การคัดกรองเบื้องต้นเพื่อหาเชื้อราสาเหตุโรคพืชสำหรับใช้เป็นสิ่งควบคุมโดยชีววิธีของผักตบชวา (*Eichhornia crassipes* (Mart.) Solms)

ชวิน อัศวเสตกุล[1] และเกรียง กาญจนวตี[2*]

[1]หลักสูตรวิทยาศาสตรบัณฑิต สาขาวิชาวิทยาศาสตร์และวิศวกรรมการจัดการ คณะวิศวกรรมศาสตร์ มหาวิทยาลัยสแตนฟอร์ด แคลิฟอร์เนีย 94305 สหรัฐอเมริกา; [2]ภาควิชาชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

[*]E-mail: Krieng.K@chula.ac.th

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บทคัดย่อ

การควบคุมโดยชีววิธีเป็นวิธีการที่ทั่วโลกยอมรับว่าเป็นวิธีจัดการผักตบชวา (*Eichhornia crassipes*) ที่มีความคุ้มค่าและนำไปใช้ได้จริง แต่การศึกษาวิจัยในเรื่องดังกล่าวมีอยู่อย่างจำกัดในประเทศไทย โดยเฉพาะอย่างยิ่งการคัดกรองเพื่อหาสิ่งควบคุมโดยชีววิธีชนิดใหม่ ผู้วิจัยได้สุ่มเก็บตัวอย่าง *E. crassipes* ที่แสดงอาการของโรคจาก 2 แหล่ง และแยกเชื้อราออกจากตัวอย่างได้ทั้งหมด 14 ไอโซเลต นำเชื้อราที่ได้เข้าสู่ใบ *E. crassipes* ที่ปราศจากโรคผ่านทางบาดแผลหรือไม่ผ่านบาดแผลเป็นเวลา 7 วัน ผู้วิจัยพบว่าเชื้อรา *Alternaria* sp. ไอโซเลต WH–06 มีความรุนแรงในการก่อโรคมากที่สุด โดยเชื้อรา *Alternaria* sp. ไอโซเลต WH–06 สามารถทำให้เกิดอาการของโรคที่เหมือนกันระหว่างการทดสอบบนใบที่ถูกและไม่ถูกตัดจากต้น อาการของโรคประกอบด้วยเนื้อเยื่อตายสีดำและสีน้ำตาลที่ล้อมรอบด้วยวงสีเหลืองที่เกิดจากภาวะพร่องคลอโรฟิลล์ (chlorosis) เมื่อศึกษาวิจัยต่อไปคาดว่าเชื้อรา *Alternaria* sp. ไอโซเลต WH–06 มีศักยภาพที่จะใช้เป็นสิ่งควบคุมโดยชีววิธีที่มีประสิทธิภาพ โดยมีความเป็นไปได้ที่จะใช้เชื้อราชนิดนี้ผ่านการจัดการศัตรูพืชแบบผสมผสาน (integrated pest management) ร่วมกับแมลงที่สามารถกิน *E. crassipes* ได้

คำสำคัญ: ผักตบชวา เชื้อราสาเหตุโรคพืช การควบคุมโดยชีววิธี ศัตรูพืชทางน้ำ

Preliminary Screening of Phytopathogenic Fungi as Biological Control Agents of Water Hyacinths (*Eichhornia crassipes* (Mart.) Solms)

Chawin Asavasaetakul[1] and Krieng Kanchanawatee[2*]

[1]Bachelor of Science Program in Management Science and Engineering, School of Engineering, Stanford University, California 94305, United States; [2]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[*]E-mail: Krieng.K@chula.ac.th

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Abstract

Biological control is globally recognized as a cost–effective and practical management mechanism of water hyacinth, *Eichhornia crassipes*, but limited research has been done in Thailand, especially for screening novel biological control agents. We collected the diseased *E. crassipes* from two sampling sites where 14 fungal isolates were isolated. Unwounded and wounded inoculation was conducted on healthy *E. crassipes* leaves in a controlled environment. Seven days after inoculation, we found that the *Alternaria* sp. isolate WH–06 was the most virulent isolate. *Alternaria* sp. isolate WH–06 was able to produce similar disease symptoms between the detached leaves and whole plants. The symptoms included black and brown necrotic tissues surrounded by a yellow halo of chlorosis. With further studies, *Alternaria* sp. isolate WH-06 has the potential to be an effective biological control agent, possibly through integrated pest management with *E. crassipes* feeding insects.

Keywords: Water hyacinth, Phytopathogenic fungi, Biological control, Aquatic pest

Introduction

Water hyacinth [*Eichhornia crassipes* (Mart.) Solms] is one of the most extensive invasive aquatic plant species worldwide, infesting waterways in every continent except Antarctica (Coetzee *et al*., 2017). Native to tropical and sub–tropical South America, *E. crassipes* was originally introduced as an ornamental plant in 1896 (Napompeth, 1994) but had since rapidly found its way into Thailand's waterways. Under suitable conditions, a population of these plants is able to double their population within one to two weeks (Taylor *et al*., 2010).

The plant's rapid proliferation continues to obstruct navigation and water flow, limits recreational use of aquatic systems, and poses a significant risk of mechanical damage to hydroelectric systems (Téllez *et al*., 2008). Additionally, *E. crassipes* also alters the physicochemical characteristics of the water by reducing the water's temperature, pH, biological oxygen demand, and nutrient levels, which may result in the death of aquatic organisms (Rai and Munshi, 1979). Furthermore, *E. crassipes* also hinder the production of rice through resource competition, restricting accessibility to nutrients and allelopathy (Smith, 1983). As such, it is considered the world's worst aquatic weed (Lata and Dubey, 2010). For this reason, the Thai Government declared that the elimination of *E. crassipes* plants as a part of the National Agenda in 2016.

Attempts to eliminate the plant through biological, chemical, and mechanical control have had varying results. The use of chemicals, at times, can be a dangerous method due to the harm it poses on the animals and humans directly using the water source without filtering. On the other hand, mechanical methods are costly, labor–Intensive, and unsustainable in the long term. Thus, the use of biological controls, if effective, may prove to be the most suitable solution, both in terms of cost and environmental safety, in the long term (Dagno *et al*., 2012).

Numerous potential microorganisms have been identified as biological control agents. Controlled experimental studies have confirmed the potential of *Acremonium zonatum*, *Alternaria eichhorniae*, and *Cercospora piaropi* to control *E. crassipes* (Charudattan, 2008; Martyn, 1985; Shabana *et al*., 1995a). One or both of the two species of *Alternaria*, *A. eichhorniae* and *A. alternata*, have been recorded on *E. crassipes* in Australia, Bangladesh, Egypt, India, Indonesia, and South Africa (Charudattan, 2001). In Egypt, along with *Alternaria alternata, Drechslera hawaiiensis and Ulocladium atrum* are also pathogens identified as producing pathogenic symptoms (El–Morsy, 2004; Elwakil *et al*., 1988). *Acremonium zonatum* has also been reported from Mexico as highly virulent with potential use as a biological control (Martinez and Charudattan, 1998). Additionally, *Myrothecium roridum* has also been reported to occur in India, Malaysia, Indonesia, possibly Mexico, and some western African countries and can be used as broad–spectrum bioherbicides (Charudattan, 2001; Walker and Tilley, 1997). *Uredo eichhorniae* (Charudattan and Conway, 1975), *Rhizoctonia* spp. (Coetzee *et al*., 2017), *Fusarium roseum* (Rintz, 1973), *Cercospora rodmanii* (Conway, 2011) *and Bipolaris stenospila* (Charudattan and Conway, 1975) have also been identified as potential biological controls. Alternatively, in Mali, out of 116 isolates, three, Mln799 (*Fusarium* sp.), Mln715 (*Cadophora* sp.), and Mlb684 (*Alternaria* sp.), were reported to have a high, moderate di-

sease incidence (Dagno *et al*., 2012). Despite limited studies conducted in Thailand, *Myrothecium roridum* was identified as a successful pathogen to control *E. crassipes* (Orawan *et al*., 2014). Unlike other biological control agents, human assistance is necessary for microbial biological control agents, especially fungi, to propagate into the epidemic level (Harding and Raizada, 2015; Hoagland *et al*., 2007). As such, this diminishes the probability of the infection of nontarget organisms.

The main objectives of this investigation were to survey and collect fungi infected *E. crassipes* plant, identify, and study the pathogenicity of the isolated fungi and test the pathogenic fungi in a controlled environment.

Research Methodology

Sampling procedure and isolation of pathogens

In this study, plants with leaf spots were collected from weed–infested sites of Bangkok (WGS84: 13°43'41"N 100°47'1"E) and Pathum Thani Province (WGS84: 14°1'53"N 100°43'56"E), Thailand, during the 2018 monsoon season (May to October). Leaf pieces (0.5 mm^2) were cut from the margins of necrotic lesions on the diseased leaves of *E. crassipes*. Individual leaf surfaces were disinfected with 10% w/v household bleach (Clorox®) and 0.1% v/v of Tween 20 for 2 min, followed by 70% ethanol for 3 min and rinsed three times with distilled sterile water. The surface–sterilized leaves were placed on the water agar and cultivated at 28±2°C for 2–3 days. The single–spore isolation technique was performed (Choi *et al*., 1999). The isolated fungi were sub–cultured on the potato dextrose agar medium.

DNA extraction and purification

The fungal cultures were grown on potato dextrose broth (Hi–media) in a shaking condition at 180 rpm for 3–5 d at 30ºC. The fungal suspension (1 mL) was collected in a 1.5–mL tube and was washed twice with sterile distilled water. After washing, 300 µL of TE–buffer, a small amount of aluminum oxide was added into the tube. The cells were lysed using a micro–mixer for 90 sec, then 300 µL of phenol–chloroform (1:1) were added and centrifuged at 10,000×*g* for 15 min. The upper layer was transferred to a new tube, and then 3 M sodium acetate (1/10 volume) and cold absolute ethanol (2 volumes) were added. The tubes were centrifuged at 10,000×*g* for 10 min, and the supernatant was discarded. The tubes were washed, respectively, with 70% and 95% ethanol and then air–dried. 30 µL of sterile ultrapure water was added to dissolve the DNA. The DNA was stored at –20ºC.

PCR Amplification

The amplification of the internal transcribed spacer (ITS) region (ITS1–5.8S–ITS2) was carried out using ITS1 (5′– TCCGTAGGT GAACCTGCGG–3′) and ITS4 (5′–TCCTCCG CTTATTGATATGC–3′) primers (White *et al*., 1990). The premix PCR kit EmeraldAmp® GT PCR Master Mix (Takara, Japan) was used to

set up the PCR mixtures by using the concentrations recommended by the manufacturer. The amplification was performed with an initial denaturation at 94°C for 1 min, followed by 35 cycles with denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min, followed by the last step at 72°C for 8 min. The PCR reactions were performed using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) thermocycler. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol.

DNA sequencing and sequence analysis

The purified PCR products were sent to the commercial Sanger sequencing service at Bioneer, Korea. The sequencing of nucleotides was performed using similar primers used in the PCR amplification. The ITS1–5.8S–ITS2 sequences were compared to the sequences deposited in GenBank using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/; Clark *et al*., 2016).

Detached leaf pathogenicity test

The detached leaf pathogenicity test was performed on fully developed *E. crassipes* leaves. The leaves were washed five times with tap water followed by 0.1% (v/v) tween-20 and sterile distilled water. Mycelium plugs cut from the edge of growing colonies on PDA were placed on the abaxial surface (Pettitt *et al*., 2011). For wounded inoculations, the leaves were wounded at three locations, via a single piercing by a sterile needle, (insect pin, 0.45 mm in diameter) per location before placing the mycelium plugs at the injury sites. Sterile cotton wool soaked with sterile water was used to provide water and a moist environment for the leaves. The inoculated leaves were placed in sterile glass Petri dishes and held at 100% RH, 28ºC with an 18–hour photoperiod for seven days. The cotton wool was changed at day 3 post–inoculation. The necrotic area of a *E. crassipes* leaf was calculated from average necrotic area of three measurements by Image J software. After the inoculation, the *E. crassipes* leaves were arranged in a completely randomized design. Each treatment was replicated four times, and the values were presented as their mean±SD. One way analysis of variance (ANOVA) with Tukey's test at $p \leq 0.05$ was used to compare the differences. SPSS version 22.0 was used for this analysis.

Whole plant pathogenicity test

The whole plant pathogenicity test was performed on healthy, fully developed *E. crassipes*. Similar to the detached leaf pathogenicity test, the plants were washed five times with tap water followed by 0.1% (v/v) tween–20 and sterile distilled water. Mycelium plugs cut from the edge of growing colonies on PDA were placed on the adaxial surface. The inoculated plants were kept in a growth room with continuous aeration at 70% RH, 28ºC with an 18-hour photoperiod for seven days.

Results

Identification of potential biocontrol agents

Fourteen fungal isolates were obtained from diseased leaves of *E. crassipes* collected from *E. crassipes* infested areas in Bangkok (WGS84: 13°43'41"N 100°47'1"E) and Pathum Thani province (WGS84: 14°1'53"N 100°43'56"E), Thailand (Fig. 1). The BLASTn analysis of the ITS1–5.8S–ITS2 sequence confirmed that the 14 single–spore fungal isolates were categorized in six different genera (Table 1). The genus with the highest occurrence was *Aspergillus* with 28.57% (four isolates). The second highest was *Coprinellus* with 21.43% (three isolates), followed by *Alternaria*, *Colletotrichum*, and *Curvularia* with 14.29% (two isolates each). The genus with the lowest occurrence was *Stagonospora* with 7.14% (one isolate). The alignment of ITS1–5.8S–ITS2 sequences among members of each genus revealed that WH–03 and WH–14 were identical to WH–05 and WH–15, respectively. This indicated that WH–03 and WH–05 were likely to be the same species, and the same applied to WH–14 and WH–15.

Fig. 1 The waterway blockage by *E. crassipes*. (A) Pathum Thani province and (B) Ladkrabang district, Bangkok were selected as the sampling sites.

Table 1 Molecular identification of fungal isolates from diseased *E. crassipes* based on ITS1–5.8S–ITS2 sequences

Sample No.	Isolate code	Tentative species	The closest species of BLASTn analysis*	% Identity
1	WH–01	*Colletotrichum* sp.	*Colletotrichum brevisporum* MT043769	100
2	WH–02	*Stagonospora* sp.	*Stagonospora bicolor* MT446144	98.3
			Stagonospora trichophoricola KY750315	
3	WH–03	*Aspergillus* sp.	*Aspergillus occultus* NR135454	100
			Aspergillus flocculosus KF305117	
			Aspergillus insulicola FR733834	
4	WH–04	*Colletotrichum* sp.	*Colletotrichum siamense* MT434664	100
			Colletotrichum asianum MT350261	
			Colletotrichum gloeosporioides MT043801	

Table 1 (continued)

Sample No.	Isolate code	Tentative species	The closest species of BLASTn analysis*	% Identity
5	WH–05	*Aspergillus* sp.	*Aspergillus occultus* NR135454	100
			Aspergillus flocculosus KF305117	
			Aspergillus insulicola FR733834	
6	WH–06	*Alternaria* sp.	*Alternaria tenuissima* MT573466	100
			Alternaria alternata MT573464	
			Alternaria burnsii MT416215	
7	WH–07	*Aspergillus* sp.	*Aspergillus versicolor* MN547369	100
			Aspergillus sydowii MN250030	
			Aspergillus flavipes KP068688	
8	WH–08	*Aspergillus* sp.	*Aspergillus oryzae* MT558944	100
			Aspergillus flavus MT558941	
			Aspergillus parasiticus CP051033	
9	WH–14	*Coprinellus* sp.	*Coprinellus radians* MK087751	99.15
10	WH–15	*Coprinellus* sp.	*Coprinellus radians* MK087751	99.15
11	WH–19	*Alternaria* sp.	*Alternaria longipes* LC269927	100
			Alternaria alternata MT555744	
			Alternaria porri MT554514	
12	WH–22	*Curvularia* sp.	*Curvularia lunata* MT541889	100
			Curvularia platzii MN540257	
			Curvularia hominis MN540252	
13	WH–25	*Coprinellus* sp.	*Coprinellus radians* MN547379	100
14	WH–27	*Curvularia* sp.	*Curvularia kusanoi* KT819137	100

*The closest species were limited to three species based on % identity.

Detached leaf Pathogenicity Test

Healthy detached leaves of *E. crassipes* were inoculated *in vitro* with all fungal isolates recovered from diseased *E. crassipes*. For wounded inoculation, the *Alternaria* sp. WH–06 exhibited the largest necrotic area after seven–day post–inoculation, followed by *Aspergillus* sp. WH–08 (Fig. 2). However, there was no statistical difference between WH-06 and WH–08. For unwounded inoculation, although the necrotic areas were clearly observed in the leaves inoculated with many fungal isolates, such as WH–03, WH–04, WH–06, WH–08, and WH–27, no statistical difference was detected among these isolates (Fig. 2). The disease symptoms of the most pathogenic fungi *Alternaria* sp. WH–06 included black and brown spots of necrotic tissues surrounded by a yellow halo of chlorosis (Fig. 3).

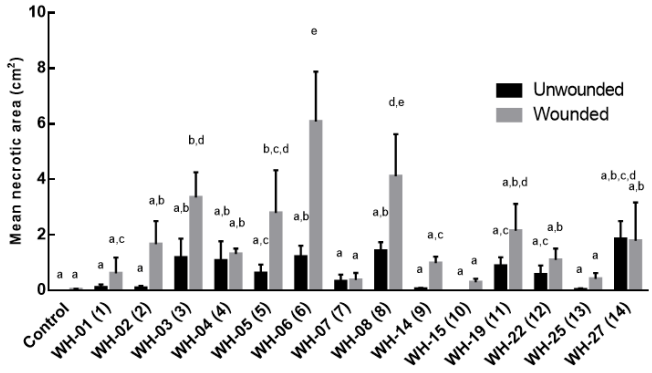

Fig. 2 Mean necrotic area on the detached *E. crassipes* leaves of different test pathogens. The necrotic area was measured seven days after wounded or unwounded inoculation in controlled environments at 100% RH and 28°C with an 18–hour photoperiod. Error bars represented SD.

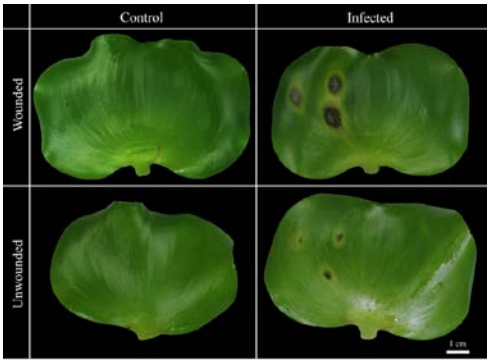

Fig. 3 Detached leaf pathogenicity test. Infection was performed by placing mycelium plugs of *Alternaria* sp. isolate WH–06 on the abaxial side of fully developed *E. crassipes* leaves. Untreated leaves were used as a control. Wounded leaves were pierced by a sterile needle. Photographs of the adaxial side were taken seven days after incubation in controlled environments at 100% RH and 28°C with an 18-hour photoperiod.

Whole plant pathogenicity test

The *Alternaria* sp. WH–06 was further investigated as a possible biocontrol agent. The pathogenicity test of the whole *E. crassipes* plant was adopted to test whether the fungi were able to infiltrate the healthy whole *E. crassipes* plant or not. The disease symptoms were quite similar to the detached leaf infected with *Alternaria* sp. WH–06.

Fig. 4 Whole plant pathogenicity test was performed by placing mycelium plugs of *Alternaria* sp. isolate WH–06 on a mature leaf of *E. crassipes* plant. After seven days of incubation in controlled environments at 100% RH and 28°C with an 18–hour photoperiod, photographs were taken on both (A) adaxial and (B) abaxial sides.

Discussion

The present study showed that the most frequently recovered genus was *Aspergillus*. Although many previous studies found that *Aspergillus* spp. were not the pathogens with the highest occurrence, studies also found that numerous members of this genus do cause diseases in *E. crassipes* (Al–Juboory and Musa, 2018; Yirefu *et al*., 2017).

For unwounded infection, many fungal isolates, such as WH–03, WH–04, WH–06, WH–08, and WH–27 exhibited an ability to infiltrate intact *E. crassipes* leaf tissues. However, there was no statistical difference between the necrotic areas of each isolate. We believe that with a longer infection time, these fungi could exhibit statistically different pathogenicity. However without wounding, it is very likely that the fungi took longer to infect and the main limitation of the detached leaf assay is the deterioration of the detached parts before the successful completion of the test (Pettitt *et al*., 2011). We found that the detached leaves did not withstand beyond seven days post–inoculation. Leaf wilt and microbial contamination were often found. Moreover, this non–statistical difference may be due to inadequate sample size.

Many studies suggested that several fungi in the genus of *Alternaria* had the potential to be biological control agents, such as *Alternaria jacinthicola* (Dagno *et al*., 2011), *Alternaria eichhorniae* (Shabana *et al*., 1995a, 1995b) and *Alternaria alternate* (Babu *et al*., 2002; El–Morsy *et al*., 2006). *Alternaria* sp. isolate WH–06 exhibited the higher virulence when wounded inoculation was performed. This suggests the possibility of the integration between fungal pathogens and insects, such as weevils *Neochetina eichhorniae* and *Neochetina bruchi*, to improve biological control of *E. crassipes* due to the facilitation of *E. crassipes* pathogenic infections by insect feeding damage (Firehun *et al*., 2013; Martínez and Gómez, 2007). This coincided with the study in Thailand that demonstrated higher disease severity on *E. crassipes* when *Alternaria* sp. was combined with weevils (Nuangmek *et al*., 2014). The *Alternaria* sp. used in the study did not cause diseases in other 60 plant species indicating high specificity to *E. crassipes* (Nuangmek and Titayavan, 2013). We strongly believe that the use of *Alternaria* sp. isolate WH–06 in combination with other insects is a potential pest–control option that can be coordinated into a broader integrated pest management (IPM) program. IPM is the integration of all appropriate pest control methods to prevent pest population accumulation based on economical and ecological considerations that minimize the effects on the human health and the environment (Barzman *et al*., 2015; Kogan, 1998). The whole plant pathogenicity test showed that *Alternaria* sp. isolate WH–06 could infect leaves of healthy whole *E. crassipes* plants and still elicit similar disease symptoms.

This confirmed the possibility of using *Alternaria* sp. isolate WH–06 as a biological control agent. However, further identification to the species level along with host range and field efficacy studies of *Alternaria* sp. isolate WH–06 are required to facilitate the implementation of the isolate as a biological control agent.

Conclusion

From our present preliminary screening, *Alternaria* sp. isolate WH–06 was found to be the most virulent isolate which successfully infected the whole *E. crassipes* plants under laboratory conditions. This suggests the potential for further field studies to establish *Alternaria* sp. as a viable biological agent for the management of *E. crassipes*.

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