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BIOACTIVITY OF MYCOTOXINS ISOLATED FROM *DRECHSLERA HAWAIIENSIS* M. B. ELLIS

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Abstract

To evaluate *in vitro* phytotoxic effects of metabolites of a fungal species, *Drechslera hawaiiensis*, the fungal species was incubated in minimal medium (M-1-D) and its culture filtrates were collected. In case of laboratory bioassays, the culture filtrates exhibited necrogenic activity as well as discoloration of *Rumex dentatus* L. leaves, an intractable weed all over the world. Toxins from culture filtrates were initially partitioned with chloroform and these were further purified with the help of thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC). Finally these toxins were purified with the help of reversed phase high performance liquid chromatography (RP-HPLC). Mass sSpectroscopy (MS) analysis showed that toxin A had molecular weight 281.2013 and that of toxin B 389.2716. These toxins exhibited bioactivity at minimum concentration of 1.0 and 2.0 mg/ml, respectively. Present study shows the occurrence of natural phytotoxic compounds in *D. hawaiiensis* that can be exploited as natural herbicides to avoid bad effects of commercial herbicides.

Introduction

Nature is full of bioactive compounds, many of these may be used as excellent alternatives to synthetic compounds already in vogue. Although synthetic compounds e.g., commercial herbicides already in use are effective but these are accompanied with so many bad effects like environmental hazards and adverse effects on grassland communities (Reeg *et al.* 2017) and wheat (Skiba *et al.* 2017). Moreover, these herbicides remain in environment and their residues have even more toxic effects (Scherr *et al.* 2017). These problems have compelled scientists to explore alternative sources of bioactive phytotoxic/herbicidal compounds which may be used as natural herbicides against a number of weeds present all over the world. At present, two options are available, either to extract bioactive compounds from plants or microbes (Harding and Raizada 2015, Souza *et al.* 2017).

Microbe-derived compounds have been proven to be good source of natural herbicides. These herbicides have novel mode of actions (MOAs) that can be a useful additions to the current repertoire of commercial herbicide MOAs (Stergiopoulos *et al.* 2013, Dayan and Duke 2014). For example, ethyl acetate extract of a fungus, *Pythium aphanidermatum* (Edison) Fitzp., inhibited seed germination as well as seedling growth of two weeds, *Amaranthus retroflexus* L. and *Digitaria sanguinalis* (L.) Scop. (Zhang *et al.* 2010). Species of *Trichoderma,* namely *T. reesei* EG. Simmons, *T. harzianum* Rifai, *T. pseudokoningii* Rafai and *T. viride* Rers. were also reported to exhibit herbicidal activity against two weeds, *Phalaris minor* Retz. and *R. dentatus*. (Javaid and Ali 2011). Macías-Rubalcava *et al.* (2017) showed that, coriloxine isolated from fungus *Xylaria feejeensis* (Berk.) Fr. exhibited phytotoxic activity against spinach. Their results showed that coriloxine derivatives could work as lead structures for the development of new herbicides.

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The incredible genus *Drechslera* is well known for the discovery of phytotoxic compounds (Jadon *et al.* 2015). Ophiobolin A produced by *Drechslera gigantea* (Heald and FA Wolf) S. lto had been reported to have herbicidal potential (Evidente *et al.* 2006a). This compound exhibited phytotoxic/herbicidal properties against many monocot and dicot weed species belonging to genera viz. *Avena*, *Phalaris*, *Chenopodium* and *Sonchus.* Moreover, initial investigations regarding presence of phytotoxic compounds in various species of *Drechslera* from Pakistan also indicated that *Drechslera* species can be exploited for the discovery of phytotoxic/herbicidal compounds. However, advanced research work pertinent to isolation and characterization of active compounds is lacking. All these *Drechslera* species depicted phytotoxic activity against various harmful weeds of wheat and *Parthenium hysterophorus* L. In pot experiments, crude extracts of all these fungal species indicated to be having herbicidal ingredients (Javaid and Adrees 2009, Akbar and Javaid 2010, Javaid *et al.* 2011). Under these circumstances, the present research work was carried out to characterize phytotoxic constituents from *D. hawaiiensis* that can be used as eco-friendly natural herbicides.

Materials and Methods

An isolate of *Drechslera hawaiiensis,* accession # FCPB-AF-1118, was collected from 1st Fungal Culture Bank, University of the Punjab, Lahore, Pakistan. Minimal growth medium was made by adopting formulation proposed by Evidente *et al.* (2006a). This medium was autoclaved, inoculated with bits of mycelia and spores of *D. hawaiiensis* and kept at 25 ºC. After 28 days of growth, extracts were passed through filter papers. The filtrate obtained in this method was regarded as crude fungal culture filtrate. Crude fungal culture filtrates of *D. hawaiiensis* obtained through filtration were pooled together to yield 4 litre of crude filtrate. This mixture was further evaporated at 45 ºC in a rotary evaporator to give 1.5 litre concentrated crude fungal culture filtrate. This was first defatted with *n*-hexane extraction followed by extraction with chloroform in a separating funnel. Extra solvents were evaporated *in vacuo* using a rotary evaporator (Heidolph) and the fraction obtained was regarded as crude fraction.

In vitro bioassays with crude fraction were performed according to procedures used by Sarpeleh *et al.* (2009) and Akbar *et al.* (2014). Young leaves of *Rumex dentatus* (30 days old plants) were used for evaluating phytotoxic activity of crude fractions. These *R. dentatus* leaves were cut into 1 cm diameter discs, which were then placed on glass slides and these glass slides were placed in Petri plates having water-moistened filter paper. This whole arrangement was made to create mini humid chamber inside each Petri plate so that leaf discs may not dry out during the course of the experiment. Four mg of the crude chloroform fraction was mixed in 100 µl of dimethylsulfoxide (DMSO) and diluted with distilled water (dH_2O) to have 1.0 ml final volume as stock solution. Then serial dilution of this stock solution was carried out with dH_2O to have less concentrated seven solutions of 2, 1,…, 0.0625 mg/ml. *R. dentatus* leaf discs surfaces were inoculated with 15 µl of each of these concentrated solutions. Ten leaf discs were used for each test concentration of crude chloroform extract. The 100 ml DMSO in dH_2O (Final volume raised to 1.0 ml) was treated as positive control. This positive control was likewise serially diluted with dH_2O to prepare subsequent lower concentrations. While distilled water treatment was served as negative control. The inoculated leaf discs within Petri dishes were kept at 25°C in the presence of light in a growth room. Appearance of symptoms (necrotic spot formation and discoloration on leaf discs of *R. dentatus*) was observed after every 8 hrs and final observations were recorded after 72 hrs. Following parameters were used for comparing bioactivities of phytotoxins.

Color scale

0 = No change; **1** = Light change; **2** = Moderate change; **3** = Severe change

Necrotic spot (N.S) scale

A = No N.S.; **B** = N.S. ≤ 1mm; **C** = N.S. ≤ 2 > 1 mm; **D** = N.S. ≤ 3 > 2 mm; **E** = N.S. ≤ 4 > 3 mm; **F**= N.S. ≤ 5 > 4 mm; **G** = N.S. ≤ 6 > 5 mm. mm= millimeters.

Bioactive toxins from crude fraction of *D. hawaiiensis* were isolated by using Thin Layer Chromatography (TLC) and initial purification through Preparative Thin Layer Chromatography (PTLC) and final purification by employing Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). Crude fraction (5 mg) was melted in 1 ml methanol. Elution was made with solvent system (chloroform: 10, ethyl acetate: 6, *n*-hexane: 84) and spots were located under UV light. Two fractions, namely **A** (R_f 0.170), **B** (R_f 0.269), were isolated from chloroform fraction of *D. hawaiiensis.* Compounds separated through silica gel in PTCL were finally eluted by methanol (CH_3OH) and extra CH_3OH was evaporated at 40 °C *in vacuo*. Compounds initially purified by PTLC were finally purified by using RP-HPLC (Lambda-Max, Model 481, LC spectrophotometer), having C_{18} column. Compounds were eluted with solvent system comprising acetonitrile having 0.1% formic acid and deionized water (DIW + 0.1% formic acid). Compounds were eluted by setting the system at gradient elution. Initial ratio of acetonitrile and deionized water (DIW) was adjusted as 10:90 and increased this ratio of acetonitrile to DIW as 100:0. The fractions were evaporated to dryness with continuous clean air flow in a fume hood.

Bioassays with purified toxins were performed by the same method as was employed in case of crude fraction but in this case lower concentrations of purified compounds were used and synthetic phytotoxic compound, 2,4-D was used to compare the potency of isolated purified compounds with synthetic one. Stock solutions of purified compounds were made by dispersing the compound in DMSO @ 2 mg/ml of various compounds. Subsequent concentrations having lower quantity of purified compounds/toxins viz. 1, 0.5, …, 0.03125 mg/ml were prepared by adding dH_2O. Positive control received DMSO at different concentrations. Treatment with pure dH_2O was utilized as a negative control. Symptoms (necrotic spot and discoloration of leaf discs) were finally recorded after 72 hrs with continuous observations for the appearance of symptoms after every 8 hrs.

Characterization of the purified toxin was made with the help of Mass sSpectrometry employing Low Resolution Electron Spray Ionization Mass Spectroscopy (LRESIMS) as well as High Resolution Electron Spray Ionization Mass Spectroscopy (HRESIMS). LRESIMS and HRESIMS spectra were recorded on a Mariner^{TM} Biospectrometry Work station by Perseptive Biosystems. Initially, phytotoxicity measurements with crude fraction/toxins were conducted. Positive reaction showing necrogenic activity and color change at leaf discs/sections of test weed plant was recorded after 72 hrs with regular observations after every 8 hrs.

Results and Discussion

In case of crude fraction, necrotic spots were observed on leaf discs of *R. dentatus* at 2.0 mg/ml concentration. Severe discoloration was also recorded at minimum concentration of 0.5 mg/ml and higher concentrations. Leaf discs inoculated with DMSO as positive control exhibited only light discoloration, while there was no effect of distilled water used as negative control (Table 1). Previously in pot experiments this effect of crude toxins had also been reported by Akbar and Javaid (2013), where crude toxins were found effective in arresting the growth of *R. dentatus* plants, but those toxins had not been isolated and characterized.

In case of phytotoxicity measurements with purified compound bioassays, two purified compounds viz. compound **A** and compound **B** from crude fraction were found potent in creating necrotic spot on leaf discs of *R. dentatus* leaf surfaces. Compound **A** was found to be active at minimum concentration of 1.0 mg/ml, while compound **B** was found to be active at the

concentration of 2.0 mg/ml only. Lower concentrations of both these compounds were found inactive in producing necrotic spots but they were able to cause discoloration at leaf discs @ 0.0625 mg/ml. In case of bioassays carried out by inoculating leaf disc with synthetic herbicide, 2,4-D, maximum size of necrotic spot was observed at lower concentration of 0.25 mg/ml. Discoloration of leaf discs was much pronounced in case of fungal isolated compounds, in contrast to 2,4-D, where only light discoloration was observed, even at highest concentration of 2.0 mg/ml (Table 2). These results are in agreement with earlier reports made where purified toxins from many species of *Drechslera* exhibited phytotoxic activity when applied on the surface of many weed species (Evidente *et al.* 2006 a,b, Akbar *et al.* 2014).

Table 1. Phytotoxic effect of crude fractions of *D. hawaiiensis* on leaf discs of *Rumex dentatus*.

DMSO conc. (µl/ml)	DMSO effect		Organic fraction conc. (mg/ml)	Effect of crude fractions	
				Chloroform	
	Color	Necrotic spot		Color	Necrotic spot
Water	0	A	Water	0	A
1.560	0	A	0.0625	1	A
3.125	1	A	0.1250	2	A
6.250	1	A	0.2500	2	A
12.500	0-1	A	0.5000	2-3	A
25.000	0-1	A	1.0000	3	A
50.000	0-1	A	2.0000	3	B
100.000	0-1	A	4.0000	3	D

Color scale: 0 = No change; 1 = Light change; 2 = Moderate change; 3 = Severe change,
Necrotic spot (N.S.) scale: A = No N.S.; B = N.S. ≤ 1mm; C = N.S. ≤ 2 > 1mm; D = N.S. ≤ 3 > 2 mm;
E = N.S. ≤ 4 > 3 mm; F = N.S. ≤ 5 > 4 mm; G = N.S. ≤ 6 > 5 mm, mm = millimeters.

Table 2. Phytotoxic activity of purified compounds from *D. hawaiiensis* on leaf discs of *Rumex dentatus*.

DMSO conc. (µl/ml)	DMSO effect		2,4-D/ compound conc. (mg/ml)	Effect of 2,4-D		Effect of purified compounds			
						A		B	
	Color	N.S		Color	N.S	Color	N.S	Color	N.S
Water	0	A	Water	0	A	0	A	0	A
0.780	0	A	0.0312	0-1	A	0	A	0	A
1.560	0	A	0.0625	0-1	A	0-1	A	0-1	A
3.125	0	A	0.1250	1	A	1	A	1	A
6.250	0	A	0.2500	1	C	1-2	A	1	A
12.500	0-1	A	0.5000	1	D	2	A	1	A
25.000	0-1	A	1.0000	1	G	2-3	D	1-2	A
50.000	0-1	A	2.0000	1	G	3	E	2	D

Color scale: 0 = No change; 1 = Light change; 2 = Moderate change; 3 = Severe change
Necrotic spot (N.S.) scale: A = No N.S.; B = N.S. ≤ 1mm; C = N.S. ≤ 2 > 1 mm; D = N.S. ≤ 3 > 2 mm; E = N.S. ≤ 4 > 3 mm; F= N.S. ≤ 5 > 4 mm; G = N.S. ≤ 6 > 5 mm,. mm = millimeters.

Mass spectrometry of chromatographic fraction/compounds **A** and **B** was carried out. Chromatographic fraction **A** was isolated as brown oil and its molecular weight was observed by LRESIMS, depicting peak at $M^+ + H^+$ 289.2. HRESIMS showed molecular weight equal to 289.20123. Chromatographic fraction **B** was isolated as white powder. Its molecular weight was observed by LRESIMS, showing peak at $M^+ + H^+$ 389.3. HRESIMS showed molecular weight equal to 389.27316. Previously some phytotoxic compounds were isolated from different species of *Drechslera* (Evidente *et al.* 2006 a, b). Three phytotoxins designated as drechslerol-A, B and C were identified from *Drechslera maydis* (Y. Nisik. & C. Miyake) Subram. & BL. Jain, a serious pathogen of maize crop. Drechslerol-A and C created necrotic spots at leaves of *Costus speciosus* (J. Koenig) Sm. at 1.6×10^{-4} M and 2.85×10^{-5} to 2.28×10^{-4} M concentration, respectively (Shukla *et al.* 1987, 1989, 1990). Drazepinone, another phytotoxic/herbicidal compound was identified from *Drechslera siccans* (Drechsler) Shoemaker (Evidente *et al.* 2005). Kenfield *et al.* (1989) had reported that curvulin and O-methylcurvulinic acid also showed phytotoxic activities on leaves of *Portulaca oleracea* L. and spiny amaranth. These compounds isolated from *Drechslera indica* (JN. Rai, Wadhwani & JP. Tewari) Mouch. exhibited necrogenic activity on leaves of these weed species. Recently, Akbar *et al.* (2014) identified a natural phytotoxic/herbicidal compound named as holadysenterine, from *Drechslera australiensis* MB. Ellis. From the present study it may concluded that *D. hawaiiensis* has phytotoxic compounds that can be used as natural herbicides.

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