

Pyrenophoric Acid, a Phytotoxic Sesquiterpenoid Penta-2,4-dienoic Acid Produced by a Potential Mycoherbicide, *Pyrenophora semeniperda*

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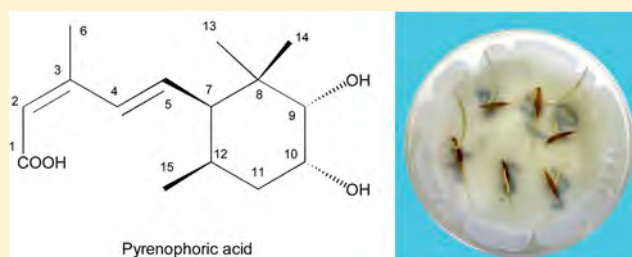
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S Supporting Information

ABSTRACT: A new phytotoxic sesquiterpenoid penta-2,4-dienoic acid, named pyrenophoric acid, was isolated from solid wheat seed culture of *Pyrenophora semeniperda*, a fungal pathogen proposed as a mycoherbicide for biocontrol of cheatgrass (*Bromus tectorum*) and other annual bromes. These bromes are serious weeds in winter cereals and also on temperate semiarid rangelands. Pyrenophoric acid was characterized as (2*Z*,4*E*)-5-[(7*S*,9*S*,10*R*,12*R*)-3,4-dihydroxy-2,2,6-trimethylcyclohexyl]-3-methylpenta-2,4-dienoic acid by spectroscopic and chemical methods. The relative stereochemistry of pyrenophoric acid was assigned using ¹H,¹H couplings and NOESY experiments, while its absolute configuration was determined by applying the advanced Mosher's method. Pyrenophoric acid is structurally quite closely related to the plant growth regulator abscisic acid. When bioassayed in a cheatgrass coleoptile elongation test at 10⁻³ M, pyrenophoric acid showed strong phytotoxicity, reducing coleoptile elongation by 51% relative to the control. In a mixture at 10⁻⁴ M, its negative effect on coleoptile elongation was additive with that of cytochalasin B, another phytotoxic compound found in the wheat seed culture extract of this fungus, demonstrating that the extract toxicity observed in earlier studies was due to the combined action of multiple phytotoxic compounds.



Pyrenophora semeniperda (Brittlebank and Adams) Shoemaker is a naturally occurring ascomycete seed pathogen that has been proposed as a potential biocontrol agent against cheatgrass (*Bromus tectorum* L.) and other annual bromes.¹ Cheatgrass, also known as downy brome, is an exotic winter annual grass weed that causes serious losses in intensive agriculture, particularly in winter cereal crops.² *B. tectorum* has also invaded millions of hectares of semiarid rangeland in western North America and is a major contributing cause of wildfires, which have immense economic and environmental costs.³

The research reported here is the latest in a series of studies carried out to determine whether *P. semeniperda* can produce novel bioactive metabolites with potential herbicidal activity. Most recently, a novel spirocyclic γ -lactam, named spirostaphylotrichin W, was isolated together with the well-known and closely related spirostaphylotrichins A, C, D, R, and V, as well as triticone E, from potato dextrose broth cultures of *P. semeniperda*. The structure and biological characterization of the new spirostaphylotrichin W has been described.⁴

P. semeniperda is also able to produce phytotoxins belonging to a different class of natural compounds when grown in solid culture on wheat seeds. Studies of an Australian strain revealed

that the fungus produced a large amount of the phytotoxic cytochalasin B, as well as cytochalasin A, cytochalasin F, deoxaphomin, and the three novel cytochalasins, Z1, Z2, and Z3.⁵ The production of these same compounds was confirmed working with solid cultures of 10 strains collected from three Utah (USA) populations, and a rapid and sensitive HPLC method for quantification of cytochalasin B in the organic extracts was developed.⁶ In a *B. tectorum* coleoptile bioassay carried out as part of this study, solid culture extracts at a concentration equivalent to 10⁻³ M cytochalasin B exhibited significantly higher toxicity (8–18% of control) than the cytochalasin B standard (34% of control). This suggested the possible presence of other phytotoxic metabolites, later confirmed by preliminary TLC investigations of organic extracts of the most active strains.

This paper reports on the chemical and biological characterization of a new sesquiterpenoid 2,4-dienoic acid with potential herbicidal activity produced by *P. semeniperda* in solid wheat seed culture. The new compound is named pyrenophoric acid

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because of its structural similarity to the well-known plant growth regulator abscisic acid.

RESULTS AND DISCUSSION

The *P. semeniperda* strain WRR10-16, one of the most active strains in the *B. tectorum* coleoptile bioassay,⁶ was again cultured on wheat, and the culture was extracted as described in the Experimental Section. A large quantity of cytochalasin B was first crystallized from the organic extract, after which the remaining mother liquors were fractionated by column chromatography. Cytochalasin F, cytochalasin Z3, and deoxaphomin as well as an additional quantity of cytochalasin B were detected in four relatively nonpolar fractions. The fifth fraction of the initial chromatography was further purified on TLC, yielding another more polar metabolite, which showed a molecular formula of C₁₅H₂₄O₄ as deduced from its HRESIMS, consistent with four hydrogen deficiencies. These results, combined with investigations of its ¹H and ¹³C NMR spectra (Table 1), showed that it was different from any cytochalasin or

Table 1. ¹H and ¹³C NMR Data for Pyrenophoric Acid (1)^{a,b}

position	δ_c^c	δ_H (J in Hz)	HMBC
1	169.1 C		H-2
2	115.0 CH	5.67 (1H) s	H-4, Me-6
3	153.0 C		H-2, H-4, H-5, Me-6
4	131.1 CH	7.53 (1H) d (15.9)	H-2, H-5, H-7, Me-6
5	136.7 CH	6.10 (1H) dd (15.9, 10.9)	H-7
6	21.5 CH ₃	2.03 (3H) d (0.93) ^d	H-2, H-4
7	55.7 CH	2.19 (1H) dd (10.9, 3.8)	H-4, H-5, Me-13, Me-14, Me-15
8	38.7 C		H-5, H-7, H-9, Me-13, Me-14
9	75.1 CH	3.48 (1H) d (3.7)	H-7, Me-13, Me-14
10	70.1 CH	4.09 (1H) q (3.7)	OH
11	38.7 CH ₂	1.78 (1H) dt (14.6, 3.7) 1.51 (1H) ddd (14.6, 12.1, 3.7)	H-7, Me-15
12	25.8 CH	2.38 (1H) m	H-7, Me-15
13	23.7 CH ₃	1.16 (3H) s	H-9, Me-14
14	27.8 CH ₃	0.92 (3H) s	H-9, Me-13
15	19.6 CH ₃	0.83 (3H) d (7.0)	
OH		3.65 bs	

^aThe chemical shifts are in δ values (ppm) from TMS. ^b2D ¹H,¹H (COSY) and ¹³C,¹H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons. ^cMultiplicities were assigned by DEPT spectrum. ^dThis is allylic coupled with H-2.

spirostaphylotrichin, but that it was a sesquiterpenoid, and, being new as described below, was named pyrenophoric acid (1, Figure 1).

The ¹H and ¹³C NMR spectra for the new compound showed the presence of the signal typical of an acid carbonyl group at δ 169.1/C (C-1) and those of *Z*-trisubstituted and *E*-disubstituted double bonds at δ 5.67/115.0 CH and 153.0 C (HC-2 and C-3), and 7.53 d/131.1 CH and 6.10 dd/136.7 CH (HC-4 and HC-5).^{7,8} These results are in agreement with the typical bands for hydroxy, acid carbonyl, and olefinic groups observed in the IR spectrum⁹ and the absorption maxima at 257 nm exhibited in the UV spectrum, typical of an extended conjugated α,β -unsaturated acid carbonyl group.¹⁰ The

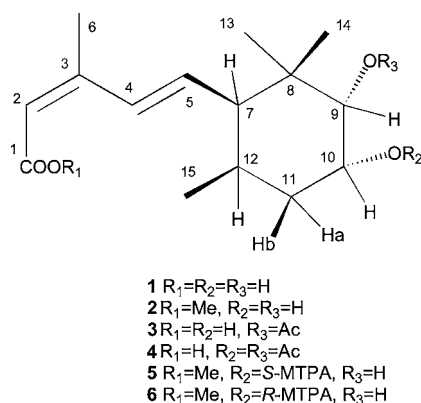


Figure 1. Structures of pyrenophoric acid (1), its acetylated derivatives (3 and 4), and the 10-*O*-S-MTPA and 10-*O*-R-MTPA esters (5 and 6) of its methyl ester (2).

trisubstituted and the disubstituted olefinic groups were determined to be bonded, respectively, to the carboxylic group and to a secondary carbon observed at δ 55.7 (HC-7) by the long-range couplings observed in the HMBC spectrum¹¹ (Table 1) between C-1 and H-2, of both C-4 and C-5 to H-7, and of C-7 with H-4 and H-5. The fragment C-1–C-4 appeared to be the tail of the sesquiterpenoid moiety, while the other two isoprene residues were rearranged into a 1,2,2,3,4,6-hexasubstituted cyclohexane ring, which represents the remaining unsaturation, with the bond between C-5 and C-7 representing the junction between the ring and the 3-methylpenta-2,4-dienoic acid side chain.

In the COSY spectrum,¹¹ the singlet of the olefinic proton H-2 at δ 5.67 coupled with a typical allylic constant ($J = 0.93$ Hz)^{7,12} with the methyl Me-6 appearing as a doublet at δ 2.03. The olefinic proton H-5, appearing as a double doublet ($J = 15.9$ and 10.9 Hz) at δ 6.10, was *trans*-coupled with the other olefinic proton H-4, with a doublet ($J = 15.9$ Hz) at δ 7.53, and also with the proton (H-7) of a secondary carbon (C-7) resonating as a double doublet ($J = 10.9$ and 3.87 Hz) at δ 2.19. The latter was also coupled with the proton (H-12) of another secondary carbon (C-12), which appeared as a complex multiplet at δ 2.38. H-12 coupled both with the adjacent methyl group (Me-15), resonating as a doublet ($J = 7.0$ Hz) at δ 0.83, and with the protons of the adjacent methylene group (H₂C-11) resonating as a double triplet ($J = 14.6, 3.7$ Hz) and a doublet of double doublets ($J = 14.6, 12.1, 3.7$ Hz) at δ 1.78 and 1.51, respectively. The latter in turn coupled with the proton (H-10) of a secondary hydroxylated carbon resonating as a quartet ($J = 3.7$ Hz) at δ 4.09, being also coupled with the proton (H-9) of the adjacent secondary hydroxylated carbon (C-9). As expected, H-9 resonated as a doublet ($J = 3.7$ Hz) at δ 3.48, being bonded to the quaternary carbon C-8 bearing two methyl groups (Me-13 and Me-14), which resonated as two singlets at δ 1.16 and 0.92, respectively. The latter results were confirmed by the long-range couplings observed in the HMBC spectrum of C-8 with H-9 and Me-13 and of Me-14 and C-9 with the same two methyl groups. The long-range couplings observed in the same spectrum from C-8, C-9, and C-12 to H-7 and from C-7 to Me-13, Me-14, and Me-15 also demonstrated that C-7 is the closing point of the cyclohexane ring, which, as above-reported, bears the penta-2,4-dienoic acid side chain.^{7,8}

Inspection of the HSQC spectrum¹¹ allowed the assignment of the chemical shifts to the remaining protonated carbons at δ 75.1, 38.7, 27.8, 25.8, 23.7, and 19.6 to C-9, C-8, C-14, C-12, C-

Table 2. ¹H NMR Data for Pyrenophoric Acid Derivatives (2–6)^a

	2	3	4	5	6
position	δ_{H} (J in Hz)	δ_{H} (J in Hz)	δ_{H} (J in Hz)	δ_{H} (J in Hz)	δ_{H} (J in Hz)
2	5.64 s	5.67 s	5.69 s	5.716 s	5.723 s
4	7.55 d (15.3)	7.54 d (15.5)	7.56 d (15.5)	6.069 d (14.9)	6.087 d (15.0)
5	6.05 dd (15.3, 10.9)	6.14 dd (15.5, 10.8)	6.12 dd (15.5, 10.9)	5.963 dd (14.9, 10.7)	5.971 dd (15.0, 10.7)
6	1.99 s	2.05 s	2.06 s	2.263 s	2.267 s
7	2.18 brd (10.9)	2.18 brd (10.8)	2.24 dd (10.9, 3.9)	1.984 brd (10.7)	2.031 dd (10.7, 3.0)
9	3.66 d (5.8)	4.83 brs	4.81 d (3.4)	3.638 d (3.5)	3.690 d (3.2)
10	4.09 brs	4.14 brs	5.36 q (3.4)	5.348 q (3.5)	5.419 q (3.2)
11	1.77 brd (14.6)	1.76 brd (14.2)	1.70 brd (14.4)	1.903 m	1.844 dt (15.3, 3.2)
	1.42 brdd (14.6, 6.6)	1.53 brdd (14.2, 13.3)	1.57 m	1.740 m	1.619 m
12	2.37 m	2.46 m	2.37 m	2.221 m	2.146 m
13	1.15 s	1.24 s	1.19 s	0.862 s	0.832 s
14	0.92 s	0.80 s	0.83 s	0.862 s	0.760 s
15	0.83 d (7.0)	0.83 d (6.7)	0.84 d (7.7)	0.691 d (6.4)	0.788 d (6.9)
OMe	3.70 s			3.705 s	3.710 s
MeCO		2.15 s	2.07 s		
MeCO			2.04 s		
OMe				3.566 s	3.586 s
Ph				7.561–7.409	7.550–7.432

^aThe chemical shifts are in δ values (ppm) from TMS.

13, and C-15, accounting for the chemical shifts to all the protons and corresponding carbons of pyrenophoric acid as reported in Table 1. On the basis of these results pyrenophoric acid was formulated as 5-(3,4-dihydroxy-2,2,6-trimethylcyclohexyl)]-3-methylpenta-2,4-dienoic acid (**1**, Figure 1).

This structure was supported by several other long-range couplings observed in the HMBC spectrum (Table 1) and by the HRESIMS data. Indeed, this latter spectrum recorded in positive mode showed the ammonium cluster $[M + \text{NH}_4]^+$ at m/z 286.2014, while when the same spectrum was recorded in negative mode, the pseudomolecular ion $[M - \text{H}]^-$ was recorded at m/z 267.1446.

The structure assigned to **1** was confirmed by preparing the corresponding methyl ester (**2**, Figure 1) by reaction with an ethereal solution of diazomethane. The IR spectrum of **2** showed the lack of bands due to a hydroxy carboxylic group and the presence of the bands of the ester groups.⁹ Its ¹H NMR (Table 2) spectrum differed from that of **1** by the presence of the singlet of the methyl ester group observed at δ 3.70. The ESIMS spectrum of **2** showed the sodium cluster $[M + \text{Na}]^+$ at m/z 305. Pyrenophoric acid was also converted into the 9-*O*-acetyl and 9,10-*O,O'*-diacetyl derivatives (**3** and **4**, Figure 1) by routine acetylation with pyridine and acetic anhydride. The IR spectrum of **3** and **4** showed the presence of the band typical of the acetyl groups. Their ¹H NMR spectrum (Table 2) differed from that of **1** only by the downfield shift ($\Delta\delta$ 0.35) of H-9 in **3** resonating as a broad singlet at δ 4.83 and by the downfield shift ($\Delta\delta$ 0.33 and 1.27) of H-9 and H-10 in **4**, appearing as a doublet ($J = 3.4$ Hz) and quartet ($J = 3.4$ Hz) at δ 4.81 and 5.36. Furthermore, the presence of the singlets of the acetyl groups was observed at δ 2.15 in **3** and 2.07 and 2.04 in **4**, respectively. Their HRESIMS spectra recorded in positive mode showed ammonium clusters $[M + \text{NH}_4]^+$ at m/z 328.2152 and m/z 370.2265, respectively.

The relative configuration of **1** was deduced from investigation of the coupling constants and the correlations observed in its ¹H NMR and NOESY¹¹ spectra. In fact the coupling observed between H-11b and H-12 (Table 1) allowed the axial localization of these two protons, and the coupling

observed between H-10 with H₂-11 allowed the equatorial localization of both H-11a and H-10. Finally, the coupling between H-12 and H-7 allowed the equatorial localization of the latter proton. These results were confirmed by the correlations observed in the NOESY spectrum (Table 3)

Table 3. NOESY Data for Pyrenophoric Acid (**1**)

irradiated	observed	irradiated	observed
H-2	Me-6	H-11b	H-5, H-9, H-10, H-11a, Me-15
H-4	H-7	H-12	H-7, H-11a, Me-13, Me-15
H-5	H-7, H-9, H-11b, Me-6, Me-14, Me-15	Me-6	H-2, H-5
H-7	H-4, H-5, H-12, Me-13	Me-13	H-12
H-9	H-5, H-10, H-11b, Me-14	Me-14	H-9
H-10	H-9, H-11b	Me-15	H-11b, H-12
H-11a	H-11b, H-12		

between H-11b and Me-15; H-10 and H-11b; H-11a and H-12; and H-12 and H-7. In addition the correlation of H-9 with H-10 allowed the axial localization of H-9. Furthermore, the double bond located between C-4 and C-5 exhibited an *E*-stereochemistry as deduced from the typical *trans*-coupling constant of 15.6 Hz^{7,12} measured in the ¹H NMR spectrum (Table 1). A *Z*-stereochemistry was assigned to the other double bond between C-2 and C-3 of the side chain on the basis of the correlation observed in the NOESY spectrum (Table 3) between H-2 and Me-6. The relative stereochemistry therefore was assigned to **1** as depicted in Figure 1. This stereochemistry was also in agreement with an inspection of its Drieding model. On the basis of these results, pyrenophoric acid was formulated as (2*Z*,4*E*)-5-[(7*S**,9*S**,10*R**,12*R**)]-3,4-dihydroxy-2,2,6-trimethylcyclohexyl)]-3-methylpenta-2,4-dienoic acid.

The absolute configuration of **1** was determined by applying an advanced Mosher's method.¹³ Pyrenophoric acid was treated with *R*-(-)- α -methoxy- α -trifluoromethylphenylacetyl (MTPA) and *S*-(+)-MTPA chlorides, to convert **1** into the correspond-

ing diastereomeric esters. Surprisingly, **1** was converted into the corresponding diastereomeric *S*-MTPA and *R*-MTPA monoesters at C-10 (**5** and **6**, respectively), but the esterification of the carboxylic group also occurred, as confirmed by the presence in the ^1H NMR spectra of both **5** and **6** of the typical singlet of the methyl ester group at δ 3.705 and 3.710, respectively, which is essentially the same chemical shift observed in **2**. The esterification of the carboxylic group was probably due to the acidic nature of **1** and to the use of methanol in the reaction procedure. The spectroscopic data for the *S*-MTPA and *R*-MTPA esters (**5** and **6**, respectively) were consistent with the structure assigned to **1**. Subtracting the chemical shift of the protons (Table 2) of the 10-*O*-*R*-MTPA (**6**) from that of 10-*O*-*S*-MTPA (**5**) esters, the $\Delta\delta$ (**5/6**) values for all of the protons were determined as reported in Figure 2.

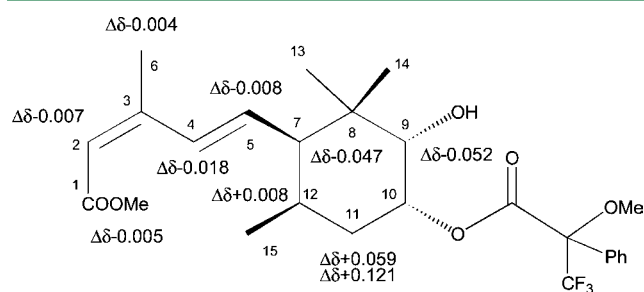


Figure 2. Structures of 10-*O*-*S*- and 10-*O*-*R*-MTPA esters of pyrenophoric acid (**5** and **6**, respectively), reporting the $\Delta\delta$ value obtained by comparison (**5/6**) of each proton system.

The positive $\Delta\delta$ values were located on the right side, and those with negative values on the left side of model A as reported in Othani et al. (1989).¹³ This model allowed the assignment of the *R*-configuration at C-10. Consequently, the *S*-configuration was assigned to C-7 and C-9, and the *R*-configuration was assigned to C-12. **1** was therefore formulated as (2*Z*,4*E*)-5-[(7*S*,9*S*,10*R*,12*R*)-3,4-dihydroxy-2,2,6-(trimethylcyclohexyl)]-3-methylpenta-2,4-dienoic acid.

Pyrenophoric acid is closely related to the well-known phytohormone abscisic acid. Both belong to the sesquiterpenoid class of natural compounds, which includes different bioactive metabolites produced by plants, fungi, and micro-

organisms,^{14–16} some with significant phytotoxic activity.¹⁷ In addition to many other functions in plants, abscisic acid regulates important physiological and developmental processes associated with seeds, including the induction of desiccation tolerance, storage product deposition, and dormancy.¹⁸ This suggests that **1** could perhaps function to impose dormancy as part of the pathogenesis on host seeds.

Abscisic acid (ABA) has also been detected in many species of ascomycetes and basidiomycetes.^{19,20} The biosynthesis and the metabolism of abscisic acid and related compounds have been extensively studied during the last 10 years.^{18,21–23} Several abscisic acid derivatives have also been synthesized for SAR study²⁴ and with the aim of enhancing synchrony of germination and emergence in plants.²⁵ Compounds related to ABA have also been isolated from both plants and fungi. Among these, three compounds, namely, 5-(3*S**,8*S**-dihydroxy-1*R**,5*S**-dimethyl-7-oxa-6-oxobicyclo[3.2.1]oct-8-yl)-, 5-(3*R**,4*R**,8*S**-trihydroxy-1*R**,5*S**-dimethyl-7-oxa-6-oxobicyclo[3.2.1]oct-8-yl)-, and 5-(3*R**,4*R**,8*S**-trihydroxy-1*R**,5*S**-dimethyl-7-oxabicyclo[3.2.1]oct-8-yl)-3-methyl-2*Z*,4*E*-pentadienoic acid isolated from *Prunus domestica* L., are very closely related to pyrenophoric acid.²⁶ Furthermore, the functional groups joined with the cyclohexane ring of pyrenophoric acid indicate a structural relationship with megastigmane derivatives isolated from *Gynostemma pentaphyllum* Makino and *Ginkgo biloba* L.^{27,28}

Coleoptile elongation bioassays using cheatgrass host seeds were carried out to determine (a) whether pyrenophoric acid exhibits phytotoxic activity, (b) whether pyrenophoric acid in combination with cytochalasin B exhibits either additive or synergistic toxicity effects, and (c) how the activity of pyrenophoric acid compares with the activity of abscisic acid. The effect of pyrenophoric acid at 10^{-3} and 10^{-4} M was compared with abscisic acid at similar concentrations, with cytochalasin B at 10^{-4} M, and with a mixture of pyrenophoric acid and cytochalasin B each at 10^{-4} M (Figure 3). Abscisic acid resulted in by far the most dramatic effect regardless of concentration; it completely inhibited postgermination coleoptile and radicle elongation, though germination (indicated by radicle protrusion) did occur, albeit belatedly (data not shown). Pyrenophoric acid also showed clear toxicity, reducing coleoptile length relative to the control by approximately half

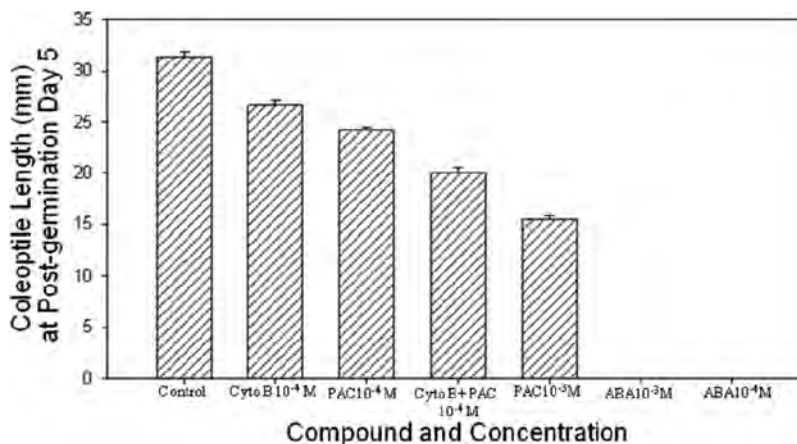


Figure 3. Results of a cheatgrass coleoptile elongation bioassay for pyrenophoric acid (PAC) at 10^{-3} and 10^{-4} M, for cytochalasin B (CytoB) at 10^{-4} M, for a mixture of pyrenophoric acid and cytochalasin B each at 10^{-4} M, and for ABA at 10^{-3} and 10^{-4} M. Mixed model ANOVA (excluding the ABA treatments, which resulted in complete suppression of coleoptile elongation) indicated that all treatments were significantly different from the control and from each other at $p < 0.001$. Error bars represent standard errors of the mean ($n = 18$ seeds).

at 10^{-3} M and by 23% at 10^{-4} M. Cytochalasin B at 10^{-4} M reduced coleoptile elongation 15%, and the combination of pyrenophoric acid and cytochalasin B at 10^{-4} M reduced coleoptile elongation 36% relative to the control. This indicates that these two compounds exhibit an additive rather than a synergistic toxicity effect, as the combined effect was essentially the sum of the effects of the two compounds applied singly. The phytotoxicity of pyrenophoric acid helps explain why organic extract phytotoxicity was not correlated with cytochalasin B concentration in the earlier study.⁶

In order to fully understand how the complex mixture of phytotoxins in the solid culture extracts of this fungus acts on seeds, quantification of pyrenophoric acid, of other cytochalasins, and of any other possible metabolites still not characterized would be necessary, in addition to the quantification of cytochalasin B already accomplished. Additional bioassays using mixtures of pyrenophoric acid and different cytochalasins will also be a powerful tool to aid in the interpretation of the results. It is clear that the action of pyrenophoric acid on seeds is quite different from that of ABA, in spite of their apparent structural similarity. Pyrenophoric acid did not slow germination, and its impact on seedling growth was much less than that of ABA. The hypothesis that it might function to halt or slow germination, a valuable adaptation in a seed pathogen, was therefore not strongly supported, and its role in pathogenesis remains unknown.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured on a Jasco P-1010 digital polarimeter; IR spectra were recorded as glassy film on a Perkin-Elmer Spectrum One FT-IR and on a Thermo Nicolet Avatar 370 FT-IR spectrometer; UV spectra were recorded in MeOH solution on Perkin-Elmer Lambda 25 UV/vis and Hewlett-Packard 8453 spectrophotometers. ¹H and ¹³C NMR spectra were recorded in CDCl₃ at 500 MHz on a Varian or at 400/100 MHz on a Bruker spectrometer. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra;¹¹ DEPT, COSY-45, HSQC, HMBC, and NOESY experiments¹¹ were performed using Bruker microprograms. HRESI and ESI mass spectra were recorded on Agilent Technologies 1100 LC/MSD TOF and on Agilent Technologies 6120 Quadrupole LC/MS instruments, respectively. Analytical and preparative TLC were performed on silica gel plates (Merck, Kieselgel 60 F₂₅₄, 0.25); compounds were visualized by exposure to UV light and/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. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063–0.200 mm).

Fungal Strain. The *P. semeniperda* strain WRR10-16 used in this study, ITS Haplotype A (GenBank accession number GQ168725), was obtained from a *B. tectorum* seed bank sample collected a few miles west of the Whiterocks enclosure on Whiterocks Road (–112.8891 long., 40.3289 lat., 1567 m elevation) in west-central Utah, USA, in November 2010. The anamorph (*Drechlera 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-sterilized, 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 MAM (modified alphacel medium) plates for conidial production. Conidia were then harvested,

tested for germinability, and stored dry at laboratory temperature in snap-cap vials.⁶

Production, Extraction, and Purification of Pyrenophoric Acid (1). For solid culture on wheat seeds, 6.6 mg of WRR10-16 conidia suspended in sterile H₂O was added to 200 g of soaked, autoclaved wheat seeds, and the mixture was placed in a sterile 1 L Erlenmeyer flask with an aluminum foil cap at 22 °C. The flask was hand-shaken periodically during the 4-week incubation period to prevent caking together of the grains. The culture was then spread in pans and air-dried for at least several weeks prior to extraction. The dried material (200 g) was then minced using a laboratory mill and extracted with 500 mL of MeOH–H₂O (1% NaCl) (1:1). The mixture was centrifuged for 1 h at 10 000 rpm. The pellet was extracted again with the same solvent mixture in the same conditions, and the two supernatants were then pooled and defatted by *n*-hexane (2 × 500 mL). The resulting aqueous phase was extracted (3 × 500 mL) with CH₂Cl₂. The combined organic extracts were dehydrated (by Na₂SO₄) and evaporated under reduced pressure to yield a brown solid residue (445.4 mg) showing significant phytotoxic activity in a *B. tectorum* coleoptile bioassay. The mixture was washed with small aliquots (5 × 1 mL) of MeOH. The solid residue was soluble in CHCl₃–MeOH (1:1) and essentially contained cytochalasin B,⁶ 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–*i*-PrOH (9:1) and EtOAc–*n*-hexane (7:3), respectively]. The solid was crystallized twice from EtOAc–*n*-hexane (1:5), giving white needles of cytochalasin B (240.2 mg). The products in the mother liquors of cytochalasin B crystallization (55.3 mg) were combined with the initial MeOH washing (for a total of 205.2 mg) and fractionated by column chromatography on silica gel, eluted with CH₂Cl₂–MeOH (93:7). Seven homogeneous fraction groups were collected. The residue (28.6 mg) of the third fraction group was further purified by TLC on silica gel, eluent CH₂Cl₂–MeOH (95:5), yielding a further amount of cytochalasin B as white needles (20.1 mg, for a total of 260.3 mg, 1.3 g/kg). The residue of the fifth fraction (23.4 mg) was further purified by TLC on silica gel, eluent CH₂Cl₂–MeOH (9:1), yielding pyrenophoric acid (**1**, *R*_f 0.36, 14.3 mg, 71.5 mg/kg) as a homogeneous amorphous solid.

Pyrenophoric acid (1): [α]_D²⁵ +10.2 (c 0.2 CHCl₃); IR ν_{\max} 3415, 1683, 1632, 1559, 1261 cm^{–1}; UV λ_{\max} nm (log ϵ) 257 (4.60); ¹H and ¹³C NMR, see Table 1; HRESIMS (+) *m/z* 286.2014 [M + NH₄]⁺ (calcd for C₁₅H₂₈NO₄ 286.2018); HRESIMS (–) *m/z* 267.1446 [M – H][–] (calcd for C₁₅H₂₃O₄ 267.1596).

Methyl Ester of Pyrenophoric Acid (2). To pyrenophoric acid (**1**, 1.7 mg) dissolved in MeOH (0.30 mL) was slowly added an ethereal solution of CH₃N₂ until a yellow color was persistent. The solvent was evaporated under an N₂ stream, and the residue (2.0 mg) purified by preparative TLC eluted with CHCl₃–*i*-PrOH (95:5), giving **2** (1.5 mg, *R*_f 0.8) as a homogeneous oil: UV λ_{\max} nm (log ϵ) 262 (4.13); IR ν_{\max} 3410, 1718, 1628, 1601, 1267 cm^{–1}; ¹H NMR, see Table 2; ESIMS (+) *m/z* 305 [M + Na]⁺.

Acetylation of Pyrenophoric Acid. Pyrenophoric acid (**1**, 3.5 mg) was acetylated with pyridine (50 μ L) and Ac₂O (50 μ L) at room temperature for 1 h. The reaction was stopped by addition of MeOH, and the azeotrope, obtained by the addition of benzene, was evaporated by an N₂ stream. The oily residue (3.6 mg) was purified by preparative TLC, eluted with CHCl₃–*i*-PrOH (95:5), to give the two 9-*O*-acetyl and 9,10-*O,O'*-diacetyl derivatives **3** and **4** of pyrenophoric acid as homogeneous compounds (*R*_f 0.63, 1.9 mg and *R*_f 0.46, 1.1 mg, respectively). Derivative **3**: IR ν_{\max} 3427, 1741, 1718, 1632, 1600, 1257 cm^{–1}; UV λ_{\max} nm (log ϵ) 259 (4.96); ¹H NMR, see Table 2; HRESIMS (+) *m/z* 328.2152 [M + NH₄]⁺ (calcd for C₁₇H₃₀NO₅ 328.2124). Derivative **4**: IR ν_{\max} 1744, 1684, 1632, 1600, 1251 cm^{–1}; UV λ_{\max} nm (log ϵ) 256 (5.13); ¹H NMR, see Table 2; HRESIMS (+) *m/z* 370.2265 [M + NH₄]⁺ (calcd for C₁₉H₃₂NO₆ 370.2230).

10-*O*-(*S*)- α -Methoxy- α -trifluoromethyl- α -phenylacetate Ester of Pyrenophoric Acid Methyl Ester (5). (R)-(-)-MPTA-Cl (10 μ L) was added to **1** (1.5 mg) dissolved in dry pyridine (20 μ L). The mixture was kept at room temperature for 1 h, and then the

reaction stopped by adding MeOH. Pyridine was removed by an N₂ stream. The residue (2.5 mg) was purified by preparative TLC, eluted with CHCl₃-*i*-PrOH (99:1), yielding **5** as a homogeneous oil (*R_f* 0.15, 1.1 mg): IR ν_{\max} 3417, 1743, 1461, 1377, 1261, 1157 cm⁻¹; UV λ_{\max} nm (log ϵ) 269 (4.56); ¹H NMR, see Table 2; HRESIMS (+) *m/z* 516.2646 [M + NH₄]⁺ (calcd for C₂₆H₃₇F₃NO₆ 516.2622).

10-O-(*R*)- α -Methoxy- α -trifluoromethyl- α -phenylacetate Ester of Pyrenophoric Acid Methyl Ester (6**).** (S)-(+)-MPTA-Cl (10 μ L) was added to **1** (1.0 mg) dissolved in dry pyridine (20 μ L). The reaction was carried out under the same conditions used for preparing **6**. The purification of the crude residue (2.2 mg) by preparative TLC eluted with CHCl₃ to give **7** as a homogeneous oil (*R_f* 0.15, 1.6 mg): IR ν_{\max} 3420, 1744, 1461, 1380, 1259, 1157 cm⁻¹; UV λ_{\max} nm (log ϵ) 268 (4.85); ¹H NMR, see Table 2; HRESIMS (+) *m/z* 516.2644 [M + NH₄]⁺ (calcd for C₂₆H₃₇F₃NO₆ 516.2622).

Cheatgrass Coleoptile Elongation Bioassay. Pyrenophoric acid (**1**), cytochalasin B, ABA (p.a. 98.5%, Sigma-Aldrich), and the mixture of **1** and cytochalasin B (1:1 molar ratio) were first dissolved in MeOH and then brought up to the assay concentration of 10⁻³ or 10⁻⁴ M with distilled water (the final content of MeOH was 1%). For each sample and concentration, 1.33 mL of the solution was pipetted into each of three 6 cm Petri dishes onto the surface of one filter paper. Seeds were incubated in 1% MeOH in the control treatment.

Six 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 h 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. Data were analyzed using mixed model analysis of variance with the compound–concentration combination as the fixed variable, Petri dish as the random variable, and day-5 coleoptile length as the response variable. Coleoptile length data were log-transformed to improve homogeneity of variance prior to analysis.

■ ASSOCIATED CONTENT

📄 Supporting Information

NMR and HRESIMS spectra of **1** are available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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