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RESEARCH ARTICLE

Optimization of *Myrothecium roridum* tode: fries phytotoxin production and bioactivity on water hyacinth (*Eichhornia crassipes*)

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ABSTRACT

Phytotoxin production by *Myrothecium roridum* Tode: Fries (IMI 394934) and *in vitro* bioactivity on water hyacinth leaves (WHL) were evaluated. Phytotoxin produced caused necrosis of WHL three days postapplication. Potato carrot broth and WHL broth formulations gave the highest phytotoxin production in culture. Phytotoxin production was light-dependent and peaked at pH 4.5. Xylose and glutamine were the best carbon and nitrogen sources, respectively. The phytotoxin was thermostable, and phytotoxicity was pH-dependent, but photoperiod-independent. Bioassay-guided fractionation indicated the active phytotoxin was roridin A. Phytotoxicity of roridin A and roridin E was similar ($p > 0.05$) to paraquat used for water hyacinth control.

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Herbicide; *Myrothecium roridum*; phytotoxin; paraquat; roridin; water hyacinth

Introduction

Water hyacinth plants have a substantial impact to the environment and socio-economic issues in the parts of the world where they occur (Mailu 2001, Villamagna and Murphy 2010, Dutta and Ray 2017). The plant forms dense impenetrable mats that interfere with navigation, fishing, irrigation, recreational use of water and power generation (Dutta and Ray 2017). Such mats provide ideal breeding conditions for mosquitoes and other waterborne disease vectors, they can impact water quality and block drainage canals (Mailu 2001); causing flooding and loss to human life and properties.

Conventional control measures such as mechanical harvesting and synthetic chemical herbicides are expensive, when applied on a large scale in a developing country (Bateman 2001), and their inherent environmental and health concerns have led to renewed interest in biocontrol of weeds. Successful biocontrol of water hyacinth using microorganisms has been reported (Charudattan 2001), but never used on a wide scale. A notable achievement in the management of water hyacinth was reported using strains of the fungus *Myrothecium roridum* alone or in combination with either one of the insects that have been used for biocontrol (*Neochetina eichhornia*) or with the chemical herbicide (2,4-D) in integrated control studies (Ponnappa 1970; Hettiarachchi *et al.* 1983; Liyanage and Gunasekera 1989; Caunter and Mohamed 1990). It is well-established that changes in climatic conditions have the potential to alter the incidence and severity of infectious plant diseases (Chakraborty *et al.* 2000; Eastburn *et al.* 2011). Thus, unlike chemical herbicides, which give similar weed control under different climatic conditions, use of a particular biocontrol strain of microorganism may be limited to regions of the world where environmental factors are similar to those of the region in which it was isolated, because of reduced virulence under other conditions.

One potential alternative to synthetic chemical herbicides in weed control is the use of phytotoxins (Saxena and Pandey 2001; Cimmino *et al.* 2015; Vurro *et al.* 2018). Phytotoxins play a role in host-pathogen interactions through disease expression evidenced by various symptoms such as necrosis, chlorosis, wilting, water soaking and eventually the death of plants (Strobel 1982, Amusa 2006). It is well documented that microbial phytotoxins often act as virulence factors for plant pathogenesis (Strobel 1974, Baker *et al.* 1997, Feys and Parker 2000). Some microbial

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phytotoxins can even act as surrogates for the pathogen itself (Baker *et al*. 1997, Amusa 2006). Most phytotoxins act by modifying physiological and biochemical processes of plant metabolism, but others may be directly toxic to plant tissues (Strobel 1974, Baker *et al*. 1997, Amusa 2006).

There is growing industrial interest in extracting phytotoxins for use as herbicides, rather than using the producer microorganism with its inherent problems of sensitivity to climate and other biotic and abiotic conditions (Strobel 1991), or using synthetic chemicals, which may have greater environmental and health concerns (Saxena and Pandey 2001, Vurro *et al*. 2018). *In vitro* production methods have been reported (Abbas *et al*. 1991, Abbas and Boyette 1992, Amusa 2006) for several phytotoxins produced by plant pathogenic fungi. We previously reported a new strain of *Myrothecium. roridum* Tode: Fries (IMI 394934) that is highly pathogenic to water hyacinth (Okunowo *et al*. 2013) and showed that aspects of the pathology is mediated by a toxin (Okunowo *et al*. 2011). The present study was initiated to determine the optimum conditions for the production of phytotoxin by the strain of *M. roridum* in culture, to characterize its phytotoxic action on water hyacinth and, if possible, to identify the phytotoxin.

Materials and methods

Materials and fungal culture

Roridin A, verrucarol and verrucarin A from *Myrothecium* spp. (purity $\geq$98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Roridin E, epi-isororidin E and roridin H were provided by the Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA. Diacetoxyscirpenol (DAS) and deoxynivalenol (DON) were purified in this laboratory from *Myrothecium* sp., culture code number: MVZ8. The commercial herbicide glyphosate was purchased from Zhejiang Xinan Chemical Industrial Group Co., Ltd (Jiande, China), and paraquat from Nanjing Red Sun Biochemical Co., Ltd. (Nanjing, Jiangsu, China). All culture media components were analytical grade from Sigma (Sigma Co., United Kingdom). This study used *M. roridum* (IMI 394934) isolated from water hyacinth in Lagos State, Nigeria (Okunowo *et al*. 2013) and deposited with the Centre for Agriculture and Bioscience International (CABI), Egham, Surrey, UK. *M. roridum* was cultured in the following media: potato carrot broth (PCB: potato, 20 g; carrot, 20 g; distilled water, 1 liter); malt extract broth (MEB: malt extract, 20 g, distilled water, 1 liter); potato dextrose broth (PDB: potato, 200 g; dextrose, 20 g; distilled water, 1 liter); potato sucrose broth (PSB: potato, 200 g; sucrose, 20 g; distilled water, 1 liter); Sabouraud broth (SB: peptone, 10 g; glucose, 40 g; distilled water, 1 liter); Czapek Dox broth (ZDB: $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 10 mg; sucrose, 30 g; distilled water, 1 liter); and water hyacinth leaf broth (WHB: water hyacinth leaves homogenized in a blender for 3 min, 145 g; and distilled water, 1 liter) was also formulated. All the prepared culture media were filtered through two layers of cheese cloth, supplemented with ampicillin (250 μg/ml) and autoclaved 20 min at 121 °C as 50 ml aliquots in 250 ml conical flasks.

Extraction and identification of Myrothecium roridum toxin

Phytotoxin production by the *M. roridum* isolate and extraction was carried out according to Charudattan and Rao (1982). Briefly, two 10 mm diameter agar medium plugs with *M. roridum* mycelium were used to inoculate a 1-liter Erlenmeyer flask containing 250 ml potato dextrose broth (PDB). The culture was grown in the laboratory for 3 weeks under a 12-h light/dark cycle at 25 ± 2 °C. The culture fluid was filtered through eight layers of cheesecloth, centrifuged at 3500×*g* for 15 min, filtered through Whatman No. 1 filter paper then through a 0.45-μm membrane filter. The culture fluid was diluted with sterile distilled water as appropriate, then used for phytotoxicity assays. Filtered culture extract was also lyophilized (MicroModulyo freeze dryer, model: MICRO MODULYO-230, Thermo Savant, Holdbrook, NY) and stored in an airtight container for use in chromatographic analysis/characterization of the active principle.

To identify the bioactive component of the culture extract, the lyophilized sample was extracted three times with 100 ml $CHCl_3$:MeOH (1:1). The extracts were combined and the solvent evaporated in a rotary evaporator. A sample (10 mg) of the extract residue was dissolved in 1 ml of methanol and fractionated by preparative thin-layer chromatography (TLC) on precoated silica gel TLC plates (0.25 mm thick; Sigma Co. United Kingdom) using two solvent systems for development, EtOAc: MeOH (9:1) (EM) and EtOAc: MeOH: Water: Acetic acid (10:1.7:1.3:0.2) (EMWA). Extract components were detected using UV light at 254 nm and 366 nm (UV lamp model 80286, Desaga, Heidelberg, Germany) and vanillin spray (Bean *et al*. 1984).

The extract was also subjected to flash chromatography on a 20 g column of 2–25 μ silica gel, eluted

first with 30 ml of CH_2Cl_2 followed by 50 ml of 10% MeOH in CH_2Cl_2 and finally by 50 ml of 20% MeOH in CH_2Cl_2 (Bean *et al*. 1984). Each fraction was evaporated and the residues fractionated by preparative thin-layer chromatography as described above except two additional development solvent systems were used, MeOH:CH_2C1_2 (8:92) (MD) and MeOH:CH_2C1_2:hexane (4:92:4) (MDH). The best resolving solvent was used to isolate each subfraction, which was assayed for phytotoxicity on water hyacinth detached leaves. Purity of the isolated phytotoxin was demonstrated by analytical thin-layer chromatography on silica gel. The purified phytotoxin preparation was tested to determine if it was a trichothecene using commercial ELISA kits for trichothecenes (Envirologix, Portland, Maine USA) as previously described (Hoagland *et al*. 2008). The trichothecene content of the purified phytotoxin preparation was determined with each ELISA trichothecene kit. The purified phytotoxin preparation was also compared to a mixture of standard trichothecenes by analytical thin-layer chromatography on silica gel.

The effect of light on toxin production and phytotoxicity of M. roridum toxin on water hyacinth detached leaves

Flasks containing 250 ml potato sucrose broth were inoculated with one 10 mm diameter agar medium disk covered with *M. roridum* mycelium. Stationary cultures were incubated 3 weeks at 25 ± 2 °C under continuous illumination from two 40 watt fluorescent tubes at an approximate distance of 40 cm (Duarte and Archer 2003). Flasks set up in the same way were incubated under darkness. Culture media were filtered as described earlier, and the filtrates stored at 5 °C in sterile bottles. For phytotoxicity assay, healthy water hyacinth plants were obtained from Badagry Creek and Ogun River in Lagos State, Nigeria. Leaves were excised and placed on moist cotton batting in a Petri plate (one per leaf). Ten microlitre of dilute (50%v/v) *M. roridum* culture extract was applied with the aid of a micropipette to 6 to 7 cm^2 upper leaf surfaces along a punctured site per leaf. Entire leaves were incubated for 72 h under a 12 h normal laboratory light conditions at 25 ± 2 °C. Uninoculated, filtered broth (10 µl) served as a negative control. Phytotoxicity was determined based on visual assessment of necrosis using a six-point graded scale and data was transformed to give an average severity index (ASI) (Duarte and Archer 2003). The ASI was derived from the following foliar symptom scale of 0–5, in which no symptoms = 0, 1–10% of foliage with necrosis = 1, 11–30% = 2, 31–70% = 3, 71–90% = 4, and 91–100% = 5. The disease scale was transformed to ASI using the formula: $ASI = \sum(\text{nki})/\text{N}$, where n = number of detached leaves corresponding to each score, i = symptom scale (i = 0 to 5). N = total number of inoculated leaves and k = summation index. The design of experiments for the optimization of cultural conditions (varying medium type, pH, carbon and nitrogen source) for phytotoxin production was one-factor-at-a-time (OFAT) approach. Each experimental treatment was conducted in six replicates and repeated twice.

The effect of culture medium pH and culture medium type on M. roridium toxin production

The effect of pH on toxin production was studied by growing *M. roridum* on potato sucrose broth with the pH adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 prior to sterilization. The pH was adjusted by adding drops of 1 M NaOH or 1 M CH_3COOH solution (Duarte and Archer, 2003).

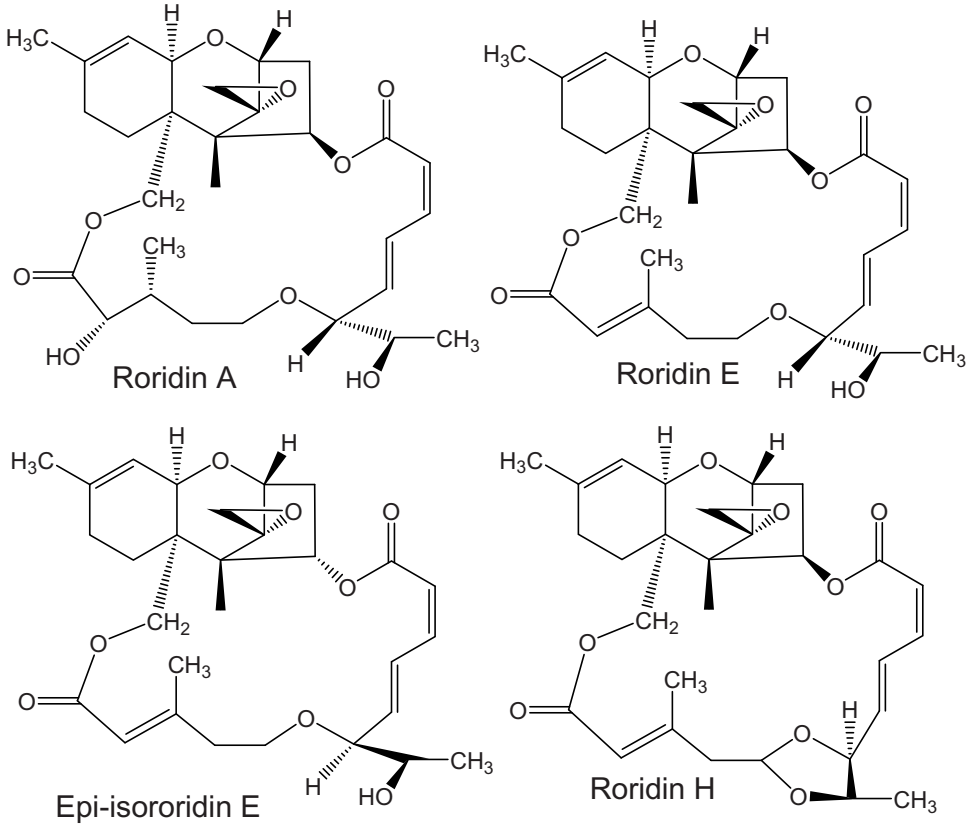
Aliquots (250 ml) of seven types of medium (PSB, PDB, SB, MB, PCB, ZDB and WHB) were placed in 1-L Erlenmeyer flasks and the pH adjusted to 5.5 prior to autoclaving. Each flask was inoculated with one 10 mm diameter agar plug covered with *M. roridum* mycelium. Stationary flasks were incubated at 25 ± 2 °C under continuous illumination for 3 weeks. Culture filtrates were tested at concentrations of 1%–100% (v/v) in water for phytotoxicity in a detached water hyacinth leaf bioassay. Uninoculated culture media adjusted to the respective pH values were used as a control. Toxicity of culture filtrates was measured as ASI (Barbosa *et al*. 2002).

The effect of carbon and nitrogen source on toxin production

Czapek-Dox broth was prepared in 1-L Erlenmeyer flasks (250 ml, pH 5.5) unaltered and with either 30g/L of fructose, glucose, xylose, maltose, galactose or lactose substituted for sucrose or with nitrogen-containing salts (NH_4NO_3, NH_4Cl, Na-glutamate, glutamine, yeast extract, and urea) substituted for $NaNO_3$. Each flask was inoculated with one 10 mm diameter agar plug covered with *M. roridum* mycelium. The cultures were grown for 3 weeks under a 12 h photoperiod at 25 ± 2 °C. Phytotoxicity of the cell-free extracts obtained was measured as Average Diameter of Necrosis with 10 µl of 70% culture filtrate.

Effect of light, pH and heat on the phytotoxicity of the M. roridum toxin

To examine the effect of light on phytotoxicity of the *M. roridum* toxin on water hyacinth leaves, 10 µl aliquots of a range of concentrations in water (2%, 1%, 0.5%, 0.25%, 0.02% and 0.01%, w/v) of nonautoclaved lyophilized culture filtrate were applied to the adaxial surface of punctured healthy water hyacinth leaves placed on moist cotton batting in Petri dishes. One set of treated leaves was incubated at 25 ± 2 °C under



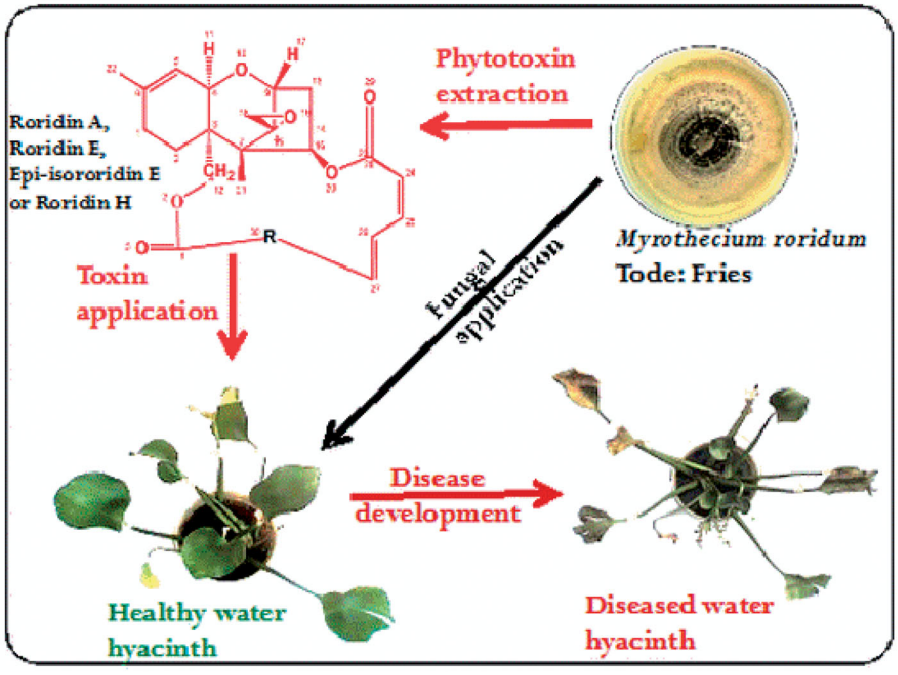


Figure 1. Structures of phytotoxic mycotoxins used in water hyacinth leaves bioassay.

12-h light/dark photoperiod, and a second set of plates was incubated in darkness.

The effect of pH on phytotoxicity was assessed with PDB culture filtrate diluted to 50% strength in 0.1 M KH_2PO_4 and K_2HPO_4 buffers of pH 4–7.5. Freshly prepared buffer solutions diluted with equal volumes of distilled water were used as a control to correct for buffer interference.

To determine heat stability of the phytotoxin from *M. roridum*, culture filtrates were produced using the following media: MEB, PCB, PDB, WHB, SB and ZDB. Each culture filtrate was diluted with water to a range of concentrations (100%, 70%, 50%, 10% and 1%, v/v), and half of each was autoclaved at 121 °C for 20 min. Aliquots (10 µl) of autoclaved and nonautoclaved culture filtrates were used in phytotoxicity assay. The experimental design for the factors (light, pH and heat) affecting the bioactivity of toxin produced was one-factor-at-a-time (OFAT). All treatments were conducted in six replicates and phytotoxicity was recorded as ASI. The experiments were repeated twice.

Phytotoxicity of M. roridum culture filtrate on detached leaves from various plants

Healthy leaves from 26 species of randomly selected plants (Table 4) cultivated in the field were used in the phytotoxicity assay described above, except that the surfaces of leaves were punctured with needles. Culture filtrates from *M. roridum* grown in potato dextrose broth was diluted with water to 50% (v/v) and aliquots (10 µl) applied to the adaxial surface of leaves punctured with needles. After 72 h incubation in a moist Petri dish at 25 ± 2 °C, toxicity of the extract was recorded as ASI. The experiments were conducted in six replicates for each plant and repeated twice.

Phytotoxicity assays using roridin standards on water hyacinth detached leaves

The phytotoxicity of roridin pure standards (Figure 1) and controls was determined *in vitro* in the water hyacinth leaf bioassay described above. Roridin A, roridin E, epi-isororidin E and roridin H were dissolved in 0.5% dimethyl sulfoxide (DMSO) at 500 µg/ml. Two commercial herbicides, glyphosate and paraquat, dissolved in 0.5% DMSO at 500 µg/ml served as positive controls, while 0.5% DMSO served as negative control. Phytotoxicity of roridin A, roridin E, glyphosate and paraquat was compared over the dose range 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml and 7.81 µg/ml. Phytotoxic activity was determined as a measure of diameter of necrotic zone. The diameter of necrosis is the average of the length of necrotic zone and width of necrotic zone measured in millimeter across point of toxin infiltration. Six replicate plates were set up for each assay. The experiments were repeated twice.

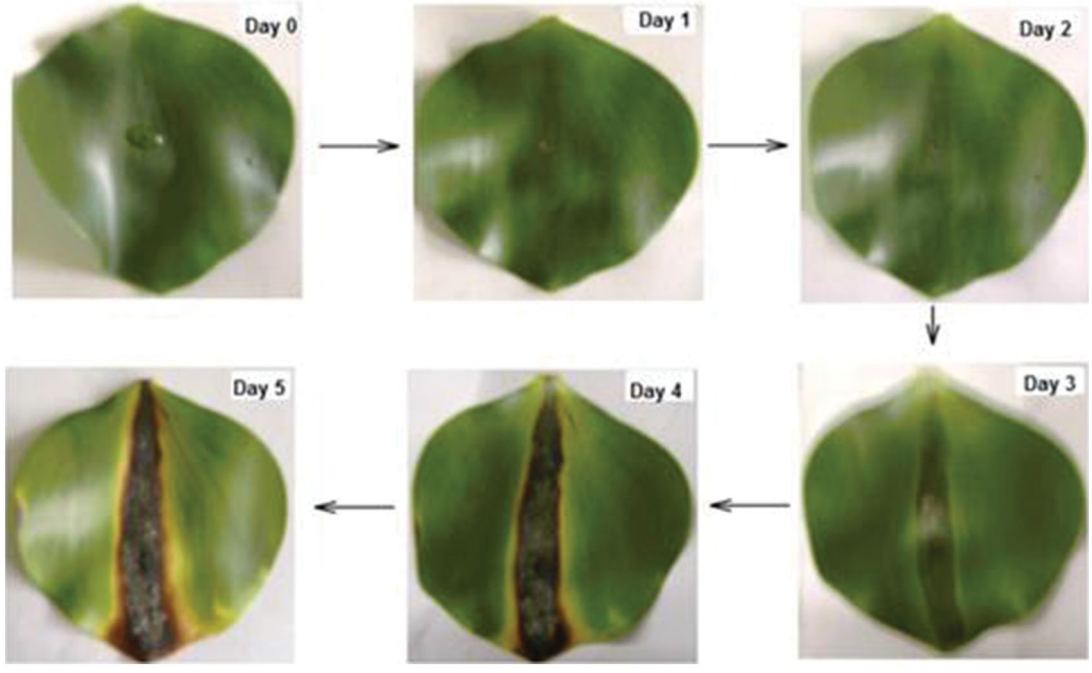


Figure 2. Lesion development on punctured water hyacinth leaves after application of *M. roridum* culture filtrate to the adaxial surface.

Table 1. Phytotoxicity of *M. roridum* culture filtrate produced in potato sucrose broth at different pH values 72 h after inoculation of water hyacinth leaves[a].

	pH of potato sucrose broth							
Toxin Concentration	4.5	5	5.5	6	6.5	7	7.5	LSD (0.05)
100%	2.33 ± 0.21	2.33 ± 0.33	2.00 ± 0.26	2.00 ± 0.26	2.00 ± 0.26	2.00 ± 0.37	0.50 ± 0.22	
70%	5.00[b] ± 0.00	3.00[c] ± 0.26	3.50[c] ± 0.22	4.50[b] ± 0.22	3.50[c] ± 0.22	4.50[b] ± 0.22	3.33[c] ± 0.33	0.61
50%	4.67 ± 0.21	2.67 ± 0.21	3.00 ± 0.26	3.00 ± 0.26	3.00 ± 0.37	3.00 ± 0.00	3.00 ± 0.26	
30%	3.00 ± 0.00	2.00 ± 0.26	3.00 ± 0.26	3.00 ± 0.37	2.00 ± 0.26	2.33 ± 0.21	2.67 ± 0.21	
10%	2.00 ± 0.26	1.50 ± 0.22	2.50 ± 0.22	2.00 ± 0.26	2.67 ± 0.33	2.33 ± 0.33	1.67 ± 0.21	
1%	1.67 ± 0.42	0.67 ± 0.21	2.50 ± 0.22	2.00 ± 0.26	0.00 ± 0.00	0.50 ± 0.22	1.50 ± 0.22	

[a]Data represent Average Severity Index (ASI) ± SEM ($n = 6$) derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%.
[b,c]Phytotoxicity of 70% culture filtrates were significantly different when subjected to ANOVA and *post hoc* Tukey's Multiple Comparison Test ($p < 0.05$). LSD, Least significant difference.

Statistical analyses

Experiments were performed with six replicates per treatment. Mean differences in phytotoxin production by various treatments (effect of varying pH, light, media, carbon and nitrogen source) were compared using one way analysis of variance (ANOVA). When significant differences existed among treatments, *post hoc* Tukey tests were employed to compare treatments. The effects of heat, light, herbicide type and concentration on phytotoxicity were examined by two-way analysis of variance with Bonferroni post-test analysis using with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) defining significance as $p < 0.05$. When ANOVA was significant at $p < 0.05$, least significant difference (LSD) was calculated to show where specific difference exists in data (Williams and Abdi 2010).

Results and discussion

Phytotoxin production by M. roridum isolate and identification

Culture filtrates from the *M. roridum* isolate IMI 394934 produced necrotic lesions at the site of application to water hyacinth leaves. Over the 5-day incubation period necrotic lesions extended on the leaves in both the directions of the vascular system and perpendicular to it (Figure 2). The necrotic lesions were similar to those produced by the fungus on water hyacinth leaves after a natural infection.

To identify the phytotoxic metabolite, crude toxin extract was fractionated on TLC plates. Only one fraction from each of the TLC plates developed in MD solvent caused necrosis on the leaves of water hyacinth similar to that caused by the fungus and the crude toxin. The fraction and roridin A standard gave a single band on TLC at $R_f = 0.51$ that reacted positively to vanillin-sulfuric acid spray reagent. The purified phytotoxic compound tested positive for trichothecene in ELISA analysis. A total of 293 mg culture extracts yielded 1.2 mg purified white crystalline powder of roridin A. The observation that detectable phytotoxicity was associated with only a single band on TLC is consistent with roridin A being the only phytotoxin produced by the isolate of *M. roridum*, but other phyotoxins, including other trichothecenes, might have been present in amounts below the limits of detection.

Effect of light on phytotoxin production by M. roridum

Toxin production by cultures of *M. roridum* was affected by illumination. The filtrate from cultures in PDB incubated under continuous illumination for 3 weeks contained an appreciable amount of phytotoxin (4.00 ± 0.26 ASI), whereas the filtrate from cultures incubated in the dark did not exhibit phytotoxicity in the water hyacinth leaf bioassay test. Phytotoxin production also occurred under 12 h light/dark cycle over a period of 3 weeks. Phytotoxicity was initially observed as dark spots 24 h after application of culture filtrate to the leaves of water hyacinth, which later turned necrotic at the point of application. The necrotic spot further extended both transversely and longitudinally across the whole leaf. Because the foliar symptoms resulting from applying culture filtrates were similar to those induced by the fungus itself, a phytotoxin produced by the fungus is implicated in the disease (Strobel 1982). *M. roridum* isolated from muskmelon fruit *(Cucumis melo)* was able to produce toxin in culture in the laboratory under ambient light conditions in a similar manner (Bean *et al.* 1984).

Effect of pH on phytotoxin production

Phytotoxin was not produced in culture medium adjusted to pH 4, but production was observed in

Table 2. Phytotoxicity of non-autoclaved culture filtrates of *M. roridum* grown in seven media, 72 h after inoculation of water hyacinth leaves[a].

	Media Types						
Toxin Concentration	MEB	PCB	PDB	PSB	WHB	SB	ZDB
100%	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.21	1.00 ± 0.26	1.67 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
70%	**3.00[b] ± 0.37**	**3.33[b] ± 0.21**	**3.00[b] ± 0.26**	**1.67[c] ± 0.21**	3.00[b] ± 0.26	**2.67[b,d] ± 0.21**	**2.33[d] ± 0.33**
50%	2.67 ± 0.33	2.67 ± 0.21	2.33 ± 0.21	1.33 ± 0.21	**3.33[b] ± 0.33**	2.00 ± 0.26	1.67 ± 0.33
10%	2.67 ± 0.33	2.00 ± 0.26	0.33 ± 0.21	0.00 ± 0.00	2.6 ± 0.21	1.33 ± 0.21	0.33 ± 0.21
1%	2.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 0.21	1.00 ± 0.26	0.00 ± 0.00

[a]Data are presented as Average Severity Index (ASI) ± SEM ($n = 6$) values derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with chlorosis and/or necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%. Data in bold are maximum ASI obtained per medium.
[b,c,d]Phytotoxicity of 70% culture filtrates were significantly different when subjected to ANOVA and *post hoc* Tukey's Multiple Comparison Test ($p < 0.05$).

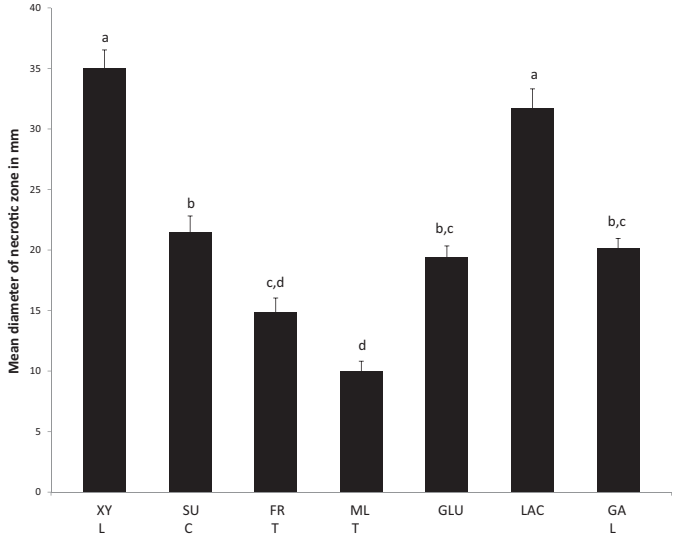


Figure 3. Effect of carbon source on the production of phytotoxin in culture by *M. roridum*. Phytotoxicity on water hyacinth leaves 72 h after application of *M. roridum* culture filtrate derived from Czapek Dox broth media containing the following carbon sources: xylose, (XYL), sucrose (SUC), fructose (FRT), maltose (MLT), glucose (GLU), lactose (LAC) or galactose (GAL). Values with the same letter are not significantly different ($p > 0.05$) ($n = 6$, means ± SEM) when subjected to one-way ANOVA and Tukey *post hoc* test. LSD, Least significant difference = 3.53.

culture medium adjusted to a pH range of 4.5 to 7.5. The ASI of the toxin produced at each pH range increased after the culture filtrate was diluted and maximum ASI was reached at 70% culture filtrate in water (Table 1). At this concentration, phytotoxicity varied significantly over the pH range ($F_{6,\ 36} = 12.68$, $p < 0.0001$). The ASI of the toxin produced at pH 4.5 was significantly greater than that obtained at pH 5.0, 5.5, 6.5 and 7.5 ($p < 0.001$). The bioactivity initially increased linearly for diluting from 100% to 70% culture filtrate and subsequently decreased with increasing dilution; this effect did not significantly differ for phytotoxin produced at different pH values ($F_{6,\ 28} = 0.5$, $p = 0.84$). Other fungi have also been reported to have an optimum culture medium pH value for toxin production; for example *Fusarium solani* f. sp. *piperis*, *Verticillium dahlia*, and *Curvularia lunata* have been reported (Nachmias *et al*. 1987, Duarte and Archer 2003) to have optimum toxin production at pH 6.0, 6.7 and 7.0, respectively.

Effect of culture media on phytotoxin production

The phytotoxic activity in each culture filtrate was significantly ($p < 0.05$) higher after dilution to 70% with distilled water for all media types tested (Table 2), with no significant difference in the dilution effect between various medium types tested ($F_{6,\ 21} = 0.61$, $p > 0.05$), except that 50% dilution was most active for PCB and WHB (Table 2). Peak phytotoxin production was significantly lower in PSB ($p < 0.001$) and SB or ZDB ($p < 0.05$). ZDB has been reported to support lower production of a phytotoxic metabolite by *Fusarium solani* (Duarte and Archer 2003). PCB and WHB may be optimal for phytotoxin production by *M. roridum* because they contain complex plant materials. Some fungi may

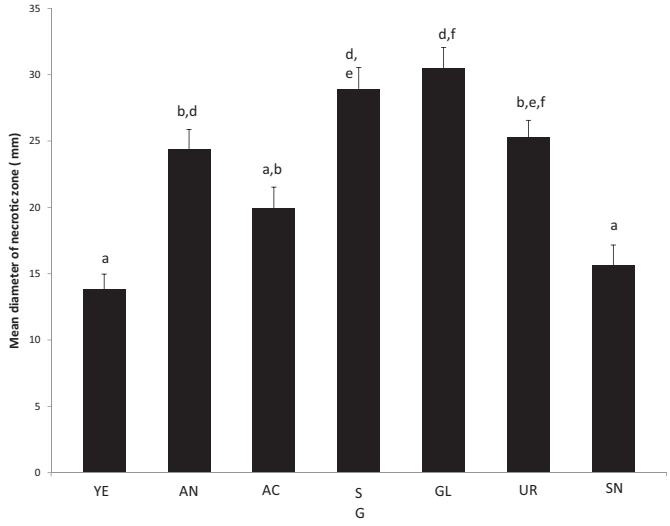


Figure 4. Effect of nitrogen source on the production of phytotoxin in culture by *M. roridum*. Phytotoxicity on water hyacinth leaves 72 h after application of *M. roridum* culture filtrate derived from Czapek Dox broth media containing one of the following nitrogen sources: yeast extract, (YEA), ammonium nitrate (AN), ammonium chloride (AC), sodium glutamate (SG), glutamine (GL), urea (UR) or sodium nitrate (SN). Values with the same letter are not significantly different ($p > 0.05$) ($n = 6$, means ± SEM) when subjected to one-way ANOVA and Tukey *post hoc* test. LSD, Least significant difference = 4.21.

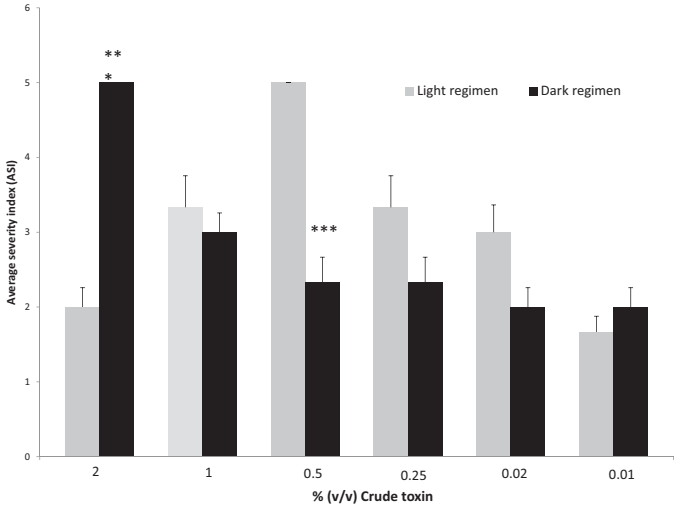


Figure 5. Effect of photoperiod on the phytotoxicity of *M. roridum* toxin measured on water hyacinth leaves. Assay values, ASI that differ significantly ($p < 0.001$) between incubation in darkness or in a 12 h light/dark photoperiod are indicated by ***. ($n = 6$, means ± SEM) when subjected to two-way ANOVA and Bonferroni *post hoc* test. LSD, Least significant difference = 1.59.

need plant-specific substances to induce phytotoxin synthesis. For example, 2-amino-1,3-propandiol (serinol) produced in sugarcane leaves has been implicated in phytotoxin induction by strains of *Helminthosporium sacchari* (Barbosa *et al.* 2002). In addition, WHB is the least expensive medium, making it the most economically attractive medium to use in toxin production.

Effect of carbon source on phytotoxin production

All carbon sources supported toxin production, but with significantly different capacity ($F_{6,\ 35} = 52.21$, $p < 0.0001$). The phytotoxic activity measured as necrotic zone diameter on leaves was highest with xylose and least with maltose (Figure 3). The maximum phytotoxic activity obtained with xylose, a pentose sugar, was not significantly greater than that obtained with lactose ($p > 0.05$), a disaccharide sugar. However, both xylose and lactose produced a significantly higher amount of the toxin than the other sugars ($p < 0.001$). Glucose is a commonly used carbon source by phytotoxin-producing fungi, but it performed poorly as a phytotoxin inducer in our study. Xylose and other sugars used in this study such as fructose,

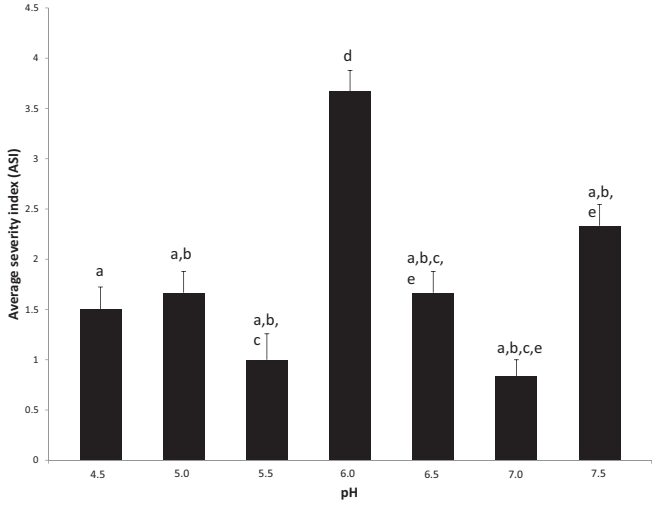


Figure 6. Effect of pH on the phytotoxicity of *M. roridum* toxin adjusted to pH values ranging from 4.5 to 7.5. ASI values with the same letter are not significantly different ($p > 0.05$) ($n = 6$, means ± SEM) when subjected to one-way ANOVA and Tukey *post hoc* test. LSD, Least significant difference = 1.12.

Table 3. Heat stability of phytotoxicity of *M. roridum* culture filtrates 72 h after application to water hyacinth leaves[a].

		Autoclaved Media Types						
Toxin	Concentration	MEB	PCB	PDB	PSB	WHB	SB	ZDB
100%		2.33 ± 0.33	1.33 ± 0.21	1.00 ± 0.26	1.33 ± 0.21	1.67 ± 0.33	1.00 ± 0.26	0.33 ± 0.21
70%		**4.33[b,d] ± 0.21**	**4.67[b] ± 0.21**	**4.67[b] ± 0.21**	**2.33[c] ± 0.21**	3.33 ± 0.33	**4.00[d] ± 0.26**	**3.33 ± 0.33**
50%		3.67 ± 0.33	3.67 ± 0.33	3.67 ± 0.33	2.33 ± 0.21	**4.00[d] ± 0.37**	3.67 ± 0.33	1.67 ± 0.21
10%		1.33 ± 0.21	3.00 ± 0.26	0.33 ± 0.21	2.33 ± 0.33	2.67 ± 0.21	0.00 ± 0.00	1.33 ± 0.21
1%		0.67 ± 0.21	2.00 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	2.33 ± 0.42	0.00 ± 0.00	1.00 ± 0.26

[a]Data represent Average Severity Index (ASI) ± SEM ($n = 6$) derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%. Data in bold are maximum ASI obtained per medium.
[b,c,d]Phytotoxicity of 70% culture filtrates were significantly different when subjected to ANOVA and *post hoc* Tukey's Multiple Comparison Test ($p < 0.05$).

galactose and sucrose have been reported to be alternative carbon sources, but they must be phosphorylated prior to interconversion in the pentose phosphate pathway or glycolytic pathway while sucrose is first hydrolyzed to its constituent sugars (Barbosa *et al.* 2002).

Effect of nitrogen source on phytotoxin production

The largest necrotic lesion on water hyacinth leaves was observed with medium containing glutamine as a nitrogen source, which caused a lesion about twice the size of the least effective nitrogen sources in the media, yeast extract and sodium nitrate, the normal nitrogen source in ZDB (Figure 4). The maximum phytotoxin production obtained with glutamine was not significantly different from that obtained with sodium glutamate, urea or ammonium nitrate ($p > 0.05$), but it was significantly greater than that obtained with sodium nitrate or yeast extract ($p < 0.001$). Phytotoxin concentration obtained from the second best nitrogen source, sodium glutamate, was significantly greater than that obtained with sodium nitrate, ammonium chloride or yeast extract ($p < 0.001$). This result suggests that an organic nitrogen source is preferred by *M. roridum* for phytotoxin production, although yeast extract supported the least phytotoxin production. Yeast extract was also reported to be a poor phytotoxin inducer in *Bipolaris euphorbiae* (Barbosa *et al.* 2002).

Effect of photoperiod on the phytotoxicity produced by M. roridum

All values of light and dark regimens had a significant effect ($F_{5,\ 30} = 18.92$, $p < 0.001$, with Bonferroni post-tests) on phytotoxicity of lyophilized toxin preparations. The concentration of toxin preparation had a significant overall effect on phytotoxicity ($F_{5,\ 30} = 12.22$, $p < 0.001$), but the light and dark regimen did not ($F_{1,\ 30} = 2.45$, $p = 0.1279$) except at concentrations of 2% and 0.5% (w/v) (Figure 5). The ASI of 2% lyophilized toxin preparation produced in darkness was significantly greater than that obtained at lower

concentrations ($p < 0.001$) (Figure 5). The ASI was maximal at 0.5% (w/v) toxin concentration on leaves under 12 h photoperiod and was significantly greater than those obtained at other dilutions ($p < 0.001$) (Figure 5). The results indicate that the toxicity of the *M. roridum* phytotoxin was greater in the light at reduced concentrations (0.02 to 1.00%, w/v) compared to toxicity produced in the dark. Some phytotoxins have been reported to be photosensitive, such as cercosporin produced by *Cercospora* sp. and perylenequinones produced by a number of important phytopathogens, all of which require photoactivation for biological activity (Daub and Chung 2007).

Effect of pH on the phytotoxicity produced by M. roridum

M. roridum phytotoxicity on water hyacinth leaves ranged from an ASI value of about 1.0 to about 3.5 over the pH range 4.5 and 7.5. Maximal ASI observed at pH 6.0 (Figure 6) was significantly greater than ASI at the other respective pH values ($p < 0.01$). *M. roridum* phytotoxicity in the water hyacinth leaf bioassay was stable over the pH range 4.5 to 7.5. Most fungi produce phytotoxins that are stable over broad pH ranges. For example, phytotoxins from *B. euphorbiae* and *Helminthosporium nodulosum* are stable over the pH range 3 to 9 and pH range 3 to 7, respectively (Barbosa *et al.* 2002).

Table 4. Phytotoxicity of *M. roridum* culture filtrate on various plant species 72 h after application to the upper surface of leaves[a].

SN	Plants (Common Names)	Botanical Names	ASI	Comment
	Acalypha	*Acalypha cordifolia*	0.00 ± 0.00	
	Aloe vera	*Aloe vera*	0.00 ± 0.00	
	Amarantus	*Amaranthus viridis*	0.00 ± 0.00	
	Banana	*Musa sapientum*	0.00 ± 0.00	
	Cowpea	*Vigna unguiculata*	2.33 ± 0.21[b]	Necrotic
	Cabbage	*Brassica oleracea*	0.00 ± 0.00	
	Carrot	*Daucus carota*	0.00 ± 0.00	
	Cassava	*Manihot esculentum*	0.00 ± 0.00	
	Cocoyam	*Colocasia esculentum*	0.00 ± 0.00	
	Corn (maize)	*Zea mays*	0.00 ± 0.00	
	Date palm	*Phoenix dactylifera*	0.00 ± 0.00	
	Long-fruited Jute	*Corchorus olitorius*	2.00 ± 0.26[b]	Necrotic
	Garden egg	*Solanum melongena*	0.00 ± 0.00	
	Groundnut (peanut)	*Arachis hypogea*	2.67 ± 0.21[b]	Necrotic
	Lemon grass	*Cymbopogun citratus*	2.33 ± 0.21[b]	Necrotic
	Lettuce	*Lactuca taraxacifolia*	0.00 ± 0.00	
	Mango	*Mangifera indica*	0.00 ± 0.00	
	Okra	*Hibiscus esculentus*	0.00 ± 0.00	
	Pawpaw	*Carica papaya*	0.00 ± 0.00	
	Pineapple	*Ananas comosus*	0.00 ± 0.00	
	Red savina	*Capsicum chinense*	0.00 ± 0.00	
	Plumed celosia	*Celosia argentea*	0.00 ± 0.00	
	Chili pepper	*Capsicum annuum*	0.00 ± 0.00	
	Tomato	*Lycopersicum esculentus*	0.00 ± 0.00	
	Watermelon	*Citrullus lanatus*	0.00 ± 0.00	
	Yam	*Dioscorea alata*	0.00 ± 0.00	
	Water hyacinth	*Eichhornia crassipes*	4.83 ± 0.17*[c]	Necrotic
	LSD (0.05)		0.62	

[a]Leaves from the indicated plants were placed on moistened cotton batting in Petri plates (one per plate) and slightly punctured with a needle at the center of the adaxial surface. A 10 µl (50% v/v) *M. roridum* PDB (potato dextrose broth) culture extract was infiltrated on the punctured site. After 72 h incubation at 25 ± 2 °C, toxicity of the extract was recorded as Average Severity Index (ASI) ± SEM ($n = 6$) derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 1 = 1–10% of foliage with necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%.

*maximum ASI achieved; significantly greater phytotoxicity than other measured values ($p < 0.001$, *post hoc* Tukey analysis). ASI values with the same superscript letter are not significantly different ($p > 0.05$). LSD, least significant difference.

Effect of heat on the phytotoxicity produced by M. roridum

The phytotoxicity of autoclaved culture filtrates (Table 3) was significantly ($F_{1, 56} = 4.23$, $p < 0.04$) higher than the corresponding nonautoclaved culture filtrate (Table 2) for all medium types, and it increased after dilution with distilled water. Maximum activity was observed at 70% (v/v) dilution in all autoclaved medium types except in WHB, which had a maximum phytotoxicity at 50% toxin concentration. The maximum ASI observed with autoclaved PCB or PDB culture filtrates at 70% concentration was significantly greater than that observed in the autoclaved PSB ($p < 0.001$) or ZDB ($p < 0.01$) culture filtrates. The phytotoxin being thermostable is typical for mycotoxins (Mehta and Brogin 2000, Duarte and Archer 2003). These results are consistent with the phytotoxin produced by *M. roridum* being heat stable and with the culture filtrates also containing activity-altering substance(s) that is sensitive to heat and dilution. This property is advantageous for a bioherbicide, in that the same process (autoclaving) would both activate the product and stabilize it for storage. The increase in activity following autoclaving might be due to destruction of metabolites in the toxin preparation that act antagonistically to the active compound, or the creation of adjuvants that could promote toxicity, such as surfactants produced by degradation of phospholipids from cell membranes. The observation that activity decreases on dilution (Table 2) is consistent with the presence in autoclaved preparations of an adjuvant that is diluted to ineffectiveness faster than the toxin is diluted.

Phytotoxicity of M. roridum culture filtrates on leaves of various plants

Leaves of four of the 26 plant species tested in addition to water hyacinth (Table 4) were susceptible to the phytotoxicity of *M. roridum* culture filtrates, and

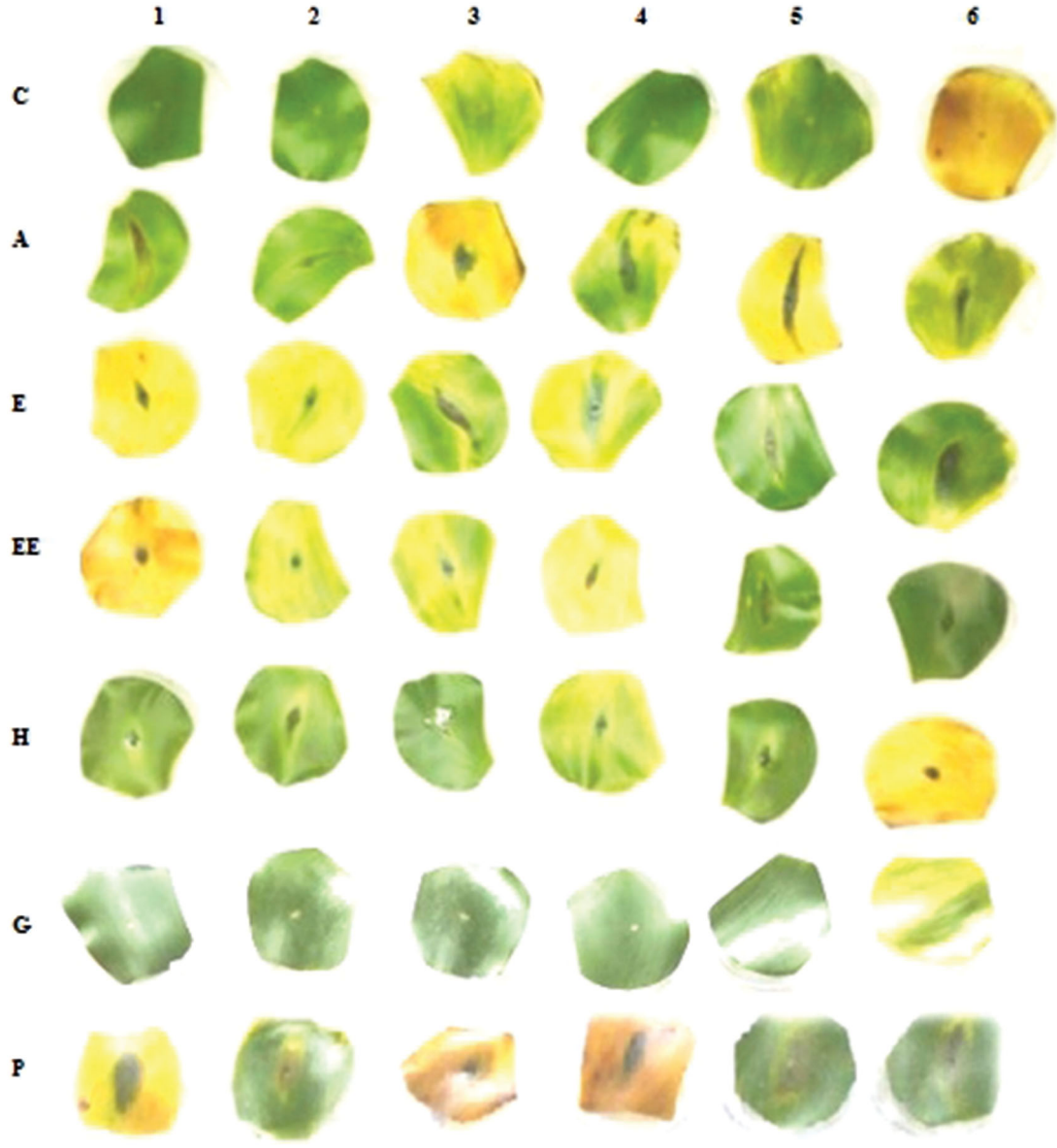


Figure 7. Disease development on water hyacinth leaf 72 h after toxin infiltration. C = Control (0.5% DMSO), A = Roridin A (500 μg/ml), E = Roridin E (500 μg/ml), EE = Epi-isororidin E (500 μg/ml), H = Roridin H (500 μg/ml), G = Glyphosate (500 μg/ml) and P = paraquat (500 μg/ml).

there were significant differences in the susceptibility to the toxin among the five susceptible plants ($F_{4,\ 25} = 28.66$, $p < 0.0001$). Water hyacinth was significantly more susceptible to *M. roridum* phytotoxicity than other susceptible plants ($p < 0.001$). We have previously reported the phytotoxicity of this isolate on water lettuce with a lower ASI of 3.0 three days post toxin infiltration (Okunowo *et al.* 2011) compared to that recorded for water hyacinth in this study. The higher susceptibility of water hyacinth to the toxin may be due to it being the source of the fungal isolate producing the phytotoxin. The susceptibility response of the affected plants to phytotoxicity of *M. roridum* in this study is consistent with that obtained with the isolate in our previous study (Okunowo *et al.* 2013). Studies have also shown that a different strain of *M. roridum*, F0252, was able to produce phytotoxin that was herbicidal to a range of weedy plants (Lee and Hong 2008). Some economically important crops such as mulberry, soyabean and muskmelon have been documented as susceptible to *M. roridum* toxin (Mackay *et al.* 1994, Murakami and Shirata 1998,Talukdar 2011). The broad spectrum activity of *M. roridum* phytotoxin on some weeds may be an advantage however; caution must be taken to avoid arial drift to non-target susceptible agricultural crops. Further work may be done to determine which of the phytotoxic metabolites of *M. roridum* is specific to water hyacinth.

Table 5. Phytotoxicity of Roridins and two commercial herbicides on water hyacinth leaves 72 h post toxin infiltration*.

Toxins	Control (5% DMSO)	Roridin A (500 μg/ml)	Roridin E (500 μg/ml)	Epi-isororidin E (500 μg/ml)	Roridin H (500 μg/ml)	Glyphosate (500 μg/ml)	Paraquat (500 μg/ml)	LSD (0.05)
ASI	0^{a}	$2.20 \pm 0.31^{b,f}$	$1.50 \pm 0.22^{b,c,d,e,f}$	$1.00 \pm 0.25^{c,d,f}$	$0.85 \pm 0.15^{a,c,d,e}$	0^{a}	$1.70 \pm 0.21^{b,c,f}$	0.57

*Data are presented as Average Severity Index (ASI) ± SEM ($n = 6$) values derived from the following foliar symptoms scale: 0–5, where 0 = no symptoms, 0.1 = less 1% of foliage with necrosis, 1 = 1–10% of foliage with necrosis, 2 = 11–30%, 3 = 31–70%, 4 = 71–90%, and 5 = 91–100%. Phytotoxicity of toxins with the same letter are not significantly different when subjected to ANOVA and *post hoc* Tukey's Multiple Comparison Test ($p > 0.05$). LSD, Least significant difference.

Table 6. Response of water hyacinth leaves 72 h post application of Roridin A, Roridin E and two commercial herbicides; paraquat and glyphosate*.

	Diameter of Necrosis (mm)		
Concentration (μg/ml)	Paraquat[q]	Roridin A[8]	Roridin E[8]
250	25.33 ± 1.92^{a}	27.00 ± 5.30^{a}	25.00 ± 3.8^{a}
125	$20.92 \pm 0.89^{a,b}$	$25.00 \pm 3.00^{a,b}$	$26.00 \pm 5.0^{a,b,c,d}$
62.5	$15.92 \pm 1.21^{b,c}$	$21.00 \pm 2.80^{a,b,c}$	$19.00 \pm 5.2^{a,c,d,e,f}$
31.25	$13.65 \pm 0.27^{c,d}$	$8.20 \pm 1.60^{d,e,f}$	$18.00 \pm 3.1^{a,d,e,f}$
15.52	7.75 ± 1.05^{e}	$6.10 \pm 0.47^{e,f}$	$6.70 \pm 0.85^{e,f}$
7.81	$4.75 \pm 1.76^{e,f}$	4.80 ± 0.28^{f}	4.80 ± 0.28^{f}
LSD (0.05)	3.77	8.08	10.41

*Data are presented as Average Diameter of necrosis ± SEM ($n = 6$) values derived from the average of the length of necrosis and width of necrosis measured in millimeter across point of toxin infiltration. Note that diameter of scratch at point of toxin infiltration is approximately 4 mm. Phytotoxicity of toxin with the same letter at varying concentrations is not significantly different when subjected to ANOVA and *post hoc* Tukey's Multiple Comparison Test ($p > 0.05$). [8]Phytotoxicity of toxins and paraquat compared to one another at varying concentrations is not significantly different when subjected to ANOVA and Bonferroni post test ($p > 0.05$). LSD, Least significant difference. Glyphosate was not phytotoxic at test concentrations.

Phytotoxicity of purified roridin A, some analogs and two commercial herbicides on water hyacinth leaves

The phytotoxicity of standard roridin A and some analogs (roridin E, epi-isororidin E and roridin H) was compared with two commercial herbicides (glyphosate and paraquat) on water hyacinth leaves. Roridin A and roridin E showed severe necrosis while epi-isororidin E and roridin H showed mild necrosis at the point of toxin infiltration extending longitudinally and laterally (Figure 7). The necrosis was typical of that caused by the crude toxin and the fungus, *Myrothecium roridum* Tode. There was no detectable necrosis in the negative vehicle control and with glyphosate at all test concentrations. Oval necrotic spots were observed with paraquat. Phytotoxicity of roridin A, roridin E and paraquat was not significantly different ($p > 0.05$). Roridin A and paraquat were significantly ($p < 0.05$) more phytotoxic than epi-isororidin E and roridin H (Table 5).

The phytotoxic responses to roridin A, roridin E and paraquat were concentration dependent, with the highest phytotoxicity at 250 μg/ml, but not significantly different from that at 125 μg/ml. The lowest concentration giving a visible necrotic spot was 62.5 μg/ml for roridin A, while that for roridin E and paraquat was 31.25 μg/ml. For further details on response of water hyacinth leaves to toxins gradient, see Supplementary Information (ESM_1, ESM_2, ESM_3 and ESM_4). Phytotoxicity was presented as diameter of necrosis (Table 6). The observed phytotoxicity was not significantly different with roridin A, roridin E and paraquat ($F_{2,90} = 0.72$, $p = 0.49$), but varied significantly with concentration ($F_{5,90} = 30.56$, $p < 0.0001$) (two-way analysis of variance with Bonferroni post-test) (Table 6).

Purified trichothecene roridin A has been reported to be phytotoxic with a wide variety of other plants inducing similar symptoms (Cole and Cox 1981, Talmage 1983). To the best of our knowledge, this is the first report of roridin A being a potent phytotoxic agent on water hyacinth. Roridin A may play a role as a virulence factor responsible for the pathogenicity of *M. roridum* on water hyacinth. This is also the first report that showed that roridin E is a potent phytotoxic agent on water hyacinth while other analogs, epi-isororidin E and H, have mild phytotoxic activity. These compounds, except roridin H, showed herbicidal potential for water hyacinth that is comparable to the commercially available herbicide, paraquat. Conversely, glyphosate failed to cause any detectable toxic response at the concentrations tested (up to 0.5 mg/ml), unlike previous reports in which higher concentrations (2.9 mg/ml and >7.2 mg/ml) caused growth retardation and death of water hyacinth, respectively (Lopez 1993, Jadhav *et al*. 2007). These results suggest that glyphosate is a weaker herbicide for water hyacinth control than roridin A, roridin E, epi-isororidin E, roridin H and paraquat. However, macrocyclic trichothecenes, including roridin A, are known to be toxic *in vitro* (Rocha *et al*. 2005) and in mammals (Wannemacher and Wiener 1977). Simple trichothecenes are known to be toxic to fish (Matejova *et al*. 2017, Zhou *et al*. 2017), but quantitative information on fish toxicity of roridin A and other macrocyclic trichothecenes is lacking. An LD_{50} of 1.0 mg/kg has been reported for intravenous injection of roridin A in mouse (Wannemacher and Wiener 1977), which is 16 times the minimum concentration (62.5 μg/ml) causing

visible toxicity on water hyacinth leaves in our study. This suggests that it may be possible to apply this compound at concentrations below which it exerts toxicity on non-host organisms. Further studies, analogous to those conducted for glyphosate and paraquat (Alberdi *et al*. 1996), will be required for either a spray-on herbicide or spore preparations for biological control of water hyacinth, focusing on the fate of roridins in water and ecotoxic effects on other aquatics.

Conclusion

This study showed that roridin A is a major component of the phytotoxin produced by a *M. roridum* isolate (IMI 394934) that is pathogenic to water hyacinth. The relative phytotoxicity of the agents tested against water hyacinth was roridin A $\approx$ roridin E $\approx$ paraquat $>$ epi-isororidin E $\approx$ roridin H $\gg$ glyphosate. Additional research will be needed to define the environmental effects of using *M. roridum* isolate IMI 394934 as either a spray-on herbicide or as spore preparations for biological control of water hyacinth.

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Disclosure statement

The authors declare that they have no conflict of interest.

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