International Journal of Agricultural Technology 2019 Vol. 15(1): 55-62
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

Screening, identification and evaluation of potential biocontrol fungi against water lettuce

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Kongjornrak, A., Teeranate, P., Thinthani, T. and Piyaboon, O. (2019). Screening, identification and evaluation of potential biocontrol fungi against water lettuce. International Journal of Agricultural Technology 15(1): 55-62.

Abstract Water lettuce is a major problem in water resources and rapid widespread. The management focused to change from herbicides to biological control using natural enemies of water lettuce including fungal pathogens. The blighted and spotted leaf of diseased water lettuce plants were collected from 8 provinces of Thailand. Thirty-nine isolates of fungal pathogens were identified using morphological characteristics. They were identified as *Myrothecium* 5 isolates, *Chaetomium* 2 isolates, *Aspergillus* 3 isolates, *Rhizopus* 3 isolates, *Nigrospora* 6 isolates, *Bipolaris* 4 isolates, *Curvularia* 2 isolates, *Epicoccum* 5 isolates and *Acremonium* 9 isolates. The fungal *Myrothecium* isolates, through the morphologically identification process, were selected to study molecular data, which were derived from sequencing the internal transcribed spacer (ITS) regions of the rDNA. The result indicated that the five *Myrothecium* isolates could be identified as *M. inundatum*. Five isolates of *M. inundatum* were tested pathogenicity for controlling water lettuce. Fungal isolates were found to be the virulent pathogenicity on water lettuce.

Keywords: biocontrol, water lettuce, fungal pathogens

Introduction

The water lettuce (*Pistia stratiotes* L.) is considered one of the top 10 world's worst weeds (Yang *et al*., 2014). Water lettuce has adverse effects on biodiversity and the environment, due to the ability of developing dense mats, impeding fishing and boat transport, as well as constituting a health hazard by sheltering disease carrying insects and snails (Mbati and Neuenschwander, 2005; Yang *et al*., 2014). Controlling water lettuce by mechanical, or chemical methods were carried out. However, both methods could not completely remove this aquatic weed effectively and sustainably (Okunowo *et al*., 2013). The pioneering survey of the mycobiota of aquatic weeds and water lettuce for finding potential biocontrol. Several researchers have surveyed and evaluated pathogens for controlling water lettuce. Barreto (1991) have surveyed fungal pathogens of water lettuce in the state of Rio de Janeiro (Brazil). The fungus

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was surveyed and collected was *Cercospora pistiae* causing leaf spot of water lettuce in Brazil (Barreto *et al*., 1999). Many species of fungi have been reported to control water lettuce, such as *Alternanthera philoxeroides, Cercospora pistiae* and *Myrothecium roridum* (Barreto *et al*., 2000; Piyaboon *et al*., 2016). Several reports indicated fugal pathogens have the potential to control aquatic weeds and water lettuce (Dunne *et al*., 1996). Fungal pathogens can produce secondary metabolites, numerous enzymes and many mycotoxins for controlling aquatic weeds and water lettuce (Okafor *et al*., 2010; Okunowo *et al*., 2010). The present study aimed to identify isolated fungi from diseased water lettuce using morphological characteristics and to select and test fungal pathogen on water lettuce for controlling water lettuce.

Materials and methods

Fungal collection and isolation

Water lettuce leaf blight disease was observed and collected from different geographical areas in 8 provinces of Thailand including, the northern region (Phayao, Chiangmai and Lampoon), north-eastern region (Ubon Ratchathani and Sisaket), central region (Nonthaburi and Nakhon Pathom), and southern region (Surat Thani). Fungal pathogens were isolated from the leaves using the tissue transplanting method. Fungal isolates were cultured on PDA (Potato Dextrose Agar) The cultures were incubated at room temperature under white fluorescent light with a 12-h photoperiod, and each fungal culture was single-spored by the hyphal tip method. A 100 µl spore suspension of fungal cultures were spreaded on water agar (WA) and incubated at room temperature for 6-8 h. When spores germinated, hyphal tips of the fungus were cut under stereo microscopes and transferred to PDA. Each pure fungal culture was maintained on PDA slants.

Morphological based identification

Each fungal isolate was examined on the basis of morphological characters such as colony color, color and shape of conidia and conidiophores to confirm genus identity and compare morphological characters as explained by Barnett and Hunter (2006).

DNA extraction and molecular based identification

Fungal isolates were randomly selected to study molecular-based identification. Pathogenic isolates were grow on PDA at 28°C under white

fluorescent light with a 12-h photoperiod for 7 days and DNA of fungi were extracted as described in Saitoh *et al*. (2006). The ITS regions of rDNA of fungal samples were amplified using the following universal primers: ITS1 (5'TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGAT ATGC-3') (White *et al*., 1990). Reactions were performed in a final volume of 40 ml with the following components: 0.2 pmole of each primer, 2.5 mM MgCl2; 0.2 mM dNTP and 1 unit of Taq DNA polymerase. The thermal cycles were as follows: 95 ^{o}C for 30 s, 35 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 1 min, and 72 ^{o}C for 1 min, and a final step of 72 ^{o}C for 10 min. After amplification, 5 ml of the PCR product was resolved by gel electrophoresis on a 1% (W/V) agarose gel and then added to 0.1 µl/ml Novel juice (Nucleic acid Gel stain, 1,000X concentrate in DMSO) in a TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). The PCR products were purified and sequenced at SolGent (Solutions for Genetic Technologies) Analysis Services. All newly generated sequences of Pathogenic isolate and fungal antagonist have been submitted to GenBank. ITS sequences were aligned with sequences obtained from the GenBank database by MEGA 7 (Tamura *et al*., 2001) using MUSCLE. Phylogenetic analyses were based on the neighbour-joining (NJ) method (Saitou and Nei, 1987) using the same program. Node support was evaluated by bootstrap analysis using 1,000 replications with the same program (Felsenstein, 1985).

Testing the efficiency of Myrothecium for controlling water lettuce

The five isolates of *Myrothecium* were selected to test the efficiency for controlling water lettuce. Each isolate was grown on PDA and was incubated at 28°C under white fluorescent light with a 12-h photoperiod for 7 days. The water lettuce plants with leaf sizes of approximately <40 cm^2 were inoculated with 10^8 spors/ml spore suspensions of the isolates using the spraying method. The experiment was conducted using a CRD with10 replications of each treatment. The experimental groups consisted of 10 treatments and a control (N= 10 water lettuce plants each). The healthy plant was inoculated with the isolates using spraying method with 5 ml of the spore suspension. At the same time, control plants were sprayed with 5 ml distilled water. The plants were placed in a growth chamber with 100% relative humidity for 24 h and then moved to their natural conditions. The disease severity was observed at 14 days after inoculation using the following rating scale: 0= no disease, 1= 1-25% of leaf blight, 2= 26-50% of leaf blight, 3= 51-75% of leaf blight, 4= 76-100% of leaf blight. The disease severities were analyzed by One-way ANOVA, followed by DMRT. A $P<0.05$ was considered to be statistically significant. Statistical analysis was performed by SPSS statistics software (version 16.0, Window).

Results

Fungal collection and isolation from diseased water lettuce

Fungal isolates were distributed in from 8 provinces of Thailand including Northern region as 17 isolates, Northeastern region as 8 isolates, Southern region as 9 isolates and Central region as 5 isolates.

Morphological based identification

Thirty-nine fungal isolates were identified on morphological characteristics as 9 genus including *Chaetomium* 2 isolates, *Aspergillus* 3 isolates, *Rhizopus* 3 isolates, *Nigrospora* 6 isolates, *Bipolaris* 4 isolates, *Curvularia* 2 isolates, *Epicoccum* 5 isolates and *Acremonium* 9 isolates and *Myrothecium* 5 isolates (Figure 1).

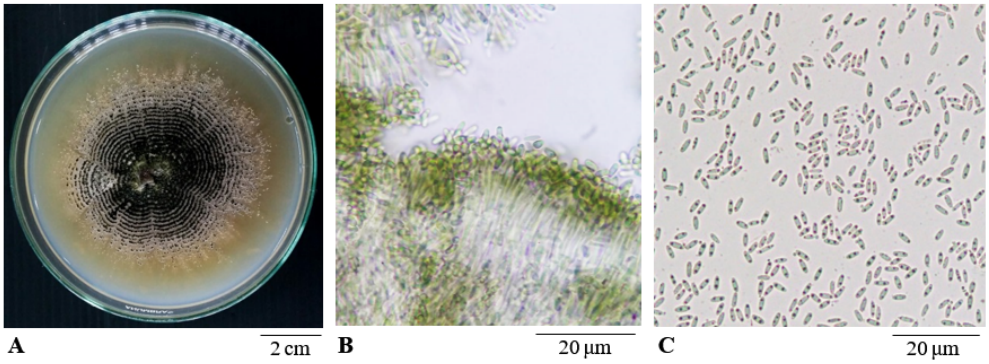

Figure 1. *Myrothecium*; concentric zones of diffused sporodochia (A), conidiophores (B, 400X) and conidia (C, 400X)

Molecular based identification by ITS sequence analysis

All sequences of five *M. inundatum* were deposited in the GenBank database as the following isolates CMDL1 (LC387254), CMSG1 (LC387255), LPPS1(LC387256), PYMU1(LC387257), and PYMU2 (LC387258). The sequence lengths of five *M. inundatum* were 572-573 bp when aligned and analyzed together with the sequences obtained from the GenBank database (NCBI) such as *M. inundatum* (HQ165763, KJ767119, KX013170 and KY827355) *M. roridum* (AB857218 JF724153, KC478534, HQ115647, KF4948828, KJ813720 and KT354921) and *Fusarium oxysporum* (AF069310). The similarity coefficient among the 5 sequences of *M. inundatum* was 99.99% when compared with the sequences of *M. inundatum* obtained from the

database. In addition, NJ clustering found that the five sequences of *M. inundatum* were in same group with other sequences of *M. inundatum* recorded in the database; this was supported by a 100 % bootstrap value, and separated from other species such as *M. roridum* and *F. oxysporum* in Figure 2. Therefore, 5 fungal isolates could be identified as *M. inundatum* based on ITS sequence.

Testing the efficiency of Myrothecium for controlling water lettuce

The five isolates of *M. inundatum* were studied for pathogenicity testing on the water lettuce under greenhouse conditions. Fungal isolates had disease severities on water lettuce. There were no statistical differences in disease severity among the 5 isolates at 14 days after inoculation (Table 1).

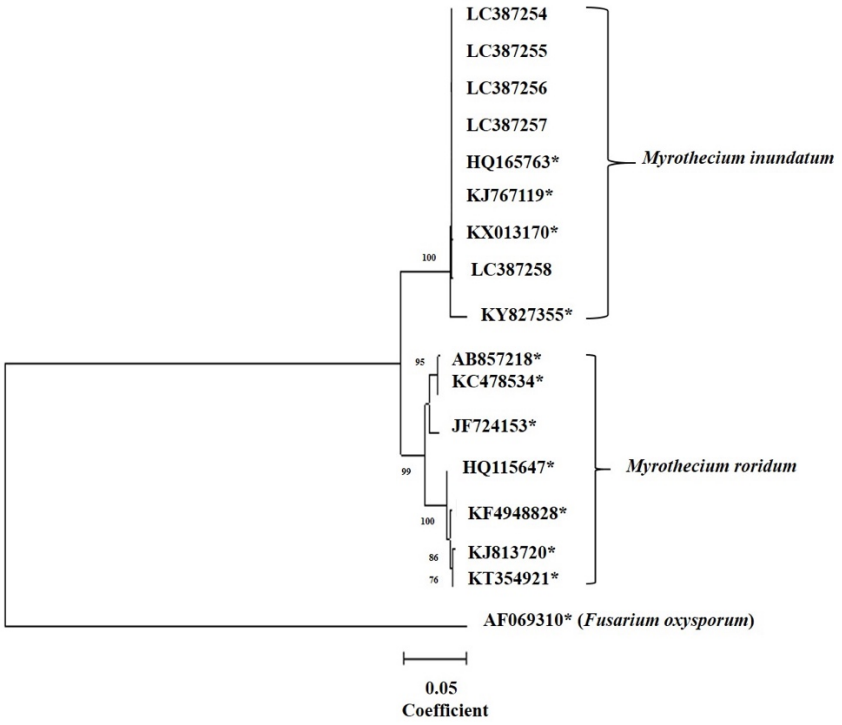

Figure 2. Phylogenetic analysis of the nucleotide sequences of the ITS region including 5.8S rDNA of 5 *M. inundatum* isolates, 11 sequences from *Myrothecium* species and one sequence from *F. oxysporum*. Percentage bootstrap support (1000 replications) is shown on branches (*= sequences obtained from the GenBank database)

Table 1. The disease severity on water lettuce produced by 5 *M. inundatum*

Myrothecium inundatum	Disease severity*
CMDL1	4.0[a**]
CMSG1	4.0[a]
LPPS1	4.0[a]
PYMU1	4.0[a]
PYMU2	3.9[a]
Control	0.0[b]

*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 in the same column followed by a common letter are not significantly different by DMRT ($P<0.05$).

Discussion

Fungal isolates were recovered from water lettuce showing leaf spot and blight disease in different geographical areas of Thailand. Previous reports indicated that leaf spot and blight disease of water lettuce caused by fungi which are widely distributed in different continents. (Barreto *et al*., 2000). This supports the investigations of Barreto *et al*. (1991), who surveyed the mycobiota of water lettuce in the state of Rio de Janeiro (Brazil).

Fungal pathogens from water lettuce could be identified as 9 genus based on morphological characters including *Chaetomium* 2 isolates, *Aspergillus* 3 isolates, *Rhizopus* 3 isolates, *Nigrospora* 6 isolates, *Bipolaris* 4 isolates, *Curvularia* 2 isolates, *Epicoccum* 5 isolates and *Acremonium* 9 isolates and *Myrothecium* 5 isolates. Similar studies confirmed that fungal pathogens were isolated from spot and blight disease of plants (Jiang *et al*., 2018; Zhang *et al*., 2016; Cui *et al*., 2018).

The five isolates of *Myrothecium* from spot and blight disease of water lettuce were identified to the species level by ITS sequencing and phylogenetic analysis. The results showed that *M. inundatum* isolates were in the same group as *M. inundatum* recorded in the GenBank database based on sequence similarity. There are many reports showing that ITS regions of rDNA can be used to confirm the species of *Myrothecium* (Chen *et al*., 2016; Fernandes *et al*., 2015). This supports the investigations of Banerjee *et al*. (2010), who studied the sequence of the ITS regions of *M. inundatum* isolated from stem pieces of *Acalyph indica* L. in India.

Five *M. inundatum* isolates from infected water lettuce is an aggressive pathogen which causes leaf blight in water lettuce in Thailand. In a previous report showed that the genus *Myrothecium* have the potential to be used as a mycoherbicide for controlling weeds (Boyette and Weaver, 2007). The genus *Myrothecium* is a prolific producer of secondary metabolites such as enzymes

and mycotoxins (Chen *et al*., 2016; Piyaboon *et al*., 2016). Similar studies confirmed that *Myrothecium* produced cell wall degrading enzymes including cellulases and xylanase (Okunowo *et al*., 2010). Moreover, *Myrothecium* species can produce trichothecene mycotoxins such as roridin A and verrucarin A which related to destroy growth and metabolism of weed plants (Hoagland *et al*., 2012; Abbas *et al*., 2002; Jarvis *et al*., 1985).

Therefore, *M. inundatum* from water lettuce could be identified based on morphological and molecular characters and *M. inundatum* can be effectively used to control water lettuce. In the next study, improvements in application of formulated *M. inundatum* will be required with the formulated fungus in order to be used in management of water lettuce in water source.

Acknowledgement

This research was financially supported by Junior Science Talent Project (JSTP), National Science and Technology Development Agency (NSTDA) for valuable scholarship, Thailand. The authors thank Department of Biology, Mahidol Wittayanusorn School, Nakhon Pathom, Thailand providing the equipment used in this research. We would like to thank Assistant Professor Dr. Jamorn Somana, Department of Biochemistry, Faculty of Science, Mahidol University for the critical reading of the manuscript.

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(Received: 3 August 2018, accepted: 30 November 2018)