Journal of Agricultural Chemistry and Environment, 2019, 8, 115-128
http://www.scirp.org/journal/jacen
ISSN Online: 2325-744X
ISSN Print: 2325-7458

A Phytotoxic and Antifungal Metabolite (Pyrichalasin H) from a Fungus Infecting *Brachiaria eruciformis* (Signal Grass)

Kumudini M. Meepagala[1*], Brandon M. Clausen[1,2], Robert D. Johnson, David E. Wedge[1], Stephen O. Duke[1]

[1]USDA-ARS, Natural Products Utilization Research Unit, University, MS, USA
[2]Sally McDonnell Barksdale Honors College, University of Mississippi, University, MS, USA
Email: *kumudini.meepagala@ars.usda.gov, *kmeepaga@olemiss.edu

How to cite this paper: Meepagala, K.M., Clausen, B.M., Johnson, R.D., Wedge, D.E. and Duke, S.O. (2019) A Phytotoxic and Antifungal Metabolite (Pyrichalasin H) from a Fungus Infecting *Brachiaria eruciformis* (Signal Grass). *Journal of Agricultural Chemistry and Environment*, **8**, 115-128.
https://doi.org/10.4236/jacen.2019.83010

Received: June 15, 2019
Accepted: August 5, 2019
Published: August 8, 2019

Abstract

Brachiaria eruciformis (sm.) Griseb, locally known as "signal grass", is a common weed in lawns and turfs in Mississippi, USA. During late spring and early summer months, leaves of *B. eruciformis* are infected with a fungus causing necrosis. The infected leaves ultimately turn brown and wither. As part of our search for potential new natural product-based agrochemicals, we studied this plant pathogen in order to investigate phytotoxic and fungitoxic metabolites produced by the fungus. The causative fungus was isolated from an infected leaf of *B. eruciformis*, cultured in potato dextrose agar plates and identified via molecular techniques as *Pyricularia grisea.* A phytotoxic compound was isolated from Czapek-Dox broth liquid culture medium and identified as pyrichalasin H by spectroscopic techniques. Pyrichalasin H was toxic to the fungal plant pathogen *Colletotrichum fragariae* in a TLC bioautography assay and phytotoxic to two monocot and one dicot plants. This is the first report of antifungal activity of pyrichalasin H against phytopathogens. Pyrichalasin H isolated from *Pyricularia grisea*, a pathogen infecting *B. eruciformis* (signal grass) was shown to be phytotoxic and fungicidal to *Colletotrichum fragariae.*

Keywords

Mycotoxin, *Brachiaria eruciformis*, Phytotoxicity, *Pyricularia grisea*, Pyrichalasin H

1. Introduction

Microbes are good sources of bioactive compounds [1]. Of particular interest in

our research are phytopathogenic fungi and their metabolites. Phytopathogenic fungi use the host plant as a source of nutrients for its growth and development. In this process, the fungi often produce toxins that are lethal to the host plant and often toxic to other plant species, and these toxins may also have insecticidal, antibacterial and antifungal activities as a result of coevolution and competition among species to survive in the biosphere [2] [3] [4].

Weeds are a major threat to crop production worldwide. Modern agricultural practices primarily rely on synthetic herbicides, due to their high efficacy, selectivity and low cost [5] [6]. Repeated use of synthetic herbicides for prolonged periods of time has facilitated the widespread evolution of herbicide resistance among weed species exposed to them [7]. Herbicide-resistant weeds have caused an increased need for new herbicides with new modes of action. Natural products offer broad chemical diversity with a wide range of bioactivities, including phytotoxicity. These compounds may have varying and multiple molecular target sites and thus can be used as novel compounds or templates for development of pest control agents [8]. This study focused on isolation of bioactive compounds from a pathogenic fungus infecting *B. eruciformis* (signal grass).

2. Materials and Methods

2.1. General Procedures

Potato dextrose agar (PDA) plates were prepared by dissolving 19.6 g of Difco™ (Detroit, MI) potato dextrose agar and 8.0 g of BD Bacto™ agar in 1 L of deionized water. The solution was autoclaved for 30 min at 120˚C. PDA solution was poured into sterile plastic petri dishes (BD Falcon™) inside a class II-A biological safety cabinet. Czapek-Dox broth media was made by dissolving 35.0 g of Czapek-Dox broth culture medium (Fluka™), 1.5 g of malt extract (BD Bacto™), and 1.5 g of yeast extract (BD Bacto™) in each 1 L of deionized water in thirty 2 L Erlenmeyer flasks. All media were sterilized by autoclave for 30 min at 120˚C. Fungal broth extracts were analyzed on 250 μm silica gel TLC plates with fluorescent indicator (Analtech, Newark, DE). UV light (254 and 365 nm). *p*-Anisaldehyde spray reagent and iodine vapor were used for visualization of compounds. Isolation and purification of metabolites were performed with a Biotage Isolera™ Flash Chromatography system (Charlotte, NC) using hexane, ethyl acetate, dichloromethane and methanol in various percentages. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer (Billerica, MA) operating at 400 MHz for ^{1}H and at 125 MHz for ^{13}C NMR. High-resolution mass spectra were obtained using JEOL ACCU TOF JMS-T1000 LC mass spectrometer (Peabody, MA).

2.2. Fungal Material

Leaves of *B. eruciformis* with necrotic symptoms were collected in Oxford, MS, USA during May 2015 (**Figure 1(a)**). Under sterile conditions, an infected leaf

(a) (b)

Figure 1. (a) Infected *Brachiaria eruciformis* leaves showing necrosis; (b): *Pyricularia grisea* growing on PDB plate.

was surface sterilized by immersing it in aqueous sodium hypochlorite (5%) solution for 30 s, followed by thoroughly rinsing with sterile deionized water. A PDA plate (half strength potato-dextrose-agar, Difco™) was inoculated by placing a small piece of leaf tissue (approx. 2 × 2 mm) cut by a sterile scalpel from an infection site. This plate was allowed to grow in a growth chamber at 24˚C under 12-h light cycle for one week. A single colony from this plate was sub-cultured on another PDA plate under similar conditions as above by placing a 0.5 cm diameter plug of the fungal colony (**Figure 1(b)**). This plate was incubated and fungal culture was allowed to grow for 7 days, and this fungal colony was used to inoculate a 500 mL Erlenmeyer flask containing 250 mL Czapek-Dox broth. The liquid culture was allowed to grow for 7 days in an orbital shaker rotating at 90 rpm at 24˚C. Aliquots of 5 mL of liquid culture broth was added to thirty 2-L flasks each containing 1 L of Czapek-Dox broth, and these culture broths were allowed to grow in an orbital shaker (80 rpm) under the same conditions as above.

2.3. Molecular Identification of Fungus

DNA sequencing and alignment of ITS region was performed by Accugenix® (Newark, DE). Consensus C169499120140715014 was aligned, using the Neighbor Joining Tree method with *Dinemasporium morbidum* as the out-group (**Figure 2**).

2.4. Extraction and Isolation of Phytotoxins in the Fungal Broth

The culture broth (Czapek-Dox) (30 L) was filtered through Miracloth (EMD Millipore. Billerica, MA) followed by filtration through filter paper (Whatman #1). The filtrate was extracted (×2) with an equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and the solvent was evaporated to obtain 5.9 g of brownish, viscous extract. TLC eluted in 80% ethyl acetate in hexane indicated presence of one major compound with several minor compounds. The crude extract was dissolved in ethyl acetate and let stand overnight to obtain a white solid (320 mg). The supernatant was concentrated and fractionated on silica gel column (SNAP 50 g Biotage column) using 5% - 100% ethyl acetate in hexane. Fractions of 27 mL were collected and similar fractions according to TLC were combined to afford 12 fractions. Fraction 8 produced a white crystalline solid (640 mg)

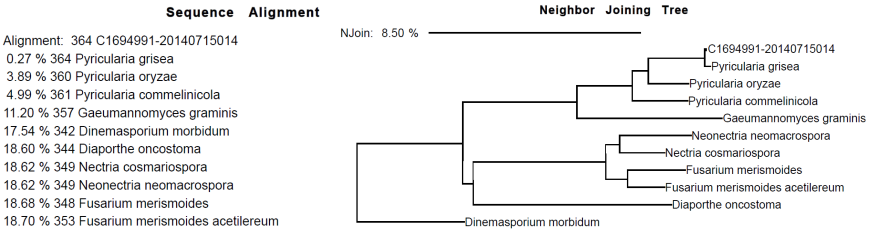


Figure 2. Sequence alignment and neighbor joining tree for the identification of *Pyricularia grisea.*

which was identical to the white solid precipitated from the crude ethyl acetate extract and was identified as pyrichalasin H (**Figure 3**) by NMR and high-resolution mass spectroscopy.

Pyrichalasin H (**1**): ESI-HRMS m/z 524.30136 [M + H] (calcd for 524.30124), $[\alpha]_D$-18.9 ($CHCl_3$ c = 0.5)mp 208˚C - 209˚C. ^{1}H NMR (400 MHz, $CDCl_3$) δ0.98 (d, J = 6.7 HZ, 3H, CH_3-11), 1.04 (d, J = 6.2 Hz, CH_3-22), 1.34 (s, CH_3-23), 1.54 (dd, J = 14, 2 Hz, Ha-17), 1.79 (m, H-16), 1.8 (m, Ha-15), 1.88 (dd, J = 14, 2 Hz, Hb-17), 2.03 (m, Hb-15), 2.11 (t, J = 4.4, H-4), 2.24 (s, OAc), 2.59 (dd, J = 14, 10, Ha-10), 2.77 (m, H-5), 2.81 (dd, J = 14, 10, Hb-10), 2.93 (dd, J = 8, 8 Hz, H-8), 3.21 (m, H-3), 3.79 (s, OMe), 3.83 (d, J = 12 Hz, H-7), 5.10 (br s, Ha-12), 5.34 (br s, Hb-12), 5.36 - 5.44 (m, H-14), 5.51 (d, J = 2 Hz), 5.54 (dd, J = 15, 2 Hz, H-20), 5.6 (s, NH), 5.74 (dd, J = 16, 9 Hz, H-13), 5.88 (d, J = 2 Hz H-19), 6.85 (d, J = 8Hz), 7.06(d, J = 8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ13.88, 14.30, 20.99, 21.14, 21.24, 26.52, 28.47, 31.17, 33.18, 42.78, 44.57, 44.61, 49.84, 51.86, 53.80, 53.83, 55.39, 60.51, 72.36, 74.35, 114.36, 114.94, 125.98, 126.60, 129.35, 130.26, 136.60, 137.98, 145.95, 158.71, 170.24, 174.18.

Acetylation of pyrichalasin H: To a solution of pyrichalasin H (200 mg, 0.382 mmol) in pyridine (4 mL), 4 eq of acetic anhydride (72 µL) was added and the mixture was stirred for 12 h at room temperature. The reaction mixture was cooled in ice, acidified with 0.5 M cold HCl solution and the product was extracted with ethyl acetate (100 mL × 2). The ethyl acetate extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated to obtain a semi-solid. The product **2** was purified by (SNAP 50 g Biotage column) using 5% - 100% ethyl acetate in hexane to afford white crystalline solid (157 mg). ESI-HRMS m/z 566.30648 [M + H] (calcd for 566.31178), $[\alpha]_D$-56.9 ($CHCl_3$ c = 0.5)mp 135˚C - 137˚C. ^{1}H NMR (400 MHz, $CDCl_3$) δ0.91 (d, J = 6.7 Hz, 3H),1.02 (d, J = 6.1 Hz, 3H), 1.26 (t, J = 7.1 Hz, 1H), 1.32 (s, 3H), 1.53 (dd, J = 14.2, 2.4 Hz, 1H), 1.66 - 1.8 (m, 3H), 1.85 (dd, J = 14.2, 2.7 Hz, 1H),1.94 (s, 3H), 2.12 (dd, J = 5.0, 3.5 Hz, 1H), 2.17 (s, 3H), 2.26 (s, 3H), 2.64 (dd, J = 13.6, 8.9 Hz, 1H), 2.86 - 2.72 (m, 2H), 3.15 (dd, J = 11.1, 9.6 Hz, 1H), 3.26 - 3.18 (m, 1H), 3.79 (s, 3H), 5.04 (t, J = 1.7 Hz, 1H), 5.34 - 5.15 (m, 3H), 5.66 - 5.43 (m, 3H), 5.89 - 5.73 (m, 2H), 6.90 - 6.78 (m, 2H), 7.12 - 7.02 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ13.82, 20.89, 21.12, 26.41, 28.39, 30.91, 31.12, 33.10, 42.66, 44.51, 44.55, 49.86, 51.73, 53.68, 53.73, 55.29, 72.20, 74.31, 77.43, 114.27, 114.81, 125.95, 126.46, 129.27, 130.10, 136.54, 137.81, 145.86, 158.63, 170.12, 173.97.

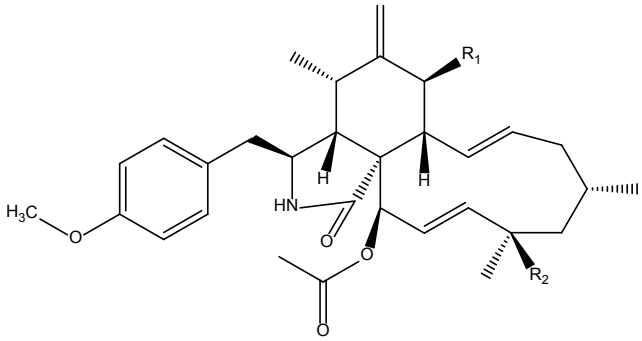


Figure 3. Structures of pyrichalasin H (**1**) and its acetate analog (**2**): R1 = R2 = OH; (**2**): R1 = OAc, R2 = OH.

2.5. Phytotoxicity Bioassay on Seed Germination

Fungal extract, column chromatography fractions and isolated compounds and their analogs were tested with the phytotoxicity bioassay described by Dayan *et al.* [9]. Phytotoxic effects of the compounds and the extract, column fractions and pure compounds were evaluated by the germination and growth effects on *Lactuca sativa* (lettuce; dicot, Crisphead cultivar from Burpee seeds, Warmister, PA) and *Agrostis stolonifera* (bentgrass; monocot, Penncross variety from Turf-Seed, Inc of Hubbard, OR) seeds in 24-well plates. The negative control was deionized sterile water and a solvent control consisted of 10% acetone in deionized water. Each 24-well plate was placed in an incubator under 16/8 h light/dark condition at 26˚C and 120 μmol·s^{-1}·m^{-2} average photosynthetically active radiation (PAR). Phytotoxic activity was ranked qualitatively on a scale of 0 to 5 after 7 days for *L. sativa* and after 10 days for *A. stolonifera* where a ranking of 0 means no difference between the control and treated seeds and a ranking of 5 indicates complete inhibition of germination, with intermediate effects (2, 3 and 4) indicating increasing effect on seedling growth and development.

2.6. Phytotoxicity Evaluation on *Lemna pausicostata*

The method described by Michel *et al.* [10] using duckweed (*Lemna pausicostata*) was used to quantitatively evaluate (e.g., accurate IC_{50} values) phytotoxicity. Two duckweed plants with three fronds each were placed in a six-well plate with 4950 μL of Hoagland's media and 50 μL of water, or the solvent, or the compound dissolved in the appropriate solvent (at a concentration of 100×). The concentration of acetone in the wells was therefore 1% by volume. The plates were incubated in the Percival incubator as described above. Duckweed plant areas were measured at day 0 and day 7 using a Lemnatec Scanalyzer PL with Lemna Launcher and Lemna Miner software (Lemna Tec GmbH, Schumanstr 19, 52, 146 Würselen, Germany). The image analysis software was used to measure and monitor frond number, total frond area as well as color classes (healthy, chlorotic and necrotic tissue). Replicate tests at varying concentrations of test compounds allowed for determination of IC_{50} values using R Studio software (Version 0.99.491).

2.7. Cellular Leakage Test on *Cucumis sativus* (Cucumber) Leaf Disks

To determine if the phytotoxic compounds cause membrane leakage, a modified method developed by Duke and Kenyon [11] was used. *Cucumis sativus* (cucumber, from Burpee seeds, Warmister, PA) plants were grown from seeds in a Conviron growth chamber (Model E7/2; Winnipeg, Canada) at 26˚C under 173 μmol·s^{-1}·m^{-2} PAR for 6 days. Fifty 4-mm disks were cut using a cork borer from *C. sativus* leaves and placed in Petri dishes along with 5 mL of 1 mM 2-(4-morpholino) ethane sulfonic acid (MES) buffer solution with 2% w/v sucrose. The pH of the solution was adjusted to 6.5 with 1 M NaOH. The test compounds were dissolved in acetone and were added to MES buffer solution such that the final concentration of acetone was 1%. Aciflurofen, a herbicide that causes rapid plasma membrane destruction in the light [12] was used as the positive control in the experiment. Electrical conductivities of the solutions in the dishes were measured using a dip cell at various time intervals (0, 1, 2, 4, 6 and 8) after exposure to the chemical. All dishes were covered with aluminum foil and kept in the dark for 18 h. The dishes were then exposed to 200 μmol·s^{-1}·m^{-2} photosynthetically active radiation (PAR) light and electrical conductivity measurements were taken at the same time intervals. Experiments were done in triplicate, and the averages were graphed as % conductivity change after treatment. The maximum leakage reading for the leaf disks was taken by measuring the conductivity of the boiled solution with leaf disks.

2.8. Effect of Pyrichalasin H on the Growth and Chlorophyll Content of Lettuce (*Lactuca sativa*) and Barley (*Hordeum vulgare* L.) Seedlings

Pyrichalasin H was also tested at varying concentrations on growth of lettuce and barley seeds.

Surface sterilized lettuce seeds and barley seeds were allowed to germinate on filter paper treated with varying concentrations of pyrichalasin H solutions. For the lettuce seeds filter paper (90 mm diameter, Whatman #1) were placed in petri dishes (100 × 15 mm; diameter × height). Sterile deionized water (1980 μL) was pipetted into each Petri dish. Then 20 μL of stock solutions of pyrichalasin H at varying concentrations dissolved in acetone was added to the surface of the water for a final concentration of 1% acetone. Finally, 50 surface-sterilized lettuce seeds were placed in the dishes, and allowed to grow for 7 days in an incubator under 16/8 light/dark condition at 26˚C and 120 μmol·s^{-1}·m^{-2} average PAR. A similar procedure was used for barley seeds (20 seeds) in a glass jar (70 mm id and 90 mm height) using 70 mm diameter filter paper covered with a glass lid. The length of shoots and roots were measured and graphed against the concentration of pyrichalasin H. The chlorophyll concentration of the shoots was determined by the method of Hiscox and Israelstarm [13]. The shoots were cut and weighed for chlorophyll analysis. The analysis was done in triplicates. The weighed tissues for each replicate were placed in glass tubes with 2 mL

DMSO. The tubes were incubated at 65˚C - 68˚C for 2h. The DMSO was transferred to another tube and the process was repeated with another 2 mL of DMSO for 30 min. DMSO extracts were combined and the concentration of chlorophyll was determined using Shimadzu UV-3101 UV-VIS NIR spectrophotometer. The absorbance values at 663 and 645 nm were taken using 1-cm plastic cuvettes. The instrument was zeroed with DMSO. The total chlorophyll concentration was determined by the following equation:

$$\text{Total Chlorophyll Concentration} = 0.0202\ A_{663} + 0.00802\ A_{645}$$

where A_{663} and A_{645} are the absorbance readings at 663 and 645 nm. Using this value, the amount of chlorophyll per fresh weight of shoots was calculated and graphed against the concentration of pyrichalasin H.

2.9. Bioautography

Silica gel TLC plate-based bioautography was carried out to identify the antifungal activity of compounds and extracts against *Colletotrichum fragariae* according to a previously published method [14]. Column fractions and pure compounds were eluted on silica gel TLC plates with appropriate solvent and were air-dried. The plates were sprayed with a spore suspension of *C. fragariae* (10^5 spores/mL) and were incubated in a moisture chamber for 3 days at 26˚C with a 12-h photoperiod. Clear zones on the TLC plates indicate the presence of antifungal constituents.

2.10. Micro-Bioassay for Quantitative Fungicide Activity

To evaluate the quantitative fungicide activity, the purified compounds were evaluated in a dose response manner (0.3, 3, and 30 μM) in a 96-well micro-bioassay against *Colletotrichum fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporium* and *Phomopsis obscurans* with comparison to commercial fungicides (captan and azoxystrobin) according to published methods [15] [16].

3. Results and Discussion

The fungus infecting *B. eruciformis* was identified by molecular analysis (by Accugenix®, Newark, DE USA) as *Pyricularia grisea.* ITS sequence alignment of consensus C169499120140715014 (**Figure 2**), using *Dinemasporium morbidum* as the out-group, showed a 99.73% match.

The results of the preliminary seed germination bioassay of the ethyl acetate extract of the culture broth and column fractions indicated that the fractions rich in pyrichalasin H Fractions 7 and 8) indicated moderate phytotoxic activity against a monocot (*L. sativa*) and a dicot (*A. stolonifera*) (**Table 1**). Bioassay-guided fractionation led to the isolation of pyrichalasin H as the phytotoxin in the culture broth. Pyrichalasin H inhibited germination of the monocot with a phytotoxicity ranking of 4 at 330 μM, whereas at the same concentration the phytotoxicity ranking for the dicot was 2. To further examine the phytotoxic

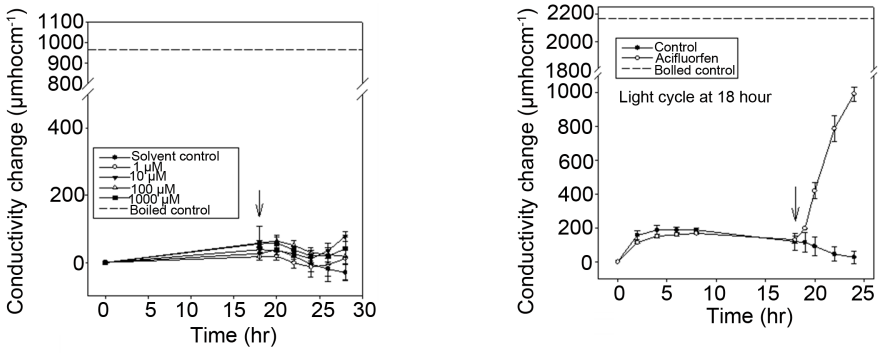
Table 1. Phytotoxicity bioassay results of crude ethyl acetate extract of the culture broth and isolated pyrichalasin H and acetate analogs (at varying concentrations).

Sample	Concentration	Lettuce	Agrostis
Culture broth EtOAc extract	1 mg/mL	3	3
Fraction 7	1 mg/mL	2	4
Fraction 8	1 mg/mL	2	4
Pyrichalasin H	0 μM	0	0
	10 μM	0	0
	33 μM	2	3
	100 μM	2	3
	330 μM	2	4
	1000 μM	2	4
Pyrichalasin Hacetate	1 mg/mL	0	0

activity and gain insight into the mode of action of pyrichalasin H, further bioassays were conducted. The cellular leakage test on cucumber cotyledon disks using pyrichalasin H as the test compound was conducted in order to see if the phytotoxic activity was due to cellular leakage caused by the test compound. The plot (**Figure 4(a)**) showed minimal to no change in conductivity even at the highest concentration of pyrichalasin H (1000 μM) that was tested. Acifluorfen, a herbicide that causes massive cellular leakage in the light was used as the positive control (**Figure 4(b)**) This observation of lack of leakage or no change in conductivity of cucumber cotyledons due to pyrichalasin H suggests that the mechanism of action of phytotoxicity does not involve disruption of the plasma membrane.

To gain more quantitative phytotoxic activity, the effect of pyrichalasin H was examined at varying concentrations on the monocot duckweed (*L. pausicostata*) (**Figure 5**). Growth of duckweed plants decreased with increasing concentration of pyrichalasin H, with an IC_{50} value of 150 μm. At higher concentrations (greater than 330 μm) pyrichalasin H started to separate from the solution making a layer of white powder in the test solution. It was also observed that duckweed plants started to show chlorosis at the concentrations 100 μm and higher, indicating that pyrichalasin H may inhibit synthesis of chlorophyll.

Pyrichalasin H was also tested at varying concentrations on growth of lettuce and barley seeds. In the concentration rage of 0.1 to 333 μM, pyrichalasin H did not affect the germination of either lettuce seeds or barley seeds. All the seeds germinated, but the shoot and root lengths were affected (**Figure 6(a)**, **Figure 6(b)** and **Figure 7**). Root lengths of both lettuce and barley were reduced by increasing concentrations of pyrichalasin H. The shoot length of lettuce seedlings was not measured but they were also affected. The chlorophyll content in the shoots was reduced by pyrichalasin H (**Figure 8(a)** and **Figure 8(b)**).



(a) (b)

Figure 4. (a) Changes of the conductivity of solutions of the cucumber leaf disks treated at varying concentrations (1, 10, 100 and 1000 μM) of pyrichalasin H in the dark and after exposure to light (arrow) at 18 h. The dotted line shows the maximum leakage value obtained by using boiled leaf disks in MES buffer. (b) The same measurements at 1 μM concentration of acifluorifen.

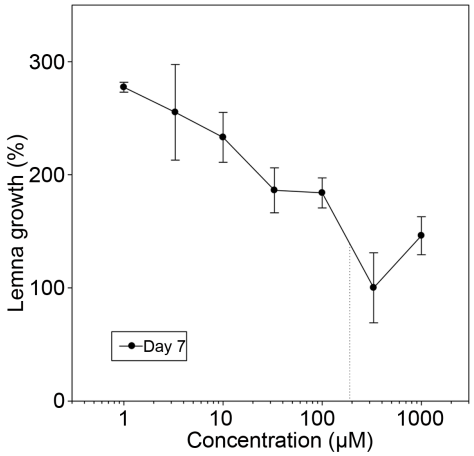
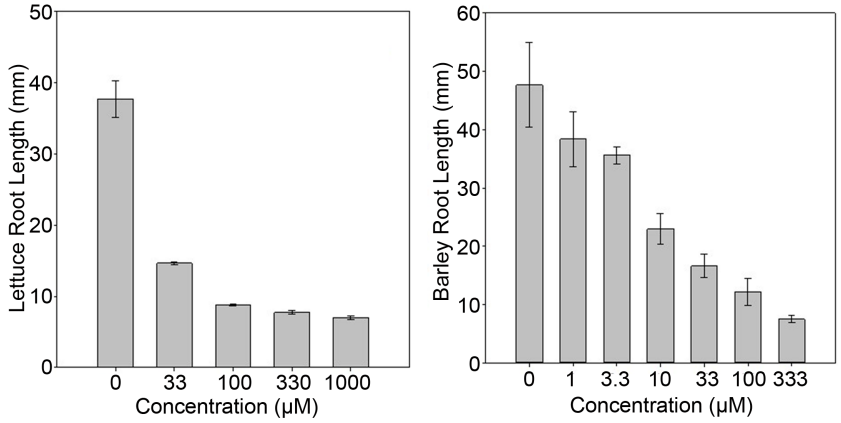


Figure 5. Effect of pyrichalasin H on *Lemna pausicostata* growth. Dotted line indicates IC_{50}. Concentrations greater than 300 μm could not be used as pyrichalasin H precipitated from the aqueous medium.



(a) (b)

Figure 6. Root length of lettuce (a) and barley (b) plants at varying concentration of pyrichalasin H measured at 7 days after treatment.

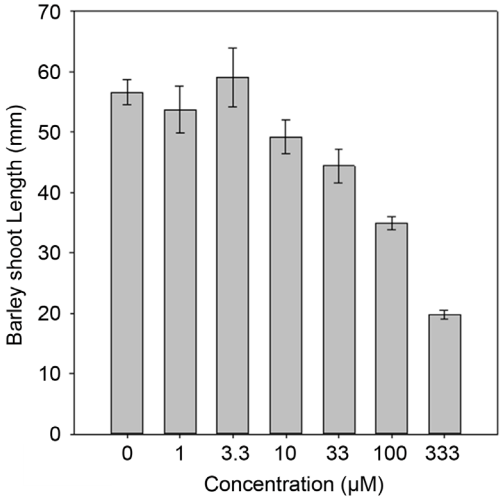


Figure 7. Shoot length of barley plants at varying concentration of pyrichalasin H measured at 7 days after treatment. Shoot length measurements were not taken for lettuce plants.

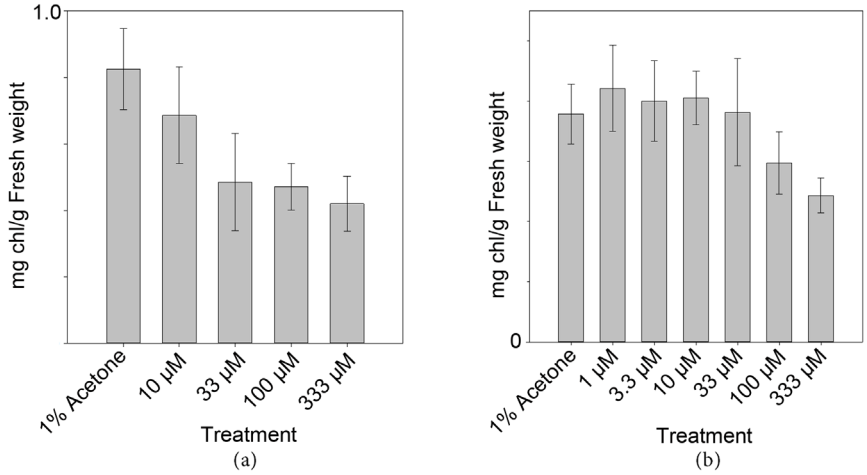


Figure 8. Chlorophyll per fresh weight of shoots lettuce (a) and barley (b) at varying concentration of pyrichalasin H measured at 7 days after treatment.

An acetate analog of pyrichalasin H was synthesized. Acetylation of pyrichalasin H using acetic anhydride in pyridine afforded only the mono acetate analog, where the secondary OH group was acetylated. The acetate analog was not phytotoxic (Table 1) in lettuce and bentgrass bioassays at 1 mg/mL concentration. Therefore, the presence of OH groups is needed for the phytotoxic activity.

Pyrichalasin H and its acetate analog were tested on TLC bioautography against *C. fragariae.* The compounds were spotted at 1 mg/mL on TLC plates. Only pyrichalasin H had significant antifungal activity (Figure 9). The acetate analog has much lower fungitoxic activity than pyrichalasin H. This is the first report of antifungal activity of pyrichalasin H. Since the acetate analogs had only marginal fungitoxicity, it is evident that the OH groups are essential for good antifungal activity. We have carried out micro-bioassay to evaluate fungicidal

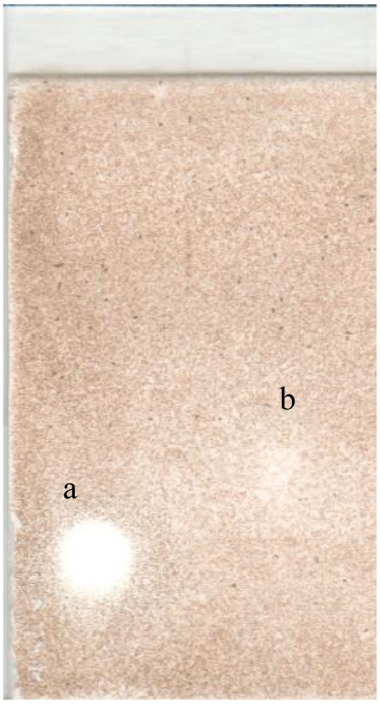


Figure 9. TLC bioautography of a: pyrichalasin H (**1**); b: pyrichalasin H monoacetate (**2**) The plate was eluted in 40% ethylacetate in hexane and sprayed with spores of *C. fragariae.*

activity in comparison to two commercial fungicides captan and azoxystrobin. The compounds were evaluated at 0.3, 3.0, 30 μm concentrations at 24 and 72 h for *Colletotrichum fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *and Fusarium oxysporium* and for 120 and 144 h for *Phomopsis obscurans*. *C. acutatum*, *F. oxysporium* and *P. obscurans* were not inhibited by pyrichalasin H (data not shown). *Botrytis cinerea* and *C. gloeosporioides* were marginally inhibitory to pyrichalasin H at 48 h after treatment and the activity diminished at 72 h (**Figure 10(a)** and **Figure 10(b)**). *C. fragariae* treated at 30 μm concentration of pyrichalasin H was inhibited about 60% and 30% at 48 h and 72 h after treatment, respectively (**Figure 10(c)**). At the same concentration of azoxystrobin and captan *C. fragariae* growth was inhibited 85% and 80% - 70% at 48 h and 72 h after treatment, respectively. *C. fragariae* was the most sensitive fungal species to pyrichalasin H among the fungi that we tested.

From the phytotoxicity bioassays, it is evident that even at the highest concentration of pyrichalasin H (1000 μm) germination inhibition was not complete. Root and shoot growth were reduced by pyrichalasin H concentrations as low as 33 μM.

Pyrichalasin H belongs to the class of cytochalasins, which are polyketide-amino acid hybrid molecules that have been isolated as fungal metabolites. More than 80 cytochalasins have been isolated from fungi belonging to various fungal genera having various biological activities such as antimicrobial, antiparasitic, cytotoic and phyototoxic activities [17] [18] [19]. Little is known of the

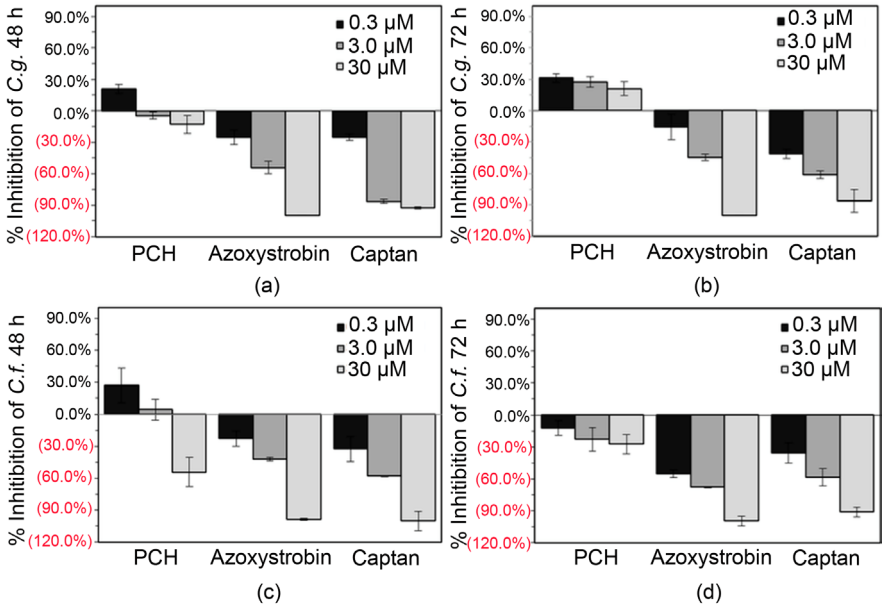


Figure 10. Microbioassay results showing % inhibition of fungal growth in comparison to that caused by commercial fungicides captan and azoxystrobin. ((a) & (b)) Inhibition caused by *C. gloeosporioides* ((c) & (d)) Inhibition caused to *C. fragariae* ((a) & (c)) results at 48 h ((b) & (d)) results at 72 h.

phytotocity of pyrichalasijn H. The amount of pyrichalain H produced by different isolates of *Pyricularia grisea* correlates positively with their virulence against *Digitaria* plants [20]. Pyrichalasin H was previously found to inhibit growth and cause curling of the shoots of rice seedlings, but produced no symptoms in the leaf [21]. Finger millet (*Eleusine coracana* L.) seeing growth is inhibited by pyrichalasin H [22]. It causes effects on plant cell morphology similar to those caused by actin polymerization inhibitors [23]. Cytochalasin H, an analog of pyrichalasin H, where the OMe group is replaced with H, is a potent inhibitor for elongation of actin fibers [24]. Cytochalasin H is also a plant growth inhibitor [25]. In our experiments, we observed that the growth of plants was not completely inhibited by pyrichalasin H at concentrations as high as 1 mM, but the elongation of shoots and roots were significantly inhibited, and chlorophyll levels were decreased at concentrations as low as 33 μM. We hypothesize that the mode of action of pyrichalasin H is similar to the mode of action of cytochalasin H, and that the effects that we have documents are secondary and/or tertiary effects of this primary effect. Experiments with pyrichalasin H on actin fiber polymerization should be conducted to confirm this hypothesis.

4. Conclusion

The causative fungus infecting *Brachiaria eruciformis* (signal grass) was isolated and identified as *Pyricularia grisea*. From the culture filtrate of *Pyricularia grisea* the phytotoxic and fungicidal constituent was identified as Pyrichalasin H via bioassay guided fractionation.

Acknowledgements

We thank Linda Robertson, Ramona Pace and William Eric Briscoe for their technical assistance.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Monciardini, P., Iorio, M., Maffioli, S., Sosio, M. and Donadio, S. (2014) Discovering New Bioactive Molecules from Microbial Sources. *Microbial Biotechnology*, **7**, 209-220. https://doi.org/10.1111/1751-7915.12123

[2] Macheleidt, J., Mattern, D.J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V. and Brakhage, A.A. (2016) Regulation and Role of Fungal Secondary Metabolites. *Annual Review of Genetics*, **50**, 371-392. https://doi.org/10.1146/annurev-genet-120215-035203

[3] Ogorek, R. (2016) Enzymatic Activity of Potential Fungal Plant Pathogens and the Effect of Their Culture Filtrates on Seed Germination and Seedling Growth of Garden Cress (*Lepidium sativum* L.). *European Journal of Plant Pathology*, **145**, 469-481. https://doi.org/10.1007/s10658-016-0860-7

[4] Kim, W., Park, J.-J., Dugan, F.M., Peever, T.L., Gang, D.R., Vandemark, G. and Chen, W. (2017) Production of the Antibiotic Secondary Metabolite Solanapyrone A by the Fungal Plant Pathogen Ascochytarabiei during Fruiting Body Formation in Saprobic Growth. *Environmental Microbiology*, **19**, 1822-1835. https://doi.org/10.1111/1462-2920.13673

[5] Duke, S.O. and Dayan, F.E. (2018) Herbicides. John Wiley & Sons, Chichester. https://doi.org/10.1002/9780470015902.a0025264

[6] Dayan, F.E., Cantrell, C.L. and Duke, S.O. (2009) Natural Products in Crop Protection. *Bioorganic & Medicinal Chemistry*, **17**, 4022-4034. https://doi.org/10.1016/j.bmc.2009.01.046

[7] Heap, I. and Duke, S.O. (2018) Overview of Glyphosate-Resistant Weeds Worldwide. *Pest Management Science*, **74**, 1040-1049. https://doi.org/10.1002/ps.4760

[8] Sparks, T.C., Hahn, D.R. and Garizi, N.V. (2017) Natural Products, Their Derivatives, Mimics and Synthetic Equivalents: Role in Agrochemical Discovery. *Pest Management Science*, **74**, 700-715. https://doi.org/10.1002/ps.4458

[9] Dayan, F.E., Romagni, J.G. and Duke, S.O. (2000) Investigating the Mode of Action of Natural Phytotoxins. *Journal of Chemical Ecology*, **2**, 2079-2094. https://doi.org/10.1023/A:1005512331061

[10] Michel, A., Johnson, R.D., Duke, S.O. and Scheffler, B.E. (2004) Dose-Response Relationships between Herbicides with Different Modes of Action and Growth of Lemnapaucicostata—An Improved Ecotoxicological Method. *Environmental Toxicology and Chemistry*, **23**, 1074-1079. https://doi.org/10.1897/03-256

[11] Duke, S.O. and Kenyon, W.H. (1993) Peroxidizing Activity Determined by Cellular Leakage. In: Boger, P. and Sandman, G., Eds., *Target Assays for Modern Herbicides and Related Phytotoxic Compounds*, Lewis, Boca Raton, 61-66.

[12] Dayan, F.E. and Duke, S.O. (2010) Protoporphyrinogen Oxidase-Inhibiting Herbicides. In: Krieger, R.I., Doull, J., Hodgson, E., Maibach, H., Reiter, L., Ross, J.,

Slikker, W.J. and Van Hemmon, J., Eds., *Haye's Handbook of Pesticide Toxicology*, 3rd Edition, Vol. 2, Academic Press, Elsevier, San Diego, 1733-1751. https://doi.org/10.1016/B978-0-12-374367-1.00081-1

[13] Hiscox, J.D. and Israelstam, G.F. (1979) A Method for the Extraction of Chlorophyll from Leaf Tissue without Maceration. *Canadian Journal of Botany*, **57**, 1332-1334. https://doi.org/10.1139/b79-163

[14] Wedge, D.E. and Nagle, D.G. (2000) A New 2D TLC Bioautography Method for the Discovery of Novel Antifungal Agents to Control Plant Pathogens. *Journal of Natural Products*, **63**, 1050-1054. https://doi.org/10.1021/np990628r

[15] Oliva, A., Meepagala, K.M., Wedge, D.E., Harries, D., Hale, A.L., Aliotta, G. and Duke, S.O. (2003) Natural Fungicides from *Ruta graveolens* L. Leaves, Including a New Quinolone Alkaloid. *Journal of Agricultural and Food Chemistry*, **51**, 890-896. https://doi.org/10.1021/jf0259361

[16] Meepagala, K.M., Schrader, K.K., Burandt, C.L., Wedge, D.E. and Duke, S.O. (2010) New Class of Algicidal Compounds and Fungicidal Activities Derived from a Chromene Amide of *Amyris texana*. *Journal of Agricultural and Food Chemistry*, **58**, 9476-9482. https://doi.org/10.1021/jf101626g

[17] Scherlach, K., Boettger, D., Remme, N. and Hertweck, C. (2010) The Chemistry and Biology of Cytochalasans. *Natural Product Reports*, **27**, 869-886. https://doi.org/10.1039/b903913a

[18] Ismaiel, A.A. and Papenbrock, J. (2015) Fungal Phytotoxins with Potential Herbicidal Activity: Chemical and Biological Characterization. *Agriculture*, **5**, 492-537. https://doi.org/10.3390/agriculture5030492

[19] Cimmino, A., Masi, M., Evidente, M., Superchi, S. and Evidente, A. (2015) Fungal Phytotoxins with Potential Herbicidal Activity: Chemical and Biological Characterization. *Natural Product Reports*, **32**, 1629-1653. https://doi.org/10.1039/C5NP00081E

[20] Tsurushima, T., Don, L.D., Kawashima, K., Murakami, J., Nakayashiki, H., Tosa, Y. and Mayama, S. (2005) Pyrichalasin H Production and Pathogenicity of Digitaria-Specific Isolates of Pyriculariagrisea. *Molecular Plant Pathology*, **6**, 605-613. https://doi.org/10.1111/j.1364-3703.2005.00309.x

[21] Nukina, M. (1987) Pyrichalasin H, a New Phytotoxic Metabolite Belonging to the Cytochalasansfrom Pyriculariagrisea (Cooke) Saccardo. *Agricultural and Biological Chemistry*, **51**, 2625-2628. https://doi.org/10.1271/bbb1961.51.2625

[22] Sanmathi, K.R.P., Shanthala, L., Anikumar, T.B. and Sudharshana, L. (2006) Phytotoxins from Pyriculariagrisea and Their Effect on Finger Millet. *Journal of Plant Biochemistry and Biotechnology*, **15**, 63-66. https://doi.org/10.1007/BF03321905

[23] Hirose, T., Izawa,Y., Koyama, K., Natori, S., Iida, K., Yahara, I., Shimaoka, S. and Maruyama (1990) The Effects of New Cytochalasins from Phomopsis sp. and the Derivatives on Cellular Structure and Actin Polymerization. *Chemical and Pharmaceutical Bulletin*, **38**, 971-974. https://doi.org/10.1248/cpb.38.971

[24] Yahara, I., Harada, F., Sekita, S., *et al*. (1982) Correlation between Effects of 24 Different Cytochalas Ins on Cellular Structures and Cellular Events and Those on Actin *in Vitro*. *The Journal of Cell Biology*, **92**, 69-78. https://doi.org/10.1083/jcb.92.1.69

[25] Cutler, H.G., Cutler, S.J. and Matesic, D. (2004) Mode of Action of Phytotoxic Fungal Metabolites. In: Macias, F.A., Galindo, J.C.G., Molinillo, J.M.G. and Cutler, H.G., Eds., *Allelopathy*: *Chemistry and Mode of Action of Allelochemicals*, CRC Press, Boca Raton, 253-270. https://doi.org/10.1201/9780203492789.ch13