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RESEARCH PAPER

Pathogenicity, host range and activities of a secondary metabolite and enzyme from *Myrothecium roridum* on water hyacinth from Thailand

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Leaf blight disease of water hyacinth was observed and collected from different geographical areas of Thailand. The disease is caused by a fungal pathogen that was identified as *Myrothecium roridum* by using its morphological characteristics. The most effective fungal strains were evaluated for pathogenicity on water hyacinth under greenhouse and natural conditions. *Myrothecium roridum* isolate, Kamphaeng Sean Campus (KKFC) 448, was found to be the most virulent. Different fungal formulations were evaluated for their level of control of water hyacinth. The results showed that spore suspensions with 10% palm oil or 1% Tween 20 caused a higher level of disease severity, compared to spores applied in water alone. The host range of KKFC 448 was evaluated by using 77 plant species that belong to 40 plant families. The fungus did not cause disease on 74 economically important plants but did produce disease signs on water hyacinth and two other aquatic weeds, duckweed and water lettuce. Leaf blight occurs on water hyacinth leaves after being treated with crude extracts of *M. roridum* and it was indicated that secondary metabolites were released from the fungal mycelia. *Myrothecium roridum* that was grown on boiled paddy rice produced β-1,4-exoglucanase, β-1,4-endoglucanase, β-glucosidase, xylanase and pectinase more than *M roridum* that was grown on potato dextrose agar. The results indicated that *M. roridum* is a pathogen of water hyacinth and the fungus is capable of producing different enzymatic activities on potato dextrose agar and boiled paddy rice, which might be important for infection.

Keywords: biological control, crude extracts, enzymatic activity, fungal formulation, leaf blight disease.

Water hyacinth (*Eichhornia crassipes*), a native aquatic weed in the Amazon Basin in South America, is an invasive species. Major epidemics of the weed have occurred in Africa, Europe, Asia and the Americas (Dagno *et al.* 2012). Water hyacinth affects the generation of electricity, irrigation and navigation and has the ability to infest a diversity of freshwater habitats, causing enormous environmental and economic problems (El-Morsy 2004). A number of the control methods for water hyacinth that are currently used are expensive and detrimental to the environment. In contrast, biological control is a promising, eco-friendly method that is used to manage a number of agricultural pests, including weeds (Okunowo *et al.* 2013). Recently, biological organisms, such as insects and fungi, have been shown to be effective against water hyacinth. Many species of fungi have been reported to

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control water hyacinth, such as *Alternaria echhorniae*, *Cercospora rodmanii* and *Myrothecium roridum* (Liyanage & Gunasekera 1989). Fungal species have been developed as biocontrol agents for controlling water hyacinth and others weeds with the intention of commercialization. For example, a mycoherbicide for water hyacinth was registered under the patent, US4097261, which was made from the fungal pathogen, *C. rodmanii* (Freeman & Charudattan 1984). Abbott laboratories in the USA developed ABG-5003, a formulation of *C. rodmanii* for controlling water hyacinth (Te Beest 1991). In addition, the mycoherbicides, Hyakill™ from *Sclerotinia sclertiorum*, Biochon™, Chontrol™ and Myco-Tech™ paste from *Chondrostereum purpureum* and Sarritor™ from *Sclerotinia minor* (de Jong & de Voogd 2006), have been developed to control water hyacinth and other weeds.

Several researchers have evaluated a number of promising biological agents that might be used as mycoherbicides against water hyacinth, including *M. roridum*, which is capable of severely damaging and suppressing water hyacinth. Previous reports have shown that *M. roridum* has the potential to control water hyacinth and other weeds (Lee *et al.* 2008; Tegene *et al.* 2012; Okunowo *et al.* 2013). *Myrothecium roridum* infects water hyacinth due to the activity of its enzymes and mycotoxins (Okunowo *et al.* 2010b). Okunowo *et al.* (2010a) reported that *M. roridum* produces cellulases and xylanases, while Aboul-Nasr *et al.* (2013) determined that *M. roridum* has the ability to produce mycotoxic compounds, such as Roridin A & E and Verrucarin A & J.

The objectives of the current study were to: (i) evaluate the pathogenicity and host range of *M. roridum* that is isolated from water hyacinth; (ii) determine the effectiveness of the crude extracts of *M. roridum* for controlling water hyacinth; and (iii) investigate enzymatic production by *M. roridum*.

METHODS

Fungal strains and the preliminary selection of effective strains

Leaf blight disease of water hyacinth plants was observed and collected from different geographical areas in Thailand: the northern, north-eastern, central and western regions. The fungal pathogens were isolated from infected leaves by using the tissue transplanting method (Piyaboon *et al.* 2014). Pure cultures were made by single-spore isolation and were maintained on potato dextrose agar (PDA) slants, including the isolates that were reported previously by Piyaboon *et al.* (2014). All the isolates were identified based on the morphological characteristics of the colonies, conidia and conidiophores (Tulloch 1972). In addition, the rDNA-internal transcribed spacers sequences of the fungal isolates were deposited in the GenBank database (Accession No. AB823651–AB823655 and No. AB857216–AB857229) and were published by Piyaboon *et al.* (2016). The preliminary selection of the effective strains was based on the colony's growth rate and spore production. Each of the pure cultures was maintained on PDA slants and deposited at the Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, at Kamphaeng Saen Campus (KKFC), Kasetsart University, , Nakhon Pathom, Thailand.

Pathogenicity test

The effective fungal strains were secondarily screened by using a pathogenicity test. Healthy water hyacinth plants, with leaves that were 50–100 cm^2 in size, were collected from natural water sources, surface-sterilized by 10% sodium hypochlorite solution for 5 min and rinsed two times with sterile distilled water. The fungal isolates were subcultured on PDA and incubated at 28°C. The photoperiods (12 h) were provided by white fluorescent lamps. Inoculation was done by spraying the leaves and petioles of water hyacinth with 1×10^8 spores mL^{-1}; the control treatment was sprayed with 10 mL of sterile distilled water. This experiment was conducted by using a completely randomized design (CRD), with 10 replications of each treatment. The plants were placed in a growth chamber with 100% relative humidity (RH) for 24 h and then moved to greenhouse conditions. The temperatures in the greenhouse ranged from 26 to 32°C, with 65–90% RH. The disease severity was observed at 7 days after inoculation by using the following rating scale: 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100% leaf blight (Piyaboon *et al.* 2014). Fungal re-isolation was conducted by using the tissue transplanting method. The infected leaves were cut into a 0.5 cm × 0.5 cm size. The samples were surface-disinfected with a 10% sodium hypochlorite solution for 5 min and then washed two times with sterilized distilled water before being plated on the PDA. The cultures were incubated at 28°C under white fluorescent lamps with a 12 h day/night cycle.

The effective strains of *M. roridum* were determined for controlling water hyacinth in a canal at Kamphaeng Saen, Kasetsart University, Nakhon Pathom, Thailand. The fungal strains were cultured on PDA at 28°C

under white fluorescent light with a 12 h photoperiod. Healthy water hyacinth plants with leaves that were 25–50 cm^2 in size were collected from the natural water source. Then, the plants were washed with a 10% sodium hypochlorite solution for 5 min and rinsed three times with sterile distilled water in order to eliminate the microorganisms and insects. The water hyacinth plants were placed in each selected site of the canal (1 m × 1 m plastic frame). The experiment was conducted by using a randomized complete block design (RCBD), with three replications of each treatment. The experiment consisted of three treatments and a control treatment (n = 30 water hyacinth plants). The treatments were inoculated with 10 mL of a spore suspension (1×10^8 spores mL^{-1}) of the most effective isolates by using the spraying method. The control plants were sprayed with 10 mL of distilled water. The level of disease severity was rated as described above.

The spore suspensions of the most effective *M. roridum* isolates were tested with different surfactants; that is, Tween 20 and palm oil. Healthy water hyacinth plants were collected from natural sources, with the size of the leaves being 100–150 cm^2. The experiment used an RCBD, with three replications of each treatment. The treatments were: (i) 1×10^8 spores mL^{-1} in distilled water; (ii) 1×10^8 spores in 1% Tween 20; and (iii) 1×10^8 spores mL^{-1} in 10% palm oil. The controls consisted of: (i) 1% Tween 20 only; (ii) 10% palm oil only; and (iii) distilled water only. The level of disease severity was rated as described above.

Host range determination

The pathogenicity of the screened isolate of *M. roridum* KKFC 448 was tested on economically important plants and weeds, including 77 plant species in 40 plant families. The experiment was conducted by using a CRD, with 10 replications of each treatment. For the experimental treatment, the 77 plant species were inoculated with the fungal isolate by using the spraying method with 10 mL of 1×10^6 spores mL^{-1}. The control plants were sprayed with 10 mL of distilled water. The plants were placed in a growth chamber with 100% RH for 24 h and then moved to natural conditions (23–32°C and 60–90% RH). Fourteen days after spraying, disease development and the host plant reactions were recorded. The disease signs were rated by visual examination as: − = "not susceptible" (healthy leaves, no disease sign observed); + = "slightly susceptible" (leaf spotting or slight chlorosis, no necrosis); + + = "susceptible" (leaf spots or leaf necrosis, 30–50% of the leaf is dead); and +++ = "highly susceptible" (severe leaf spotting or necrosis, >50% of the leaf is dead), following Okunowo *et al.* (2013).

Crude extraction and plant inoculation

Fungal crude extracts

The crude extraction of the *M. roridum* strains was done by following the method of Sibounnavong *et al.* (2012). The fungal isolates were cultured in potato dextrose broth (PDB) and incubated at room temperature (27–30°C) for 30 days. The fungal mycelia and spore mats were removed from the PDB by cheesecloth filtration and air-dried at a room temperature of ~27–30°C for 3–5 days. The fresh and dry weights of the mycelia and spore mats were recorded. The dried mycelia and spore mats of the fungus were ground separately with an electrical blender and dissolved in hexane at 1:10 (g/v) for extraction, then incubated at room temperature for 7 days. The fungal extracts were separated from the marc by filtration through filter paper (No.1; Whatman, Maidstone, UK). The marc was further extracted with ethyl acetate and finally with methanol by using the same hexane extraction procedure. The hexane, ethyl acetate and methanol fractions were separately extracted by using a rotary vacuum evaporator (IKA, Germany). Each crude extract was weighed, placed in a small bottle and then stored at 4°C for testing.

Plant inoculation with the crude extracts

The crude extracts were assayed for controlling water hyacinth. Each crude extract first was dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted to the desired final concentrations with distilled water (final concentration of DMSO, 10%). One-hundred microliter drops of the assay solutions were applied to 25–50 cm^2 healthy water hyacinth leaves. The experiment was conducted by using a CRD, with 28 treatments and three replications. Twenty-seven of the treatments represented the different solvent extracts: crude hexane extracts, crude ethyl acetate extracts and crude methanol extracts at concentrations ranging from 500 to 100,000 p.p.m. The control plants were treated with 10 mL of 10% DMSO. The level of disease severity was monitored at 3 day intervals following inoculation and rated by using the scale indicated above (Piyaboon *et al.* 2014).

Evaluation of the enzymatic activity

Enzymatic production

Myrothecium roridum was studied for enzymatic production by using PDA and boiled paddy rice as the growth

media. The enzymes were produced by cultivation of the fungus at room temperature (27–30°C) for 21 days. At the end of the incubation period, spore suspensions were harvested by centrifugation at 10,000 *g* for 20 min. The culture supernatants were produced from 13 g of fresh spores and used in the enzyme assay. The enzymatic activity was expressed as "unit per mL" (U mL^{-1}), which represents the micromol of sugar released per min.

Determination of the enzymatic activity

β-1,4-Endoglucanase activity The level of β-1,4-endogluc anase activity was determined according to the method of Okunowo *et al.* (2010a), using carboxymethylcellulose (CMC) as the substrate, and the formation of the reducing sugars was measured by the reaction with dinitrosalicylic acid (DNS). 1 mL of 1% CMC in 0.05 M sodium acetate buffer (pH = 5.0) were placed in a test tube and 1 mL of culture supernatant was added. The reaction mixture was incubated at 50°C for 30 min. The reaction was terminated by adding 1 mL of DNS. The test tubes were heated at 100°C in a boiling water bath for 5 min and cooled to room temperature. Then, 10 mL of distilled water were added. The color was read at 540 nm by using a spectrophotometer (T60 UV-VIS; PG Instruments, Ltd., the United States). The reducing sugar concentration was quantified by using a glucose concentration curve as the standard.

β-1,4-Exoglucanase activity. The level of β-1,4-exoglucanase activity was determined by using microcrystalline cellulose (Avicel; Switzerland) as the substrate and was measured by using DNS. The reaction mixtures that contained 1 mL of 1% Avicel in 0.05 M sodium acetate buffer (pH = 5.0) and 1 mL of culture supernatant were incubated at 50°C for 30 min. After incubation, 1 mL DNS was added to the solution in order to stop the reaction and the test tubes were put in a boiling water bath for 5 min. The colored solution was cooled to room temperature and then 10 mL of distilled water were added. A spectrophotometer was used to measure the β-1,4-exoglucanase activity at 540 nm and glucose was used as a standard to determine the amount of reducing sugar that was released.

β-Glucosidase activity. The level of β-glucosidase activity was determined by following the procedure of Okunowo *et al.* (2010a). The reaction mixture consisted of 0.5 mL of 0.5% cellobiose in 0.05 Msodium acetate buffer (pH = 5.0) with 0.1 mL of the culture supernatantand was incubated at 50°C for 10 min. After incubation, 2 mL of the assay reagents containing glucose oxidase peroxidase reagent (Sigma-Aldrich, USA) and o-dianisidine reagent (Sigma) were added and incubated at 37°C for 30 min. Then, 12 N sulfuric acid was added to the solution to stop the reaction. The concentration of glucose that was released was read by optical density at 540 nm.

Xylanase activity. The level of xylanase activity was assayed by 1 mL of 1% birch wood xylan as the substrate in 0.05 Msodium acetate buffer (pH = 5.0) and 1 mL of the culture supernatant at 50°C for 30 min. The reaction mixture was stopped by the addition of 1 mL of DNS, put in a boiling water bath for 5 min and then cooled to room temperature. Next, 10 mL of distilled water were added to the reaction. The optical density of the samples was measured at 540 nm. The amount of reducing sugar that was released was determined by using xylose as the standard.

Pectinase activity. The level of pectinase activity was measured following Okafor *et al.* (2010) by using citrus pectin as the substrate. The reaction mixture consisted of 1 mL of 1% citrus pectin in 0.05 M sodium acetate buffer (pH = 5.0) and 1 mL of culture supernatant and was incubated at 50°C for 30 min in a water bath. The reaction was stopped with 1 mL of DNS. The mixture was boiled for 5 min, cooled to room temperature and then 10 mL of distilled water were added. The color was measured at 540 nm. A standard curve of galacturonic acid was used to compare the reducing sugars of the samples.

Protein determination

The protein content of the samples was determined by the method of Lowry *et al.* (1951) and bovine serum albumin was used as the standard. Five milliliters of alkaline solution (2% $C_4H_4KNaO_6$, 1% $CuSO_4$ and 2% Na_2CO_3) were added to each of the sample solutions, which were thoroughly mixed before incubation at room temperature for 10 min. Then, 0.5 mL of the Folin Ciocalteu phenol reagent was added and the samples were mixed and incubated at room temperature for 30 min. In order to determine the protein concentration, the reaction in each sample was read at 750 nm.

Statistical analysis

All the experiments in these studies were assessed by an ANOVA. Significant differences among the treatment

Fig. 1. Signs of diseased water hyacinth leaves from Chiang Mai province in the northern region of Thailand. (a) Round-to-teardrop-shaped leaf spots and blight and (b) conidial masses of *Myrothecium roridum* KKFC 448 on a blighted leaf.

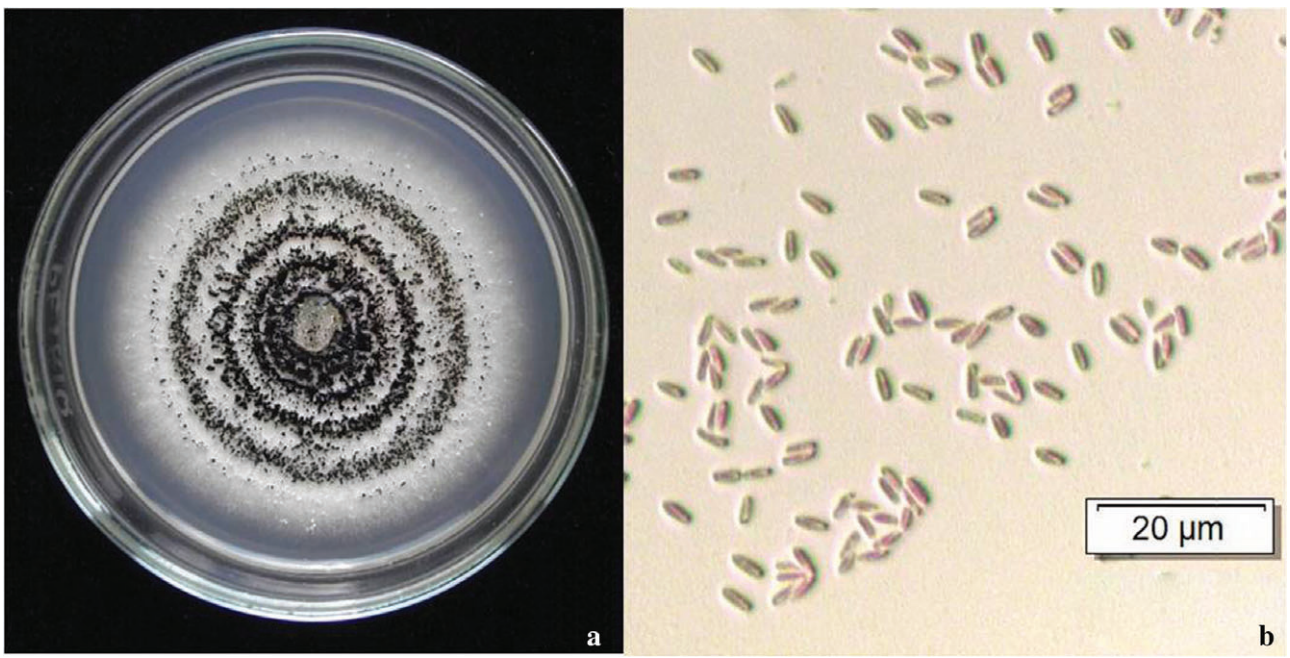

Fig. 2. Morphological characteristics of *Myrothecium roridum* KKFC 448 on potato dextrose agar. (a) Concentric zones diffused in sporodochia and (b) conidia (400×).

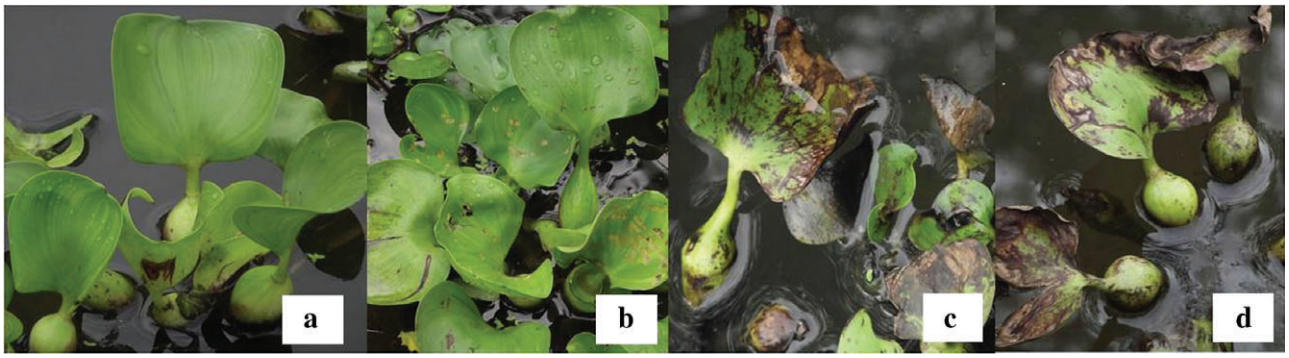

Fig. 3. Disease signs on water hyacinth that was affected by the various isolates of *Myrothecium roridum* in a natural water source. (a) Control treatment, (b) KKFC 447, (c) KKFC 448 and (d) KKFC 496.

means were compared by using Duncan's multiple range test at $P < 0.05$. The statistical analysis was carried out with SPSS for Windows statistical software, v. 16.0, Chicago, IL, USA.

RESULTS

Fungal collection and isolation

The characteristics of leaf blight disease included round-to-teardrop-shaped leaf spots and blights with conidial masses (Fig. 1). The sporodochia on the leaves were sessile, polymorphic in a surface view and the size was very variable: 60–750 μm in diameter and 40–150 μm deep. The spore masses were wet when young, drying to hard, shiny black, convex and surrounded by a fringe of entangled white hyphae. On the PDA that was used in this study, sporulation that was often in concentric zones consisted of sporodochia and spore masses that were initially viscous and green (Fig. 2a). The conidiophores were subhyaline to colored, branched and with terminal conidia. The conidia were rod-shaped with rounded ends, colorless-to-pale olive green to black when in mass and 3.32–4.88 μm × 0.75–2.14 μm in size (Fig. 2b), with similar characteristics to the species in a previous study (Tulloch 1972). The results showed that 137 isolates from the blighted water hyacinth were identified as *M. roridum*, based on the morphological characteristics.

Selection of the effective strains and the pathogenicity test

Ten isolates of *M. roridum*, including KKFC 447, KKFC 448, KKFC 457, KKFC 462, KKFC 480, KKFC 483, KKFC 496, KKFC 497, KKFC 509 and KKFC 519, showed a high colonial growth rate and spore production. Then, these fungal isolates were chosen for pathogenicity testing on water hyacinth under greenhouse conditions (26–32°C, 65–90% RH). The selected *M. roridum* isolates infected and produced disease signs on the water hyacinth leaves. The initial disease signs were small necrotic spots that were followed by leaf blight with conidial masses 7 days after inoculation. The *M. roridum* isolates, KKFC 447, KKFC 448 and KKFC 496, caused the significantly highest disease severity on water hyacinth. These three strains were tested for their ability to infect water hyacinth in a natural water resource (Fig. 3). The levels of disease severity that were observed at 14 days after inoculation on water hyacinth with isolates KKFC 447, KKFC 448 and KKFC 496 were 0.3, 2.4 and 2.2, respectively. These isolates were re-isolated from the lesions of the inoculated leaves, fulfilling Koch's postulate.

However, the *M. roridum* strains, KKFC 448 and KKFC 496, had no significant difference on disease severity (2.4 and 2.2, respectively). When these two strains were screened and compared on spore production, the results showed that the amount of spore

Table 1. Effect of fungal formulations on *Myrothecium roridum* leaf blight in water hyacinth under natural conditions

Treatment	Disease severity†
Spore suspension	1.2b‡
Spore suspension +1% Tween 20	3.0a
Spore suspension +10% palm oil	2.9a
Distilled water	0.0c
1% Tween 20 only	0.0c
10% palm oil only	0.0c

†Disease severity was rated by using the following scale: 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100% leaf blight; ‡means in the same column followed by a common letter are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

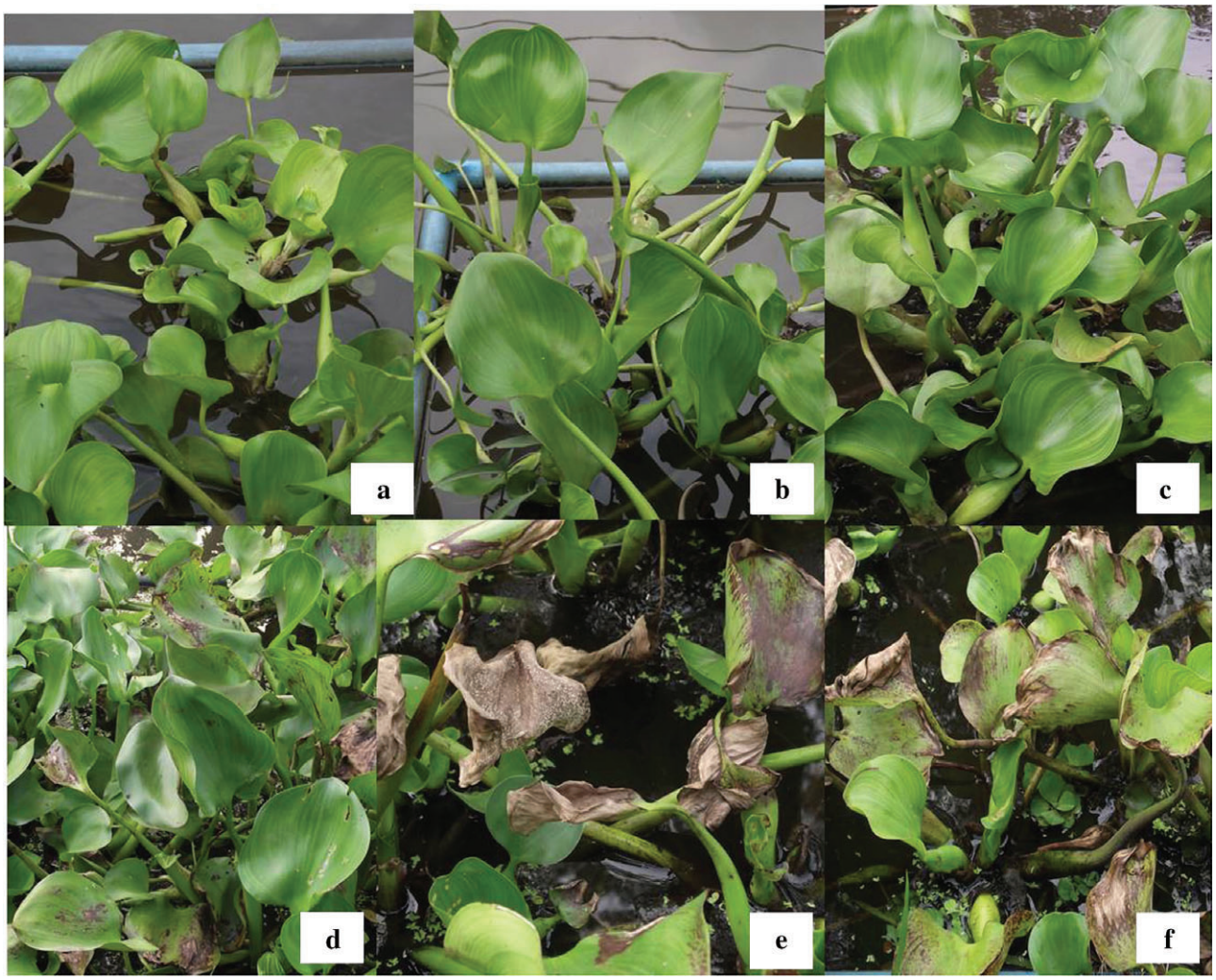

Fig. 4. Effects of the surfactants on the severity of leaf blight that was caused by *Myrothecium roridum* KKFC 448. (a) Control treatment (distilled water only), (b) control treatment (1% Tween 20 only), (c) control treatment (10% palm oil only), (d) spore suspensions in distilled water, (e) spore suspensions + 1% Tween 20 and (f) spore suspensions + 10% palm oil.

production of KKFC 448 was higher than that of KKFC 496. Therefore, *M. roridum* strain KKFC 448 was the most effective strain and was used for mycoherbicide development. Fungal spore suspensions in 10% palm oil or 1% Tween 20 caused noticeable leaf necrosis at day 2 postinoculation, but a spore suspension that was applied in distilled water caused only slight leaf necrosis at day 2 postinoculation. Table 1 shows the level of disease severity that was recorded 14 days after inoculation. An analysis of the fungal disease's progression indicated that the spore suspension with 10% palm oil and the spore suspension with 1% Tween 20 had higher levels of disease severity than did the spore suspension in distilled water (Fig. 4). The controls that were treated with 10% palm oil only, 1% Tween 20 only and distilled water alone developed no sign of disease. The experiment indicated that the disease severity was increased when a spore suspension was mixed with either palm oil or Tween 20.

Host range determination

The determination of the host range of *M. roridum* on various agricultural plants showed that 74 plant species were not hosts of the fungus, as leaf blight disease was not observed (Table 2). However, water hyacinth was infected by the *M. roridum* strain. The disease signs on water hyacinth were severe necrosis and >50% of leaf

Table 2. Host specificity of *Myrothecium roridum* at 14 days postinoculation

Plant family	Common name	Scientific name	Disease rating
Alliaceae	Green shallot	*Allium cepa*	–
Amaranthaceae	Cockscomb	*Celosia argentea*	–
Amaryllidaceae	Garlic chives	*Allium tuberosum*	–
Anthericaceae	Chinese fan palm	*Cholorophytum comosum*	–
Apocynaceae	Madagascar periwinkle	*Catharanthus roseus*	–
Araceae	Duckweed	*Lemna minor*	+
	Water lettuce	*Pistia stratiotes*	++
Asteraceae	Lettuce	*Lactuca sativa*	–
	Sunflower	*Helianthus annuus*	–
	Red oak lettuce	*Lactuca sativa* var. *crispa*	–
	Jerusalem artichoke	*Helianthus tuberosus*	–
Asclepiapaceae	Cowslip creeper	*Telosma minor*	–
Brassicaceae	Cauliflower	*Brassica oleracea* var. *botrytis*	–
	Broccoli	*Brassica oleraceae* var. italica	–
	Cabbage	*Brassica oleracea* var. capitata	–
	Ceylon spinach	*Basella alba*	–
	Chinese kale	*Brassica albroglabra*	–
	Chinese cabbage	*Brassica rapa*	–
	Flowering cabbage	*Brassica chinensis*	–
Caricaceae	Papaya	*Carica papaya*	–
Chenopodiaceae	Spinach	*Spinacia oleracea*	–
Cicereae	Butterfly pea	*Clitoria ternatea*	–
Compositae	Marigold	*Tagetes erecta*	–
Cucurbitaceae	Watermelon	*Citrullus lanatus*	–
	Pumpkin	*Cucurbita moschata*	–
	Cantaloupe	*Cucumis melo*	–
	Bitter cucumber	*Momordica charantia*	–
	Cucumber	*Cucumis sativus*	–
	Spring bitter cucumber	*Momordica cochinchinensis*	–
	Angled loofah	*Luffa acutangula*	–
	White gourd	*Benincasa hispida*	–
Cruciferae	Chinese cabbage	*Brassica chinensis*	–
	Bitter cucumber-Chinese	*Momordica charantia*	–
Euphorbiaceae	Sweetleaf bush	*Sauropus androgynus*	–
Fabacea	Peanut	*Arachis hypogaea*	–
	Green bean	*Vigna radiata*	–
	Yardlong bean	*Vigna unguiculata*	–
	Winged bean	*Psophocarpus tetragonolobus*	–
Gramineae	Sorghum	*Sorghum bicolor*	–
Lamiaceae	Common basil	*Ocimum basilicum*	–
	Hoary basil	*Ocimum citriodourum*	–
	Kitchen mint	*Melissa officinalis*	–
	Holy basil	*Ocimum sanctum*	–
	Mint	*Mentha* spp.	–
	Tree basil	*Ocimum gratissimum*	–
Leguminosae	Sesban	*Sesbania grandiflora*	–
Malvaceae	Okra lady's finger	*Abelmoschus esculentus*	–
	Jamaica sorrel	*Hibiscus sabdariffa*	–
Mimoseae	Impala lily	*Acacia pennata*	–

Table 2 (continued)

Plant family	Common name	Scientific name	Disease rating
Moringaceae	Horseradish	*Moringa oleifera*	–
Musaceae	Rose apple	*Musa sapientum*	–
Myrtaceae	Guava	*Psidium guajava*	–
	Sword fern	*Syzygium samarangense*	–
Nymphaeaceae	Water lily	*Nymphaea stellata*	–
Oleaceae	Arabian jasmine	*Jasminum sambac*	–
Oleandraceae	Fishbone fern	*Nephrolepis cordifolia*	–
Orchidaceae	Dendrobium hybrids	*Dendrobium orchids*	–
Palmae	Fountain palm	*Livistona chinensis*	–
Pandanaceae	Pandom wangi	*Pandanus amaryllifolius*	–
Passifloraceae	Passionfruit	*Passiflora edulis*	–
Piperaceae	Wildbetal leafbush	*Piper sarmentosum*	–
Poaceae	Rice	*Oryza sativa*	–
	Corn	*Zea mays*	–
	Lemongrass	*Cymbopogon citratus*	–
Polygonaceae	Vietnamese coriander	*Persicaria odorata*	–
Pontederiaceae	Water hyacinth	*Eichhornia crassipes*	+++
Rosaceae	Rose	*Rosa hybrida*	–
Rubiaceae	Indian mulberry	*Morinda citrifolia*	–
	Common lime	*Citrus aurantifolia*	–
Solanaceae	Bang chang chilli pepper	*Capsicum annuum*	–
	Tomato	*Solanum lycopersicon*	–
	Potato tree	*Solanum melongena*	–
	Eggplant	*Solanum melongena*	–
	Brinjal	*Solanum aculeatissimum*	–
	Banana pepper	*Capsicum annuum*	–
Umbelliferae	Dill	*Anethum graveolens*	–
	Stinkweed	*Eryngium foetidum*	–

Fungal spore suspension used was 1×10^6 spores mL^{-1}. Disease rating scale: −, not susceptible (leaves healthy, no disease sign observed); +, slightly susceptible (slight leaf spotting or slight chlorosis, no necrosis); ++, susceptible (leaf spots or leaf necrosis, 30–50% of the leaf was dead); +++, highly susceptible (severe leaf spotting or necrosis, >50% of the leaf was dead).

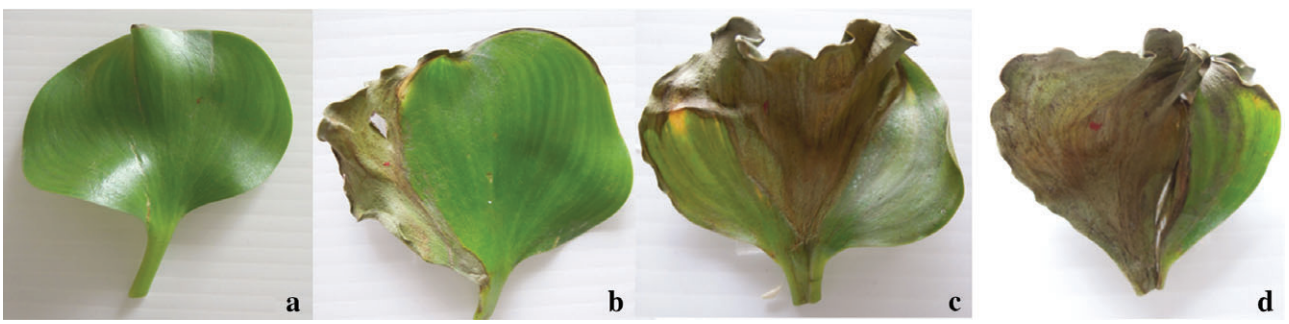

Fig. 5. Effects of 10,000 p.p.m. crude extracts of *Myrothecium roridum* from different solvents on water hyacinth. (a) Control, (b) crude hexane extract, (c) crude ethyl acetate extract and (d) crude methanol extract.

blight area. Water lettuce was infected by this susceptible fungus and showed leaf necrosis and 30–50% death of the plants, while duckweed was slightly susceptible to the fungus, resulting in slight chlorosis without a necrosis lesion. The *M. roridum* strain could infect water hyacinth and other water weeds, such as water lettuce and duckweed, although water lettuce and duckweed were less susceptible than water hyacinth.

Crude extraction and plant inoculation

Early leaf blight signs appeared on the water hyacinth leaves 1 day after treatment with the crude extracts. Three days later, leaf blight and dead tissue were evident throughout the treated areas. The crude extracts of *M. roridum* from ethyl acetate, methanol and hexane at concentrations that ranged from 500 to 100,000 p.p.m. caused leaf blight signs (Fig. 5), except that 500 p.p.m. of a crude extract from hexane caused no disease sign. The crude extracts from ethyl acetate and methanol showed the highest amount of leaf blight on the water hyacinth leaves 3 days after being treated at 25,000–100,000 p.p.m., while the crude extracts from hexane showed the highest amount of leaf blight on the water hyacinth leaves at 50,000–100,000 p.p.m.

Evaluation of the enzymes

Myrothecium roridum was tested for the production of three types of cellulase, β-1,4-exoglucanase, β-1,4-endoglucanase and β-glucosidase, when grown on PDA and boiled paddy rice. In addition, the fungus was tested for the production of xylanase and pectinase on the same media. The results showed that the enzyme activity of β-1,4-exoglucanase, β-1,4-endoglucanase, β-glucosidase, xylanase and pectinase were 0.015 ± 0.005, 0.016 ± 0.002, 0.019 ± 0.001, 0.237 ± 0.022 and 0.056 ± 0.005 U mL^{-1}, respectively, for *M. roridum* that was grown on the PDA. In contrast, the enzyme activity of β-1,4-exoglucanase, β-1,4-endoglucanase, β-glucosidase, xylanase and pectinase were 0.034 ± 0.004, 0.120 ± 0.017, 0.156 ± 0.01, 2.245 ± 0.158 and 0.114 ± 0.119 U mL^{-1}, respectively, for *M. roridum* that was grown on the boiled paddy rice (Table 3). Therefore, the boiled paddy rice appeared to enhance significant protein and enzymatic production, compared to the PDA.

DISCUSSION

A number of isolates of *M. roridum* was recovered from water hyacinth that was exhibiting leaf blight disease in different geographical areas of Thailand. There have been previous reports on leaf blight disease of water

Table 3. Enzymatic activity of *Myrothecium roridum* on potato dextrose agar and boiled paddy rice†

Carbon source	Total protein (μg mL^{-1})	Enzymatic activity (U mL^{-1})				
		β-1,4-Exoglucanase	β-1,4-Endoglucanase	β-Glucosidase	Xylanase	Pectinase
Potato dextrose agar	258.16± 1.520b‡	0.015 ± 0.005b	0.016 ± 0.002b	0.019 ± 0.001b	0.237 ± 0.022b	0.056 ± 0.005b
Boiled paddy rice	1,252.93± 3.061a	0.034 ± 0.004a	0.120 ± 0.017a	0.156 ± 0.01a	2.245 ± 0.158a	0.114 ± 0.119a

†Data represent the mean ± standard deviation of three replicates; ‡the means in the same column followed by a common letter are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

hyacinth that are caused by the genera of *Myrothecium*, which are widely distributed in different continents, including Thailand (Evans & Reeder 2001). Similarly to the current study, the isolates of *M. roridum* have been reported to have specificity to water hyacinth and to produce enzymes and mycotoxins (Aboul-Nasr *et al.* 2013). A recent report showed that *M. roridum* produced cell wall-degrading enzymes (Okunowo *et al.* 2010a) and macrocyclic trichothecene mycotoxins, such as Verrucarin A & J and Roridin A & E (Jarvis *et al.* 1985; Abbas *et al.* 2002). Verrucarin A is a germination inhibitor of several problematic weeds (Lee *et al.* 2008) and Roridin A causes rapid effects that are associated with detachment of the protoplasm from the cell wall of plants (Hoagland *et al.* 2012). Moreover, fungal isolates can decrease the fresh weight of water hyacinth plants. Similarly, studies confirmed that fungal pathogens that were isolated in Ethiopia reduced the water hyacinth biomass (Tegene *et al.* 2012).

The isolate of *M. roridum* that was used in this research has a host specificity to aquatic weeds, such as duckweed, water lettuce and water hyacinth. Okunowo *et al.* (2013) similarly found that an indigenous strain of *M. roridum* from water hyacinth in Nigeria could infect duckweed and water lettuce. In field use, *M. roridum* will be applied by spraying onto water hyacinth. The fungal strain that was tested in the current research would not be expected to affect important agricultural plants in Thailand. Therefore, this isolate should be viewed as a potential biocontrol agent for managing water hyacinth infestations.

The efficacy of fungal isolates against water hyacinth under natural conditions was lower than under greenhouse conditions, apparently related to environmental factors that influence plant infection and disease development under field conditions, such as water activity, solar irradiation, temperature and climate variability (Dagno *et al.* 2011a). Dagno *et al.* (2011b) studied the effect of water activity and temperature on the spore germination and mycelial growth rates of *Fusarium sacchari*, *Cadophora malorum* and *Alternaria* sp. for controlling water hyacinth in Mali. The study concluded that the water activity and temperature influenced the percentage of viable conidia and the mycelial growth rate of the fungal isolates.

The application of the fungal pathogen by using a spore suspension with surfactants, such as Tween 20 and palm oil, resulted in higher levels of disease on water hyacinth than without them. It has been suggested that surfactants provide a better distribution of inoculum on plant surfaces and increase the infection of plants by pathogens via the direct penetration of microbial cells through the stomata (Hoagland *et al.* 2007). Dagno *et al.* (2011a) also reported that *C. malorum* + palm oil and *Alternaria jacinthicola* + palm oil produced a higher level of disease severity than those fungi without the surfactant on water hyacinth in greenhouse and field conditions.

The crude extracts of *M. roridum* caused leaf blight signs on water hyacinth because of the natural metabolites in the crude extracts. It is possible that such natural metabolites in the crude extracts can influence the disease development of *M. roridum* in water hyacinth. Okunowo *et al.* (2010b) suggested the possibility that many of the natural metabolites that are produced by this fungus could induce similar disease signs as the fungus on water hyacinth.

The ability of *M. roridum* to produce cellulase, xylanase and pectinase was demonstrated by using enzymatic activity assays. The cellulose- and xylan-degrading potential of *M. roridum* was confirmed by Okunowo *et al.* (2010a) and they found that pathogenic *M. roridum* Tode (IMI 394934) from water hyacinth is capable of producing cellulase and xylanase. In addition, the plant pathogenic fungus *Myrothecium verrucaria* was able to produce cellulase, xylanase and pectinase in submerged cultures (Afifi 2003; Moreira *et al.* 2005). This research was related to the mechanical forces that are exerted by pathogens on host tissues (Agrios 1997). The extracellular enzymes that are secreted by fungi are able to macerate tissues and degrade cell wall components, which is important in facilitating penetration and tissue colonization. Evaluation of the production of cellulase, xylanase and pectinase from two different carbon sources indicated that the level of enzymatic production by *M. roridum* in boiled paddy rice was higher than that in PDA. Other research also demonstrated that different carbon sources had different levels of efficiency in stimulating fungal enzymatic production (Silva *et al.* 2006). Enzymatic production by *M. roridum* is apparently favored by such polysaccharides in boiled paddy rice as cellulose, hemicellulose and amylopectin (Beg *et al.* 2000). In contrast, PDA is composed of monosaccharides, such as glucose, which are less suitable for enzymatic production by the fungus.

CONCLUSION

Myrothecium roridum strains were isolated from water hyacinth that was exhibiting leaf blight, which are highly virulent and cause severe disease of water hyacinth. *Myrothecium roridum* strains were identified that can efficiently control water hyacinth when applied as fungal formulations and fungal crude extracts, probably because of extracellular enzymes and secondary

metabolites. However, only enzymatic activity was described in this article. The activity of mycotoxins should be measured in a further study. A host range study indicated that the strain of *M. roridum* caused disease on water hyacinth and two invasive aquatic weeds but not on the other 74 important cultivated plants, indicating that *M. roridum* has the potential as a biocontrol agent for water hyacinth. In order to use the strain of *M. roridum* as a mycoherbicide, additional host range testing using other plant species in the family Araceae, which the invasive weeds belong to, should be conducted.

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