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Curvularin and Dehydrocurvularin as Phytotoxic Constituents from *Curvularia intermedia* Infecting *Pandanus amaryllifolius*

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Abstract

Microbes are good sources of biologically active compounds that can be used as pharmaceuticals and agrochemicals. As part of our continuous efforts in search for biopesticides from natural sources, a fungus is isolated from leaves of *Pandanus amaryllifolius* that shows severe necrosis. This fungus is cultured in potato dextrose agar and identified as *Curvularia intermedia.* The ethyl acetate extract of the liquid culture medium is phytotoxic. Identification and bioassay of *αβ*-dehydrocurvularin (1) and curvularin (2) from this fungus are described. Monoacetyldehydrocurvularin (3), and diacetyldehydrocurvularin (4) are synthesized from 1. Phytoxicity rankings in a variety of bioassays are 1 > 2 > 4 > 3.

Keywords

***Curvularia intermedia*, *Pandanus amaryllifolius*, Phytotoxins, Curvularin, Dehydrocurvularin**

1. Introduction

Fungi are good sources for biopesticide and pharmaceutical discovery [1]-[8]. Plant pathogenic fungi in particular produce toxins that are phytotoxic and thus can be used as bioherbicides or can be used as lead molecules to develop more potent molecules than the naturally occurring molecules [7]-[9]. *Pandanus amaryllifolius* is a monocotyledonous species in the Pandanaceae family. The leaves of this plant are used in cooking to impart desired aroma and flavor and are used in the food industry [10] [11]. Leaves of this plant growing in Oxford, MS,

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USA are infected with a fungus that causes wilting of the leaves and eventual plant death. A fungus is cultured from lesions of this plant in potato dextrose agar medium being identified as *Curvularia intermedia* by molecular techniques. The ethyl acetate extract of the liquid culture medium of this fungus is phytotoxic. In this paper, isolation, identification and bioassay of phytotoxic metabolites from *C. intermedia* are described.

2. Experimental

2.1. General Experimental Procedures

Extracts, isolated compounds and column chromatography fractions were analyzed on silica gel TLC plates GF with fluorescent indicator (250 micron, Analtech, Newark, DE, USA). Iodine vapor, UV light (at 254 and 365 nm), and anisaldehyde spray reagents were used for the detection of compounds. Column chromatography was carried out with Biotage flash purification system using SNAP silica columns with mixtures of hexane and ethyl acetate in varying amounts. All solvents were reagent grade and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded either on a Bruker AMX NMR spectrometer operating at 600 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR. High-resolution electrospray ionization mass spectrometry (HR-ESIMS) was measured by using a JEOL ACCU TOF JMS-T1000 mass spectrometer. Optical rotations were determined on an Autopol IV Automatic Polarimeter model 589 - 546 (Rudolph Research Analytical; NJ, USA).

2.2. Fungal Material

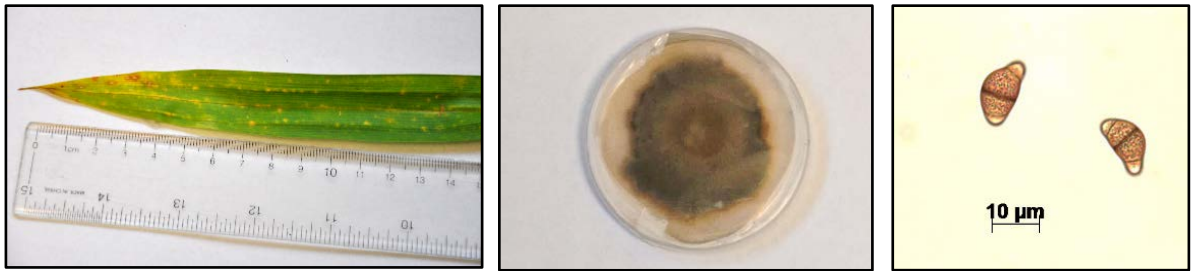
Infected *P. amaryllifolius* leaves were collected locally in Oxford, Mississippi (**Figure 1**). Part of an infected leaf (about 6 cm long) was surface sterilized by dipping in 0.5% (v/v) sodium hypochlorite in deionized water for 5 min and then rinsing with sterile deionized water three times. Half strength PDA plate was inoculated with the fungus using a small piece (about 2 mm × 2 mm) of the surface-sterilized leaf tissue from an infection site and allowed to grow for 4 days to obtain a single colony. A circular plug (0.5 cm radius) from the single colony was taken by a sterile drinking straw and used to inoculate another PDA plate. After allowing this to grow for 4 days, 10 straw plugs from this plate were added to 250 mL of PDB in a 500 mL flask and was allowed to grow for 7 days in a shaker at 120 rpm at 25˚C under continuous light. After growing for 10 days, the liquid culture (5 mL aliquots per flask) was used to inoculate fifteen 2000 mL flasks containing 1000 mL of PDB.

2.3. Molecular Identification of the Fungus

DNA Sequencing of the fungus and alignment of the ITS region was performed at Accugenix® (Newark, DE). Consensus C1823058-20150121028 was aligned constructing the neighbor joining tree method with *Drechlera nodulosa* as the outgroup.

2.4. Extraction and Isolation

PDB culture broth (15 L total) cultured for 14 days was filtered through Miracloth (EMD Millipore, Billerica, MA), followed by filtration through filter paper (Fisher brand P8, Particle Retention: 20 - 25 µm, diameter 20.5 cm, coarse). The filtrate was extracted twice with equal volume of ethyl acetate. Ethyl acetate extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure at 40˚C to obtain brown viscous



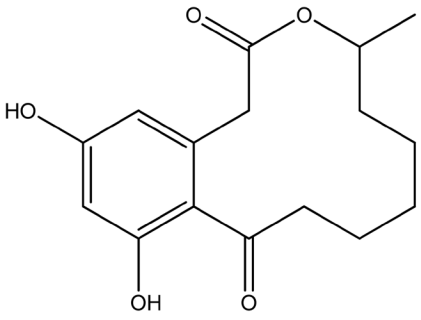
(a) (b) (c)

Figure 1. *Pandanus amaryllifolius* leaf infected with the fungus (a), fungus growing on PDA plate (b) and (c) fungal spores.

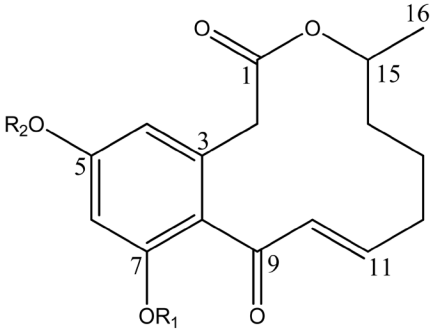
extract of 8.2 g which produced a brown crystalline solid. TLC of the extract indicated that culture medium was rich in two major compounds. The fungal broth crude extract (8 g) was subjected to Biotage Flash column chromatography using (SNAP Cartridge, KP-SIL, 100 g) silica column using 5% - 100% ethylacetate in hexane over 40 column volumes followed by 5% methanol in ethyl acetate over 4 column volumes. Fractions of 27 mL were collected, assessed by TLC, and similar fractions were combined to yield nine fractions.

αβ-dehydrocurvularin (**1**) (**Figure 2**) Fraction 3 (820 mg) on further purification by silica gel flash column chromategraphy using biotage flash column chromatography system eluting with 0% - 35% ethylacetate in hexane yielded a pale yellow crystalline solid upon crystallization with CH_2Cl_2 in hexane (520 mg); ^{1}H NMR (acetone-d_6, 600 MHz) δ 6.78 (d, J = 15.5 Hz, 1 H), 6.57 (dq, J = 15.5, 4.8 Hz, 1 H), 6.36 (d, J = 2.4 Hz, 1 H), 6.31 (d, J = 2.4 Hz, 1 H), 4.73 (m, 1 H), 4.08 (d, J = 17.3 Hz, 1 H), 3.61 (d, J = 17.7 Hz, 1 H), 2.42 (m, 1 H), 2.35 (m, 1 H), 1.99 (m, 1 H), 1.85 (m, 1 H), 1.67 (m, 1 H), 1.62 (m, 1 H), 1.19 (d, J = 6.4 Hz, 3 H); ^{13}C NMR (d_6-acetone, 150 MHz) δ 197.7, 172.3, 166.5, 163.6, 150.1, 140.0, 133.1, 116.2, 114.2, 103.3, 73.4, 44.2, 35.3, 33.7, 25.5, 22.8; HRMS (ESI-TOF) m/z 291.123248[M+H]$^+$ (calcd for $C_{16}H_{19}O_5$, 291.123250); $[\alpha]_D$ -49.7 (c, 0.3 in MeOH). NMR data are in agreement with those published in the literature [12].

Curvularin (**2**): (**Figure 2**) Fraction 5 (brownish yellow) was evaporated and crystallized using CH_2Cl_2 in methanol (approximately 4%) to obtain off white crystals (2.9 g). ^{1}H NMR (600 MHZ, acetone-d_6) 8.89 (s br), 6.38 (d, J = 1.8 Hz, 1 H), 6.34 (d, J = 2.4 Hz, 1 H), 4.91 (s br, 1 H), 3.77 (d, J = 12 Hz, 1 H), 3.71 (d, J = 12 HZ, 1 H), 3.11 (ddd, 3.0, 8.7, 15.6 HZ, 1 H), 2.77 (ddd, J = 2.9, 9.8. 15.6 Hz), 1.74 (m, 1 H), 1.59 (m, 1 H), 1.53 (m, 1 H), 1.43 (m, 2 H), 1.29 (m, 2 H), 1.11 (d, J = 6 Hz, 3 H); ^{13}C NMR (d_6-acetone, 150 MHz) δ 206.5, 171.1, 160.1, 158.3, 137.0, 121.3, 112.3, 102.6, 72.65, 44.0, 39.7, 32.9, 27.5, 24.5, 23.5, 20.6; HRMS (ESI-TOF) m/z 293.138906[M+H]$^+$ (calcd for $C_{16}H_{21}O_5$, 293.138900); $[\alpha]_D$ -29.1(c, 0.4 in MeOH). NMR data are in agreement with those published in the literature [13].



curvularin



R_1= H ; R_2=H :dehydrocurvularin (**1**)
R_1= OAc; R_2=H: monoacetyldehydrocurvularin (**3**)
R_1= OAc; R_2= OAc:diacetyldehydrocurvularin (**4**)

Figure 2. Fungal metabolites (curvularin and 1) isolated from the ethyl acetate extract of the culture medium and two synthesized analogs (3 and 4).

Acetylation of αβ-dehydrocurvularin (**3**, **4**): (**Figure 2**) To 300 mg of (**1**) in pyridine (5 mL) acetic anhydride (2eq in 3 mL of pyridine) was added and the reaction mixture was stirred for 2 hr. The solution was acidified with 2 M HCl and extracted with CH_2Cl_2 and the organic layer was dried over anhy. Na_2SO_4 to obtain a brownish oil. The products were purified by silica gel flash column chromatography using 0% - 30% ethylacetate in hexane to afford **3** (more polar) (90 mg) compound: ^{1}H NMR (600 MHZ, $CDCl_3$) δ 6.98 (d, J = 14.5 Hz, 1 H), 6.87 (dq, J = 15.5, 4.8 Hz, 1 H), 6.4 (d, J = 2.4 Hz, 1 H), 6.3 (d, J = 2.4 Hz, 1 H),5.35 (s br, 1H), 4.13 (m, 1 H), 3.6 (d, J = 17.3 Hz, 1 H), 3.3.5 (d, J = 17.7 Hz, 1 H), 2.28 (s, 3 H), 1.95 - 1.97 (m, 2 H), 1.41 (m, 2 H), 1.28 - 1.38 (m, 2 H), 1.35 (d, J = 6.4, 3 H) HRMS (ESI-TOF) m/z 333.133818$[M+H]^+$ (calcd for $C_{18}H_{21}O_6$, 333.133815).

Diacetyldehydrocurvularin (**4**) (170 mg): ^{1}H NMR (600 MHZ, $CDCl_3$) δ 7.8 (d, J = 1.5 Hz, 1 H), 7.3 (d, J = 1.5 Hz, 1 H), 6.97 (d, J = 14.5 Hz, 1 H), 6.87 (dq, J = 15, 4.8 Hz, 1 H), 4.13 (m, 1 H), 3.52 (d, J = 17.3 Hz, 1 H), 3.44 (d, J = 17.7 Hz, 1 H), 2.28 (s, 6 H), 1.95 - 1.97 (m, 2 H), 1.4 (m, 2 H), 1.28 - 1.38 (m, 2 H), 1.35(d, J = 6.5 Hz, 3 H): HRMS(ESI-TOF) m/z 375.144377$[M+H]^+$ (calcd for $C_{20}H_{23}O_7$, 375.144380).

2.5. Bioassays with *Lactuca sativa* L. and *Agrostis stolonifera* L.

Seeds of lettuce (*L. sativa*—Iceberg A Crisphead cultivar from Burpee Seeds, Warminster, PA) and bentgrass (*A. stolonifera*—Penncross variety obtained from Turf-Seed, Inc of Hubbard, Oregon) were surface sterilized with a 0.5% to 1% (v/v) sodium hypochlorite solution for approximately 10 min, rinsed with deionized water and dried in a sterile environment. The ethyl acetate extract of the fungal broth, column chromatography fractions and pure compounds were tested for phytotoxicity on the monocot bentgrass and dicot lettuce according to published methods [14]. Under sterile environment, a filter paper disk (Whatman Grade 1, 1.5 cm) was placed in each well of a 24-well plate. The control wells contained 200 ml of deionized water. The control + solvent well contained 180 ml of water and 20 ml of the solvent. All sample wells contained 180 ml of water and 20 ml of the appropriate dilution of the sample. Water was pipetted into the well before the sample or solvent. Test samples were dissolved in acetone and final concentrations in the wells were 1 mg/mL for the ethyl acetate extract and 1, 3, 10, 33, 100, 330, and 1000 µM for pure compounds.

For the bioassay five lettuce seeds or 10 mg of bentgrass seeds were placed in each well before sealing the plate with Parafilm. The plates were incubated either for 5 days (lettuce) or 12 days (bengrass) in a Percival Scientific CU-36L5 incubator under continuous light conditions at 26˚C and 120 µmol·s^{-1}·m^{-2} average photosynthetically active radiation (PAR). A qualitative estimate of phytotoxicity was made by assigning a rating of 0 for no effect (sample well plants looked identical to the control + solvent well plants; seeds had germinated and resulting seedlings had grown normally), 2 for less than 50% germination inhibition, 3 for about 50% germination inhibition, 4 for more than 50% germination inhibition, and 5 for no germination of the seeds. Each experiment was repeated three times.

2.6. Bioassays with *Lemna paucicostata* Hegelm

The bioassay with duckweed (*L. paucicostata*) was carried out according to publish methods [15]. Duckweed-stocks were grown from a single colony consisting of a mother and two daughter fronds in 100 ml of Hoagland's No. 2 Basal Salt Mixture (Sigma H2395) (1.6 g/L) with added iron (1 mL of 1000X Fe-EDTA solution to 1 L of Hoagland media, pH adjusted to 5.5) in sterile jars with vented lids in a Percival Scientific CU-36L5 incubator under continuous light conditions at 26˚C and 120 µmol·s^{-1}·m^{-2} average PAR. The iron solution (1000X) contained 18.355 g/L of Fe-EDTA.

Media were changed every 2 to 3 days or new stocks were prepared in fresh media. Plant doubling time was approximately 24 to 36 hr. Both screening and replicate series tests were conducted using non-pyrogenic polystyrene sterile six-well plates (CoStar 3506, Corning Incorporated). Each well contained 4950 µL of the Hoagland's media plus 50 µL of water, or the solvent, or the compound dissolved in the appropriate solvent (at a concentration of 100X). Final concentration of the solvent was therefore approximately 1% by volume. A graphic template of the six-well plates was used for LemnaTec (LemnaTec, Würselen, Germany) image analysis software. Each well was inoculated with two 3-frond plants of the same age (4 to 5 day old) and approximate size and incubated in the Percival incubator as described above.

Plant frond areas were measured at time intervals (0 - 7 days) using LemnaTec image analysis methodology that records frond number, total frond area, as well as color classes that indicate chlorotic or necrotic effect [15].

2.7. Cellular Leakage Tests

Cucumber (*Cucumis sativus* L. Burpee Seeds, Warminster, PA) and maize (*Zea mays* L. Burpee Seeds, Warminster, PA) seeds were sown in flats in Miracle Grow potting soil in a Conviron growth chamber (model E7/2; Winnipeg, Manitoba, Canada) under 173 μmol·s^{-1}·m^{-2} continuous PAR at 26˚C for 6 days and for 30 days, respectively. Using a modification of the method of Duke and Kenyon [16], under dim green light, 4-mm diameter disks were cut from cotyledons of 6-day-old cucumber plants with a cork borer. Similar disks were used with one month-old maize leaves. Fifty disks were placed in polystyrene Petri dishes (6 cm in diameter) with 5 ml of 1 mM MES buffer [2-(4-morpholino) ethane sulfonic acid] supplemented with 2% sucrose (by weight) and were adjusted to pH 6.5 with 1 N NaOH. The MES buffer contained an appropriate amount of diluent or test compound (1, 10, 100, and 1000 μM). Test compounds were in stock acetone solutions that were added directly to the buffer. The final concentration of acetone in all dishes was 1%. Electrical conductivity readings of the solutions were taken with a dip cell and a model 1056 VWR Scientific digital conductivity meter to measure cellular leakage under dim green light at an initial 0 h time. The dishes were then covered with aluminum foil and placed in darkness for a total of 18 h after exposure to the chemical. Another electrical conductivity reading was taken in dim light after the dark period before placing the dishes in the light (200 μmol·s^{-1}·m^{-2} PAR). Readings in the light period were taken at 2, 4, 6, 8 and 10 h. All results were plotted as % changes in conductivity after treatment with test compounds began. The experiment was performed in triplicate. A maximum potential leakage reading was taken for the solution with the same number of cucumber and maize disks boiled for 8 min in MES buffer. SigmaPlot (version 12.0) was used to generate the error bars for the plots.

2.8. HPLC Analysis and Rate of Production of Curvularin *αβ*-Dehydrocurvularin

HPLC analysis was carried out using an Agilent 1260HPLC system coupled to a diode array detector, quarternary pump equipped with Luna 10 micron C18 (2) (250 × 4.6 mm column) with isocratic elution with 40:60 methanol: water; with a flow rate 1 mL/min. In order to understand the rate of metabolite production, time dependent analysis of the fungal broth was carried out. An Erlenmeyer flask (500 mL) containing 250 mL PDB was inoculated with five straw plugs (approximately 0.5 cm diameter) from a PDA plate that has been inoculated previously and grown for 4 days. The flask containing PDB was allowed to grow in a shaker at 120 rpm at 24˚C for 4 days. The liquid culture broth was filtered through sterile miracloth and the filtrate was used to inoculate (5mL in each flask) three 2-L flasks containing 1L of PDB in each flask and was allowed to grow in a shaker at 120 rpm at 24˚C under continuous light. From each flask 2 mL of fungal culture broth was taken under a sterile hood and combined with 2 mL of ethyl acetate and were mixed in a vortex mixer for 10 seconds. From the ethyl acetate layer 1mL of the solution was collected and this procedure was repeated continuously for 18 days. The samples were kept in the freezer until use. For HPLC analysis, 500 μL from the ethyl acetate layer from each sample was transferred to a vial and ethyl acetate was evaporated at 40˚C under reduced pressure. To each sample 200 μL of methanol was added and mixed well. 100 μL from each sample was then used for HPLC analysis. All samples were eluted in the HPLC with 40% methanol in water as described above. Standard curves for curvularin (R^2 = 0.99) and *αβ*-dehydrocurvularin (R^2 = 0.99) were plotted using isolated purified compounds in the concentration ranges of 0.0020 to 0.5 mg/mL.

3. Results and Discussion

The fungus was identified using DNA analysis. Consensus C1823058-20150121028 was aligned with 363 base pairs constructing the neighbor joining tree method with *Drechlera nodulosa* as the outgroup (**Figure 3**). The sequence matched 100.00% to *Curvularia intermedia*.

The ethyl acetate extract of the culture filtrate inhibited germination and growth of dicot and monocot (lettuce and bentgrass) at 1 mg/mL with rankings of 3 and 2, respectively (**Table 1**). TLC of the ethyl acetate extract showed the presence of two major metabolites.

Fractions 3 and 5 of the Biotage column chromatography of the ethyl acetate extract were phytotoxic in the seed germination bioassay with rankings of 4 and 3 for fraction 3 and 3 and 2 for fraction 5 at 1 mg/mL, respectively, for lettuce and bentgrass. Further purification by crystallization of fraction 3 with 4:1 hexane in ethyl acetate afforded 820 mg of *αβ*-dehydrocurvularinas pale yellow crystals. Fraction 5 was crystallized with 1:3 of ethylacetate in hexane to afford curvularin as off white crystals (2.9 g). The identity of these compounds was established by NMR, HRESI mass spectra, and optical rotation. Curvularin and dehydrocurvularin are closely

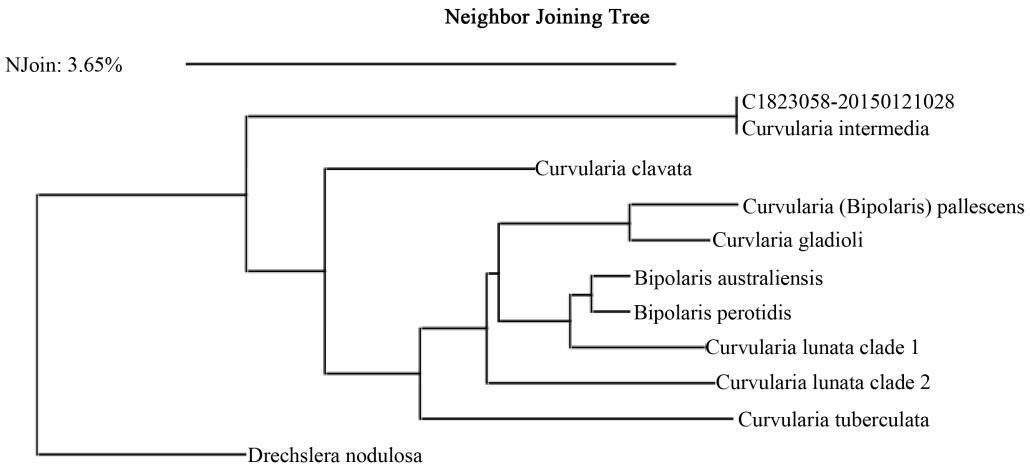


Figure 3. Neighbor joining tree showing the identification of *Curvularia intermedia.*

Table 1. Seed germination and growth bioassay of fungal broth extract, column fractions, curvularin and dehydrocurvularin. The samples were dissolved in10% acetone in water at a concentration of 1 mg/mL. Rankings were done at 7 days after treatment.

Sample ID	Lettuce	Bentgrass
Ethyl acetate extract	3	2
Fraction 3	4	3
Fraction 5	3	2
αβ-dehydrocurvularin (1)	4	4
Curvularin (2)	2	0

related phenolic compounds differing only by the presence of a double bond between C-10 and C-11 in *αβ*-dehydrocurvularin, whereas curvularin has none in the corresponding position. In the seed germination bioassay at 1 mg/mL, *αβ*-dehydrocurvularin was more effective inhibitor towards lettuce and bentgrass, with rankings of 4 and 4, respectively, whereas curvularin had weak activity with rankings of 2 and 0 (**Table 1**). Curvularin and *αβ*-dehydrocurvularin were further tested for effects on seed germination and seedling growth in a dose dependent manner. In this assay *αβ*-dehydrocurvularin had a phytotoxicity ranking of 4 at 1000 µM on both bentgrass and lettuce. In the same assay curvularin had rankings of 3 and 1 on lettuce and bentgrass, respectively (**Table 2**), indicating that curvularrin is more phytotoxic for dicots. Acetylation of dehydrocurvularin gave two products; monoacetyl and diacetyl analogs. These two analogs were less phytotoxic than *αβ*-dehydrocurvularin with rankings of 1 and 3 for dicots and 1 and 1 for monocts respectively (**Table 2**) in the seed germination bioassay.

The fungus *Curvularia intermedia* was isolated from *Pandanus amaryllifolius*, a monocotyledonous plant. The infected leaves showed wilting leading to brownish, dry and brittle leaves. In order to better understand the selectivity of *αβ*-dehydrocurvularin and curvularin on plant types and the mode of phytotoxicity, cellular leakage of cucumber cotyledon and maize leaf disks treated with these two compounds was measured (**Figure 4**). This bioassay can be used to determine light requirements for herbicidal activity and also to see if there is a rapid effect on the plasma membrane of leaf tissues [16] [17]. Solutions with boiled cucumber cotyledon and maize leaf disks in MES buffer were used to measure the maximum possible conductivity reading caused by cellular leakage in each type of leaf tissues. Dehydrocurvularin at 100 and 1000 µM caused leakage in cucumber cotyledon disks in the dark but the rate of leakage was higher, similar to light-dependent leakage caused by ligh-dependent herbicides such as the protoporphyinogen oxidase inhibitor acifluorfen [16]. Maize leaf disks were less sensitive to dehydrocurvularin causing similar leakage only at 1000 µM. Curvularin caused leakage only with cucumber cotyledon disks at 1000 µM, but showed no light dependence. Maize leaf disks showed no significant cellular electrolyte leakage in the presence of curvularin. This result obtained in the electrolyte leakage bioassay

Table 2. Seed germination and growth bioassay ranking of curvularin and dehydrocurvularin and synthetic analogs. The samples were dissolved in 10% acetone in water at a concentration of 1 mg/mL. Rankings were done at 7 days after treatment.

Sample ID	Concentration µM	Lettuce	Bentgrass
αβ-dehydrocurvularin (1)	0	0	0
	1	0	0
	3	1	0
	10	1	0
	33	1	0
	100	1	0
	333	3	3
	1000	4	4
Curvularin (2)	0	0	0
	1	0	0
	3	1	0
	10	2	0
	33	2	0
	100	2	0
	333	2	1
	1000	3	1
Monoacetyl-dehydrocurvularin (3)	10	0	0
	100	0	0
	1000	1	1
Diacetyldehydrocurvularin (4)	10	2	0
	100	2	0
	1000	3	1

indicated that dehydrocurvularin is more selective towards dicots and curvularin is active as a phytotoxin at high concentrations on dicots.

The duckweed bioassay was used to more quantitatively test the phytoxicity of curvularin and dehydrocurvularin (**Figure 5**). At 333 µM, dehydrocurvularin completely inhibited growth of duckweed, and bleaching or chlorosis of the plants was observed. At 100 µM and below dehydrocurvularin was not effective on duckweed. When curvularin was tested under similar conditions, phytotoxic effects were less severe and even at the highest concentration tested (1000 µM), duckweed growth was not completely inhibited (**Figure 5(b)**).

Curvularin is the major metabolite in the fungal culture broth (**Figure 6**), but *αβ*-dehydrocurvularin is the more phytotoxic metabolite. In order to understand the rate of biosynthesis and to determine the optimal time to harvest the culture broth to obtain the active metabolite, a time-dependent study of production of these two metabolites in PDB culture broth was carried out. Production of curvularin and *αβ*-dehydrocurvularin was monitored continuously by HPLC for 18 days after inoculation of PDB culture medium (**Figure 7(a)** and **Figure 7(b)**). Maximum production of *αβ*-dehydrocurvularin occurred at or near day 7, and after day 7 there was a steady decline in concentration of *αβ*-dehydrocurvularin concentration. The maximum production of curvularin occurred at day 10 - 11, whereas the concentration of dehydrocurvularin was at a minimum around 10 - 15 days. From this observation it is not clear if *αβ*-dehydrocurvularin is converted to curvularin or if it is being used or consumed by the fungus to make other metabolites. The TLC of the crude extract of the culture broth indicated the

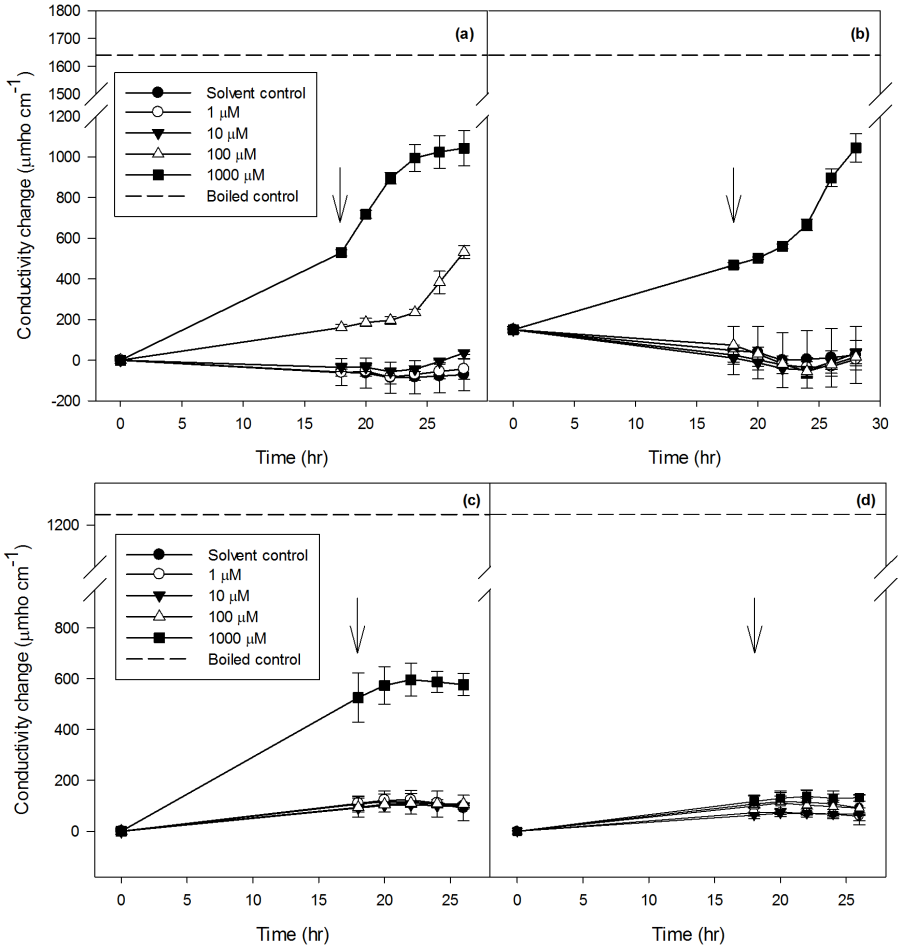


Figure 4. Conductivity changes of the solution of the cucumber cotyledon disks (a) and (c) and maize leaf treated with dehydrocurvularin ((a) and (b) respectively) curvularin ((c) and (d) respectively) at disks varying concentrations under darkness for 18 h, followed by light (arrow). The dotted line represents the maximum leakage value determined by boiled leaf disks in MES buffer. Each treatment was carried out in triplicate. Error bars are ± one standard error of the mean.

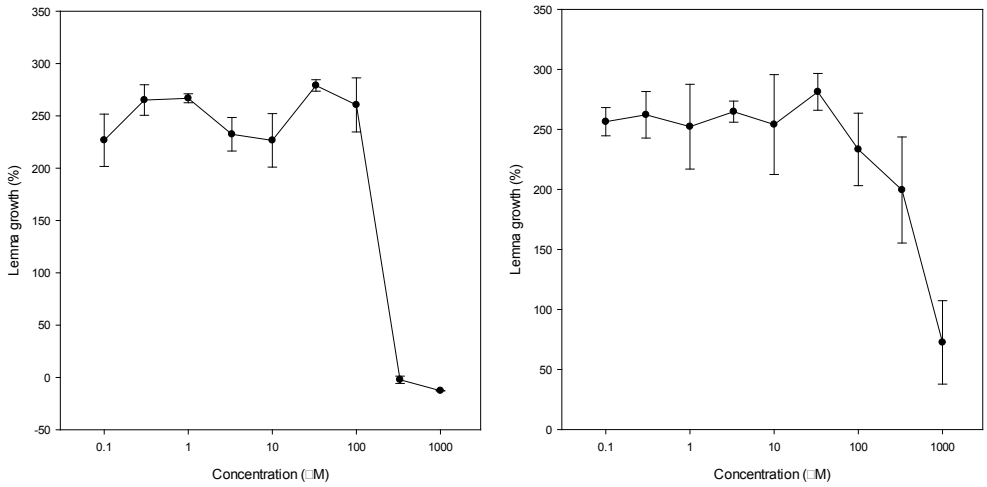


Figure 5. Effect of dehydrocurvularin (a) and curvularin (b) on the growth (% of initial frond area) of duckweed at varying concentrations (control is at 1 μM) after 7 days of exposure. Each treatment was carried out in triplicate. Error bars are ± one standard error of the mean. The LC50 for dehydrocurvularin and curvularin were ~226 and >500 μM, respectively.

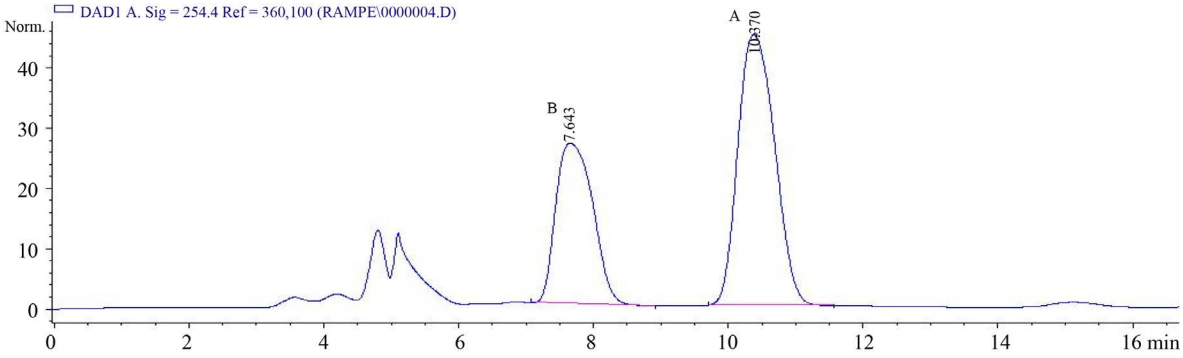


Figure 6. HPLC chromatogram of ethyl acetate extract of culture filtrate of *C. intermedia* monitored at 254 nm. A: curvularin A; B: dehydrocurvularin.

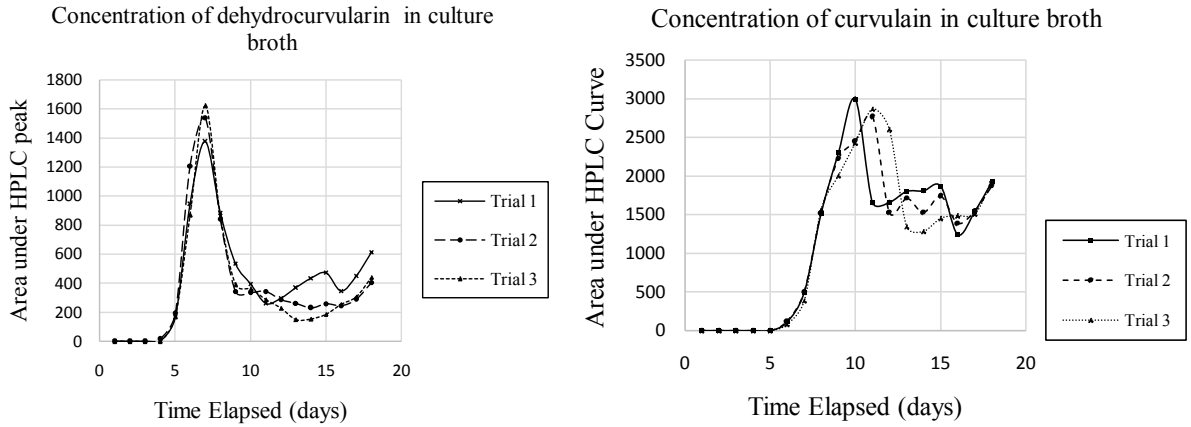


Figure 7. Time dependent production of dehydrocurvularin (a) and curvularin (b) in culture broth.

presence of very polar constituents that would not elute on silica gel. In order to understand the fate of these metabolites more time dependent biosynthetic studies and analyzing the polar constituents will be needed. From HPLC analysis of the ethyl acetate extract of the culture broth the relative concentrations of curvularin and *αβ*-dehydrocurvulari were estimated to be 42% and 12% of the fungal culture broth ethyl acetate extract respectively.

Occurrence of curvularin and dehydrocurvularin has been reported from a variety of microbes such as *Curvularia*, [18] *Penicillium*, [19] *Cochliobolus*, [20] *Altenaria*, [21]-[23] *Aspergillus* [24] and *Nectria* [25] species. Phytotoxicity of both these metabolites has been reported previously [22].

4. Conclusions

In our study, we have shown that *αβ*-dehydrocurvularin is more effective as a phytotoxin than curvularin. The fungus *C. intermedia* has been evaluated as a microbial herbicide for control of *Digitaria sanguinalis* (large crab grass) in crops such as soybean, cotton and peanut [25]. It has also been proposed that the plant pathogenic fungus *C. eragrostidis* can be used as a bioherbicide for control of *Digitaria sanguinalis* [26]. The study conducted by Jiang *et al*. [18] has shown that *αβ*-dehydrocurvularin does not cause necrosis on maize and soybean but causes necrosis on a variety of weeds. Our electrolytic leakage assay on cucumber cotyledon and maize leaf disks indicates that maize leaves are affected by *αβ*-dehydrocurvularin. The difference in the two observations can probably be due to the difference in working with the whole plant versus working with leaf disks. It can also be due to the fact that maize plants need a longer incubation period (>7 days) before the pathogen starts excreting phytotoxins. *αβ*-dehydrocurvularin and curvularin belong to benzenediol lactones which are biosynthesized by iterative polyketide synthase [27] [28]. *αβ*-dehydrocurvularin is a dihydroxyphenylacetic acid lactone or a resorcinolic macrolide with a 12-membered ring with a double bond between C10 and C11. Other than phyto-

toxic activity *αβ*-dehydrocurvularin is known to inhibit heat shock protein 90 (Hsp90) in colon cancer cells and modulates the immune system [29] [30].

In summary, we have shown the phytotoxicity of *C. intermedia* isolated from infected, necrotic leaves *P. amaryllifolius*is apparently due primarily to *αβ*-dehydrocurvularin and to a lesser extent to curvularin. We have also shown that *αβ*-dehydrocurvularin causes plant cell membrane disruption. This is most likely the mode of action of *αβ*-dehydrocurvularin as a phytotoxin. Further field studies and environmental and mammalian toxicity studies should be conducted prior to considering the use of fungal spores or the extracts of the culture medium of *C. intermedia* for weed management.

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