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Bioactive potential of *Myrothecium inundatum* against microbial plant pathogens and free-floating aquatic weeds

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Abstract. *Myrothecium inundatum* is a fungus capable of producing bioactive potential metabolites. This research aims to study the bioactive efficiency of *M. inundatum* isolated from diseased water lettuce. Five isolates of *M. inundatum* were isolated from blighted leaf of disease in the northern region province of Thailand. The first method, the dual culture test, was used to test the efficiency of *M. inundatum* for inhibiting phytopathogenic bacteria and phytopathogenic fungi. The result indicated that total *M. inundatum* could inhibit *Xanthomonas* sp. and *R. solanaceae* and total *M. inundatum* could inhibit *Nigrospora* sp., *B. maydis*, *B. oryzae*. On the other hand, 5 isolates of *M. inundatum* could not inhibit *Sclerotium* sp. In addition, *M. inundatum* were tested for pathogenicity on free-floating aquatic weeds. The result showed that 5 isolates of *M. inundatum* could cause disease severity in water lettuce and water hyacinth, but 5 isolates *M. inundatum* could not cause disease severity in duckweed. Therefore, bioactive potential of *M. indunatum* can be used in the development and be valued in agriculture and environment.

Keywords: Free-floating aquatic weeds, phytopathogenic bacteria, phytopathogenic fungi

1. Introduction

Thailand is one of the world's top major food crops (Office of Agricultural Economics 2016). Nowadays, the problem in economic crop production is caused by disease-causing microorganisms including fungi, bacteria and viruses (Heinrichs et al. 1979). In addition, the problems of free-floating aquatic weeds are mainly caused by water hyacinth, duckweed and water lettuce (Holm et al. 1977). At present, a common method, which is the chemical control, is used to manage disease-causing microorganisms and free-floating aquatic weeds. This is because the use of chemicals is an easy, fast and efficient method. The 2,4-D and other chemical herbicides are efficient in controlling water hyacinth in Huai wai reservoir in Loei province of Thailand (Klaaigaew 2013). However, chemicals are not sustainable over the long-term, as they are expensive and damaging to non-targeted organisms in soil and water of agricultural areas and environment (Plant et al. 2005). Biological control methods have been identified as the best option for successful and cost-effective management with long-term sustainability. The use of microorganisms as biological control agents (BCAs) was developed worldwide in the 1960s (Hernández-Rosas et al. 2020). In Thailand, biological control was initiated in the early1970s by the National Biological Control Research Center (NBCRC). Many species of fungi have been reported for control of plant pathogens and aquatic weeds. Fungi as for biological control are used to produce biological control agents such as enzyme antibiotic and mycotoxin (Dagno et

al. 2012). Many species of fungi have been reported for control of plant pathogens and aquatic weeds including fungus, *Myrothecium*. *Myrothecium* species belonging to the family Stachybotryaceae; with over 30 species, consist of fungal saprophytes and weak pathogens with a worldwide distribution. Researches showed several species of *Myrothecium* are prolific producers of vast and biologically active secondary metabolites. Biological activities and origin of these novel antibacterial, antifungal and antiviral compounds. The bioactive secondary metabolites of *Myrothecium* are enzymes, antibiotics, sesquiterpenoids, triterpenes, diterpenoids, cyclopeptides, and many macrocyclic trichothecene mycotoxins such as verrucarins and roridins (Elkhateeb and Daba 2019).

The present study aimed to evaluate the biological activities of *M. inundatum* for controlling free-floating aquatic weeds and antimicrobial activity which is selected as an alternative for human and environmental benefits to reduce the use of the hazardous chemicals. Moreover, this induces the development and adds value of the natural resources in the country.

2. Materials and Methods

2.1 The antagonistic fungi

The 5 isolates of antagonistic *M. inundatum* were isolated from water lettuce collected in the northern region province of Thailand including Chiang Mai (2 samples), Lamphun (2 samples) and Phayao (1 sample). Each fungal culture was single-spored by the hyphal tip method (Senanayake et al. 2020). Aliquots of 100 µl of the pathogen suspension (10^2 spores/ml) were spread on water agar (WA) and incubated for 6-8 hours at room temperature. When spores germinated, hyphal tips were cut under stereo microscopes and transferred to potato dextrose agar (PDA). Each pure fungal culture was maintained on PDA slants.

2.2 In vitro antibacterial activity

The ability of five *M. inundatu* isolates to inhibit the growth of twophytopathogenic bacteria was assessed *in vitro*: *Rasonia solanaceae* and *Xanthomonas* sp. using dual-culture assay according to the modified method described by Hassan et al. (2021). For antagonistic capability assessment, *M. inundatum* isolates were cultured together with the bacterial pathogens on potato dextrose agar (PDA) medium. A 0.6 cm mycelial plug was taken from a seven-day-old *M. inundatum* culture on one side of the plate. On the opposite side of the plate, five-day-old bacterial pathogen culture was streaked on the same media at 9 cm from the plate edge and incubated at 28°C for 21 days. The experiments were conducted in a randomized complete block design (CRD) with five replications and was repeated twice. Each bacterial pathogen was used as a treatment with *M. inunatum* isolate combination. However, the positive control treatment was each bacterial pathogen without the *M. inundatum* isolate which used 10 µg/ml chloramphenicol in 0.6 cm diameter paper disc. Antifungal activity of *M. inunatum* was calculated for each culture expressed as percentage growth inhibition (PGI) (Zambrano et al. 2021), calculated as PGI = (R1 - R2)/R1 × 100. R1 was the radius of the control pathogen and R2 was the radius of the pathogen in confrontation with each isolate. Data were subjected to analysis of One-way ANOVA, followed by Duncan's multiple range test (DMRT). A $P<0.01$ was considered to be statistically significant.

2.3 In vitro antifungal activity

The antagonistic effects of *M. inunatum* isolates on phytopathogenic fungi as *Nigrospora* sp., *Sclerotium* sp., *Bipolaris*

maydis, and *B. oryzae* were investigated using the dual culture method. Each agar plug of a 7-day-old antagonistic isolate colony was placed on the side of 9 cm Petri dishes, with an agar plug of each fungal pathogen (0.6 cm diameter) placed on the opposite side 5 cm from the antagonistic fungus (0.6 cm diameter).

The negative control contained only pathogens, while the positive control contained 20 µg/ml amphotericin B (Sigma, USA) in a 0.6 cm diameter paper disc. Cultures were incubated at 28°C for 21 days with a 12-hour photoperiod under white fluorescent light. The experiment was designed according to a complete randomized block (CRD) with five replicates and repeated twice. The percent growth inhibition (PGI) of pathogen was also calculated as percentage inhibition (%) = R1 − R2/R1× 100, R1 = the distance (measured in cm) from the point of inoculation to the colony margin in control plate, R2 = the distance of fungal growth from the point of inoculation to the colony margin in treated plate in the direction of the antagonist. Test of variance was calculated using Analysis of variance (One-ANOVA) and the means were compared using DMRT at $P \leq 0.01$.

2.4 Pathogenicity test on free-floating aquatic weeds

The five isolates of *M. inundatum* were tested for their ability to control free-floating aquatic weeds. Healthy water hyacinth (*Eichhornia crassipes*), duckweed (*Lemna minor*) and water lettuce (*Pistia stratiotes*) were collected from natural water sources. Plant samples were sterilized with a 10% sodium hypochlorite solution for 5 min and rinsed two times with sterile distilled water. Inoculum production was initiated by suspending a mycelial plug (5 mm in diameter), cut from 5-day-old cultures in PDA medium. After a 14-day incubation period under white fluorescent lamps were used to provide a 12-hour photoperiod. The fungal spores were diluted with sterile distilled water and the spore concentration was adjusted to 10^8 spores/ml using a hemocytometer. Inoculation was done by spraying leaves and petioles of free-floating aquatic weeds with 1×10^8 spores/ml; the control treatment was sprayed with 10 ml sterile distilled water. This experiment was conducted using a completely randomized design (CRD) with 10 replications of each treatment. The plants were moved to the greenhouse after spending 24 hours in a growth chamber with 100% relative humidity. Temperatures in the greenhouse ranged from 26 to 32°C with 65-90% relative humidity (RH). The disease severity was evaluated at 7 days after inoculation using the following rating scale; 0= 0%, 1= 1-25%, 2= 26-50%, 3= 51-75%, 4= 76-100% leaf blight (Kongjornrak et al. 2019). ANOVA was used to assess the disease severity in all repeated experiments. Duncan's multiple range test (DMRT) was used to analyze and group the means at $P \leq 0.05$.

3. Results

3.1 In vitro antibacterial activity

The 5 isolates of *M. inundatum* isolates were able to suppress the growth of *R. solanaceae* and *Xanthomonas* sp. on PDA (Table 1). The percent inhibition of radial growth varied from 47.5% to 61.0%. *M. inundatum* isolates inhibited radial growth of *Xanthomonas* sp. and the percent inhibition of radial growth varied from 60.0% to 65.7%. *Myrothecium inundatum* isolates also inhibited the radial growth of *R. solanaceae*. Moreover, the percent inhibition of radial growth of *R. solanaceae* and *Xanthomonas* sp. from five antagonistic isolates were not

significantly different from 10 µg/ml chloramphenicol.

Table 1. The percentages of the inhibition of fungal plant pathogens by five *M. inundatum* isolates

Treatment	The percentages of the inhibition	
	R. Solanaceae	***Xanthomonas* sp.**
Negative control	0.0±0.0[b]*	0.0±0.0[b]*
Positive control (10 µg/ml chloramphenicol)	57.1±12.9[a]	48.0±9.9[a]
M. inundatum isolate 1	60.0±11.3[a]	52.5±9.0[a]
M. inundatum isolate 2	65.7±5.7[a]	61.0±1.4[a]
M. inundatum isolate 3	64.4±5.7[a]	47.5±11.5[a]
M. inundatum isolate 4	62.2±7.0[a]	56.5±9.3[a]
M. inundatum isolate 5	65.2±2.4[a]	53.5±12.6[a]

*Means±SD in the same column followed by a common letter were not significantly different by DMRT ($P \leq 0.01$).

3.2 In vitro antifungal activity

The five isolates of *M. inundatum* showed the inhibitory activities against all tested fungi except for *Sclerotium* **sp**. (Table 2). The obtained results indicated that no significant differences in the inhibition growth of *Nigrospora* sp., *B. maydis*, and *B. oryzae* by five fungal isolates. However, the effect on the percentages of the inhibition of five antagonistic isolates and 20 µg/ml amphotericin B was significantly different in Table 2.

Table 2. The percentages of the inhibition of fungal plant pathogens by five *M. inundatum* isolates

Treatment	The percentages of the inhibition			
	B. maydis	*B. oryzae*	*Nigrospora* sp.	*Sclerotium* sp.
Negative control	0.0±0.0[c]*	0.0±0.0[c]	0.0±0.0[c]	0.0±0.0[a]
Positive control (20 µg/ml amphotericin B)	31.1±3.1[b]	21.2±3.1[b]	19.0±6.4[b]	0.0±0.0[a]
M. inundatum isolate 1	49.0±3.7[a]	52.6±4.5[a]	32.4±2.1[a]	0.0±0.0[a]
M. inundatum isolate 2	44.5±4.6[a]	49.3±1.5[a]	31.9±4.6[a]	0.0±0.0[a]
M. inundatum isolate 3	49.3±3.3[a]	47.3±6.3[a]	31.4±2.6[a]	0.0±0.0[a]
M. inundatum isolate 4	48.3±9.5[a]	41.6±12.9[a]	38.7±6.8[a]	0.0±0.0[a]
M. inundatum isolate 5	42.4±6.7[a]	40.3±8.9[a]	26.9±13.4[a]	0.0±0.0[a]

*Means±SD in the same column followed by a common letter were not significantly different by DMRT ($P \leq 0.01$).

3.3 Pathogenicity test on free-floating aquatic weeds

These fungal isolates were studied for pathogenicity testing on free-floating aquatic weeds under greenhouse conditions. The *M. inundatum* isolates infected and produced symptoms of disease on water hyacinth leaves (Figure A-B). *Myrothecium inundatum* isolate 1, isolate 4 and isolate 5 had the significantly highest disease severities on water hyacinth (Table 3). In addition, fungal isolates could also infect water lettuce (Figure C-D), observing as leaf blight, and *M. inundatum* isolate 1, isolate 2, isolate 3 and isolate 4 had the significantly highest disease severities (Table 3). However, none of *M. inundatum* isolates were unable to infect duckweed (Figure E-F).

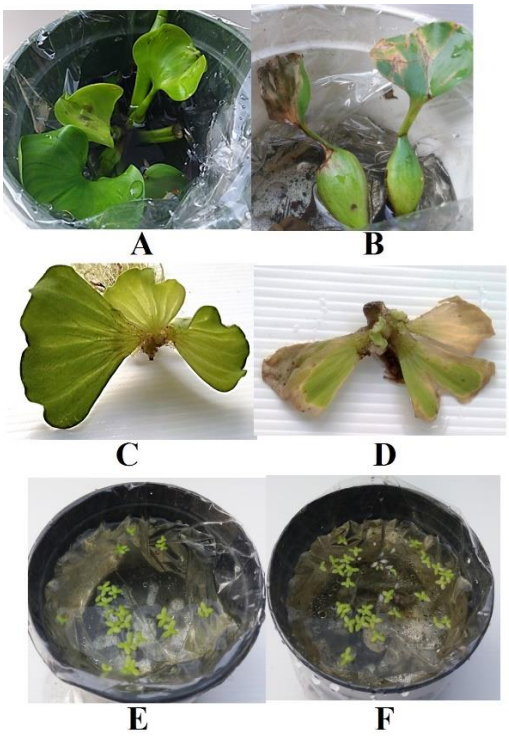

Figure 1 Leaf blight symptoms on free-floating aquatic weeds;
(A) control treatment on water hyacinth, (B) leaf blightsymptoms on water hyacinth,
(C) control treatment onwater lettuce, (D) leaf blight symptoms on water lettuce
(E) control treatment on duckweed, (F) none of leaf blightsymptoms on duckweed

Table 3. The disease severity on water hyacinth and water lettuce produced by 5 isolates of *M. inundatum* under greenhouse condition

Treatment	**Disease severity***	
	water hyacinth	**water lettuce**
Negative control	0.0±0.0^{e**}	0.0±0.0^{d**}
Positive control	4.0±0.0^{a}	4.0±±0.0^{a}
M. inundatum isolate 1	1.2±0.45^{bc}	3.67±0.58^{a}
M. inundatum isolate 2	0.4±0.55^{de}	2.67±1.16^{b}
M. inundatum isolate 3	0.6±0.55^{cde}	3.00±1.00^{ab}
M. inundatum isolate 4	1.4±0.89^{b}	3.33±0.58^{ab}
M. inundatum isolate 5	0.8±0.45^{bcd}	1.33±0.58^{c}

*Disease severity was rated using the following scale: 0= 0%, 1= 1-25%, 2= 26-50%, 3= 51-75%, 4= 76-100% leaf blight.

**Means±SD in the same column followed by a common letter were not significantly different by DMRT ($P \leq 0.05$).

4. Discussion

Similar to previous reports, the potent *M. inundatum* isolates inhibited the growth of *R. solanaceae* and *Xanthomonas* sp. because several researchers have already reported similar antibacterial activity of *Myrothecium* species against bacterial pathogens. The *Myrothecium* crude extracts has been previously reported against a wide range of pathogenic bacteria such as *Escherichia coli, Salmonella Typhi*, *Klebsiela pneumoniae* and *Bacillus cereus* (Elkhateeb and Daba 2019). *Myrothecium* species produced secondary metabolites or enzymes. Muhsin et al. (2012) reported that the purified compound of *M. verrucaria* isolated from soil in southern Iraq that could inhibit the bacterial strains *E. coli* and *Staphylococcus aureus*.

The isolates of *M. inundatum* were able to produce antifungal activity (Naz et al. 2015). Similar results were observed by Anyanwu et al. (1994), in which they studied the antagonistic activity of five *Myrothecium* species against fungi such as *Fusarium*, *Trichoderma*, *Stachybotrys*, *Cladosporium*, *Penicillium*, *Aspergillus* and *Alternaria*. *Myrothecium* species which are produced by biologically active secondary metabolites such as enzymes, antibiotics and mycotoxins against fungi (Elkhateeb and Daba, 2019). Vidhate et al. (2015) reported that produces mycolytic enzymes mainly chitinases, proteases, β -1-3 glucanases and lipases capable of degrading fungal cell walls. Moreover, mycotoxins or antibiotics from *Myrothecium* controlled fungal pathogens (Elkhateeb and Daba 2019). Mondol et al. (2015) exhibited that trichothecenes from *M. roridum* M10 have widespread toxicological effects throughout the cells, and have been inhibited in cell structure and metabolism including inhibition of cell structure and metabolism, as well as inhibition of RNA and DNA synthesis in eukaryotes.

The isolate of *M. inundatum* has a host specificity to free-floating aquatic weeds such as water lettuce and water hyacinth. In contrast, the study by Piyaboon et al. (2016) showed that *M. roridum* have been reported to be specific to water hyacinth, water lettuce and duckweed. Therefore, the *M. inundatum* is a candidate biocontrol agent against free-floating aquatic weeds. *Mycothecium* produced the secondary metabolites such as mycotoxins and enzymes. The enzymes of *Myrothecium* are able to macerate tissues and degrade cell wall components of weeds. The potential of cellulase, xylanase and pectinase are confirmed by Piyaboon et al. (2016) as *M. roridum* producing three types of cellulase (β-1,4-exoglucanase, β-1,4-endoglucanase and β-glucosidase), xylanase and pectinase. Other researches also demonstrated that the fungus *M. verrucaria* was able to produce cellulase, xylanase and pectinase (Moreira et al. 2005). It has been suggested that trichothecene mycotoxins such as verrucarins and roridins of *Myrothecium* demonstrated growth inhibitory activity against weeds. A recent report showed that verrucarin A was a germination inhibitor of several problematic weeds, and roridin A caused rapid effects associated with detachment of the protoplasm from the cell wall of plants (Piyaboon et al., 2016).

Summary, *Myrothecium inundatum* were isolated from blighted leaf of diseased water lettuce. The capability of *M. inundatum* was active against bacterial plant pathogens as *Xanthomonas* sp. and *R. solanaceae* and *M. inundatum* was active against fungal plant pathogens as *Nigrospora* sp., *B. maydis*, *B. oryzae* except *Sclerotium* sp. Moreover, *M. inundatum* could cause disease severity in water lettuce and water hyacinth but *M. inundatum* could not cause disease severity

in duckweed. Therefore, bioactive potential of *M. inundatum* can be used for the development of novel active antimicrobial agents and free-floating aquatic weeds in agriculture and environment.

It can be determined from our findings that *M. inundatum* contain various bioactive components that have the ability to fight against antimicrobial agents and herbicide. This research could be helpful in the development and adds value of the natural resources in the country. However, further research is still needed to establish a correlation between the better herbicide and antimicrobial properties of extracts of these *M. inundatum* under greenhouses and plantations.

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