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Exploration of Major Fungal Pathogens Associated with Water Hyacinth (*Eichhornia crassipes* (C. Mart) Solms) and Evaluation of their Potential as Mycoherbicides by Proving Pathogenicity

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A B S T R A C T

Eichhornia crassipes (C. Mart) Solms is one of the gregarious and noxious aquatic weed which was invasive to Asian countries. It was spread throughout the country and also infestation of water hyacinth can be seen in many parts of Tamil Nadu along the waterways. The present study was carried out to isolate different fungal pathogens associated with water hyacinth infesting water bodies of Thoothukudi district in Tamil Nadu and to identify the biocontrol potential of the pathogens against water hyacinth based upon their pathogenecity. Twelve fungal pathogens were isolated and subjected to *in vitro* detached leaf assay and whole plant assay to prove their pathogenicity. The isolated pathogens were produced various symptoms such as leaf spot, blight, chlorosis, necrosis and rotting on the infected leaves. Out of the 12 fungal isolates obtained from the diseased water hyacinth plants, all isolates were pathogenic and the isolates WH-5 and WH-11 found to be more pathogenic to the plants than the other isolates. Based on the morphological and molecular identification, both the isolates belong to the genus *Fusarium.* The isolates WH-5 and WH-11 caused 60.77 and 66.45 per cent of infection on the plants respectively and having potential to be an effective biocontrol agent for the management of water hyacinth.

K e y w o r d s

Water hyacinth, Aquatic weed, Fungal pathogens, *Fusarium* species, ITS-rDNA and biocontrol

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Introduction

Water hyacinth (*Eichhornia crassipes* (C. Mart) Solms) is one of the invasive and noxious aquatic weed all over the world. According to the International Union for conservation of Nature (IUCN), it was considered as one of the 100 most problematic invasive weed species and top ten worst weed that infested all over the world (Patel, 2012). It belongs to the family *Pontederiaceae* and it is a perennial, free

floating aquatic plant and reproduces mainly by means of stolons irrespective of seeds (Henderson, 2001). It was native to South America and first infestation of water hyacinth was reported in Egypt during the period 1879-1892 as an ornamental plant. In 1896, it was introduced in Bengal regions of India as an ornamental plant for its beautiful flowers and leaf shape. It was considered as a terror of Bengal and now it has spread throughout the country. Because of its high reproducing ability and ability to infest wide range of freshwater habitats, it causes serious problems on the environment. It leads to reduction of oxygen level that results in loss of water quality, blocks sunlight, provides breeding ground for the pests and vectors like mosquitoes that causes many vector borne diseases like malaria, filariasis and encephalitis on humans and it also causes detrimental effects on aquatic organisms and affects fisheries, irrigation, navigation, water supply and total ecology of the infested water bodies (Narayanan, 2007).

Water hyacinth management can be done through various methods including mechanical or physical, chemical and biological control. Among the three methods, biological control has been found to be an effective and economic method to control water hyacinth by using their natural enemies (Firehun *et al*., 2013). Many fungal pathogens have been identified as an effective biocontrol agent worldwide and several species of *Alternaria, Cercospora, Acremonium Myrothecium* have been studied intensively in experimental conditions (Charudattan, 2001; Shabana *et al*., 1995; Martinez and Gutierrez, 2001; Praveena and Naseema, 2004). The aim of the present study is to isolate and identify the effective biocontrol agent for the management of water hyacinth and development of the mycoherbicide formulations.

Materials and Methods

Isolation

Diseased water hyacinth plants showing symptoms like chlorosis, leaf spot, leaf blight and necrosis were collected and brought to the laboratory. The collected plants were washed under running tap water for 10-15 min. to remove surface debris. The diseased portions were dissected about 1-2cm along with healthy tissues using sterile blade. Then the dissected leaf pieces were placed in 10% sodium hypochlorite about 1min for surface sterilization and rinsed thrice with sterile distilled water. After surface sterilization, leaf tissues were placed on a blotting paper to become dry and aseptically transferred into the petriplates containing potato dextrose agar medium and incubated for 5-7 days under room temperature. The inoculated plates were regularly observed for appearance of fungal colonies.

Sub culturing and maintenance of the isolates

The fungal colonies observed in the isolation plates were aseptically transferred into fresh PDA plates for sub culturing and then the pure culture of fungal colonies were maintained by inoculating them into a sterilized PDA slants and stored at 4°C.

Pathogenicity test

The fungal isolates were tested for their ability to produce symptoms in healthy plants. The pathogenicity test was conducted by using two methods *viz., in vitro* detached leaf assay and whole plant assay.

(a) *In vitro* detached leaf assay

Healthy leaves were collected from water hyacinth plants and washed under running

water. The collected leaves were surface sterilized by using 70% ethanol and placed in sterile petriplates containing wet germination paper. Then the leaves are painted with tween-80 and wounded artificially with sterile needle. The fungal isolates were inoculated on the leaves at a spore concentration of 1×10^6/ml and incubated for 7 days. Inoculated leaves were observed for every 24h and disease progression of each isolates was determined using Area under disease progressive curve (AUDPC). Then the AUDPC values were calculated by using the formula

AUDPC =

$$\sum_{i}^{n-1}\left[\left\{\frac{Yi+Y(i+1)}{2}\right\}\times(t(i+1)-ti\right]$$

Whereas

Yi = disease severity on the i^{th} observations
ti = day or hours on the i^{th} observations
n = number of observations on which symptoms recorded.

(b) Whole plant assay

Healthy water hyacinth plants were collected and placed in 500ml glass beaker containing water and acclimatized to the glass house conditions. The plants were wounded artificially by pin prick method and sprayed with spore suspension of fungal isolates at a concentration of 1×10^6/ml along with Tween 80 as an adhesive. Then the inoculated plants were covered with polythene bags to maintain humidity. The inoculated plants were observed for the production of disease symptoms regularly up to 30 days.

Disease severity of fungal isolates were observed for every 24h and intensity of infection was determined using the score chart developed by Freeman and Charudattan (1984) and designated as: – (no symptom: healthy plant), + (mild symptom: plant showing slight symptoms up to 15% of leaf area), ++ (moderate symptom: plant showing definite bigger patches of diseased areas from 16% to 59% of leaf area) and +++ (severe symptom: enlarged lesions covering 60–100% of leaf area). Disease was scored using a 0 to 5 scale rating system were 0 = no symptoms; 1 = 1-10%; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; 5 = ≥75% of leaf area covered by disease symptoms. Then the disease index was calculated by using the formula:

Per cent Disease Index (PDI) =

$$\frac{sum\ of\ all\ individual\ ratings\times100}{total\ number\ of\ leaves\ scored\times maximum\ score}$$

Morphological and molecular identification of virulent fungal isolates

For morphological identification, the mycelium color, texture, spore color, shape and size of the fungal isolates were observed under microscope. For molecular identification, the genomic DNA was isolated from 2-3 day old culture of the isolate and subjected to PCR amplification.

The internal transcribed spacer regions of the nuclear rDNA were amplified using ITS1 and ITS4 primers. The amplification was carried out in a total volume of 25µl reaction mixture containing target DNA, double distilled water, PCR master mix, primers ITS1 and ITS4. Initial denaturing step was carried at 94°C for five min followed by 30 cycles of denaturation at 94°C for two min. annealing at 57°C for one min and elongation at 72°C for two min.

The unpurified PCR products were submitted to the Bioserve Biotechnologies (India) Pvt. Ltd, Hyderabad for sequencing and the nucleotide sequence was aligned through nucleotide blast program.

Results and Discussion

Isolation

Diseased water hyacinth plants were collected from the water bodies around Agricultural College and Research Institute, Killikulam. The collected plants showed different types of symptoms like chlorosis, necrosis, leaf spot, leaf blight and rotting of plants. From the diseased plants 12 fungal isolates were obtained. Some of the isolates were identified tentatively and they belong to the genera *Curvularia, Fusarium, Helminthosporium* and *Aspergillus.*

Pathogenicity test

In vitro detached leaf assay

In the *in vitro* detached leaf assay, all the 12 isolates produced the disease symptoms in which the isolates WH- 1, 2, 3, 4, 5, 6, 7, 8 and 11 exhibited symptoms about 48 hours of post inoculation (HPI) and the isolates WH-9, 10 and 12 exhibited symptoms about 72 HPI (Table 1). During the assay, various symptoms were produced which includes chlorosis, leaf spot, leaf blight and drying of the leaves. The isolates WH-6 and WH-11 exhibited higher AUDPC value about 5200 and having more pathogenic potential to plants and the isolate WH-12 exhibited lower AUDPC value about 2720 and having less pathogenic potential to the plants.

Whole plant assay

Then all the 12 isolates were subjected to whole plant assay for pathogenecity test, in which the isolate WH-11 produced higher PDI value of 66.45% showing brown spots on the leaves, marginal browning and chlorotic symptoms initially which turned into brown elongated spots with yellow halo. Then the symptoms extends to the petiole and finally results in rotting of the plant and reduce its reproducing ability within 20 days after inoculation. The intensity of infection to the water hyacinth plants ranged from 31.51% to 66.45% (Table 2). The isolate WH-11 produced severe symptoms and followed by isolate WH-5. Hence the isolates WH-5 and WH-11 were found to be virulent isolates having more pathogenic potential to the plants (Fig. 1).

Morphological and molecular identification of virulent fungal isolates

The morphological identification of the virulent isolates WH-5 and WH-11 showed white cottony mycelial growth on the PDA medium with hyaline, septate mycelium, and produces 3-4 septate sickle shaped conidia. The DNA banding of the isolates WH-5 and WH-11 ranged between 500-700bp. (Fig. 2). The sequences of the isolates WH-5 and WH-11 thus obtained was showing 93.52 % sequence similarity with *Fusarium* species. Both the morphological and molecular identification showed that they belong to the genus *Fusarium*.

Some species of the genus *Fusarium* were reported as major and common pathogens that infect water hyacinth plants all over the world (Evans and Reeder, 2001). In 1986, Jamil and Rajagopal conducted a survey on the fungal pathogens of water hyacinth from Andhra Pradesh and reported *Fusarium oxysporum* Schlet that infects water hyacinth. Santhi (1994) reported *Fusarium equisiti* and *F. pallidoroseum* had an effective biocontrol potential against water hyacinth. Yirefu *et al.*, (2017) reported that *F. oxysporum*, *F. equiseti* were found to be highly pathogenic to water hyacinth from the rift valley of Ethiopia. In 2012, Ray and Hill conducted a survey from different water bodies of South Africa and reported several isolates of *Fusarium* species *viz., F. avenaceum, F. oxysporum, F. solani* and found to be more pathogenic under controlled conditions.

Table.1 Effect of fungal isolates on water hyacinth during *in vitro* detached leaf assay

Isolates	Leaf area damaged after different hours post inoculation (HPI)						
	48	72	96	120	144	168	AUDPC Value
WH-1	6.67	20	26.67	26.67	46.67	53.33	3600
WH-2	6.67	20	33.33	33.33	53.33	60	4160
WH-3	13.33	20	26.67	33.33	53.33	66.67	4160
WH-4	6.67	13.33	26.67	33.33	40	60	3520
WH-5	6.67	20	33.33	40	46.67	66.67	4240
WH-6	20	33.33	33.33	46.67	60	60	5200
WH-7	6.67	13.33	26.67	40	53.33	66.67	4080
WH-8	6.67	13.33	26.67	40	46.67	53.33	3760
WH-9	0	6.67	20	26.67	33.33	46.67	2640
WH-10	0	13.33	20	26.67	40	46.67	2960
WH-11	13.33	20	33.33	46.67	66.67	86.67	5200
WH-12	0	13.33	20	26.67	26.67	46.67	2720

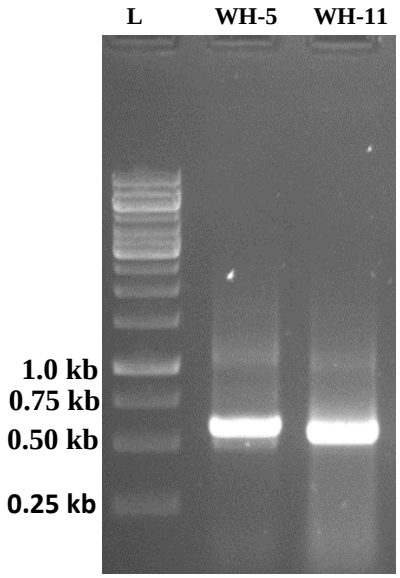
Table.2 Intensity of infection and symptoms produced by the fungal isolates

Isolates	Percent Disease Index (PDI) (%)	Intensity of infection *	Symptoms produced by pathogen
WH-1	33.42	++	Blighting on leaves and petiole
WH-2	37.07	++	Spots on leaves and petiole
WH-3	43.28	++	Blighting on leaves and petiole
WH-4	35.26	++	Leaf spot and chlorosis
WH-5	60.77	+++	Spots on leaves and petiole
WH-6	40.59	++	Spots on leaves and petiole
WH-7	41.17	++	Leaf blight
WH-8	34.85	++	Blighting on leaves and petiole
WH-9	33.41	++	Leaf spot
WH-10	31.51	++	Leaf spot
WH-11	66.45	+++	Spots on leaves and petiole
WH-12	33.20	++	Leaf spot

* Intensity of infection: – (no symptom: healthy plant), + (mild symptom: plant showing slight symptoms up to 15% of leaf area), ++ (moderate symptom: plant showing definite bigger patches of diseased areas from 16% to 59% of leaf area) and +++ (severe symptom: enlarged lesions covering 60–100% of leaf area).

Fig.1 Pathogenicity of the isolates WH-5 and WH-11

Fig.2 PCR Amplification of the fungal isolates



L – Ladder (1kb)
WH-5 & WH-11 – fungal isolates

The present study concluded that *Fusarium* species was found to be the most promising biocontrol agent against water hyacinth and also it can be promoted as a mycoherbicide in biocontrol programmes. The isolated *Fusarium* species was performing well under controlled greenhouse conditions and further studies were carried out to identify their safety against non-target crops, water quality and their effect on soil health.

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