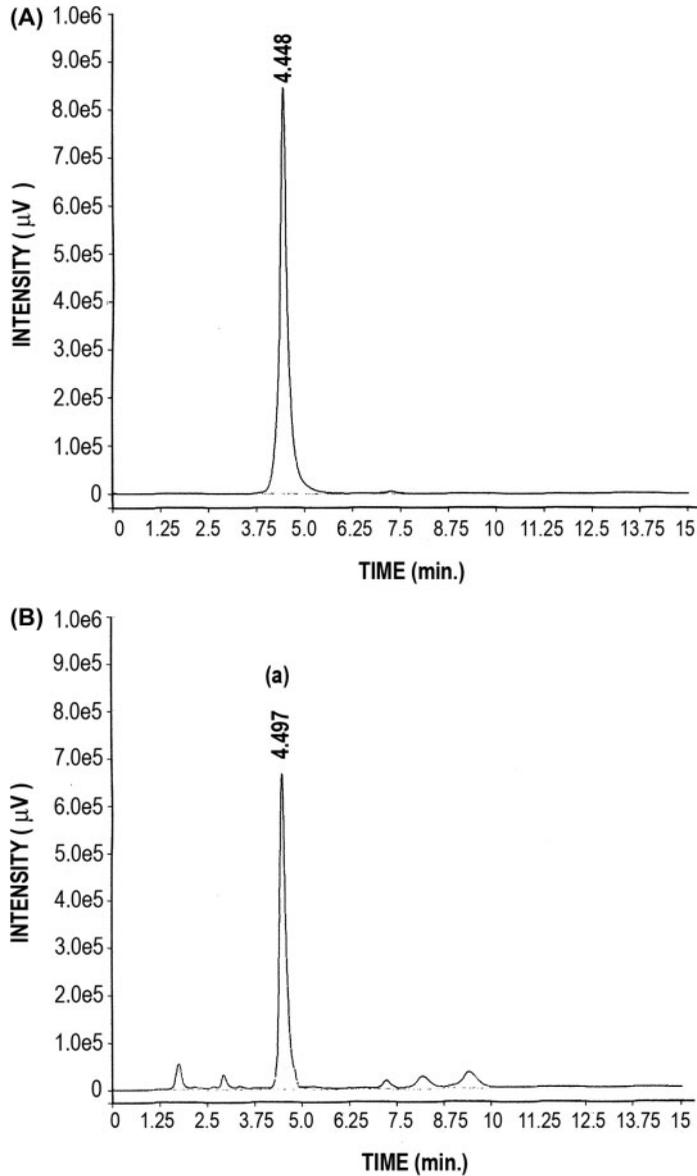


Figure 2. (A) HPLC profile of the cytochalasin B standard using a high-density C-18 stationary phase and isocratic elution with MeCN–H₂O (6:4 v/v) at a flow rate of 1 mL min⁻¹. (B) HPLC profile of strain TMC 1026 organic extract obtained under the same conditions. Cytochalasin B peak in the organic extract is indicated as (a).

presented in Figure 2b. The chromatographic peak (a) in the sample was coincident to the 4.44 min retention time of the cytochalasin B standard (Figure 2a). The retention times were highly reproducible, varying less than 0.50 min. For all strains, substances absorbing at 260 nm were eluted within the first 10 min. No further peaks appeared when samples were eluted with a higher percentage of water in the mixture,

but the retention times increased. Features of the metabolite peak in samples in comparison with those of the purified standard suggest no other substances are present as overlapping peaks. Using the described HPLC conditions, cytochalasin B could be quantitatively and reproducibly detected at concentrations as low as 0.001 μg . Furthermore, the recovery of cytochalasin B added to the wheat seed culture of TMC1034 was $96 \pm 3.8\%$, indicating that both extraction and analytical methods are adequate for the quantitative characterisation of fungal metabolites in culture.

The cytochalasin B content in the organic extracts of the 10 strains tested ranged between 534.8 ± 7.7 and 2256.4 ± 8.7 mg kg^{-1} (expressed as the concentration in the original wheat seed culture; Table 2). The quantity of organic extract obtained from 200 g of wheat seed culture ranged from 222 to 711 mg, while the concentration of cytochalasin B in these organic extracts varied from 39.4% to 70.2% (Table 2, Figure 3a). The toxin content in the organic extract of the standard strain (WRK1022) as determined by HPLC (298.2 ± 1.5 mg) was somewhat higher than that obtained by chemical purification (292.2 mg) of the same strain grown in identical conditions. It can be expected that some loss of the target metabolite would occur during the complex purification process.

For the PDB filtrate organic extracts from the 10 strains, the presence or absence of cytochalasin B was first determined using TLC. None of the filtrate organic extracts contained cytochalasin B, making further quantification unnecessary.

3.2. Seedling bioassays

When the cytochalasin B standard was tested in a seedling bioassay at 10^{-4} M, it was only mildly toxic, reducing coleoptile elongation to 87% of the 1% MeOH control (Figure 3b). At concentrations equivalent to 10^{-4} M cytochalasin B, organic extracts from the 10 strains of *P. semeniperda* were also only mildly toxic and were not significantly different from each other, from the cytochalasin B standard, or from the 1% MeOH control. At an equivalent concentration of 10^{-3} M, much higher toxicity

Table 2. Mycelial growth rate on quarter strength PDA at 20°C and cytochalasin B production in solid wheat seed culture for 10 *Pyrenophora semeniperda* strains.

Strain	Mycelial growth rate (mm day ⁻¹)	Organic extract ^a (mg)	Cytochalasin B detected (mg) ^a	% of cytochalasin B in the organic extract	Production of cytochalasin B (mg kg ⁻¹)
TMC1014	4.9	221.5	107.0 ± 1.5^b	48.3	534.8 ± 7.7
TMC1022	4.9	364.0	169.9 ± 1.3	46.7	849.5 ± 6.6
TMC1026	2.9	710.9	451.3 ± 1.7	63.5	2256.4 ± 8.7
TMC1030	3.4	538.4	381.4 ± 0.9	70.8	1907.2 ± 4.3
TMC1034	2.3	339.9	168.2 ± 0.6	49.5	841.2 ± 3.0
WRK1004	4.2	642.2	342.2 ± 1.3	53.3	1711.0 ± 6.5
WRK1022	5.1	456.2	298.2 ± 1.5	65.4	1491.2 ± 7.5
WRR1016	5.1	445.4	261.9 ± 0.5	58.8	1309.7 ± 2.3
WRR1023	3.6	674.0	265.7 ± 2.1	39.4	1328.4 ± 10.3
WRR1029	3.7	561.5	347.2 ± 2.8	61.8	1735.8 ± 14.2

^aAmount from 200 g of wheat culture.

^bData are mean \pm SE ($n = 3$).

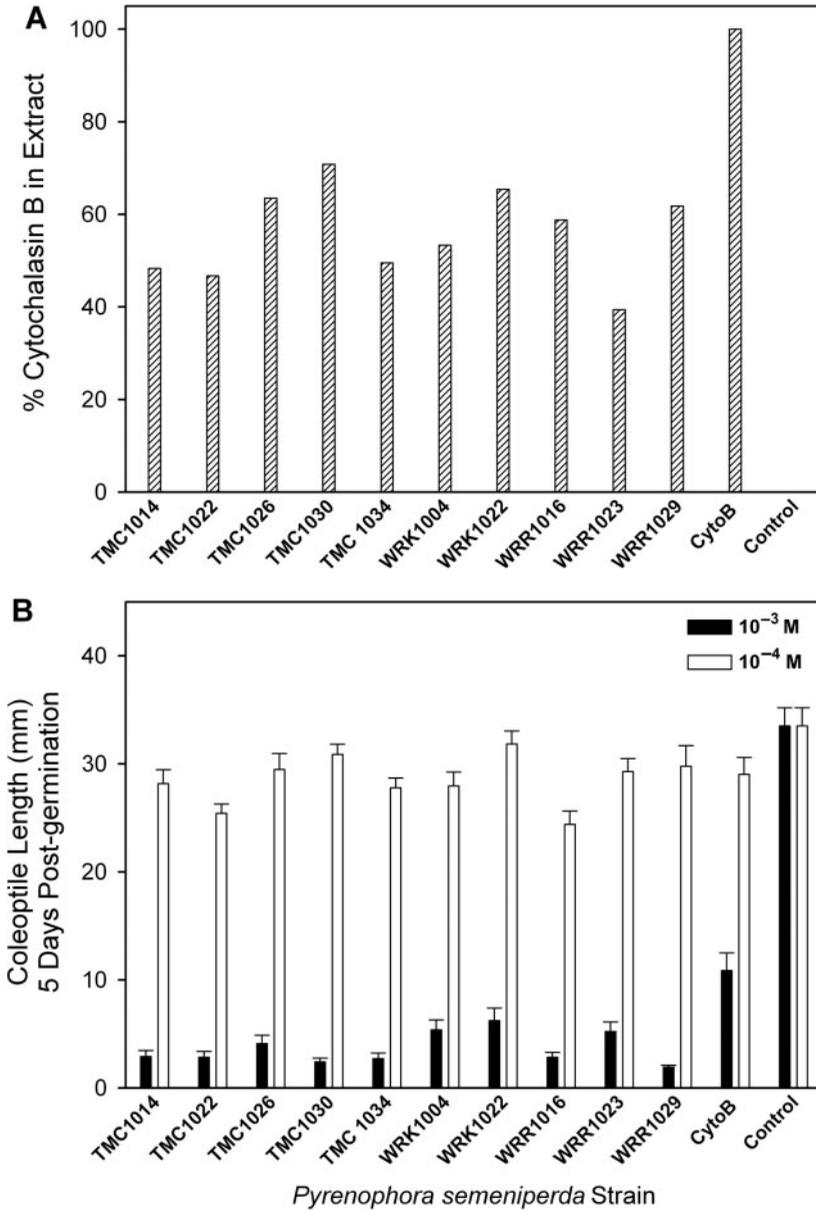


Figure 3. (A) Percentage of cytochalasin B in the organic extracts of 10 *P. semeniperda* strains produced in solid wheat seed culture. (B) Mean 5-day *B. tectorum* coleoptile lengths in bioassays with organic extracts of 10 *P. semeniperda* strains at concentrations equivalent to 10^{-3} and 10^{-4} M cytochalasin B, with mean coleoptile lengths for the 1% MeOH control and the cytochalasin B standards for comparison. Error bars for mean coleoptile length are standard errors of the mean ($n = 36$).

was observed (Figure 3b). The cytochalasin B standard reduced coleoptile elongation to 34% of the control, a significant reduction. Extracts from the 10 pathogen strains showed even more pronounced toxicity, reducing coleoptile elongation to 8–18% of the control. All 10 extracts reduced coleoptile elongation significantly more than the cytochalasin B standard, suggesting the presence of additional phytotoxic compounds. Coleoptile elongation reduction did not differ significantly among strains. There was no significant correlation between cytochalasin B concentrations in the organic extracts (Figure 3a) and their phytotoxicity in the coleoptile elongation bioassay at either 10^{-3} or 10^{-4} M (Figure 3b).

Seedling bioassays of organic extracts of the filtrates produced in PDB culture also indicated high toxicity, particularly for some strains, with coleoptile elongation reduction values varying from 3% to 82% of the 1% MeOH control (data not shown). As these extracts did not contain cytochalasin B, their toxicity must have been due to the presence of other bioactive metabolites. The aqueous remainder solutions from the filtrate extractions did not exhibit any phytotoxic activity, confirming that the bioactive compounds present were lipophilic rather than hydrophilic in nature.

3.3. Cytochalasin B production and mycelial growth rate

There was a weak negative relationship between cytochalasin B production in solid wheat seed culture and mycelial growth rate on quarter strength PDA, but this correlation was not significant, largely because of the outlier TMC1034, which had both a slow growth rate and low cytochalasin B production. When this outlier was removed, the negative correlation between mycelial growth rate and cytochalasin B production became significant ($r = 0.745$, d.f. = 7, $P < 0.05$). We found a similar outlier, with both slow growth rate and low virulence, in our earlier study (Meyer et al., 2010).

4. Discussion

The method presented here for quantification of cytochalasin B in complex mixtures including organic extracts of fungal cultures shows considerable promise as a rapid screening protocol for virulence in *P. semeniperda*. A principal advantage to the method is that it is not necessary to purify the compounds of interest in order to quantify them. Methodologies like this could be developed for other secondary metabolites and, therefore, have application to the study of many fungal pathosystems. This approach could be applied both as a practical screening tool and as a research tool for understanding the role of various fungal secondary metabolites in pathogenesis.

There was no direct relationship between cytochalasin B concentration in organic extracts and phytotoxicity in the seedling coleoptile elongation bioassay. Toxicity of cytochalasin B was surprisingly low compared to results reported earlier in which a 10^{-4} M solution decreased root length of wheat seedlings to 62% and tomato seedlings to 28% of the control (Evidente et al., 2002). Even at 10^{-3} M cytochalasin B had virtually no effect on *B. tectorum* seed germination in our experiment (i.e., seeds germinated normally), although it clearly affected subsequent seedling growth. This could be because the host seeds have coverings that effectively exclude this toxin or because they have higher inherent physiological tolerance or a stronger defence

response than species used in earlier bioassays, possibly because of a long shared evolutionary history with the pathogen. It is almost certain that *in vivo* delivery of this toxin by the pathogen inside the seed would be more effective than exogenous application.

We qualitatively examined the solid culture extracts using TLC for the presence of other phytotoxic compounds that could explain the increased toxicity of these extracts relative to equivalent concentrations of cytochalasin B. All 10 strains produced cytochalasin F, cytochalasin Z3 and deoxaphomin, while WRK1022 also produced cytochalasin A. Several of the strains also produced a more polar unknown metabolite. All these compounds appeared to be produced in small quantities relative to the production of cytochalasin B, but it is possible that they interacted synergistically to increase the toxicity of the extracts.

Preliminary TLC investigations of the culture filtrate organic extracts showed the presence of different metabolites. Much more work will be needed to purify, chemically and biologically characterise and establish the role of these metabolites in *P. semeniperda* pathogenesis.

We did not relate cytochalasin B production directly to virulence on non-dormant seeds in this study. However, we were able to demonstrate a significant negative correlation between cytochalasin B production and mycelial growth rate, which is known from our earlier study to itself be negatively correlated with virulence on non-dormant seeds (Meyer et al., 2010). More studies will be needed to establish the degree to which these traits are correlated in a larger group of strains and to determine the usefulness of chemical assay as a screening tool. The rapid method for determining cytochalasin B production in culture that we have developed will be a key component of these future studies.

We confirmed that cytochalasin B is not produced by this fungus under all cultural conditions. In the earlier study, there was no production of cytochalasin B in liquid Fries medium (Evidente et al., 2002). Similarly, we found in the current study that none of the strains produced cytochalasin B in PDB culture. This raises the interesting question of how the cytochalasin B biosynthetic pathway is induced in culture. Now that we have a rapid method for quantifying cytochalasin B production, this question has become more experimentally tractable. In addition, by successfully inducing cytochalasin B in liquid culture, we would be able to avoid the possible confounding of mycelial growth rate and phytotoxin production per unit of mycelium that is a potential source of error in the present study. It is not possible to measure mycelial growth rate directly in wheat seed culture, but in liquid culture, both phytotoxin production and mycelial growth rate could be measured directly on the same experimental units. And finally, in liquid culture, it may be possible to perform gene expression studies to determine whether the biosynthetic pathway for cytochalasin production in our organism is similar to that elucidated for the apple pathogen *Penicillium expansum* (Schümann & Hertweck, 2007).

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