Evaluation of microbial bioherbicides for *Eichhornia crassipes* to rejuvenate water bodies of Punjab

A

thesis submitted

in the partial fulfilment of the requirements for the award of degree of

DOCTOR OF PHILOSOPHY

IN

CIVIL ENGINEERING

Submitted By

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February 2017

CERTIFICATE

Certified that the thesis "Evaluation of microbial bioherbicides for *Eichhornia crassipes* to rejuvenate water bodies of Punjab" submitted by Mr. Birinderjit Singh, Reg. no. 950802002 in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in the Department of Civil Engineering, Thapar University, Patiala, Punjab is a record of candidate's own independent and original research work carried out by himself under our supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "Evaluation of Microbial Bioherbicides for *Eichhornia crassipes* to rejuvenate water bodies of Punjab " in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy at Department of Civil Engineering, Thapar University, Patiala is an authentic record of my own work during the period from September 2011 to February 2017, under the supervision of Prof. Maneek Kumar, Department of Civil Engineering, Thapar University and Prof. Sanjai Saxena, Department of Biotechnology, Thapar University, Patiala, Punjab. This report has not been submitted for the award of any degree or certificate in this or any other university.

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ACKNOWLEDGMENT

In my entire service as an environmental engineer, I have won several awards, received innumerable guests of Honour, presented on great many platforms both in India and abroad, met world renowned fellow environmentalist and worked at the senior most environmental engineering post in Punjab ; But nothing compares to the sense of achievement and satisfaction while completing this research dissertation. I gratefully acknowledge the permission granted to me by my then employer Punjab Pollution Control Board without which this research could never have happened.

I would like to express my sincere gratitude to the Thapar University and its visionary director **Prof. Dr. Prakash Gopalan** for letting me fulfil my dream to kill this invasive weed *Eichhornia Crassipes* for rejuvenation of water bodies of Punjab.

There have been many people who have either admired or cursed me for pursuing this study at the fag end of my professional career. But there were some who guided me during this hard time and showed me the way to achieve it. **Prof. Dr, Maneek Kumar**, Dean Students Affairs, Thapar University, Patiala, Punjab is one of such illustrious figure who motivated me to carry out this research and without his encouragement, the present work would never have seen day's light. I am eternally grateful to him.

During the journey of this study, the personal and professional changes have been exciting, profound and seismic. It was **Prof. Dr. Sanjoi Saxena**, Department of Bio-technology, Thapar University, Patiala, Punjab, a generous and dynamic teacher who could bring about such professional changes in an old man like me. He is the main inspiration behind converting an Environmental Engineer into a bit of mycologist. I am indebted to him and can never ever thank him enough.

I am beholden to other members of the doctoral committee of the Thapar University; Prof. **Dr. Naveen Kavatra**, Head of Civil Engineering Department, Thapar University, Patiala, Punjab, **Dr. Shveta Goyal**, Asst. Professor, Department of Civil Engineering, Thapar University, Patiala, Punjab and **Dr. Anita Rajor**, Asst. Professor, School of Energy and Environmental Science, Thapar University, Patiala, Punjab for their constructive suggestions and critical remarks during six monthly review presentations.

I am fortunate to have the blessed support of my young colleagues Dr. Vineet Mesharam, Dr. Mahiti Gupta, Miss Neha Kapoor and Mr. Vegish Dwibedi Ph.D. scholars in the department of Biotechnology for helping me in designing and planning experimental protocol and providing me the necessary guidance in carrying out the laboratory work.

My thanks are due to **Dr. Charanjit Singh**, Scientific Officer, **Shri Janak Raj**, Junior Scientific Officer and **Dr. Gurpreet Singh**, Junior Scientific officer, Punjab Pollution Control Board Head Office Laboratory at Patiala in helping me in conducting physico-chemical analysis of water samples.

Pursuing doctorate degree was the most challenging task I have done so far. It was possible only with the support and encouragement of my parents, children and wife who has been a consistent source of support and encouragement.

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Dedicated

to

My Father

List of Publications

1. **Singh B**, Saxena S, Meshram V and Kumar M (2016): Mycoflora of *Eichhornia crassipes* infesting Harike wetland and their pathogenicity for prospective application as biological control agents. *Mycobiology*. 44(2): 85-92. ISSN 2092-9323. IF: 0.57.
2. **Singh B**, Saxena S, Meshram V and Kumar M (2016): *Diplodia mutila* as a new pathogen on water hyacinth *Eichhornia crassipes* in Harike wetland. *Journal of Biopesticides*, 9 (2): 180-188. ISSN 0974-391X. IF: 0.55

POSTER PRESENTATIONS

1. **Birinderjit Singh**, Sanjai Saxena, Maneek Kumar and Vineet Meshram (2013): Potential of fungal pathogens for biocontrol of Eichhornia crassipes. 39th Meeting of the Mycological Society of India & National Seminar on Current Perspectives of Fungi in Healthcare and Environment (KAVAASTHA), Department of Microbiology and Biotechnology, Jnanabharathi Campus, Bangalore from 13-14th Mar. 2013

2. **Birinderjit Singh,** Sanjai Saxena, Maneek Kumar and Vineet Meshram (2014): Lasiodplodia tasuga: Host specific biocontrol agent of Eichhornia crassipes. International symposium Role of fungi & microbes in 21st century A global scenario, India Mycological Society, Kolkata, West Bengal, 20-22 Feb 2014.

3. **Birinderjit Singh**, Vineet Meshram and Saxena S (2015): Mycoflora of Eichhornia crassipes infesting Harike wetland and their pathogenicity for prospective application as biological control agents. DAE-BRNS life science symposium (LSS-2015): Advances in microbiology of food, agriculture, health and environment, Feb 3-5, 2015, Bhabha Atomic Research Centre (BARC), Mumbai, Maharashtra

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S. no	Symbol	
1.	$	US dollar
2.	%	percentage
3.	~	approximately
4.	’	minutes
5.	±	plus minus
6.	≥	equals to or greater than
7.	°	degree
8.	°C	degree celsius
9.	µg	microgram
10.	µl	microliter
11.	µm	micrometer
12.	bp	base pair
13.	cm	centimeter
14.	g	gram
15.	h	hour
16.	l	litre
17.	mA	milliampere
18.	mg	milligram
19.	min	minute
20.	ml	milliliter
21.	mm	millimeter
22.	mM	millimolar
23.	psi	pounds per square inch
24.	rpm	revolutions per minute
25.	s	second
26.	v	volume
27.	µS/cm	microSiemens per centimeter

List of Abbreviations

S. no	Abbreviations	Full form
1.	2,4-D	2,4 dichlorophenoxy acetic acid
2.	ANOVA	Analysis of Variance
3.	AUDPC	Area Under Progressive Curve
4.	BCA	Biological Control Agent
5.	BLAST	Basic Local Alignment Search Tool
6.	BOD	Biological Oxygen Demand
7.	CMA	Corn Meal Agar
8.	COD	Chemical Oxygen Demand
9.	DMSO	Dimethyl Sulfoxide
10.	DNA	Deoxyribonucleic acid
11.	dNTP	deoxy Nucleotide Triphosphate
12.	DO	Dissolved Oxygen
13.	EEA	European Economic Area
14.	FAS	Ferrous Ammonium Sulphate
15.	GDP	Gross Domestic Product
16.	Hpi	Hours Post inoculation
17.	Hpt	Hours post treatment
18.	HST	Host Selective Toxins
19.	ISI	Indian Standard Institute
20.	ITS	Internal Transcribed Spacer
21.	IUCN	International Union for Conservation of Nature
22.	LAD	Leaf Area Damage
23.	MDCA	3,6 dichloro-2- pyridine carboxylic acid
24.	MEA	Malt Extract Agar
25.	NCBI	National Centre for Biotechnology Information
26.	NTO	Non Target Organisms
27.	PDA	Potato Dextrose Agar
28.	pH	Power of Hydrogen
29.	PLA	Pine Leaf Agar
30.	PSCST	Punjab State Council for Science and Technology
31.	RB	Richard's Broth
32.	RNA	Ribonucleic acid
33.	SD	Standard Deviation
34.	SDW	Sterile Distilled Water
35.	SNA	Synthetischer Nahourstoffarmer Agar
36.	TBR	Tree Bisection Regrafting
37.	TDS	Total Dissolved Solids
38.	TSS	Total Suspended Solids
39.	USEP	United States Environment Protection Agency
40.	WII	Wildlife Institute of India

EXECUTIVE SUMMARY

The present study was oriented to develop microbial bioherbicides for controlling the infestation of water hyacinth (*Eichhornia crassipes*) in the internationally acclaimed manmade wetland, Harike in Punjab, India. The idea was to develop a mycoherbicide using augmentative/ innundative approach for which mycoflora inhabiting the diseased *Eichhornia* from the wetland were recovered by using standard mycopathological techniques. Of the 52 isolates, only 9 isolates exhibited significant pathogenicity by assessment of their spore suspension (1x 10^6 spores/ml) using *in vitro* detached leaf bioassay. However, only two isolates #8BJSSL and #19BJSSL exhibited complete leaf area damage after 168 hpi and disease development in terms of AUDPC. On further evaluation of these spores on whole plants, #19BJSSL exhibited higher pathogenicity. Using morphological and molecular techniques these were identified as *Phaeoacremonium italicum* (#8BJSSL) and *Diplodia mutila* (#19BJSSL). This is the first report of occurrence of these fungi as pathogens of *Eichhornia crassipes* in the world. Further, culture filtrates of #19BJSSL and #8BJSSL were evaluated for their phytotoxic potential by *in vitro* leaf puncher assays wherein #19BJSSL exhibited significantly higher phytotoxicity. Hence culture filtrate of #19BJSSL was further extracted using organic solvents by liquid- liquid extraction procedure and again assessed for their phytotoxic potential using *in vitro* leaf puncher assay. The best phytotoxicity was exhibited by the hexane fraction. However, in the whole plant assays of culture filtrate, hexane and chloroform fraction of #19BJSSL, the culture filtrate exhibited the highest phytotoxicity followed by the hexane fraction and chloroform fraction. The highest phytotoxicity of the culture filtrate is attributed to synergistic effect of Alkaloids and terpenoids which present in different concentration in hexane and chloroform fraction affecting the phytotoxic activity. Using food poison assay the concentration of hexane fraction compatible to the spores of *Diplodia mutila* (#19BJSSL) was found to be 2.5 µg/ml which was used for preparation of a formulation with the spores for enhancement of the mycoherbicidal potential of *Diplodia mutila* for the control of *E. crassipes*. The bioherbicidal formulation (2.5 µg/ml hexane fraction+ 1x 10^6spores/ml) was found to induce the pathogenesis by 24 hours causing 13 % damage as compared to spores of wherein the initial symptoms of infestation occurred after 48 hours. Further, the bioherbicidal formulation exhibited 100% mortality of the test plants after 192hpt while the spore suspension only induced 84% death. Thus it was concluded that by use of crude phytotoxins with spores of *Diplodia mutila* there was a significant enhancement in the mortality of water hyacinth. Further the spores of *Diplodia mutila* were sprayed on non-target, economically important plants and duckweed. No disease development was observed in these plants till one month indicating the safety as well as suggesting the host specificity of *Diplodia mutila* (#19BJSSL) for *Eichhornia crassipes*. There was deterioration in the water quality of experimental tubs which is predominantly attributed to the dead and decaying biomass also enhancing the BOD, COD, TDS, Electrical conductivity, total coliforms and fecal coliforms. However there was a significant reduction in the evapotranspiration rate i.e. by 7% thus conserving water.

Thus, from the present study it is concluded that #19BJSSL (*Diplodia mutila*) and its bioherbicidal formulation with crude phytotoxins offer a plausible alternative to control and manage the infestation of *Eichhornia crassipes* in Harike wetland. However, this method has to be integrated with the mechanical method for immediately removing the dead plant biomass, so as to prevent the deterioration of water quality thereby rejuvenating it.

Chapter 1

Introduction

1. BACKGROUND

1.1 Weeds

Weed is any wild plant which is growing at an unwanted location. This is generally regarded as an intruding plant species which competes with the other cultivated plants for space, light and nutrients. Some weeds also produce compounds which are toxic to other beneficial species of plants and reduce their population. The toxic chemicals are referred to as allelochemicals. Navas (1991) has given the broad definition of weed as 'A plant that forms populations that are able to enter habitats cultivated, markedly disturbed or occupied by man, and potentially depress or displace the resident plant populations which are deliberately cultivated or are of ecological and/or aesthetic interests".

1.2 Weed eradication: a necessity

Weeds interfere in agricultural practices as they affect the agricultural production both quantitatively as well as qualitatively. They affect the crop yield and simultaneously increase the cost of crop production. Apart from this, weeds have severe human health hazard as many of them are potent allergens which may lead to systemic and skin allergenic problems. The common characteristics of weeds include (a) a abundant seed production (b) rapid population establishment and spread and capacity to occupy sites which have intervention of humans.

Weeds have been found to be responsible for interference in terrestrial as well as aquatic resources. They create problems in the maintenance and inspection of various defence, electrical, railway and airport installations besides being a potential fire hazard in forests and cause health hazards like skin allergy, nasal diseases, etc. It has been estimated that an additional income of Rs. 1, 05,036 crores (Rs 1050360 millions) per annum would be generated

if proper weed management strategies are adopted. This saving could be further enhanced if the indirect impact of weeds on aquatic systems, forestry and industrial production are also included. It is further estimated that around Rs. 100 billion is annually spent on weed management practices across India in arable land alone. The concept of weed management is as old as the discovery of agriculture in India. However, the strategies of weed management have been changing all through these years (NRCWS, 2007)

In case of aquatic weed like *Eichhornia,* there is an increased loss of water due to excessive evapo-transpiration, interference of navigation in waterways, affect fishing potential and quality of water in the canals to be used either for drinking or for irrigation purpose (Praveena and Naseema, 2004; Tellez et al., 2008). India has approximately 7 million hectares of water bodies such as lakes, reservoirs, ponds, tanks and brackish water apart from 1.7 lakh km under rivers and canals (MoWR, GoI, 2016).

1.3 Eichhornia crassipes- as one of the world's worst aquatic weed

Eichhornia crassipes is a floating water weed which has spread throughout the globe in water bodies (El-Morsy et al., 2004). It is commonly known as water hyacinth and its vernacular names in India are buti and Jalkumbhi (Baral and Vidya, 2011). It is spread in more than 50 countries and 5 continents of the world and is creating a significant problem in Africa and Southeast Asia (Cohen and Carlton, 1995). Water hyacinth belongs to the Pontederiaceae family which vigorously spreads through vegetative reproduction (Gopal, 1987). In India, water hyacinth first appeared in Bengal in early 1890, and has currently spread throughout the country with exceptions in more arid western parts of Rajasthan, in the rugged region of north and in Kashmir. In Punjab, it has infested all ponds, drains, road side stagnations and travels

through rivers to wetlands and enters hydrological cycle and ultimately creates an impact in our food chain by affecting the drinking water sources (Varshney et al., 2008).

Biologically, *Eichhornia crassipes* is described as an erect, free-floating stoloniferous, perennial aquatic herb native of South America (Brazil) which grows to height of 1 m and possesses buoyant leaves, which vary in sizes according to growth conditions. The dark green leaves blades are circular to elliptical in shape attached to the spongy, inflated petiole. Leaves are produced singly from the apical main stem and are arranged spirally around the stem, so that as the stem elongates the leaves move progressively lower on the stem and begin to senesce (Fig 1.1). Leaf production is constant and proportional to leaf mortality, so that each plant tends to maintain a constant number of leaves (Center, 1981). Underneath the water, is a thick, heavily branched, dark fibrous root system. Water hyacinth has striking light blue to violet flowers located on a terminal spike (Gopal, 1987).

Fig. 1.1: *Eichhornia crassipes*: a flowering aquatic weed

Water hyacinth is a very aggressive invader and can rapidly multiply to 120,000 plants from 2 plants in 120 days. This aquatic herb is hardy exhibiting flexible temperature with minimum at 12 °C (54°F) tolerance. The maximum growth temperature is 33-35 °C (92-95 °F) while the optimal growth temperature is between 25-30 °C. In shallow water, the plant may become anchored and produce flowers and seeds, with flowering being stimulated by temperature rather than day length. In deeper water, vegetative reproduction occurs through runners, called ramets or daughter plants, and these become individual “mother” plants once they have broken off at the stolon that connects them to the original mother plant. In suitable

habitats (usually where the water is highly eutrophic), the population may double in number every 11 to 18 days. The seeds of *Eichhornia* withstand long, dry spells and remain viable even after 15 years (Edwards and Musil, 1975). This is one of the prime reasons of *Eichhornia* to become invasive in any water body due to its rapid vegetative reproduction and growth in the absence of any natural enemies. Further it has an ability to re-infest from seed or flood borne plants (Harley, 1990).

As the growth rate is uncontrollable, the ability to infest a wide range of freshwater habitats have led to enormous environmental, agricultural and public health problems (Pimental et al., 2005; Rahel and Olden, 2008; Pysek and Richardson, 2010). Water hyacinth reduces the availability of water for irrigation, aquaculture, potable water apart from creating obstruction of navigation in waterways and canals. It has also been listed as top 100 dangerous invasive species of the world (Tellez et al., 2008).

1.4 Water hyacinth as an aquatic weed in Harike

Wetlands are very sensitive and important ecosystems. Fish and Wild Life Service of United States has defined wetland as “Lands transitional between terrestrial and aquatic systems where the water table is usually at or near the surface or the land is covered by shallow water”. As defined in terms of physical geography, a wetland is an environment “at the interface between truly terrestrial ecosystems and aquatic systems making them inherently different from each other yet highly dependent on both” (Mitsch and Gosselink, 2007). It acts as nature's kidney. Wetland eco system helps the environment process toxins, excess fertilizers and maintains the health of our aquatic ecology.

Punjab is a landlocked state in the Union of India situated between 29°30'-32°32'N longitude and 73°55'-76°50'E latitude with a triangular shaped configuration. Satluj and Beas rivers are two main rivers out of five, left in Punjab after India's Partition in 1947 and these rivers are its main source of water. In 1952, a barrage was constructed downstream of the confluence of these rivers at Harike, a small township located on Amritsar - Ferozepur highway (Singh et al., 2016a,b). The purpose to build this barrage was to store water to meet the agriculture and drinking water requirements of southern Punjab and part of Rajasthan. This barrage after its construction submerged vast area under water and gifted us 'Harike wetland'. It is a 41 sq. km. area including a big water reservoir enclosing island of marsh and permanently inundated land supporting a profusion of vegetative growth (Fig 1.2). It is one of the largest fresh water wetland in Northern India (Ladhar et al., 1994). Harike wetland is also very

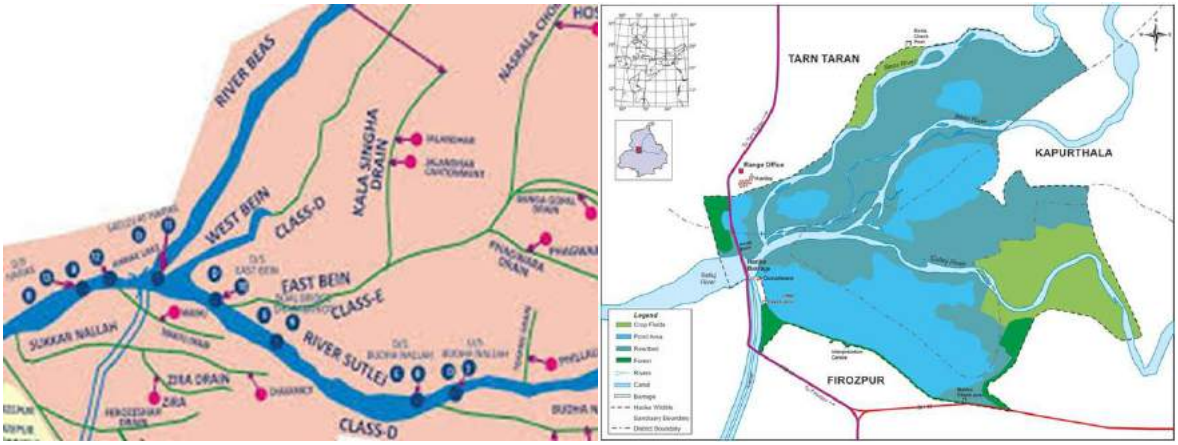

Fig.1.2: (a) Represents the confluence of the rivers Satluj and Beas (b) Map showing the spread of Harike wetland its location in Punjab as well as in India. Source: Fig. 1(a): http://floodlist.com/asia/india-flood-gates-pakistan, Fig 1(b): www.wwfindia.org

important for Punjab as it plays a vital role in the hydrological cycle of southern Punjab in particular and the whole Punjab in general. It also helps in absorbing minor floods as well as a main source of water recharging in this region. It was given immense importance during the Ramsar Convention held in Iran in 1971 following which it was declared as a Ramsar site of

significance in 1990. Harike is a winter home to more than 20,000 waterfowls every year. High diversity of fish is found in the wetland apart from seven species of turtles. Apart from being a representative of biodiversity of the North India Flood Plain System, Harike holds enormous socio-economic value as the fish that is found here is renowned in the state of Punjab as a delicacy. As many as 175 species of plants are found in and around the wetland (PSCST, 2001).

Today, Harike, under the pressure of growing human population is facing several problems i.e., increasing silt load, weed infestation, pollution and over exploitation of wetland resources. Discharge of untreated domestic as well as industrial waste water from catchment cities and towns into drains and rivers which feed the wetland have resulted in wide spread weed infestation and proliferation. The ecological crisis has reached an alarming stage and experts now estimate its lifespan to be less than 80 years. The major reason of this degradation is weed infestation, nearly 80 % of the open water surface has got covered with water hyacinth and its 33 islands are hardly visible (WII, 2005). At Harike lake, for most part of the year, little or no open water is visible; instead, what predominates is the lush green growth of *Eichhornia (*PSCST, 2001). Hence, there is an immense need to check the growth of *Eichhornia crassipes* (water hyacinth) in this internationally important Ramsar wetland which is a winter abode for migratory birds, possess unique diversity of fish and fresh water turtles apart from being a drinking water source to southern Punjab and state of Rajasthan (Moza and MIsra, 2008).

1.5 Present strategies of aquatic weed management

Water hyacinth is being controlled by variety of methods which are broadly classified as physical, chemical and biological control methods. Physical methods generally involve the use of manual and mechanical means by using diversion booms (nets). Chemical methods involve

the use of herbicides like terbutryn, amitrole, diquat, paraquat, glyphosate and 2, 4-D. However, the use of these herbicides also kills the beneficial aquatic flora apart from biomagnification of herbicides in ecological chain, thereby, threatening all life forms including humans. Biological control involves the use of host specific living forms which kill or damage the target weed and suppresses it below sub-economic level and sub-lethal level without complete eradication. Use of arthropods and fungi has been attempted in controlling the water hyacinth (Julien et al., 2001; El-Morsy et al., 2004; Ray and Hill, 2012).

A number of state agencies like the Forest and Wildlife Department, Irrigation Department, Soil Conservation Department, Punjab Pollution Control Board, Punjab Energy Development agency and various research institutions are actively involved in conservation activities related to the Harike wetland. There have been various attempts to remove water hyacinth from Harike lake using manual/mechanical methods but it has not met with success and have proved to be a cost intensive exercise.

Chemical control of water hyacinth is fast and efficacious; however, there is a serious societal concern of using chemical herbicides in control of aquatic weeds in lakes and water bodies which provide water for drinking purposes (UNEP, 2013). Hence, applications of chemical pesticides in these water bodies are not recommended. US has already banned 2, 4-D and diquat for use as an herbicide for water hyacinth in 2007 while India banned the use of 2, 4-D in 2011. Thus, there is a demand for new ecofriendly interventions to control and check the spread of this invasive aquatic weed species.

Biological control generally involves the use of a living organism which is host specific in nature and intends to bring down the interfering species to sub-economic level without

complete eradication. The insects which have been exploited for the control of water hyacinth consist of two *Neochetina sp.* (Weevil) and a moth (*Samoedes albiguttalis*). Huge reduction in population of water hyacinth through insect's biological control has been observed at limited sites. Magnificent success has been achieved at Hebbel tank in Bangalore (India) causing 95% control within a span of two years, Loktak Lake in Manipur and several ponds in Jabalpur However, Kangeri tank in Bangalore is also an example where weevil release have been a total failure. Hence variations in results on application of arthropods for biological control of water hyacinth has been frequently observed but reasons of failure of arthropods in biological control have not been accounted for (Pathak and Kanan, 2011; Jayan and Satyanathan, 2012)

Surveys have demonstrated that there exists a rich mycobiota associated with *E. crassipes* across the globe which could be explored for their potential to be used for biological control of water hyacinth (Babu et al., 2002; El-Morsy et al., 2004). *Myrothecium roridum* has been found to be associated with *E. crassipes* in Amazon whereas *Alternaria eichhorniae* had been found to be associated with its counterpart in India (Charudattan, 2000; Babu et al., 2002). Further *Alternaria eichhorniae* from Egypt and India have higher virulence as compared to the *Alternaria eichhorniae* in USA. Thus innundative approach would be more appropriate in exploring the potential of fungi as biological control agents of *Eichhornia crassipes*. Till date, no work has been done to know the natural plant pathogens associated with the infesting *Eichhornia crassipes* in Harike Wetland. Hence, in the present study, we propose to study the different fungal pathogens associated with *Eichhornia* infesting in this wetland and evaluate them for their possible use as an innundative bioherbicide to check the spread of *Eichhornia* in this internationally acclaimed wetland Harike and to rejuvenate it to its original health.

Chapter 2

Present Approach

2. AIM

As water is attaining the status of precious commodity throughout the globe, there is immense need to protect the ground water levels and reduce evaporation rate from the water bodies for its conservation. Water hyacinth is responsible for high evapotranspiration rate which is 3-7 times of normal evaporation rate due to huge leaf area thereby causing loss of water from water bodies. Water hyacinth has been termed as an invasive habitat modifier since its infestation in water bodies leads to displacement of native plant species in the aquatic system, apart from posing a severe threat to aquatic life (Aneja and Srinivas, 1990; Tellez et al., 2008; Dango et al., 2012). As already highlighted that manual methods are cost intensive as well as ineffective while chemical methods are not environment friendly and pose a health hazard to non-target organisms. Therefore, there is a great need to explore newer ecofriendly interventions to manage the invasion by this aquatic weed (Charudattan and Dinoor, 2000).

Biological control using arthropods have been attempted in South India and have met limited success. In Punjab also, control of water hyacinth was attempted using *Neochetina* sp. (Weevil) and a moth (*Samoedes albiguttalis)* (Sushilkumar, 2011). However, the indigenous mycoflora existing in the water hyacinth infesting Harike wetland has not been explored for the development of an innundative biological control method. Therefore, an overall aim of this study is to identify most virulent mycoflora available in Punjab wetland which can control water hyacinth and prepare the most effective formulation of the same. Hence, this thesis aims at the 'Evaluation of microbial bioherbicides for *Eichhornia crassipes* to rejuvenate the water bodies of Punjab'. Accordingly, objectives of the present study are:

i) Screening the epiphytic microflora of *Eicchornia crassipes* from the leaves; stem; stumps and roots for their selective pathogenicity using *in vitro* leaves, cut shoot and hydroponic bioassays.

ii) Screening of bioactive compounds of the selected biological control agents for improving the bio herbicidal efficacy as a bio control formulation.

iii) Efficacy evaluation of assessment of the biological control agent / bio control formulation.

Chapter 3

Review of Literature

3. REVIEW OF LITERATURE

3. 1 Water hyacinth: a global invasive species

Invasive alien species are a major global challenge as they alter ecosystem services and processes. Such noxious wild species reduce the abundance and richness of native species and decrease the genetic diversity of ecosystems creating pressure on world's biodiversity and thus demand an urgent action. (Hejda et al., 2009; Rands et al., 2010; Vila et al., 2011). These are also responsible for economic losses which amount to US$ 120 billion in countries like USA (Pimental et al., 2005; Kettunen et al., 2009) and 0.3% of total GDP in case of South Africa which may touch upto 5% GDP if the invasive plants are allowed to grow at their full potential (Wilgen and Lange, 2011). Aquatic weeds are those unwanted plants, growing in water and at least complete a part of their life cycle in water. The economic importance of such weeds becomes very high as they pollute drinking water when they die and decay in water body (Varshney et al., 2008).

Water hyacinth or *Eichhornia crassipes* Mart. (Solms) sports a position of top 100 global invasive species as listed by International Union of Conservation of Nature (IUCN) (Tellez et al., 2008) and recognized as top 10 worst weeds of the world (Shanab et al., 2010; Gichuki et al., 2012; Patel, 2012). It is monocotyledonous, belonging to the family Pontederiaceae. German naturalist C. von Martius discovered the species in 1823 and named it as *Pontederia crassipes*. It was Solms who included it in the *Eichhornia* genus, 60 years later in 1883 as earlier Kuntz described it in 1829 (Tellez et al., 2008).

Water hyacinth is a perennial, mat-forming aquatic plant, free floating or anchored in shallow water, usually 100-200 mm high but up to 1 m when growing in dense mats. The floating plants have feathery and long roots. The leaves of water hyacinth are dark green, shiny, rosette structured with distinctive erect swollen bladder-like petioles while the flowers are pale violet or blue, in flowered spike with individual flower measuring about 50 mm in diameter. The fruits consist of capsules with very fine seeds (Hendersen, 2001). The flowering of water hyacinth occurs throughout the year with an annual

production of 3000 seeds. The seeds are long lived up to 20 years. The seeds may not be viable at all sites, and therefore, water hyacinth colonizes in new areas by vegetative reproduction and propagation through horizontally growing stolons (Gopal, 1987)

Today, water hyacinth is found in both tropical and subtropical regions of the world. The major cause of its spread has been human activities. *Eichhornia crassipes* is a native of South America comprising Argentina, Brazil, Paraguay, Uruguay, Bolivia, Ecuador, Colombia, Chile, Guyana Surinam, and Venezuela. It has subsequently extended to Panama, Nicaragua, Honduras and El Salvador in Central America (Gopal, 1987).

It was introduced in New Orleans, Louisiana at the Cotton States Exposition in North America in 1884. Subsequently it spread across the South eastern state to Florida in 1895 and California in 1904 (Center et al., 2002). Today it has spread to Alabama, Arkansas, Arizona, California, Colorado, Florida, Georgia, Hawaii, Kentucky, Louisiana, Missouri, Mississippi, North Carolina, New York, Oregon, South Carolina, Tennessee, Texas, Virginia and Washington i.e approximately 50% of the United States of America (USA).

Water hyacinth is locally established in Azores (France) and Corsica (Italy) while causal records of its presence have been reported from Belgium, the Czech Republic, Hungary, the Netherlands and Romania (EEA, 2012). The most threatened countries with water hyacinth infestation in Europe are Spain and Portugal (DellaGreca et al*.,* 2009).

In Asia, the plant was introduced in the end of 19th Century. In India it was introduced in Bengal in 1890 mainly as an ornamental plant in the botanical gardens. A recent study has indicated that majority of Indian states are infested with water hyacinth exceptions being arid western part of Rajasthan and rugged regions of north and in Kashmir. In Kerala, Sasthamkotta lake, Vembanad- Kol wetland and Asthamundi lake, East Kolkata wetlands in West Bengal, Bhoj Wetland in Madhya Pradesh, Upper Ganga river in Uttar Pradesh, Chandertal Wetland in Himachal Pradesh, Harike wetland, Kanjili

wetland and Ropar Wetland in Punjab, Kolleru lake in Andhra Pradesh, Loktak Lake in Manipur are infested with Water hyacinth (Narayanan et al., 2007). From Indian subcontinent it subsequently spread to Indonesia and to Japan.

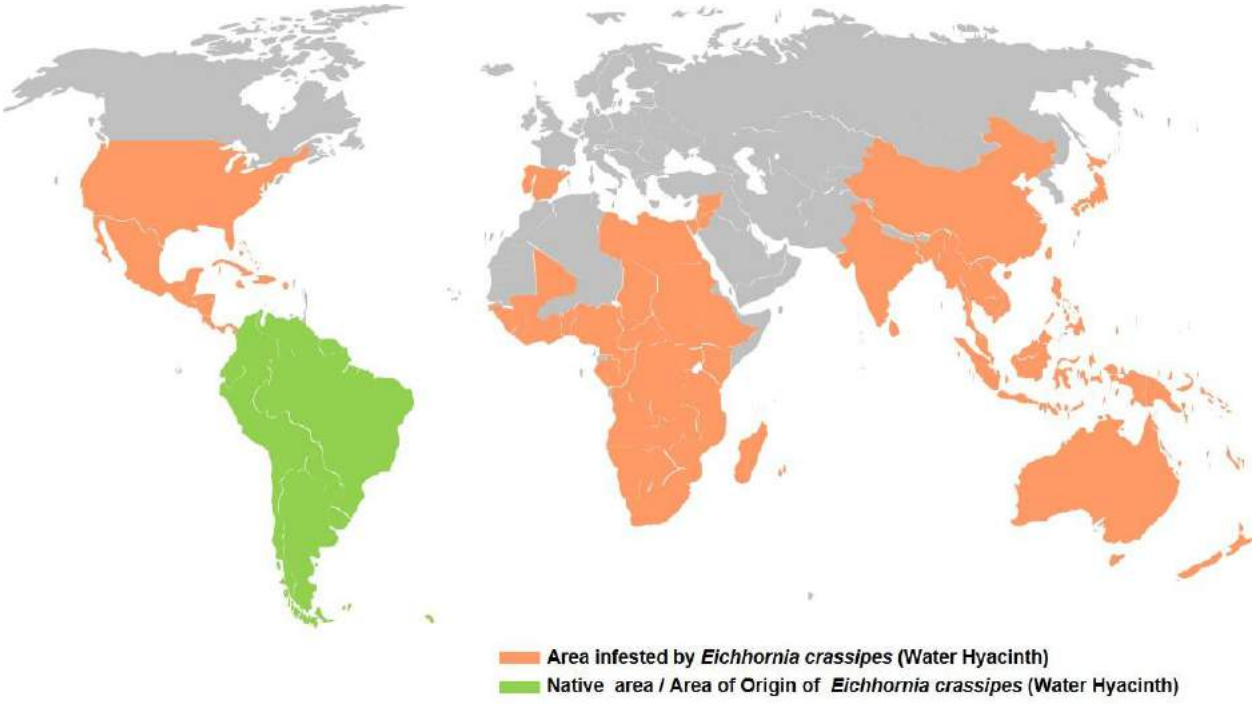

Fig. 3.1 Global map of infestation of *Eichhornia crassipes* (Redrawn from UNEP/DEWA from Tellez et al., 2008)

Water hyacinth is also responsible for economic, social and environmental problems in southern China (Choo et al., 2006). Today, water hyacinth has invaded over 50 countries on five continents (Fig 3.1). The recent change in global climatic conditions indicates that its distribution to higher latitudes may expand with the change in global temperature (Hellmann et al., 2008; Rahel and Olden, 2008).

3.2 Environmental impact of water hyacinth

Invasion of *Eichhornia crassipes* into non-native areas has resulted in tremendous impact on the environment. Water hyacinth poses (a) a serious impact or loss to the biodiversity of the water system; (b) it affects the water quality by decreasing the transfer of oxygen from air to water surface as well as decreases the oxygen production by other aquatic plants and algae; (c) promotes breeding of pests and

vectors which are detrimental to human health; (d) it blocks waterways thereby hampering agriculture, recreation, fisheries and hydroelectric power generation.

3.2.1 Effect on Biodiversity

In freshwater bodies, water hyacinth affects the ecological stability of the aquatic ecosystem by routing other aquatic species growing in its vicinity, thereby, posing a threat to aquatic biodiversity. Generally, water hyacinth establishes itself in water bodies where there is a lack of aquatic vegetation; however it still competes with submerged vegetation and phytoplanktons for light and nutrients, thus diminishing their chances of growth and survival (Mitchell, 1985). Inhibition of planktonic green algae in a Portuguese lake has been reported due to infestation of water hyacinth (Almeida et al., 2006). It has also been reported that there is a substantial increase in populations of phytoplanktons and cyanobacteria after the removal of water hyacinth from the water bodies (Lugo et al., 1998; Mangas-Ramirez and Elias-Gutierrez, 2004).

Zooplanktons distribution in aquatic system is generally dependent on light intensity, temperature, chlorophyll, dissolved oxygen and food availability. Reduced phytoplankton productivity may decrease the zooplankton availability due to decrease in food availability. However, no specific trend has been recorded with respect to zooplanktons distributions and diversity due to infestation by *Eichhornia crassipes* (Villamagna and Murphy, 2010). Aquatic phytomacrofauna has been found to decrease with the infestation of water hyacinth which results due to reduction in periphyton food resources due to limitation in light penetration. The movement of phytomacrofauna gets restricted due to mats of water hyacinth which restrict penetration of light, limit periphyton growth, nutrient exchange and water circulation (Dvorak and Bes, 1982; Carpenter and Lodge, 1986; Beckett et al., 1992, Sloey et al., 1997; Mehra et al., 1999).

With respect to food, there is little correlation between water hyacinth and herbivorous fish as water hyacinth is poor in protein (Cowx, 2003). Predominantly, the diversity and abundance of fish

community is entirely dependent on the structure of food web and extent of water hyacinth infestation. As water hyacinth provides a very complex structure near the surface of water similar to a forest canopy, there is a little chance of vegetative growth below its surface, thus this habitat complexity and heterogeneity affects the assemblages of fishes (Meerhoff et al., 2007).

Water hyacinth mats have been implicated to reduce the predation and catchability of fish leading to their abundance in certain cases (Kateregga and Sterner, 2009). However, they may also hinder in the breeding, nursery and feeding grounds (Twongo and Howard, 1998). Dissolved oxygen levels below 4.8 mg/l are considered detrimental for fish survival as per USEPA (United States Environment Protection Agency).

Macro-invertebrates like snails, arachnids and amphipods have exhibited a positive correlation between floating aquatic vegetation including water hyacinth. In native range, water hyacinth has been reported to be an important substrate in invertebrate colonization (de Marco et al., 2001; Toft et al., 2003; Rocha-Ramirez et al., 2007). Macro invertebrate colonization has been reported in Lake Okeechobee, Florida (USA); costal lagoon of Mexico and Lake Victoria (Uganda) (O' Hara, 1967; Masifwa et al., 2001; Rocha-Ramirez et al., 2007). As such, there are no direct studies and evidences that indicate a correlation between mats of water hyacinth and community of aquatic birds. However, their prevalence and feeding on water hyacinth mats is more frequently observed as this is in agreement with the prevalence of higher densities of macro-invertebrates with the water hyacinth mats as compared to other macrophyte stands or open water (Svingen and Anderson, 1998; Masifwa et al., 2001; Toft et al., 2003). The common aquatic birds foraging water hyacinth mats are Great Egret (*Ardea alba* L.), Snowy Egret (*Egretta thula* Molina), Tri-colored Heron (*Egretta tricolor* Muller), and Great Blue Heron (*Ardea herodias* L.) and a direct food source for American Coots (*Fulica americana* Gmelin) (Villamagna, 2009). In Harike also, apart from migratory birds, the wetland's aquatic birds comprise of Great Egret, Black

crowned night Heron, Painted Stork (*Mycteria leucocephala*), *Eurasian Spoonbill* and *Eurasian Coot* (*Fulica atra*) forage on water hyacinth mats (PSCST, 2001).

3.2.2. Effect on water quality

There has been a considerable attention on the water quality of aquatic systems, infested with water hyacinth. Water hyacinth cover lower temperatures, pH, bicarbonate alkalinity, and increases free CO_2 content of the water body (Rai and Duttamunshi, 1979; Goel, 1980). The mats of water hyacinth prevent oxygen exchange between the air and the water surface (Hunt and Christiansen, 2000) apart from interference of oxygen production by other plants and alga due to its allelopathic nature. This drastically affects the dissolved oxygen (DO) concentration of the waterbody. In a specific case study at Sacramento-San Joaquin Delta, California (USA), it was observed that areas with water hyacinth infestation has poorest DO concentration when compared with other areas infested by aquatic plants like *Myriophyllum spicatum* L., *Hydrilla verticillata*, and *Potamogeton* spp.. It was found that water hyacinth infested areas reported a DO less than 5 mg/l (Toft et al., 2003).

Further deterioration of DO content occurs when the plant dies and the biomass sinks at the bottom of the aquatic body. The low DO content is a driver for release of phosphorus in the waterbody which is the primary cause of eutrophication. This release of the phosphorus is further responsible for the vigorous growth of water hyacinth and algal blooms (Bicudo et al., 2007). Shredding of water hyacinth from the water body significantly enhanced the nitrogen and phosphorus in the water column (Greenfield et al., 2007). Similarly, Marshall (1997) recorded an increase in nitrogen and phosphorus in the water column subsequent to biological control in Lake Chivero in 1990. The lake was infested by over 30 % of water hyacinth prior to application of biological control. The death and decay of water hyacinth in the aquatic system is also affecting the potability of water due to change in Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) and thus making water treatment for drinking a cost intensive process (Patel, 2012; Mironga et al., 2011).

3.2.3. Effect on Human Health

The floating mats of water hyacinth enhance turbidity of the surrounding water due to decay and death of older plants rendering the water unfit for human consumption. The fibrous free floating roots; semi submerged leaves and stems of water hyacinth hinder the water current and help in breeding of organisms which serve as vectors of human diseases (Mack and Smith, 2011). Breeding of anopheles mosquito, a vector of *Plasmodium falciparum* is responsible for chronic or falciparum malaria, has been reported from Lake Victoria (Minakawa et al., 2008). It has also been reported that water hyacinth is also a perfect breeding ground of Mansonioides mosquitoes which are vectors of human lymphatic filariasis causing nematode Brugia (Chandra et al., 2006; Varshney et al., 2008).

There are some reports wherein it has been clearly indicated that water hyacinth infestation in ponds serve as breeding grounds of vectors like Culex mosquitoes which spread the Japanese encephalitis (JE) diseases in humans as well as in domesticated animals (Center for Health Protection, Hong Kong, 2016). The habitat created by water hyacinth infestation also promotes the breeding of snails of genera *Biomphalaria* and *Bulinus* which host flukes responsible for the disease schistosomiasis (bilharzia) and filariasis (Borokini and Babalola, 2012). Mitchell (1974) has already reported that the mats of water hyacinth serve as resting sites as well as food area (algae and detritus) for the snails.

It has also been reported that water hyacinth harbors the causative agent of Cholera, *Vibrio cholerae*. The number of cases of cholera in Nyanza Province, Kenya which borders with lake Victoria has been correlated to the two pulses of abundance of water hyacinth (1997-2000 and 2006-2008). The incidence of cholera per capita was much higher than in Kenya as a whole (Feikin et al., 2010). The probable hypothesis is that water hyacinth is providing refuge to *V. cholera* carrying copepods or provides nidus to the bacterium in its fibrous roots. Alternatively, it has also been hypothesized that shoreline packed with *Eichhornia crassipes* may concentrate contaminated sewage close to shore thereby increasing the opportunity for infection (Rejmakova et al., 2006).

3.2.4. Socio-economic impediments of Water hyacinth

The dense mats of water hyacinth are disrupting socioeconomic as well as subsistence activities such as boat and ship navigation, restricted access to water for recreation, fisheries and tourism. The weed has covered the most intricate system of waterways along the south-western and south-eastern coasts of Nigeria (Kusermiju et al., 1985). Further, it has entered the river Niger and Kainji lake a decade back where it has started posing a threat to Kainji dam which is a source of hydroelectricity to Nigeria and Niger Republic. The other places where water hyacinth has played havoc in hydroelectric power generation are Kafue Gorge in Zambia where it is blocking the turbines apart from water loss due to high evapotranspiration rate. It has lead to a loss of approximately US$ 15 million annually for the power company (ZEO, 2008). Similarly annual cost of cleaning intake screens at the Owens Fall Hydroelectric power plant, Jinja (Uganda) is more the 1 million US$ per annum (Mailu, 2001). In China, the river Huangpu in Shanghai district is blocked by water hyacinth mats and hinders navigational activities in fall and winter each year (Gao and Li, 2004). Similarly about 60 % of the Bailianhe reservoir in Hubei province in China is covered by water hyacinth and interferes with the functions such as irrigation, hydroelectric power generation, navigation and recreation (Xu et al., 2003).

In India, navigation in Brahmaputra River has been affected by infestation of this weed. It has also blocked irrigation channels and obstructed the flow of water in crop fields (Patel, 2012). In west Bengal it has been reported to cause heavy losses in paddy by directly suppressing the crop; inhibiting rice germination and interfering in harvesting. Apart from that, it gets entangled in the boat's propeller thereby, posing difficulty in navigation, thus hampering fishing. As water hyacinth impedes the water flow by 40 to 95 % in irrigation channels, it may also result in severe flooding (Varshney et al., 2008).

3.3 Control strategies of water hyacinth

3.3.1 Mechanical control

Manual harvesting is labor intensive (transportation of labour, removal of the weed from water surface and its haulage, and labour management). Working under water is cumbersome and a tiring task. Moreover, mechanical harvesting is capital intensive with cost components comprising of acquisition of harvesting nets, repairs and maintenance of machine, removal of the weed from water and haulage, and acquisition of skill labor. The cost of mechanical/ manual control may vary upto US$ 600-1200 per hectare (Malik, 2007; Villamagna and Murphy, 2010) and thus proves to be uneconomical for larger areas of infestation. Dagno et al., (2007) reported that mechanical management of the weed in Mali is costing approximately US$ 80,000–100,000 per annum. The advantage of mechanical control is that it immediately opens physical space (habitat) for fish, boat traffic, fishing, recreation, and for remediation of damaged canals and barrage.

Despite the fact that mechanical removal of the weeds is effective to a considerable extent, infestations recur because the shredded branches of the weeds are carried by waves to other unaffected areas and there they reestablish and start proliferating (Shanab et al., 2010). Another important problem is storage and disposal of harvested biomass.

3.3.2 Chemical Control

Chemical control is capital intensive with cost components on acquisition, application and equipment. The economic efficiency of a chemical in controlling the weed depends upon the percentage of its active ingredients, rates of application at which the chemical is effective and its unit price. Mechanical harvesting is more economically efficient than manual harvesting but not as efficient as chemical control. Weedicides like Glyphosate (Roundup), Diquat, and 2, 4-D amine have been exploited in controlling water hyacinth chemically (Villamagna and Murphy, 2010); however, there is a need of repeated applications to cover up large areas in a shorter duration. Such repeated applications in

shorter durations in a large area could lead to de-oxygenation and threat to aquatic life. There are regulatory constraints of using these herbicides in water as they have been found to be possessing residual toxicity, thereby, inducing variety of maladies including cancers in non-target organisms (Malik, 2007). Further, considering the huge area of infestations of aquatic bodies by water hyacinth, it is unlikely that it can be controlled by chemical means alone (Borokini and Babalola, 2012). Further, use of chemicals in water bodies has significant socio-economic impact if the consumption of water is for drinking and cooking of food (Dagno et al., 2012).

3.3.3 Biological Control

Biological control appears to be a plausible ecofriendly alternative to control water hyacinth. The advantage of biological control is that it avoids the introduction of toxic chemicals., It is not labor or equipment intensive and has a potential to be self-sustaining method if the introduced biological control agent can reproduce successfully in the new environment without causing further ecological effects (Hershenhorn et al., 2016). The critical parameter for the success of biological control program depends upon the virulence and host specificity. It should maintain viable population for a longer duration on the host. Common biological control options for water hyacinth include various insect species and plant pathogens.

Neochetina eichhorniae and *N. bruchi* are two commonly used weevil species from the plant's native range which have been experimented as BCA (Biological Control agent) of water hyacinth. However, tremendous variations have been observed with respect to duration required to achieve the control which is attributed to spatial temporal distribution of water hyacinth in water bodies. Hence, the desirable attribute required for effective control similar to the chemical control is possible only if BCA proliferation rate is either as rapid or more as compared to water hyacinth. Hence, microbes primarily fungi and bacteria offer more opportunities over insects in control of water hyacinth.

3.4 Mycoflora associated with water hyacinth

The concept of using endemic or native pathogens of weeds *en masse* could be destructive to its host at a particular growths stage was demonstrated by Daniel et al., (1974). Thus, the application of the innundative dose of inoculum and its proper application timing would shorten the lag period of infection development based on inoculum buildup and its distribution which is essential for epiphytotics. Thus the two prime aspects of the mycoherbicide approach are (a) to *in vitro* culture of the pathogenic microorganisms in large quantities for use as an inoculum (b) innundative application of the inoculum to achieve rapid endemic buildup for high level of disease development in the host. The concept of mycoherbicide, therefore, is defined as use of plant pathogenic fungi in an innundative strategy to control weeds in a manner, similar to chemical herbicides but by living products which control specific weeds as effectively as chemicals (Charudattan, 1991).

Detailed review of literature has indicated that approximately 60 potential pathogens are associated with water hyacinth out of which 54 are reported from those countries or regions where water hyacinth has been designated as invasive alien species Table 3.1 summarizes the mycobiota associated with *Eichhornia crassipes* (Evans and Reeder, 2001). From this list, it can be easily deduced that only a few of the fungi possess the potential to be used as an innundative biological control agents. It has been found in surveys that ubiquitous pathogens appear to be having a higher possibility of being used as a potential biological control agent. *Alternaria eichhorniae*, *Acremonium zonatum* and *Cercospora rodamanii* have been recorded most frequently during the surveys in USA, South Africa and India (Freeman and Charudattan, 1974; Evans, 1987; Morris et al., 1999; Maricela et al., 2015), however, they are absent or rare in upper Amazon area. Recently *Myrothecium roridum* has been evaluated for potential use as mycoherbicide for water hyacinth (Piyaboon et al., 2016). A strain of *Alternaria alternata* has been reported from the ponds in Kurukshetra (Haryana), India (Aneja et al., 2014).

Similarly, there are very limited or no reports on mycobiota associated with water hyacinth infesting the Ramsar wetlands globally. Hence, it is imperative to explore the mycobiota at these sites and they may be quite different from the mycobiota of water hyacinth infesting lakes, ponds, rivers and navigational waterways.

3.5 Bioassays for mycoherbicidal potential

To assess the herbicidal potential of BCA's, *in vitro* and *in vivo* bioassays are developed to assess their efficacy. These assays help in selecting the appropriate BCA based on their pathogenicity to the leaves of the target plants. Subsequently, the most pathogenic isolate is selected based on the average leaf area damaged as well as on area under disease progressive curve (AUDPC) values. *In vitro* detached leaf bioassay has been the most frequently used assay to select the fungal pathogens of weeds as well as confirm the Koch's postulates. The bioassays also help in determination of the final concentration of the inoculum used to develop a suitable formulation which can be tested in field trails.

3.5.1 *In vitro* detached leaf bioassay for pathogenicity and phytotoxicity

In vitro detached leaf assay is commonly carried out to assess the pathogenic potential of the candidate fungi by using detached leaves of the target weed / host plant. *In vitro* detached leaf assay was used to assess the disease severity induced by different fungi for the biological control of Johnson grass (*Sorghum halepense*) and was rated on a five point scale based on the extent of the lead area damaged (LAD) (Chiang et al., 1989).

Fusarium avenaceum, *Colletotrichum dematium* and *Phomopsis* species have been evaluated for their pathogenic potential to *Rubus* sp. using *in vitro* detached leaf bioassay (Oleskevich et al., 1998). *In vitro* detached leaf bioassay using healthy leaves of *Lantana camara* was used to identify the mycoherbicidal potential of *Alternaria alternata* LC#508 (MTCC5432) (Saxena and Pandey, 2002). Pathogenicity of five isolates of *Corynespora cassiicola* to *Lantana camara* was carried out by *in vitro* detached leaf bioassay (Pereira et al., 2003). *In vitro* leaf section test was also carried out to evaluate the

Table 3.1 Country wise distribution of the mycobiota associated with *Eichhornia crassipes.*

Country	Fungi	Country	Fungi
Argentina	*Uredo eichhorniae*	India	*Acremonium zonatum*
Australia	*Acremonium crotocigenum*		*Alternaria eichhorniae*
	Acremonium implicatum		*Cercospora piaropi*
	Acremonium strictum		*Cercospora rodmanii*
	Acremonium zonatum		*Cochliobolus bicolor*
	Fusarium acuminatus		*Curvularia clavata*
	Fusarium graminearum		*Cylindrocladium scoparium*
	Fusarium oxysporum		*Fusarium equiseti*
	Fusarium solani		*Fusarium sulphureum*
	Gliocladium roseum		*Myrothecium roridum*
	Phoma sorghina		*Pestalatiopsis palmarum*
	Rhizoctonia oryzae- sativae		*Marasmiellus inoderma*
Brazil	*Uredo eichhorniae*		*Rhizoctonia solani*
	Bipolaris species		*Rhizoctonia sp.*
Burma	*Myrothecium*		*Thanateophorus cucumeris*
China	*Thanateophorus cucumeris*		*Curvularia lunata*
Dominican	*Leptosphaeria eichhorniae*	Malaysia	*Helminthosporium sp.*
Republic	*Doassansia eichhorniae*		*Myrothecium roridum*
Ghana	*Alternaria eichhorniae*		*Chaetomella species*
		Panama	*Acremonium zonatum*
Hong Kong	*Cladosporium oxysporum*		*Leptosphaeria eichhorniae*
	Pestalatiopsis adusta		*Rhizoctonia solani*
Indonesia	*Tulasnella grisea*	Sri Lanka	*Curvularia lunata*
Kenya	*Alternaria eichhorniae*		*Cercospora piaropi*
Nigeria	*Phyllosticta species*		*Glomerella cingulata*
Pakistan	*Acremonium zonatum*	Thailand	*Alternaria eichhorniae*
Philippines	*Myrothecium roridum*		*Blakeslea trispora*
South Africa	*Alternaria eichhorniae*		*Myrothecium roridum*
	Fusidium species		*Rhizoctonia solani*
Trinidad	*Didymella exigua*	USA	*Acremonium zonatum*
Taiwan	*Pestalatiopsis adusta*		*Alternaria eichhorniae*
	Thanateophorus cucumeris		*Bipolaris species*
Sudan	*Acremonium sclerotigenum*		*Cephalotrichum species*
	Acremonium zonatum		*Cercospora piaropi*
	Curvularia lunata		*Curvularia affinis*
	Coleophoma species		*Curvularia penniseti*
	Drechslera spicifera		*Didymella exigua*
	Fusarium equiseti		*Exserohilum prolatum*
	Fusarium monoliforme		*Leptosphaerulina sp.*
	Phoma sorghina		*Memnoniella subsimplex*
	Spegazzinia tessarthra		*Mycosphaerella tassiana*
Egypt	*Alternaria alternata*		*Phoma sp.*
	Alternaria eichhorniae		*Stemphylium vesicarium*
	Bipolaris urochloae		*Mycoleptodiscus terrestris*
	Curvularia lunata		*Rhizoctonia sp.*
Zimbabwe	*Alternaria eichhorniae*		*Phythium sp.*

Adopted : Evans and Reeder (2001)

potential of *Curvularia lunata* strain B6 as a mycoherbicidal candidate to control Barnyard grass (*Echinochloa crus-galli (L.) Beauv.)* in rice crops (Jing et al., 2013). *Fusarium oxysporum* 07 strain, which is a cowpea pathogenic isolated has been evaluated for its pathogenicity against the weed *Peporomia wightiana* by using 6-8 cm^2 pieces of detached leaves (Namasivayam and Aruna, 2012).

Dagno et al. (2012) has used 8 cm^2 fragments of healthy leaves of water hyacinth plants for *in vitro* evaluation of the pathogenicity of fungal isolates for their prospective use as biological control agent. Ray and Hill (2012) have placed 2 mm disc of actively growing fungus along with medium on healthy leaves of *Eichhornia crassipes* to assess their pathogenic potential for their possible use as a bioherbicide. Thus *in vitro* detached leaf bioassay is a rapid, economical and reliable method for testing the pathogenicity of fungi against the target weeds apart from providing the basis of host- pathogen interactions in dicot and monocot plants.

Plant pathogenic fungi produce a variety of toxic secondary metabolites when grown in liquid cultures which play a significant role in development of disease and in the process of pathogenesis. They produce symptoms such as chlorosis, wilting, necrosis and leaf spots. These phytotoxins have been classified as Host selective toxins (HST's) and Non- Host selective toxins (non- HST's) based on their role in pathogenicity. Hence, *In vitro* detached leaf bioassay is also used for screening variety of cell free culture filtrates of phytopathogenic fungi to assess their role in pathogenesis by testing them on target as well as non-target plants. In case, these cell free culture filtrates (CFCF) exhibit the above effects such as chlorosis, wilting, necrosis or leaf spots on non- target plant, then the phytotoxin so produced in non-HST. Both HST's and Non-HST's are being screened for their possible use as biorational agrochemical or for enhancing the mycoherbicidal potential of the biocontrol agent by developing suitable formulations.

Crude culture filtrates allow easy bioassay and screening for toxic effects on plants, cuttings, leaf discs or even cell suspension cultures of the host species (Isaac, 1991). Crude culture filtrates have been used as selective agents in numerous disease resistance studies in which they exhibit phytotoxic activity

(Chen and Swart, 2002; Svabova and Lebeda, 2005). The tests are performed either in detached mode (*in vitro*) or in undetached mode by making small puncture on leaves with fine capillaries (leaf puncture assay) (Evidente et al., 1995). Cell free culture filtrate of *Alternaria eichhorniae* induced necrosis in detached leaves of water hyacinth. This was attributed to the presence of two phytotoxins; Bostrycin and 4-deoxyBostrycin (Charudattan and Rao, 1982). *In vitro* detached leaf bioassay was also used during isolation and characterization of Brefeldin A and α, β- dehydrocurvularin produced by *Alternaria zinnia*, a biocontrol agent of *Xanthium occidentale* (Vurro et al., 1998). Culture filtrate of *Colletotrichum dematium* FGCC # 20 was also tested for its phytotoxic potential against *Parthenium hysterophrous* by using detached leaf bioassay (Singh et al., 2010). SS-toxin produced necrotic lesions on detached garlic leaves, similar to that caused by *Stemphylium solani* (Zheng et al., 2010). Culture filtrates of four *Drechslera* spp. namely, *D. hawaiiensis* Ellis; *D. holmii* (Luttr.) Subramanian and Jain; *D. biseptata* (Sacc.and Roum.) Richardson and Fraser and *D. australiensis (*Bugnicourt) Subramanian and Jain exhibited selective phytotoxic activity against *Chenopodium album* and *Avena fatua*, which are cumbersome weeds in wheat cultivation (Akbar and Javaid, 2012).

3.5.2 Hydroponic assay

Hydroponic assays have been used to evaluate the mycoherbicidal as well as phytotoxic activity of the biological control agents of weeds. Spore solutions, culture filtrates, organic fractions of culture filtrates and pure compounds have been used to assess the effect of the biological control agent on weed plants. The seedlings are grown in plant growth chamber /greenhouse in small volumes of nutrient solutions for ex. Hoagland's solution. Subsequently they are exposed to test solutions for sufficient duration to observe the phytotoxicity (Evidente et al., 2002; Kastanias and Chrysayi- Tokousbalides, 2005). These are then retransferred to the nutrient solutions for additional period to see to post treatment phytotoxic / herbicidal activity. Similarly, seedling cuttings can also be used to assess the bioherbicidal and phytotoxic activity of mycoherbicidal agents. In these tests, the seedlings are cut and apical part of the

stems are dipped in the test solutions, subsequently transferred to nutrient solution and observed for the appearance of symptoms such as wilting, browning, chlorosis, necrosis, internal tissue collapse. This is a quantitative method as the degree of damage/ death can be assessed (Evidente et al., 2005; Cabras et al., 2006)

Hydroponic seeding bioassay using 4 days old dark grown seedlings have been used for assessing the bioherbicidal efficacy of spore formulations of *Colletotrichum truncatum* and *Alternaria cassia* on hemp sesbania (*Sesbania exaltata*) and sicklepod (*Cassia obtusifolia*), respectively (Hoagland, 1995). Seedling assays have been used for the evaluation of *Myrothecium verrucaria* as a bioherbicidal agent to control hemp sasbania and sickelpod (Hoagland et al., 2007). The effects of 2-(3 H)-benzoxazolinone (BOA), an allelochemical against different grain crops have also been evaluated using hydroponic seedling bioassays (Belz and Hurle, 2004).

Disease severity of *Plectosporium tabacinum* against *Hydrilla verticillata* has been tested using *in vitro* cut shoot bioassay in which the cut shoots were maintained on Hoagland's solution (Smither-Kopperl et al., 1999). Saxena and Pandey (2002) used *in vitro* cut shoot bioassay to assess the mycoherbicidal potential of *Alternaria alternata* against *Lantana camara*. *In vitro* cut shoot or detached shoot bioassay has been used for the evaluation of *Colletotrichum gloeosporioides* for the control of dwarf mistletoe parasitizing western hemlock (Askew et al., 2009). Cut shoot of Eurasian Watermilfoil (*Myriophyllum spicatum L*.) has been used for screening pathogenicity over 222 fungal isolates for possible use as a mycoherbicide (Shearer et al., 2011)

Hydroponic assay has been used as the main assay for evaluation of bioherbicide candidates of aquatic weeds also. The pathogenicity of *Alternaria alternata* for the control of *Eichhornia crassipes* has been tested by using whole plants in 1 ft deep earthen pots and placed in environmental growth chamber to provide proper conditions (Babu et al., 2002). The effectiveness of *Myrothecium roridum* has been tested using whole plant assay in *Eicchornia crassipes* was placed in 1 ft deep pot having

Hoagland's solution (Okunowo et al., 2013). Similarly the mycoherbicidal potential of *Acremonium zonatum* was also tested using whole plant bioassay for the biological control of *Eichhornia crassipes* (Opande et al., 2013)

3.6 Bioherbicide formulation and efficacy

One of the primary and critical difference between bioherbicides and herbicides is that in the former active ingredients are living organisms as compared to chemicals in the later. These living organisms normally require time to develop or reproduce *en masse* after application in order to control the target weed or pest (Charudattan and Dinoor, 2000).

Formulation and application methods are of paramount importance in determining the effectiveness of pathogens as mycoherbicides (Boyette and Templeton, 1981). The mode of action through which a biocontrol agent suppresses its target includes production of toxins, parasitism or competition, each mode of action requires a different set of formulations (Rhodes, 1990). Adding substances that increase the efficacy of the biocontrol agent, e.g., polymers that extend leaf wetness period, amendment with pathogen-produced extracellular phytotoxins, or other plant-stress inducing factors, is desirable (Saxena and Pandey, 2009). Different formulations have been tested under *in vitro* and *in vivo* trails to optimize the application and efficacy so that bioherbicidal strategy effectively complements the chemical herbicidal control.

Sodium alginate is commonly used in many food products (Connick, 1979) and any residues in plants or water would not be toxic to non-target organisms. In a selective study alginate formulations of *Alternaria eichhorniae* 5 (isolate Ae5), a virulent Egyptian isolate, were compared on water hyacinth (*Eichhornia crassipes*). The fungus was formulated alginate pellets containing mycelium alone, mycelium plus culture filtrate or culture filtrate alone. Each formulation was applied with and without a hydrophilic humectant (Evergreen 500). These formulations were evaluated for disease incidence (DI), and disease severity (DS), to select the most efficacious formulation- i.e alginate formulations

supplemented with the hydrophilic polymer (Shabana et al., 1997). Oil emulsions of isolate Ae5 have also been evaluated and it was observed that all invert and oil emulsion formulations induced higher levels of disease on water hyacinth plants than the aqueous formulation under dew-free conditions (Shabana et al., 2005). Shabana and Mohamed, (2005) combined *Alternaria eichhorniae* isolate 5 with 3,6-dichloro-2-pyridinecarboxylic acid (MDCA) to weaken water hyacinth defenses, which increased disease severity and has the potential for greater biocontrol.

Formulation of *Alternaria alternata* spores in an oil emulsion (10% oil in water) enhanced its efficacy to control the water hyacinth plants. The necrotic leaf area of the inoculated plants increased as a result of longer duration of maintenance of relative humidity there by leading to 80% plant tissue death when the spore concentration was $1x10^6$ spores/ ml in the formulation (El-Morsy, 2004). Saxena and Pandey, (2009) have reported the use of crude phytotoxins along with different spore concentrations for enhancing the mycoherbicidal potential of *Alternaria alternata* LC#508 outstandingly. The concept of evaluating the crude spent broth exhibiting phytotoxicity produced by the fungal biological control agent is new and has not been tested so far with the idea of enhancing the mycoherbicidal potential in terms of disease severity. The added advantage is that the spent broth can serve as osmoticum thus providing relevant conditions of storage and limiting the use of surfactants and hydrants. Recently it has been reported that a spore concentration of 10^9 conidia/ml in 20% canola oil emulsion of *Alternaria alternata* (Fr.) Keisseler at temperature of 25°C and 30°C induced 100% mortality in *Chenopodium album* plants (Siddiqui et al., 2011). Thus, it must be stressed that an effective formulation is desirous for enhancing the mycoherbicidal efficiency as well as improve the shelf storage conditions.

3.7 Toxicological safety of the bioherbicide formulation

Environmental safety of the biopesticides is of prime importance for the deployment of bioherbicides in the environment since, it should not induce adverse effects on macro and microcosms. Risk assessment

entails the following actions (i) effects assessment (ii) hazard identification (iii) dose response assessment (iv) exposure assessment and (v) risk characterization (i.e. estimation of incidence and severity of adverse effects likely to occur due to the actual or predicted source). In this way, proper data on mode of action and other microbiological properties, the host range and the natural distribution may preclude the exposure to the non-target organisms (NTO's). In aquatic systems, several aquatic plants are suggested that include algae, aquatic bacteria, marine and freshwater diatoms. Apart from the above features there is a need to assess the change in water quality (physical, biological and chemical changes) (Singh et al., 2003)

Subject to through host- range testing, a very few, if any detrimental effects occur on the release of fungal herbicides to control weeds (Barton, 2004). Additional animal, avian, fish and daphnia testing is also required in many countries before the herbicides can be registered. *Mycoleptodiscus terrestris* was tested for its use as a mycoherbidicide for Eurasian watermilfoil (*Myriophyllum spicatum*), an exotic submerged aquatic weed in North America. Of the 16 non target aquatic species tested, *Mycoleptodiscus terrestris* was pathogenic to *Hydrilla verticillata* (hydrilla), *Myriophyllum aquaticum* (parrotfeather), and *Ceratophyllum demersum* (coontail). However, the plant mortality was comparable to Eurasian water milfoil only in hydrilla whereas the remaining 13 submerged, floating, and emergent aquatic species were unaffected (Verma and Charudattan, 1993). Similarly, *Phoma macrostoma* was tested against 94 plant species as well as target and non-target weeds. It was found that 57 species were resistant to *Phoma macrostoma* (Bailey et al., 2011).

3.8 Commercially available mycoherbicides

Mycoherbicides have been commercialized, however the number of products which have reached the market is quite limited due to several developmental constraints. Some bottlenecks are difficulty in producing large amount of inoculum through fermentation processes, high stability of bioherbicidal formulations during shelf storage, impediments imposed by climate after their application since they

require high humidity to germinate and infect the plant, apart from the small size of markets. These constraints can be overcomed by the development of an appropriate formulation which can be developed keeping in the mind the type of application of the biological control agent. The developed and commercialized mycoherbicides have been listed in Table 3.2.

Table 3.2 Commercially registered bioherbicidal agents

Biological Control Agent	**Commercial Name**	**Target Weed**	**Company/ Country where used**
Phytophthora palmivora	DeVine®	*Morrenia odorata*	USA (1981)
Colletotrichum gloesporioides f.sp. aeschynomene	COLLEGO®	Northern Jointvetch	Encore Technologies USA (1982)
Alternaria cassia	CASST™	Sickle pod and coffee senna (*Cassia* sp.)	USA (1983)
Cercospora rodmanii	ABG-5003	*Water hyacinth (Eicchornia crassipes)*	USA (1991)
Colletotrichum gloesporioides	BIOMAL®	*Malva pusilla*	USA (1992)
Cylindrobasidium leave	STUMPTOUT™	Acacia species	South Africa (1997)
Chondrostereum purpureum	BIOCHON™	Woody weeds like *Prunus serotina*	Netherlands (1997)
Xanthomonas campestris pv poae	COMPERICO™	*Poa annua* (Turf grass in golf courses)	Japan (1997)
Colletotrichum acutatum	HAKATAK	*Hakea gummosis* & *H. sericea*	South Africa (1999)
Puccinia thlaspeos	WOAD WARRIOR	Dyers woad *(Isastis tinctoria)* in farms, rangeland, waste areas, & roadsides	USA (2002)
Chondrostereum purpureum	CHONTROL™ ECOCLEAR™	Alders, aspen & other hard-woods	Canada (2004)
Chondrostereum purpureum	MYCOTECH™ PASTE	Deciduous tree species in rights of way & forests	Canada (2004)
Alternaria destruens	SMOLDER ™	Cuscutta	USA (2005)
Sclerotinia minor	SARRITOR®	Dandelion (*Taraxacum officinale*) in lawns / turf	Canada (2007)

Chapter 4

Materials & Methods

4. MATERIALS AND METHODS

4.1 Plant sample collection

Infected plant parts (stolons, swollen bases and leaves) with necrotic spots were collected from the Harike Wetland area, Taran Taran, Punjab (geographical coordinates 31° 28' N 74° 58' E latitude and 31° 28' N 74° 58' E longitude) during rainy and early winter season of year 2011. The plant parts were kept in sterile bags, brought to the laboratory and processed within 24 hours.

4.2 Isolation of pathogenic fungi from water hyacinth

The infected plant parts were first washed thoroughly under running tap water for 15 min for the removal of the surface debris. These were then surface sterilized using 1 % sodium hypochlorite solution for 1-2 min followed by 30 % ethanol for 1 min and then washed with sterile distilled water (SDW) thrice and then air dried aseptically under the laminar air flow. These surface sterilized infected plant samples were then cut into 2-4 mm segments aseptically using a sterile scalpel and plated on pre-made PDA (Potato dextrose Agar) plates supplemented with streptomycin. Maximum of eight segments were inoculated per plate and incubated at 26±1°C for 7 days. The emerging fungal isolates were then sub-cultured over fresh PDA plate as pure cultures using the hyphal tip technique or the single spore method and subsequently stored on a PDA slant containing 15 % glycerol (Babu et al., 2003; El-Morsy, 2004; El-Morsy et al., 2006; Ray and Hill, 2012).

4.2.1 Preparation of PDA Plates

Premade-PDA (HiMedia, Mumbai) was used for preparation of PDA plates. Briefly, 39 g of PDA mixture was dispersed in 1 l single distilled water (SDW) and stirred thoroughly. Then, 150 ml

of the solution was dispensed in 250 ml Erlenmeyer flasks and autoclaved at 121°C, 15 psi for 15 minutes. Subsequently, the flasks were removed from the autoclave and cooled. Then under sterile conditions the molten sterile PDA was dispensed into sterile Petri dishes (90 mm, Borosil, India) and allowed to solidify at room temperature. After solidification, Petri dishes were sealed with cling film and stored in incubator at a temperature of 26 ± 2°C until further use.

4.2.2 Sub-culturing

The fungal cultures so obtained were re-cultured on fresh PDA plates to confirm their purity as single cultures. The plates were incubated at 26 ± 2°C for maximum of 7-10 days.

4.2.3 Maintenance of pure isolates

PDA slants of the pure cultures were prepared by inoculating 7-day old culture of pure culture previously grown on Petri dishes and subsequently incubating them at 26 ± 2°C for maximum of 4 days. These were then stored at 8-10 °C till further use.

4.3 Inoculum production and *in vitro* detached leaf bioassay

The fungal isolates were inoculated from the stock cultures on PDA medium and incubated for 6-7 days at a temperature of 26 ± 2 °C. Subsequently, the spores were harvested by flooding the 7- days old culture plate with sterile distilled water (SDW). Thereafter, spore concentrations of 1 x 10^6 spores/ml were prepared using a haemocytometer. The concentration of the surfactant (Tween 80, Merck, USA) was 0.5 % per 100 ml of the final spore suspension.

A set of healthy water hyacinth leaves were collected from *Eicchornia crassipes* plants maintained under the greenhouse conditions. Three leaves per plant from 10-15 plants approximately were used for the preliminary pathogenicity assay. 10 ml of the each spore concentration was used for spraying healthy *Eichhornia* leaves which were subsequently placed

in a mist chamber prepared aseptically in 90 mm Petri dishes and incubated at 28°C for 7 days under 12 h photoperiod (7350 lx). Leaves were rated for disease severity every 24 h on a five point scale. All the tests were performed in triplicates (Jimenez and Lopez, 2001; Saxena and Pandey, 2002; El-Morsy et al., 2006).

4.4 Hydroponic bioassay for assessment of pathogenicity on whole plants

Two week old plants having 3-4 leaves were transferred in glass containers containing Hoagland solution ($(CaNO_3)_2.4H_20$ - 0.5g; KNO_3 – 0.255g; $MgSO_4.7H_20$-0.245 g; KH_2PO_4- 0.57 g, Ferrous tartrate – 0.00025 g; Distilled Water – 500 ml) with an illumination of 12 h daily for a period of one week for acclimatization under laboratory conditions. These were then sprayed with fungal inoculum of a spore concentration to 1 x 10^6 spores /ml. The glass containers were covered with sterile bags for next 48 h and then they were brought to normal laboratory conditions for next 5-6 days at 28°C. The experimental set had nine replicates and the control set had six replicates. The control set received only sterile water (SDW). Plants were observed daily for the disease severity as per Chaing et al. 1989 until all plants died. The experiment was repeated thrice (Chiang et al., 1989; Saxena and Pandey, 2002; Opande et al., 2013).

4.5 Morphotaxonomy of the selected fungal pathogen

The fungal isolate was grown over different media including Pine Leaf Agar (PLA) and Synthetischer Nahrstoffarmer agar (SNA) and Corn Meal Agar (CMA) (Hi Media, India) and incubated at 26 °C for 3-4 weeks in dark. The fungus was identified based upon morphological characteristics like colony size, texture, color and microscopic characters like the hyphae, conidiophores and spores. The microscopic characters were studied using a Nikon eclipse 50i microscope coupled with CCD camera and measurements carried out using NIS element

software. Atleast, 30 observations were made per structure (Barnett and Hunter, 1998; Burgess and Husband, 2006).

4.6 Molecular identification of the selected fungal pathogens

4.6.1 Fungal DNA isolation, Gene amplification

The fungal genomic DNA extraction was carried out by using Wizard® Genomic DNA purification kit (Promega, USA). For the DNA isolation, 2-3 mycelial discs (10 mm) of 4 -5 day old fungal culture were scooped out and crushed to very fine powder in mortar and pestle by using liquid nitrogen. The powder was immediately transferred to the 2 ml micro-centrifuge tube and the further extraction was carried out as per manufacturer's instructions.

The ITS1, 5.8S, ITS2 rDNA sequence was amplified using the primers ITS1 (5' TCCGTA GGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990). Amplification was performed in 25 µl reaction mixture containing 25 ng of extracted genomic DNA, 0.8 µM of each primer (ITS1 and ITS4), 0.2 mM of dNTP, 1.5 mM $MgCl_2$, 1.5 U of *Taq* DNA polymerase in 10 X *Taq* buffer. The thermal cycling conditions consisted of initial denaturation at 96°C for 5 min followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 sec, 72 °C for 45 s followed by final extension at 72 °C for 5 min.

Whereas the β-tubulin gene sequence was amplified using bena-T1 (5′AACATGCGTGAGATTGTAAGT 3′), bena-T22 (5′ TCTGGATGTTGTTGGGAATCC3′) primer pair. Amplification was performed in a 25 µl reaction mixture volume consisting of 25 ng of extracted genomic DNA, 0.8 µM of each primer pair, 2.5 mM of dNTPs, 1.5 mM $MgCl_2$, 1.5 U of *Taq* DNA polymerase. The thermal cycling parameters was 96 ˚C for 5 min followed by 35 cycles of 95˚C for 60 s, 58 ˚C for 90 s, 72 ˚C for 90 s followed by final extension at 72 ˚C for 7 min (Damm et al.,

2008; Carlucci et al., 2015). The amplified products were purified by using the Wizard® SV gel and PCR clean up system kit (Promega, USA). Gel imaging was performed under UV light in Bio-Rad Gel documentation system using Quantity-1-D analysis software (Bio-Rad, USA). Purified products were directly sent for sequencing to Chromus Biotech, Bangalore. The Final sequence was obtained by assembling the obtained sequences using Sequencher ver. 5 (www.genecodes.com). Sequences obtained in the present work were submitted in the NCBI database.

4.6.2 Sequence assembly, alignment and phylogenetic analysis

The final sequences of both the isolates (#8BJSSL and #19BJSSL) were subjected for similarity search by using the BLAST algorithm against the non-redundant database maintained by the National Center for Biotechnology Information (http:www.ncbi.nlm.nih.gov). The sequences showing highest similarity for each locus were selected and aligned with the respective sequences obtained in the present work by using ClustalW. The phylogenetic relationship of the isolate was inferred by constructing phylogenetic tree using maximum parsimony method in MEGA 5.2 (Tamura et al., 2011). The most parsimonious tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 15 nucleotide sequences. Clade stability was assessed by bootstrap analysis with 1000 bootstrap replicates.

4.7 Production of the culture filtrate for phytotoxicity evaluation

The fungal isolates which exhibited potential pathogenicity in the *in vitro* detached leaf bioassay and hydroponic bioassay were further subjected for production of culture filtrates to

assess their phytotoxic potential. Briefly, mycelial plugs (5 mm diameter) of 7-day old fungal culture was inoculated into 100 ml of pre-sterilized Richard's broth (Sucrose: 50 g, Potassium dihydrogen orthophosphate: 8 g, Potassium nitrate: 10 g, Magnesium sulphate: 2.5 g, Ferric chloride: 0.02 g, Distilled water 1000 ml, pH 4.5-4.6) in 250 ml Erlenmeyer flasks (Schott Duran, Germany). These were then incubated in an orbital shaker at 28 ± 2 °C, 120 rpm for 15 days. The mycelium was separated from the liquid medium initially with a sterile muslin cloth followed by Whatmann No. 4 filter paper and finally though 0.22 µm nitrocellulose membrane to make it cell free. The cell free broth was frozen at -4°C till further use (Babu et al., 2003; Raviraja et al., 2006; Sangeetha and Rawal, 2008; Saxena and Pandey, 2009).

4.8 *In vitro* leaf puncher assay

Fresh and healthy leaves were kept in a moist chamber prepared in 90 mm sterile Petri dishes. Leaves were punched using sterile needle. About, 200 µl of the cell free culture broth, as well as the four organic residues (100 µl) produced were dispensed over the leaves. The plates were sealed using parafilm and incubated at 26°C ± 2°C The experimental set had 9 replicates and the control set had three replicates. The control set received Richard's broth and DMSO. Leaves were observed daily for the disease severity on five point scale. The experiment was repeated thrice (Saxena and Pandey, 2002; Pathak and Kanan, 2011).

4.9 Production of the culture filtrate and solvent extraction for phytotoxic evaluation

The fungal isolates which exhibited potential pathogenicity in the *in vitro* detached leaf bioassay and hydroponic bioassay were further subjected for production of culture filtrates to assess their phytotoxic potential. Briefly, mycelial plugs of (5 mm diameter) of 7 days old fungal culture was inoculated into 100 ml of pre-sterilized Richard's broth (Sucrose: 50 g, Potassium

dihydrogen orthophosphate: 8 g, Potassium nitrate: 10 g, Magnesium sulphate: 2.5 g, Ferric chloride: 0.02 g, Distilled water 1000 ml, pH 4.5-4.6) in 250 ml Erlenmeyer flasks (Schott Duran, Germany). These were then incubated in an orbital shaker at 28 ± 2 °C, 120 revolutions per minute (rpm) for 15 days. The mycelium was separated from the liquid medium initially with a sterile muslin cloth followed by Whatmann No. 4 filter paper and finally though 0.22 µm nitrocellulose membrane to make it cell free. The cell free broth was frozen at - 4°C till further use (Babu et al., 2003; Raviraja et al., 2006; Sangeetha and Rawal, 2008; Saxena and Pandey 2009).

Liquid-liquid extraction procedure was adopted to extract the spent broth of fungal isolates. The aqueous layer was sequentially extracted using different solvents like ethyl acetate, dichloromethane, chloroform and hexane (Merck GR, USA). The extraction procedure comprised of liquid- liquid partitioning of the aqueous layer with single solvent three times and then pooling the solvent layers so obtained. Subsequently, the aqueous layer was extracted in a similar manner by other organic solvents. All the organic solvent layers were dehydrated using anhydrous sodium sulphate. The organic layer from each solvent was then transferred into a pre-weighed crucible to obtain residue after evaporation. After removal of solvent, stock solutions of extracts (1 mg/ml) were prepared in 10% DMSO and stored at –20°C till use (Vicente et al., 2001).

4.10 *In vitro* Leaf puncher assay of the solvent residues

Fresh and healthy leaves were kept in a moist chamber prepared in 90 mm sterile Petri dishes. Leaves were punched using sterile needle. 200 µl of the cell free culture broth, as well as four organic residues (100 µl) produced were dispensed over the leaves. The plates were sealed

using parafilm and incubated at 26°C ± 2°C The experimental set had 9 replicates and the control set had three replicates. The control set received Richard's broth and DMSO. Leaves were observed daily for the disease severity on five point scale. The experiment was repeated thrice (Pathak and Kanan, 2011; Saxena and Pandey, 2002).

4.11 Hydroponic bioassay of water hyacinth with culture filtrates and crude organic residues

In this assay, two week old healthy water hyacinth plants having 3-4 leaves were transferred to sterile tissue culture bottles containing 200 ml of Hoagland solution ($(CaNO_3)_2.4H_20$- 0.5g; KNO_3 – 0.255g; $MgSO_4.7H_20$-0.245 g; KH_2PO_4- 0.57 g, Ferrous tartarate – 0.00025 g; Distilled Water – 500 ml) with 12 h of photoperiod for period of one week for acclimatization under laboratory conditions. These were then sprayed with 2 ml culture filtrate and crude residues (1 mg/ml) of hexane and chloroform. The tissue culture bottles were covered with sterile bags for next 48 h. subsequently, their covers were removed and they were transferred to normal laboratory conditions for next 5-6 days at 28°C. The experimental set had nine replicates and the control set had six replicates. The control set received only physiological saline. Plants were observed daily for the disease severity as per Chaing et al., (1989). The experiment was repeated thrice (Chang et al., 1989; Saxena and Pandey, 2002; Ray and Hill, 2012).

4.12 Characterization of the bioactive extract

Characterization of the culture filtrates and solvent residues so obtained were tested to assess the nature of the compound in the spent broth and in chloroform and hexane extracts. Tests were performed to check the presence of alkaloids, flavonoids, steroids, terpenoids, saponins, resins, tannins, anthroquinone, phlobatamine, glycosils, carbohydrate, proteins and esters. (Egwaikhide and Gimba, 2007).

4.12.1 Test for alkaloids

Two methods were used to confirm the presence of alkaloids in culture filtrate and crude residue. In the first method, few drops of Marquis Reagent (100 ml of concentrated (95–98%) sulfuric acid to 5 ml of 40% formaldehyde) were added to 100 µl of culture filtrate and crude residue (1 mg/ml in SDW). Turbidity or red precipitation indicated the presence of alkaloids (Harborne, 1973).

Similarly, 100 µl of culture filtrate and crude residue were separately mixed with few drops of Dragendorff's reagent (Bismuth sub-nitrate-1.7 g, glacial acetic acid-20 ml, water-80 ml and 50 % solution of potassium iodide in water-100 ml). Formation of turbidity or red precipitation indicated the presence of alkaloids (Oloyede, 2005).

4.12.2 Test for anthraquinones

To test the presence of anthraquinones in culture filtrate and crude residue, 500 µl of benzene was added to 100 µl of each test sample. Subsequently, 300 µl of 10% ammonia solution was added followed by vigorous shaking. The occurrence of a violet color in lower ammonical phase indicates the presence of free hydroxyl anthraquinones (Trease and Evans, 1996).

4.12.3 Test for tannins

Briefly, few drops of ferric chloride reagent were added to 100 µl of culture filtrate and crude residue separately. The presence of a blue black precipitate indicates the presence of tannins (Trease and Evans, 1996).

4.12.4 Test for saponins

The ability of saponins to produce froth in aqueous solution was used as evidence to the presence of saponins in the extract. About, 100 µl of test sample was mixed with 200 µl of

distilled water in a micro-centrifuge tube and subjected to vigorous vortexing. The appearance of froth upon slight warming of the vortexed mixture indicated the presence of saponins (Oloyede, 2005).

4.12.5 Test for flavonoids

The presence of flavonoids was tested by mixing 100 µl of test sample with 500 µl of water and ethanol mixture (1:1) and centrifuged. The solution was further mixed with 500 µl of concentrated HCl and 100 mg of zinc turnings. The presence of flavonoids was indicated by the appearance of pink/ magenta color within two minutes which could be extracted with butanol (Aynehchi et al., 1981).

4.12.6 Test for glycosides and glycolipids

To test the presence of glycosides and glycolipids, diphenylamine reagent was used. Diphenylamine reagent was prepared by adding 10 ml of 10% diphenylamine in ethanol, 100 ml HCl and 80 ml glacial acetic acid. This reagent was added to 100 µl of test sample, followed by heating for 30-40 min at 110 °C. Appearance of blue spots/coloration indicates the presence of glycosides/ glycolipids (Narasimhan et al., 1982).

4.12.7 Test for carbohydrates

To test the presence of carbohydrates, 100 µl of the test sample was mixed with few drops of Molisch reagent (α-napthol in ethanol). After mixing, few drops of concentrated sulfuric acid were slowly added along the walls, without mixing, to form a layer. Appearance of a purple ring at the interface between the acid and test layers indicates presence of carbohydrates (Sawhney et al., 2011).

4.12.8 Test for amino acids

To confirm the presence of amino acids, ninhydrin solution was separately added to 100 µl of test sample in a micro–centrifuge and then kept in boiling water bath for 5 min. Appearance of blue purple coloration confirms the presence of amino acids (Sawhney et al., 2011).

4.12.9 Test for fats

Briefly, 100 µl of crude residue was mixed with sudan dye IV. Formation of red coloration confirms the presence of fats (Zhu et al., 2015).

4.12.10 Test for terpenoids and steroids

1 mg of crude residue was suspended in 1 ml of chloroform in micro-centrifuge tube, placed in an ice bath for 10 minutes. Subsequently, 1 ml of acetic acid was added followed by a few drops of concentrated sulphuric acid along the walls. Appearance of pink or pinkish brown ring/ colour indicates the presence of terpenoids and the appearance of bluish green or a rapid change from pink to blue color indicates the presence of steroids (Kantamreddi et al., 2010).

4.13 *In vitro* whole plant bioassay of the solvent extracts

As explained previously in section 4.11, Four to six week old plants were grown in tubs and acclimatized under laboratory conditions. These were then sprayed with 10 ml of solvent extracts residues of chloroform and hexane (1mg/ml). The experimental set had nine replicates and the control set had six replicates. The control set received only SDW, DMSO. Plants were observed daily for the disease severity on five point scale as per Chaing et al., (1989). The experiment was repeated thrice (Chiang et al., 1989; Saxena and Pandey, 2002).

4.14 *In vitro* whole plant bioassay for spore suspension

In whole plant bioassay, assessment of *in vitro* potential of spore suspension, 4-6 week old plants were grown in tubs (40 cm diameter, 12 cm depth and 10 plants/tub) with water and 12 h illumination for a period of one week for acclimatization under laboratory conditions. These were then sprayed with 10 ml of spore suspension (1×10^6 spores/ml) per plant. The experimental set had nine replicates and the control set had six replicates. The control set received only SDW. Plants were observed daily for the disease severity on five point scale as per Chaing et al., (1989). The experiment was repeated thrice (Chiang et al., 1989; Saxena and Pandey, 2002).

4.15 Food Poison assay

Food poison assay was done to evaluate the toxic concentration of crude residue of the selected test fungus. Stock solution of crude organic residue having concentration of 100 mg/ml was prepared in 10 % dimethyl sulphoxide (DMSO). The stock solution was added in appropriate concentrations to PDA medium in order achieve a final concentration of 10–100 µg /20 ml of PDA which was then aseptically poured into sterile 90 mm Petri dishes in triplicates and allowed to solidify. The control plates were dispensed with only 10 % DMSO. The control and test plates were then inoculated with 5 mm disc of 7-day old culture of selected test fungus upside down in the center of Petri dishes and incubated at 26±2°C till the Petri dishes in control set were maximally covered i.e. 7 days. The mycelial growth (mm) of the test fungus was measured diametrically in different treatments and inhibition calculated using the formula:

$$I\% = [(C - T)/C] \times 100$$

Where, I refers to Inhibition; C refers to the diameter of fungi in the control and T refers to diameter of fungi in the test (Saxena and Pandey, 2009).

4.16 *In vitro* whole plant bioassay for spore suspension

In whole plant bioassay under for assessment of *in vitro* potential of spore suspension, 4-6 week old plants were grown in tubs (40 cm diameter, 12 cm depth and 10 plants/tub) with water, with 12 h illumination for a period of one week for acclimatization under laboratory conditions. These were then sprayed with 10 ml of spore suspension (1×10^6 spores/ml) per plant. The experimental set had nine replicates and the control set had six replicates. The control set received only SDW. Plants were observed daily for the disease severity on five point scale as per Chaing et al. (1989. The experiment was repeated thrice (Chiang et al., 1989; Saxena and Pandey, 2002).

4.17 *In vitro* whole plant bioassay of culture filtrate

As discussed in section 4.11, in this assay also 4-6 week old plants were grown in tubs (40 cm diameter, 12 cm depth and 10 plants/tub) with illumination of 12 h daily for a period of one week for acclimatization under laboratory conditions. These were then sprayed with 10 ml of culture filtrate of the test fungi (#19BJSSL) per plant. The experimental set had nine replicates and the control set had six replicates. The control set received only SDW. Plants were observed daily for the disease severity on five point scale as per Chaing et al., 1989. The experiment was repeated thrice (Chiang et al., 1989; Saxena and Pandey, 2002).

4.18 *In vitro* assessment of formulations by whole plant bioassay

Based on the results of the food poison assay, a formulation of spore and crude organic residues was prepared. It consisted a consortium of crude residue in concentration 50 µg/ml

and spores concentration of $1x10^6$ spores/ml. Four to six week old plants grown in tubs (40 cm diameter, 12 cm depth and 10 plants per tub) with an illumination of 12 h daily were kept for acclimatization for a period of one week under laboratory conditions. These were then sprayed with 10 ml of effective formulation of spores and crude residue per plant. The experimental set had six replicates and the control set had three replicates. The control set received only 10% DMSO. Plants were observed daily for the disease severity on five point scale as per Chaing et al., (1989). The experiment was repeated thrice and all data was presented as mean ± SD (Babu et al., 2002; Saxena and Pandey, 2009).

4.19 Host specificity testing

Host specificity of the selected fungal species was evaluated by spraying the spores suspension over six seasonal crops i.e. wheat, potato, eggplant, brassica, beet root and cauliflower and duckweed. Fungal spores (1×10^6 spore/ml) of selected fungal isolates were sprayed over the plants and kept under normal laboratory conditions for 15-20 days. Plants were observed daily for the occurrence of disease symptoms. The experimental set had 9 replicates and the control set had three (3) replicates. The control set received SDW. The experiment was repeated thrice (Dhingra and Sinclair, 1994; Babu et al., 2002).

4.20 Physical, chemical and bacteriological characteristics of water after *in vitro* whole plant bioassay

The residual water samples were collected after completion of *in vitro* whole plant bioassay in a sterile bottle. Physical, chemical and bacteriological characteristics i.e. Dissolved Oxygen (DO), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), pH, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), conductivity, color, optical density, MPN and pathogen

concentration of residual water were analyzed using standard methods as per ISI (Indian Standard Institute) and APHA (America Public Health Association) standard at Punjab Pollution Control board Laboratory, Patiala (El-Morsy et al., 2006).

4.19.1 Biological Oxygen Demand (BOD)

Sterile Distilled Water (5 l) was aerated with air bubbler (Shengzhe-BS 310). Then phosphate buffer solution (5 ml), magnesium sulphate (5 ml), calcium chloride (5 ml) and ferric chloride (5 ml) were added in it. The pH of sample was adjusted to pH 7.0 and aerated for 4 hours. Then the sample and dilution water were taken into 1:1 ratio, and mixed well. Two BOD bottles were filled with these mixed samples. The First BOD bottle was analyzed immediately for dissolved oxygen, while second BOD bottle was kept in incubator (Trans International, India) at 27 ^{o}C for 3 days and then analyzed for dissolved oxygen.

For determination of dissolved oxygen 2 ml of $MnSO_4$, 2ml of alkali iodide azide solution and 2 ml of H_2SO_4 were added in sample in BOD bottle and mixed well. Then, 200 ml sample was taken and titrated with standard sodium thiosulphate by using starch solution as indicator BOD of sample was calculated as per following calculations.

$$BOD\ (mg/l) = (I_s\text{-}F_s) – (I_B\text{-}F_B) \times \text{Dilution factor}$$

Where, I_s = Dissolved Oxygen of the sample on 1st day, F_s = Dissolved Oxygen of the sample after 3rd day, I_B= Dissolved Oxygen of the sample on 1st day, F_B= = Dissolved Oxygen of the sample after 3rd day

4.19.2 Chemical Oxygen Demand (COD)

20 ml of sample was taken in digestion tube, 0.4 gm of $HgSO_4$, 10ml of H_2SO_4 were added in it. Then 10 ml of 0.04167 M $k_2Cr_2O_7$ solution was added and mixed well then 20 ml more H_2SO_4

was added in it. The flask was attached to condenser and refluxed for 2 h in COD digester (Macro Scientific Works (P) Ltd., India) at 150 ^{o}C. After 2 h, reflux condenser was disconnected. The mixture was diluted to about twice it volume with distilled water. Further, it was kept at room temperature and titrated it with excess $k_2Cr_2O_7$ with ferrous ammonium sulphate (0.1 N) using 2-3 drops of ferroin indicator. The end point of titration was observed by color change from blue green to reddish brown that persist from 1 minute or longer. COD of sample was calculated as per following calculations.

COD (mg/l) = A-BxNx8000/ml of sample

Where, A = ml FAS used for blank, B = ml FAS used for sample, M = Molarity of FAS, n8000 = mili equivalent weight of oxygen x 1000 ml/l.

4.19.3 pH

pH of sample was measured by using pH meter-orion 940. 50 ml of sample was taken in 100 ml beaker. Electrode probe of pH meter was washed with distilled water and dried with tissue paper. This electrode probe was put in the sample and noted the pH value from the display.

4.19.4 Total Dissolved Solid (TDS)

The sample was filtrated by using glass fiber filter (pore size: 1.2 µm). This filtrate was washed thrice with 10 ml of reagent grade water. Total filtrate was transferred into beaker which is previously dried and weighted. Then, it was dried at 180 ^{o}C to evaporate water content. The final weight of beaker was taken after cooling it desiccator. The TDS was calculated as following calculation.

TDS (mg/l) = (A-B) x1000/S

Where, A = Final weight of the beaker containing residues (mg), B = Initial weight of empty beaker (mg), S = Sample taken (ml)

4.19.5 Conductivity

Conductivity of sample was measured by using themo-orion conductivity meter equipped with dip type conductivity cell. The instrument was calibrated by using conductivity CRM. Probe was rinsed with distilled water and put in the sample solution. The value of conductivity was observed from the display.

4.19.6 Dissolved Oxygen (DO)

For determination of dissolved oxygen, 2 ml of $MnSO_4$, 2 ml of alkali iodide azide solution and 2 ml of H_2SO_4 were added in sample in BOD bottle and mixed well. Then, 200 ml sample was taken and titrated with standard sodium thiosulphate solution to pale straw colour. At this time, few drops of starch were added and titrated it to disappearance of blue colour. The DO of sample was calculated by following calculations.

For titration of 200 ml sample, each 1 ml of 0.025 M $Na_2 S_2O_3$ = 1 mg/l of D.O.

4.19.7 Total Suspended Solid (TSS)

a) Selection of sample size

Appropriate volume of sample was selected such that it yield the non-filtrate dried residue between 2.5 – 200 mg.

b) Membrane Filtration Assembly

The GF/c filter paper (whatman) and petridish were dried at 103-105^oC in oven for one hour and cooled in desiccator then weighted to get initial weight.

c) The stirred sample was passed through membrane filter assembly with applying vaccum. The filter paper with residue was removed carefully and put in the petridish and the same was dried on 103-105^{o}Cin oven for one hour. Then cooled in desicator and weighted until constant reading is obtained. TSS was calculated using the following calculations.

$$TSS\ (mg/l) = (A-B) \times 1000/S$$

Where, A = weight of filter paper and crude residue (mg), B = weight of filter paper only (mg), S = Volume of sample (ml)

4.19.8 MPN

a) Presumptive Phase (*Total coliform*)

Lorryl Tryptose broth was used in presumptive portion of multiple tube test. Fermentation tubes were arranged in the rows of 5 tubes in a rack of test tube rack. Each tube in a set of 5 was inoculated with sample volumes (in increasing decimal dilution). Mixed the test portion in medium by gentle agitation. The tubes were incubated at 35 ^{o}C and after 24 hours swirled each tube examine for growth and production of gas in the inner vial. Production of gas constitutes a positive presumptive reaction. The absence of acidic growth/gas formation at the end of 48 hour of incubation constitutes a negative test.

b) Confirmed Phase *(Fecal coliform)*

Brilliant green lactrose bile broth was used in confirmation phase. All the primary tubes showing acidic growth/gas formation were subjected to the confirm phase. The primary tube showing gas was shaken gently to resuspend the growth. The growth of microorganism was inoculated in a fermentation tube containing brilliant green lactose bile broth. This step is repeated for all positive presumptive tubes. The fermentation tubes incubated for 48 hours at

35^{o}C. Formation of gas in any amount in the inverted vial of the brilliant green lactose bile broth fermentation tube at any time within 48 hours constitute a positive confirmed phase. MPN value was calculated from the number of positive brilliant green bile tubes using statistical table.

Chapter 5

Results

5.1 Isolation of mycoflora associated with diseased *Eichhornia crassipes*

A total of 52 isolates belonging to Ascomycota and Zygomycota were predominantly isolated during sampling of diseased water hyacinth plants from Harike wetland (Table 5.1). These isolates were tentatively identified on the basis of morphological and microscopic characteristics (Barnett and Hunter, 1998). The highest occurrence was *Alternaria* species (17.3%) followed by *Fusarium* sp. (11%), *Aspergillus* sp. (7.7%), *Penicillium* sp. (7.7%) and Zygomycetes (7.7%). Around 25% fungal isolates could not be definitively identified on the basis of morphological and microscopic characteristics (Fig 5.1). Only a single isolate of *Curvularia* sp., *Arthrinium* sp. *Phaeoacremonium* sp., *Didymella* sp., *Trichoderma* sp., *Acremonium* sp. and *Nigrospora* sp. were reported during the isolation.

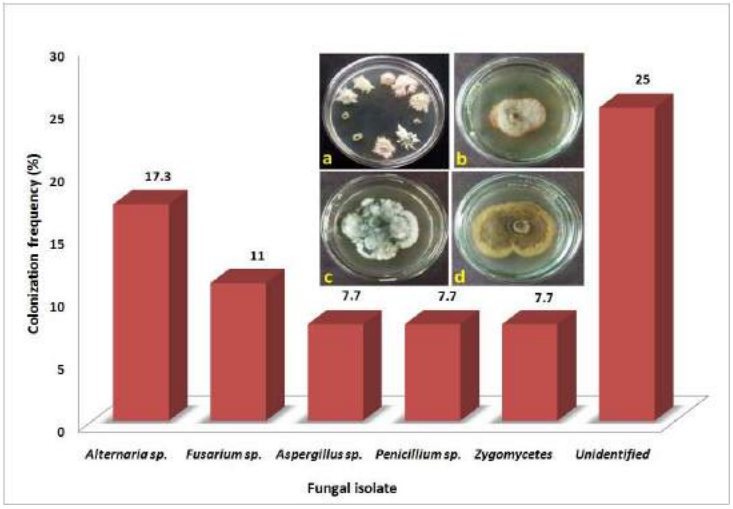

Fig 5.1 Fungal isolates from water hyacinth infesting Harike wetland, Punjab. In the inset (a) Isolates emerging from host tissue, (b) *Fusarium* sp. (c) *Penicillium* sp. (d) *Alternaria* sp.

5.2. Preliminary screening of pathogenic potential of isolates using detached leaf bioassay

In the preliminary screening assay of the isolates obtained from the infected samples of the water hyacinth infesting Harike Wetland, 46 % were non- pathogenic as they did not induce any visible symptoms of infection. Out of 52 isolates, only 9 isolates exhibited a leaf area damage (LAD) of ≥60 % and above after 168 hpi. #8BJSSL and #19BJSSL exhibited complete LAD after 168 hpi i.e. 7 days which was closely followed by #7BJSSL and #5BJSSL with 88.3 ± 2.9% and

86.7 ± 2.9% LAD respectively (Fig 5.2). 2- way repeated measures ANOVA confirmed that culture type as well as incubation time played a significant role in the pathogenicity of *Eichhornia crassipes* leaves ($F(357,728)=115.34$, $p<0.0001$) followed by Tukey's post-hoc analysis. Further, #19BJSSL exhibited the highest AUDPC (Area Under disease progress curve) value of 8640 ± 447.8 followed by #8BJSSL with 8104 ± 542.5, #7BJSSL, #10BJSSL and #5BJSSL (Table 5.1; Fig 5.3). Thus based on the LAD after 168hpi and AUDPC values, #19BJSSL was considered as the most pathogenic isolate followed by #8BJSSL. Hence, both #19BJSSL and #8BJSSL were selected to further assess their potential for their possible use as innundative biocontrol agent for *Eichhornia crassipes*.

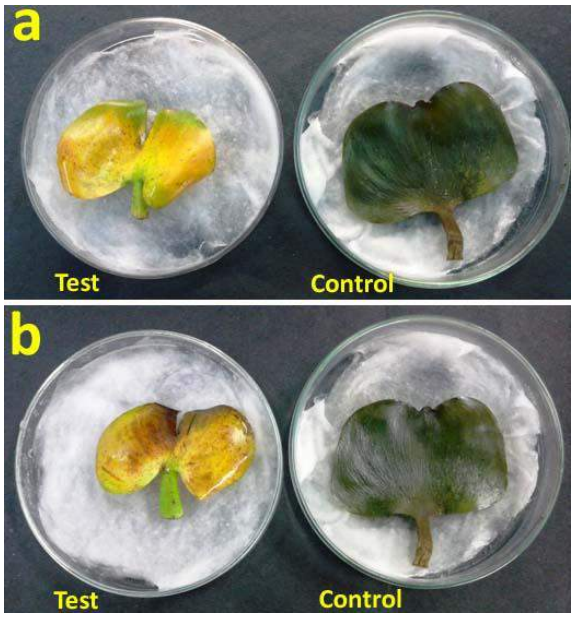

Fig 5.2 Leaf area damage caused by spores (1x 10^6 spores/ml) of (a) #19BJSSL and (b) #8BJSSL in *in vitro* detached leaf bioassay after 168 hpi.

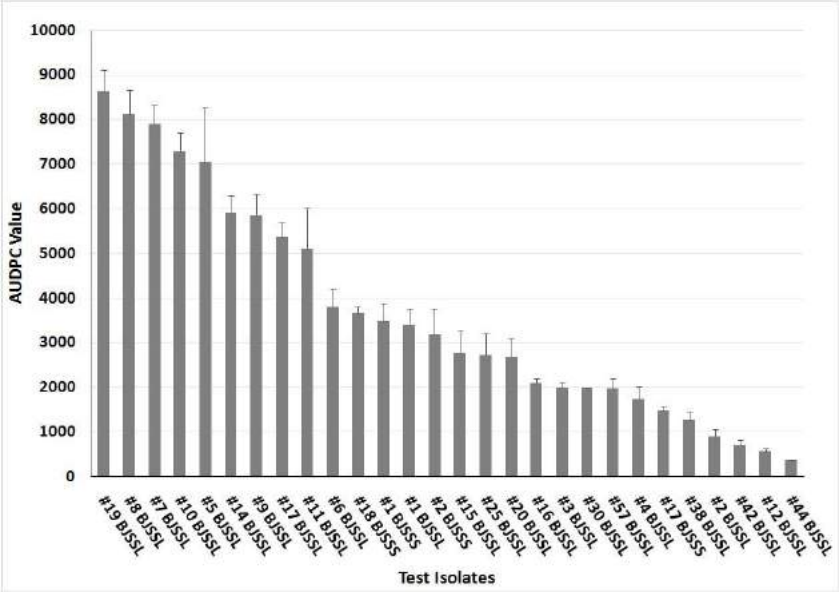

Fig 5.3 Area Under Diseases Progressive Curve (AUDPC) values of test isolates for selection of a potential mycoherbicidal candidates

Table 5.1 Fungal Isolates from *Eichhornia crassipes* infesting Harike wetland and their pathogenic potential by *in vitro* detached leaf bioassay using a standard inoculum ($1x10^6$ spores/ml)

Culture Code	Tentative Identification	Leaf Area Damaged (%) at different hours post inoculation (hpi)[ab]						AUDPC*
		48	72	96	120	144	168	
#1 BJSSL	*Alternaria sp.*	10± 0.0 ^{d}	18.3 ± 2.9^{c}	23.3 ± 2.9^{c}	30.0 ± 5.0^{b}	38.3± 2.9 ^{a}	43.3 ± 2.9^{a}	3400 ± 362.5
#2 BJSSL	*Aspergillus sp.*	0^{b}	0^{b}	8.3 ± 2.9^{a}	10.0 ± 0.0^{a}	11.7 ± 2.9^{a}	13.3 ± 2.9^{a}	880 ± 173.2
#3 BJSSL	*Fusarium sp.*	0^{c}	10.0 ± 0.0^{b}	15.0 ± 0.0^{a}	21.7 ± 2.9^{a}	23.3 ± 2.9^{a}	25.0 ± 0.0^{a}	1980 ± 138.6
#4 BJSSL	*Fusarium sp.*	0^{c}	5.0 ± 0.0^{c}	11.7 ± 2.9^{b}	20.0 ± 5.0^{a}	23.3 ± 2.9^{a}	23.3 ± 2.9^{a}	1720 ± 293.2
#5 BJSSL	*Alternaria sp.*	16.7± 2.9^{e}	31.7±10.4^{d}	50.0 ± 13.2^{c}	70.0± 13.2^{b}	81.7 ± 10.4^{a}	86.7 ± 2.9^{a}	7040 ± 1238.5
#6 BJSSL	*Zygomycetes*	0^{d}	10.0 ± 5.0^{c}	28.3 ± 5.8^{b}	46.7 ± 2.9^{a}	48.3 ± 2.9^{a}	50.0 ± 0.0^{a}	3800 ± 397.1
#7 BJSSL	*Botryosphaeria sp.*	20 ± 5.0^{e}	40.0 ± 5.0^{d}	61.0 ± 3.6^{c}	80.0 ± 0.0^{b}	83.3 ± 2.9^{ab}	88.3 ± 2.9^{a}	7884 ± 430.5
#8 BJSSL	***Phaeoacremonium sp.***	**15 ± 0.0^{f}**	**31.7 ± 7.6^{e}**	**60.0 ± 10.4^{d}**	**86.7 ± 2.9^{c}**	**94.3 ± 2.1^{b}**	**100.0 ± 0.0^{a}**	**8104 ± 542.5**
#9 BJSSL	*Fusarium sp.*	11.7 ± 2.9^{f}	28.3 ± 2.9^{e}	45.0 ± 5.0^{d}	55.0 ± 5.0^{c}	66.7 ± 2.9^{b}	73.3 ± 2.9^{a}	5840 ± 482.5
#10 BJSSL	*Fusarium sp.*	35.0 ± 5.0^{e}	45.0 ± 0.0^{d}	55.0 ± 5.0^{c}	63.3 ± 2.9^{b}	68.3 ± 2.9^{ab}	73.3 ± 2.9^{a}	7280 ± 413.2
#11 BJSSL	*Acremonium sp.*	10.0 ± 5.0^{f}	21.7 ± 10.4^{e}	36.7 ± 12.6^{d}	48.3 ± 5.8^{c}	61.7 ± 2.9^{b}	68.3 ± 2.9^{a}	5100 ± 914.3
#12 BJSSL	*Nigrospora sp.*	0^{b}	0^{b}	5.0 ± 0.0^{ab}	5.0 ± 0.0^{ab}	8.3 ± 2.9^{a}	10.0 ± 0.0^{a}	560 ± 69.3
#13 BJSSL	*Mycelia sterilia*	0	0	0	0	0	0	0
#14 BJSSL	*Alternaria sp.*	21.7 ± 2.9^{e}	31.7± 2.9^{d}	43.3 ± 2.9^{c}	51.7 ± 2.9^{b}	63.3 ± 2.9^{a}	68.3 ± 2.9^{a}	5900 ± 381.1
#15 BJSSL	*Penicillium sp.*	0^{f}	6.7 ± 2.9^{e}	16.7 ± 2.9^{d}	26.7 ± 7.6^{c}	40.0 ± 5.0^{b}	50.0 ± 5.0^{a}	2760 ± 501.9
#16 BJSSL	Unidentified	5 ± 0.0^{ef}	10 ± 0.0^{de}	15.0 ± 0.0^{cd}	20.0 ± 0.0^{bc}	23.3 ± 2.9^{ab}	26.7 ± 2.9^{a}	2080 ± 103.9
#17 BJSSL	*Penicillium sp.*	0^{f}	27.7 ± 2.5^{e}	41.7 ± 2.9^{d}	54.3 ± 4.0^{c}	63.0 ± 2.6^{b}	73.7 ± 3.2^{a}	5364 ± 328.7
#18 BJSSL	*Mycelia sterilia*	0	0	0	0	0	0	0
#19 BJSSL	***Botryosphaeria sp.***	**25.7± 4.0^{e}**	**40.0 ± 5.0^{d}**	**60.0 ± 5.0^{c}**	**88.3± 2.9^{b}**	**96.0 ± 1.7^{a}**	**100.0 ± 0.0^{a}**	**8640 ± 447.8**
#20 BJSSL	*Alternaria sp.*	0^{f}	10.0 ± 5.0^{e}	20.0 ± 5.0^{d}	26.7 ± 2.9^{c}	33.3 ± 2.9^{b}	43.3 ± 2.9^{a}	2680 ± 413.2
#21 BJSSL	*Alternaria sp.*	0	0	0	0	0	0	0
#23 BJSSL	*Aspergillus sp.*	0	0	0	0	0	0	0
#25 BJSSL	Un-identified	6.7 ± 5.8^{e}	13.3 ± 2.9^{d}	21.7 ± 5.8^{c}	25.0 ± 0.0^{bc}	28.3 ± 2.9^{b}	36.7 ± 5.8^{a}	2720 ± 485.0
#27 BJSSL	*Fusarium sp.*	0	0	0	0	0	0	0
#28 BJSSL	Un-identified	0	0	0	0	0	0	0
#29 BJSSL	*Fusarium sp.*	0	0	0	0	0	0	0
#30 BJSSL	Un-identified	5 ± 0.0^{de}	10.0 ± 0.0^{cd}	15.0 ± 0.0^{bc}	20.0 ± 0.0^{ab}	20.0 ± 0.0^{ab}	25.0 ± 0.0^{a}	1980 ± 0.0
#34 BJSSL	*Curvularia sp.*	0	0	0	0	0	0	0
#38 BJSSL	*Artheminium sp.*	0^{d}	0^{d}	0^{d}	13.3 ± 2.9^{c}	23.3 ± 2.9^{b}	31.7 ± 2.9^{a}	1260 ± 173.2
#42 BJSSL	*Alternaria sp.*	0^{d}	0^{d}	0^{d}	5.0 ± 0.0^{c}	13.3 ± 2.9^{b}	21.7 ± 2.9^{a}	700 ± 103.9
#44 BJSSL	*Penicillium sp.*	0^{b}	0^{b}	0^{b}	5.0 ± 0.0^{ab}	5.0 ± 0.0^{ab}	10.0 ± 0.0^{a}	360 ± 0.0
#47 BJSSL	Un-identified	0	0	0	0	0	0	0
#52 BJSSL	*Didymella sp*	0	0	0	0	0	0	0
#54 BJSSL	*Trichoderma sp.*	0	0	0	0	0	0	0
#55 BJSSL	Un-identified	0	0	0	0	0	0	0
#57 BJSSL	*Alternaria sp.*	0^{d}	0^{d}	15.0 ± 0.0^{c}	20.0 ± 5.0^{c}	28.3 ± 2.9^{b}	36.7 ± 2.9^{a}	1960 ± 223.9
#58 BJSSL	Un-identified	0	0	0	0	0	0	0
#63 BJSSL	Un-identified	0	0	0	0	0	0	0
#72 BJSSL	*Zygomycetes*	0	0	0	0	0	0	0
#83 BJSSL	*Zygomycetes*	0	0	0	0	0	0	0
#1 BJSSS	Un-identified	11.7 ± 2.9^{d}	16.7± 2.9^{d}	24.3 ± 4.0^{c}	31.7 ± 2.9^{b}	38.3 ± 2.9^{a}	43.3 ± 2.9^{a}	3464± 408.8
#2 BJSSS	Un-identified	10.0 ± 8.0^{e}	20.0 ± 5.0^{d}	25.0 ± 5.0^{cd}	28.3 ± 2.9^{bc}	31.7 ± 2.9^{ab}	35.0 ± 0.0^{a}	3180 ± 586.4
#4 BJSSS	Un-identified	0	0	0	0	0	0	0
#5 BJSSS	Un-identified	0	0	0	0	0	0	0
#6 BJSSS	Un-identified	0	0	0	0	0	0	0
#8 BJSSS	*Zygomycetes*	0	0	0	0	0	0	0
#14 BJSSS	*Aspergillus sp.*	0	0	0	0	0	0	0
#15 BJSSS	Un-identified	0	0	0	0	0	0	0
#16 BJSSS	*Penicillium sp.*	0	)	0	0	0	0	0
#17 BJSSS	*Alternaria sp.*	0^{d}	0^{d}	5.0 ± 0.0^{d}	15.0 ± 0.0^{c}	21.7 ± 2.9^{b}	38.3 ± 2.9^{a}	1469 ± 103.9
#18 BJSSS	*Alternaria sp.*	0^{f}	15.0 ±0.0^{e}	26.7 ± 2.9^{d}	35.0 ± 0.0^{c}	48.3 ± 2.9^{b}	55.0 ± 0.0^{a}	3660 ± 138.6
#19 BJSSS	*Aspergillus niger*	0	0	0	0	0	0	0

[ab] The values provided in the table are Mean ± SD of triplicate readings. Mean values represented by same alphabets in each row are not significantly different by Tukey's post hoc test at $p<0.05$.* - represents the Mean ± SD of Area under disease Progression Curve (AUDPC).

5.3 Assessment of pathogenicity of selected fungal isolates using whole plant hydroponic bioassay

In this bioassay on whole plants, the onset of diseases caused by the spore suspension ($1x10^6$ spores/ml) of #8BJSSL and #19BJSSL began at 48 hpi. Approximately, 20% higher mortality / damage were observed in plants sprayed with #19BJSSL spore suspension (~58%) as compared to #8BJSSL (~38%) after 120 hpi. After 192 hpi, #19 BJSSL exhibited a higher mortality/ damage of *Eichhornia crassipes* i.e. 84% while # 8BJSSL exhibited only 62.2% mortality / damage to the test plants. Two way ANOVA analysis also indicated a significant role of incubation time and culture type on disease development during the assay ($F(8,144) = 29.82$, $p<0.0001$). The AUDPC values also substantiated the above observations as #19BJSSL exhibited a higher AUDPC value of 7565.7± 628.2 compared to 5231.1 ± 780.5 of # 8BJSSL (Fig 5.4- Fig 5.5). Thus, based on the percentage damage/ mortality of the whole plants and AUDPC values, #19 BJSSL was selected as a mycoherbicidal candidate for *Eichhornia crassipes*.

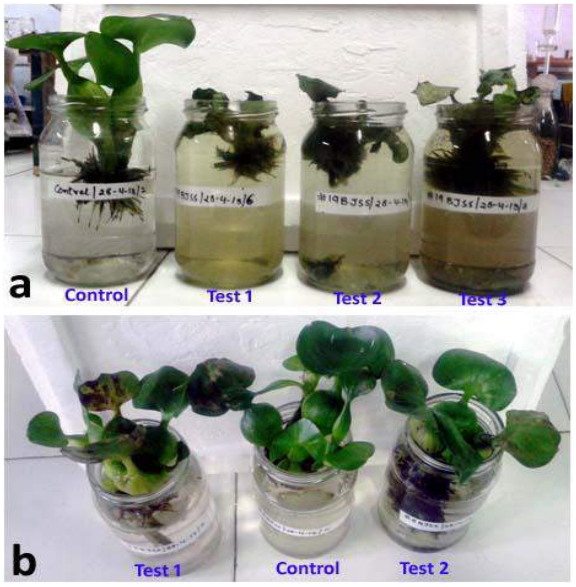

Fig 5.4 Plant mortality / damage caused by spores ($1x\ 10^6$ spores/ml) of (a) #19BJSSL and (b) #8BJSSL in whole plant hydroponics bioassay after 192 Hpi 192 hpi.

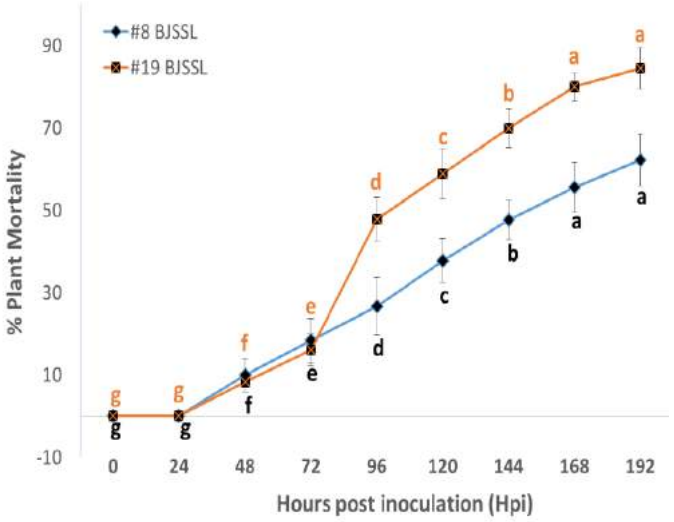

Fig 5.5 Plant mortality / damage caused by spores ($1x\ 10^6$ spores/ml) of #8BJSSL and #19BJSSL using whole plant hydroponics bioassay. Means with different alphabets are significantly different by Tukey's post hoc analysis at $p<0.05$

5.4 Identification of #8 BJSSL and #19 BJSSL

Both morphotaxonomic and molecular taxonomic methods were used for the identification of #8BJSSL and #19 BJSSL

5.4.1 Morphotaxonomic studies of #8 BJSSL

Colonies of # 8 BJSSL over PDA were moderately growing reaching a diameter of12 ± 1.2 mm in 14 days at 28^{o}C. Colonies were white in color, reverse vinaceous, flat, cottony at the centre and powdery with felty margins (Fig 5.6a). Hyphae was found singly or in bundle of 6-7 (Fig 5.6b). Conidiophores borne on the aerial mycelium were usually single and unbranched having an inflated bottom and tapering towards the edge (Fig 5.6c-5.6g). Certain hyphal coils were also observed (Fig 5.6h). The dimensions of the conidia were (3.78) 6.22 (10.15) × (1.52) 2.03 (3.23) µm and were allantiod to obovate in shape (Fig 5.6l).

Over MEA medium, colonies reach a diameter of 39.67 ± 1.15 mm after 14 days at 28 °C. Colonies were olive grey to vinaceous colored, flat and felty margined (Fig 5.6j). Mycelium consisting of branched septate hyphae measuring (0.99) 1.64 ± 0.37 (2.64) µm that occur singly or as bundle of 6-8(Fig 5.6k). The dimensions of conidiophore was [(3.35) 7.43 ± 2.48 (12.08)] × [(1) 1.54 ± 0.42 (2.89)] µm which were born on the aerial mycelium and were usually single, unbranched, cylindrical, slightly inflated with narrow collarate (Fig 5.6l-5.6o). Adelophialides were terminal, monophialadic, smooth and collarate (Fig 5.6m-5.6n). Several hyphal coils were also observed (Fig 5.6g). Conidia [(2.48) 3.72 ± 0.69 (5.09)] × [(0.93) 1.14 ± 0.73 (1.87)] were single celled, allantoid to oblong-ellipsoidal in shape (Fig 5.6q).

Colonies on CMA medium were moderately growing (41.33 ± 2.08 mm), vinaceous colored, flat, with a felty margin (Fig 5.6r). Mycelium consisting of long cylindrical and septate

hyphae measuring (1.2) 2.22 ± 0.43 (2.96) µm that occurred singly or as synmeta of 5-6 (Fig 5.6s).

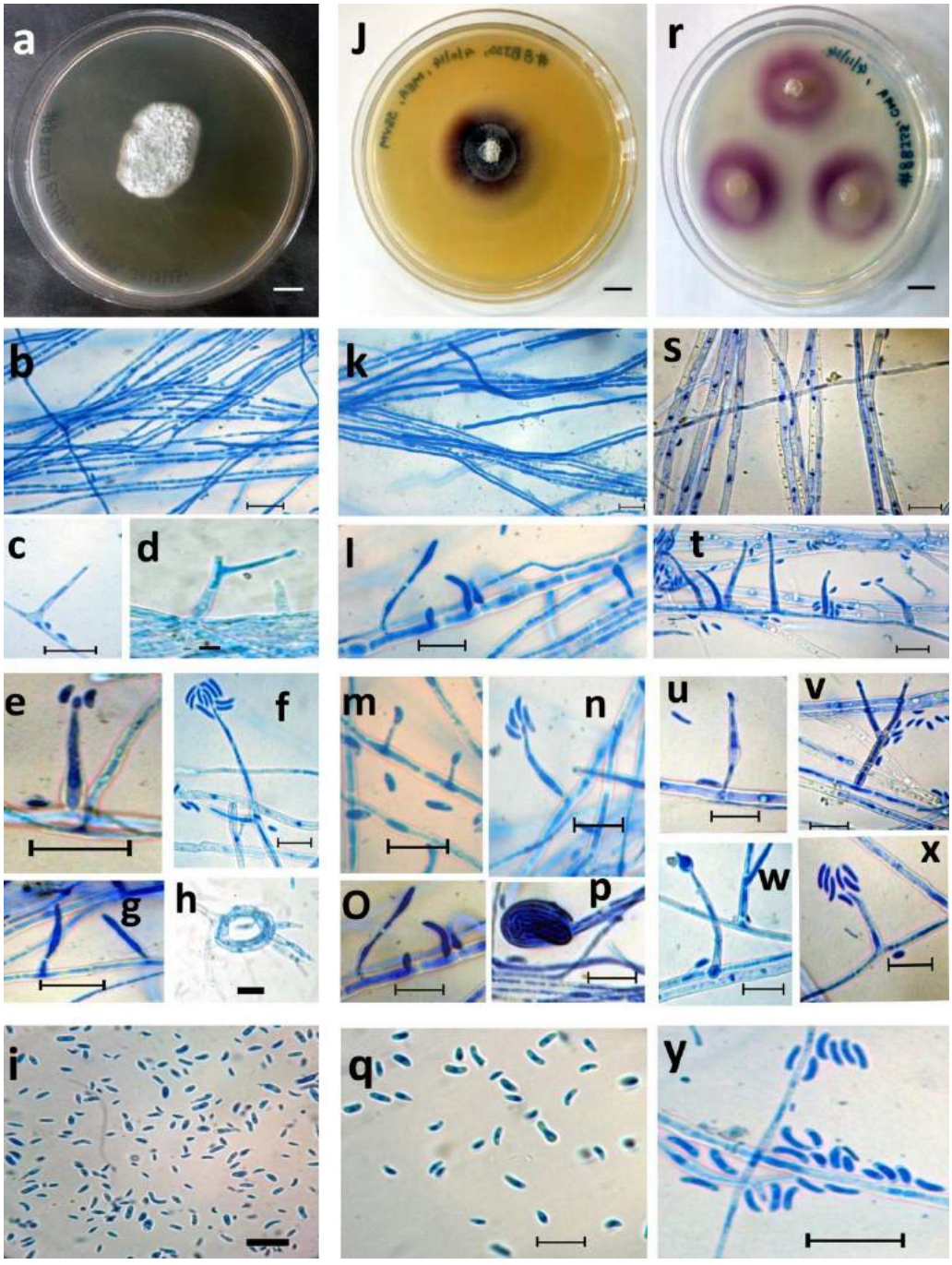

Fig. 5.6. Morphological and microscopic structures of *Phaeoacremonium italicum* (#8 BJSSL). a, j, r: Fourteen-day old colonies at 28°C on potato dextrose agar (PDA) (a), malt extract agar (MEA) (j), and corn meal agar (CMA) (r) (scale bar=10mm). b-i, Structures on the surface of PDA: b, Hyphae over PDA medium; c, g, Unbranched conidiophore; d, Branched conidiophore; e, f, Adelophialides with conidia; h, Hyphal coils; i, Conidia; k-q, Microscopic characters produced over MEA; k, Hyphae; l, o, unbranched conidiophores; m, n, Adelophialides with conidia; p, Hyphal coil; q, Conidia; s-y, Features produced over CMA; s, Hyphae; t, u, Unbranched conidia; v, Branched conidiophore; w-x, Adelophialides with conidia; y, Conidia (scale bar = 10 µm).

The conidiophore measured [(6.96) 17.43 ± 4.67 (27.83)] × [(1.03) 1.75 ± 0.41 (2.17)] µm which arise from the aerial mycelium or the submerged hyphae, usually single, long, branched, slightly inflated at the bottom and collarate at the edge (Fig 5.6t-5.6v). Adelophialides were terminal and collarate (Fig 5.6w-5.6x) conidia. The conidia measured [(2.29) 3.8 ± 0.91 (6.94)] × [(0.76) 1.11 ± 0.22 (1.52)] µm and were single celled and allantoid in shape (Fig 5.6y). Thus, based on the above features, the isolate was predicted to be *Pheoacremonium italicum*. However, for further confirmation we carried out molecular phylogenetic analysis.

5.4.2 Morphotaxonomic identification of #19BJSSL

Over PLA, the fungus was moderately growing forming a downy green to brown colour colonies. No pigment or odour was produced by the fungus. Asexual reproductive bodies were formed in 3 weeks of incubation. Hyphae (5.68 ± 0.76 µm) (Fig 5.7a) broad, thick, brown coloured over which the conidia were formed in clusters. Conidiophores arise from the mycelium. Conidia (14.2±3.9 × 12.4±4.41 µm) oval to polygonal in shape, blue when immature later turning to brown on maturation (Fig 5.7b,c).

Over CMA, the fungus was fast growing; initially white later turning to slight cream coloured, forming aerial mycelium (Fig 5.7d). After 2-3 weeks, asexual reproductive structures were formed. Hyphae (5.17±0.82 µm) broad, brown in colour, septate, thick and branched. Conidia (14.07 ± 3.06 × 11.16 ± 1.94 µm) were oval to round in shaped, double walled, single celled, found in chain or clusters, immature conidia was blue coloured whereas the mature conidia was brown in colour, double walled, multinucleate (Fig 5.7e). The above features suggest that the fungus belongs to *Botryosphaeria* species.

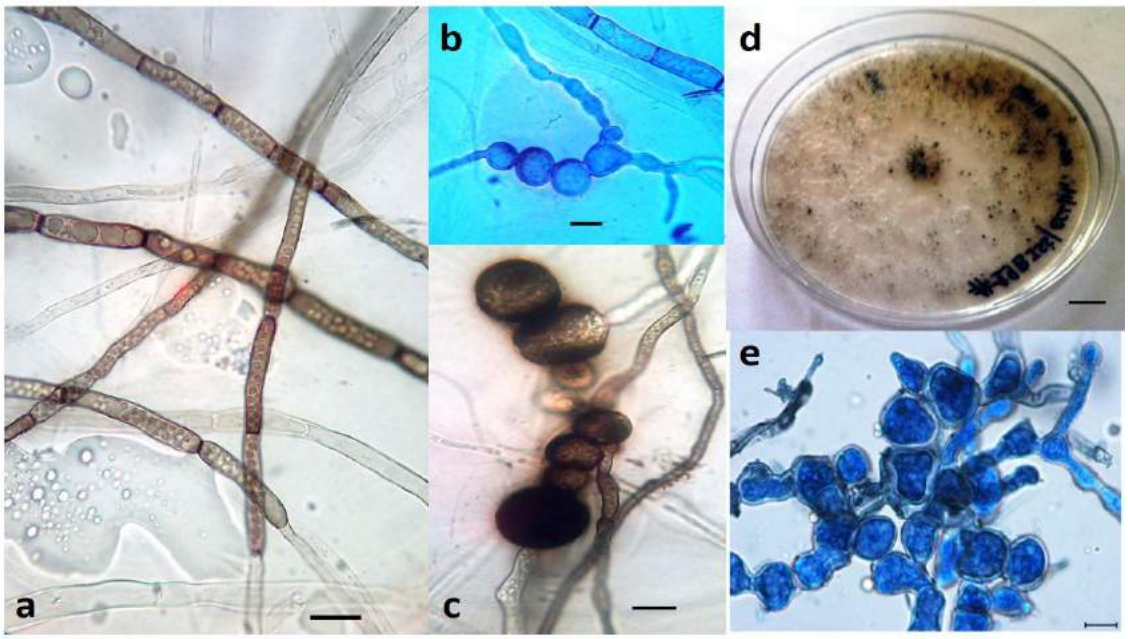

Fig. 5.7 (a) Appearance of asexual reproductive bodies after three weeks of incubation similar to *Botryosphaeria* species (b-c) development of conidia from the mycelium in *Botryosphaeria* species which are oval to polygonal in shape and turn brown on getting matured (d) Appearance #19BJSSL on CMA medium (e) Asexual reproductive structures of #19BJSSL on CMA medium

5.4.3 Molecular taxonomy of #8 BJSSL

To further confirm the isolate #8BJSSL to be *Phaeoacremonium* species, we carried out genomic DNA isolation of which the β-tubulin gene region was amplified using specific primers. The sequence data so obtained was submitted in GenBank with accession No. **KP696755.** BLAST

Table 5.2 BLAST search summary of homology analysis of β-tubulin gene of #8BJSS

Name of species	**Accession No.**	**e-Value**	**Identity (%)**
Phaeoacremonium italicum strain Pm19	KJ534074	0.0	99
Phaeoacremonium alvesii strain IRNHM-KPH63	KU737518	0.0	99
Phaeoacremonium alvesii isolate A21	KF790535	0.0	99
Phaeoacremonium alvesii strain STEU 6988	JQ038914	0.0	99
Phaeoacremonium alvesii strain 125ss2Pal	EU883990	0.0	99
Phaeoacremonium alvesii strain CBS 113590	AY579304	0.0	99
Phaeoacremonium italicum strain MFLUCC 13-0336	KU194225	0.0	99
Phaeoacremonium alvesii isolate IRNHM-ALV103	KP322595	0.0	98
Phaeoacremonium alvesii isolate IRNHM-ALV101	KM111549	0.0	98
Phaeoacremonium alvesii strain IRNHM-KPH61	KU737517	0.0	98
Phaeoacremonium rubrigenum strain IRNHM-KPH62-2	KU737514	0.0	98
Phaeoacremonium rubrigenum strain IRNHM-KPH62	KU737513	0.0	98
Phaeoacremonium rubrigenum strain IRNHM-KPH117	KR867714	0.0	98

analysis of the β-tubulin gene region gave similarity to *Phaeoacremonium italicum* as the first hit (Table 5.2).

The Maximum Parsimony tree generated based on β-tubulin region resolved into 4 different clades of *Pheoacremonium* species. Clade I comprised of eight strains of *P. italicum* and #8BJSSL. Clade II grouped three strains of *P. alvesii*, Clade III grouped *P. ruberigenum* species and Clade IV clustered three strains of *P. scolyti*. The tree was rooted with *Lasiodiplodia gonubiensis* (Fig 5.8). Based on the phylogenetic tree of β-tubulin the correct speciation of the selected isolated #8BJSSL was confirmed as *P. italicum* (Singh et al., 2016a).

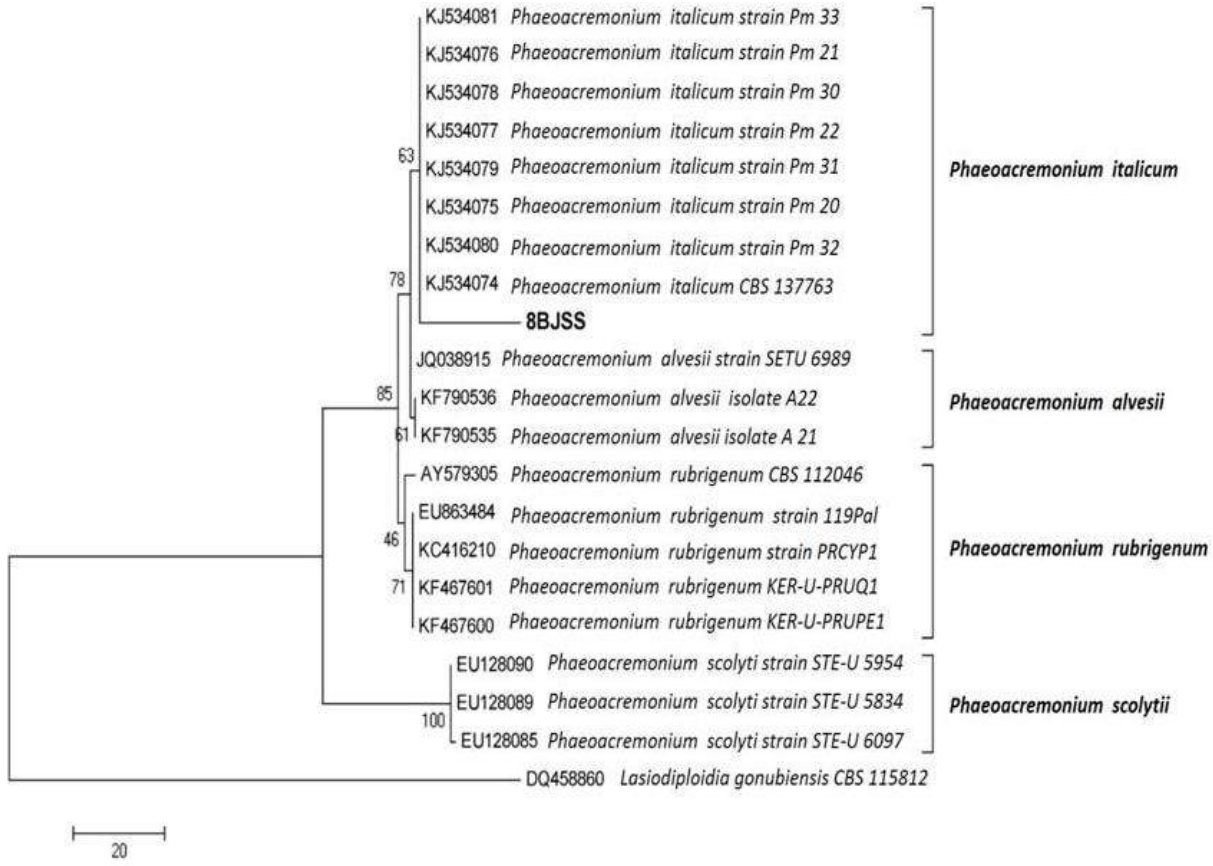

Fig 5.8 Maximum parsimony tree of #8BJSSL using Close Neighbor Interchange logarithm with search level 3. The confidence interval of internal nodes was assessed by employing bootstrap analysis (1,000 replicates)

5.4.4 Molecular taxonomy of #19BJSSL

In case of #19BJSSL, the morphological studies broadly indicated it to be a member of *Botryosphaeriaceae* family, hence amplification of ITS region (ITS1-5.8s-ITS2) of its genomic DNA was imperative to identify the genus as well as the species. The ITS amplicon sequence so obtained was submitted in GenBank with accession no. **HG738871.** BLAST analysis of the ITS region of #19BJSSL exhibited 98% sequence similarity with *Sphaeropsis* sp., *Diplodia* sp. and *Botryosphaeria* sp. (Table 5.3).

Table 5.3– List of isolates and accession number of their ITS sequences from BLAST used in the phylogenetic analysis of *Botryosphaeria* species #19BJSS (GenBank Accession HG 738871)

S.No.	Name	Isolate number	Accession number*	Host	Place
01	*Diplodia mutila*	A24-AA-2	KP026317	*Amelanchir annifolia*	Canada
02	*Diplodia mutila*	1476	KM580526	*Vitis vinifera*	Chile
03	*Diplodia mutila*	4D33	KF778779	*Juglans regia*	USA
04	*Diplodia mutila*	KER-U-DMAPR1	KF535901	*Prunus armenica*	Iran
05	*Diplodia mutila*	BEI36	KT954166	*Vitis vinifera*	France
06	*Diplodia seriata*	DA221	KC960900	Pear	China
07	*Diplodia seriata*	KER-U-DSGG2	KC535906	*Prunus domestica*	Iran
08	*Diplodia scrobiculata*	CBS109944	DQ458899	*Pinus greggi*	Mexico
09	*Diplodia mutila*	BSDP1	KC789072	Date Palm	Iran
11	*S. sapinea*	CBS 393.84	DQ458895	*Pinus nigra*	Netherlands
12	*S. sapinea*	WA0000019144	JX981458	*Lycopodium sp.*	Poland
13	*B. rhodina*	CSS	GU226856	*Vitis vinifera*	China
14	*B. rhodina*	3-5	GU323603	-	China
15	*B.eucalyptorum*	MUCC387	DQ131571	*Eucalyptus sp.*	Australia
16	*B.eucalypticola*	MUCC388	DQ131571	*Eucalyptus globus*	Australia
17	*A.niger*	BGD22	HM107005	Rhizophora stylosa	China

*NCBI GenBank Accession.

The Neighbor-Joining tree obtained by taking 17 sequences comprising of *Diplodia mutila, Diplodia seriata*, *S. sapinea, B. rhodina* and *B. eucalyptorum* was divided into three clades viz. Clade I, II and III. Clade I was further sectioned into two sub-clades- Sub-clade I which

clustered with #19BJSSL-ITS sequence along with six strains of *Diplodia mutila* with high a bootstrap support. Sub-clade II grouped strains of *D. seriata* and *S. sapinea*. However, *B. rhodina* and *B. eucalyptorum* formed their respective clades namely clade II and III. *Aspergillus niger* was chosen as out-group to root the tree (Fig 5.9). Thus, #19BJSSL was identified as *Diplodia mutila*. The occurrence of *Diplodia mutila* as a pathogen of water hyacinth is the very first report (Singh et al., 2016a).

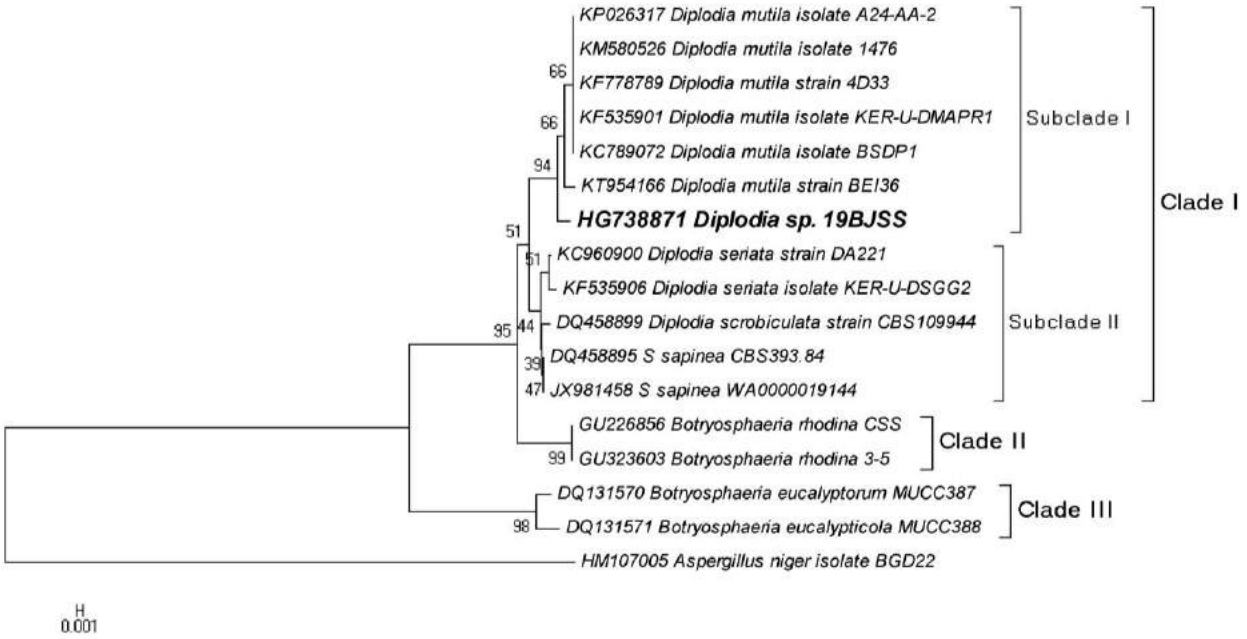

Fig. 5.9 Phylogenetic placement of #19BJSSL as *Diplodia mutila*

5.5 Phytotoxic potential of the selected isolates by *in vitro* leaf puncher assay

The initial symptoms of phytotoxicity of the culture filtrate were observed at 48 hpt in both #8BJSSL and #19BJSSL. However the damage of the leaf was much higher in case of #19BJSSL (>60%) as compared to #8BJSSL. Further, after 120 hpt, 100% leaf area damage was observed in #19BJSSL while 73% was observed in the case of #8BJSSL (Fig 5.10-Fig 5.11). Hence, #19BJSSL exhibited higher phytotoxic potential. This was further confirmed by the whole plant hydroponics bioassay.

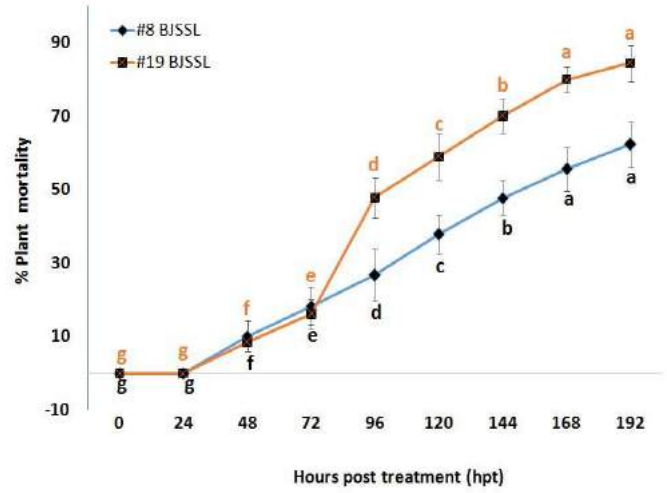

Fig 5.10 Phytotoxicity of culture filtrates of #8BJSSL and #19BJSSL by *in vitro* leaf puncher assay. All values are Mean ± SD of triplicate readings. Means with different alphabets are significantly different by Tukey's post hoc analysis at $p<0.05$

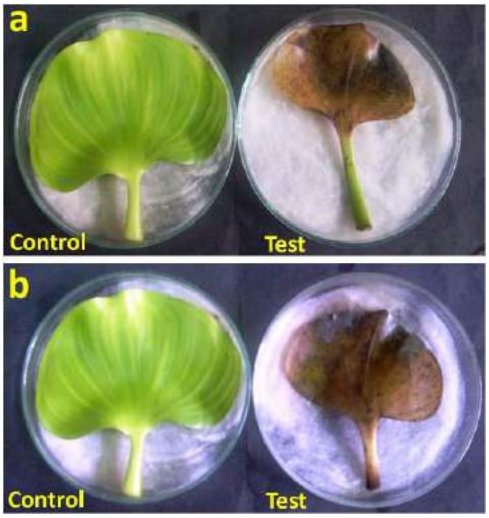

Fig 5.11 Phytotoxicity of culture filtrates of (a) #8BJSSL and (b) #19BJSSL by *in vitro* leaf puncher assay after 192 hpt.

5.6 Phytotoxic activity of solvent residues of #19BJSSL culture filtrate

As evident from *in vitro* leaf puncher assay, culture filtrate of #19BJSSL exhibited higher phytotoxicity, hence solvent residues obtained from the culture filtrate was individually evaluated for their phytotoxic potential by *in vitro* leaf puncture assay. The best phytotoxicity was expressed by hexane fraction, followed by chloroform. Two way ANOVA ($F(21, 56) = 25.53$, $p< 0.0001$ also indicated that the interaction of the solvent residue and time plays a significant role in phytotoxicity (Fig 5.12-Fig 5.13). Hence

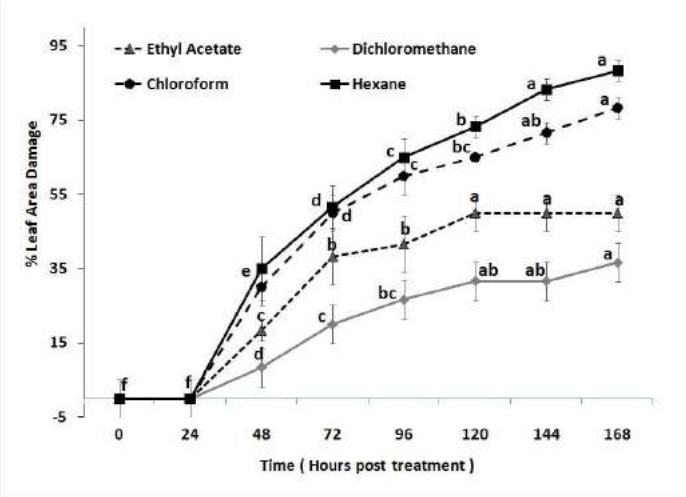

Fig 5.12 *In vitro* leaf puncher assay of different solvent residues of culture filtrate of #19BJSSL for assessment of phytotoxic potential. All values are Mean ± SD of triplicate values. Means with different alphabets are significantly different by Tukey's post hoc analysis at $p<0.05$.

hexane and chloroform residues were further taken up for phytotoxic potential by whole plant bioassay.

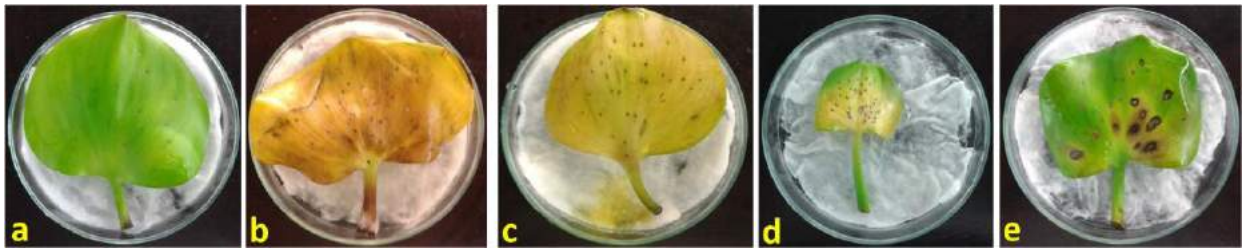

Fig 5.13 *In vitro* leaf puncher assay of different solvent residues of culture filtrate of #19BJSSL for assessment of phytotoxic potential after 168 hpt. (a) control, (b) hexane residue, (c) chloroform residue, (d) ethyl acetate residue and (e) dichloromethane residue

5.7 Whole plant hydroponic assay of the culture filtrate and potential solvent residues of #19BJSSL

In the hydroponic whole plant assay, culture filtrate, chloroform and hexane residues exhibited phytotoxic symptoms after 48 hpt. However, the highest mortality at 48 hpt was observed in hexane residue. Culture filtrate of #19BJSSL exhibited highest damage/ mortality in the whole plant assay causing 97% death /damage followed by hexane fraction with 85% death/damage to the plant at 168 hpt. The least phytotoxic damage /mortality was observed in the plants sprayed with chloroform residue. A significant interaction between the solvent residues/culture filtrate with time played an important role in the mortality of the whole plants as per two way ANOVA (F (16,120) =16.17,

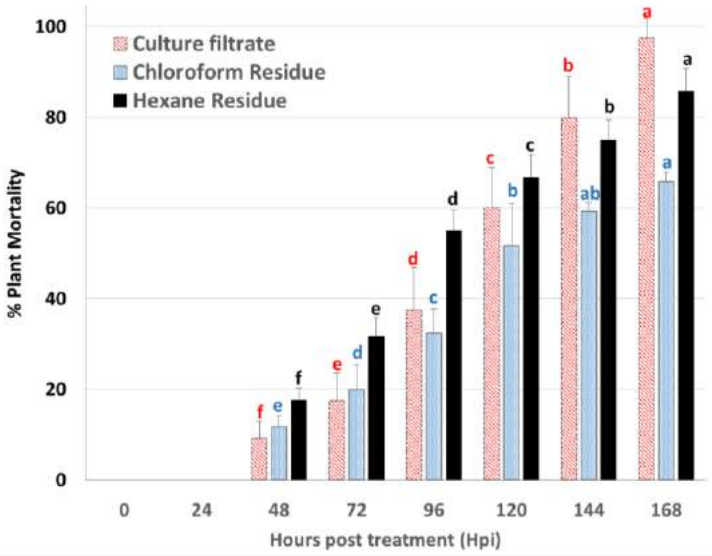

Fig 5.14 Comparative phytotoxicity evaluation of culture filtrate, hexane residue and chloroform residue by whole plant hydroponics assay. All values are Mean ± SD of triplicate values.Means with different alphabets are significantly different

p<0.0001, followed by Tukey's post hoc analysis (Fig 5.14-Fig 5.15). Hence, hexane residue of culture filtrate of #19BJSSL was selected for further studies.

Fig 5.15 Comparative phytotoxicity evaluation of (b) culture filtrate, (c) hexane residue and (d) chloroform residue by whole plant hydroponics assay after 168 hpt. (a) No damage was observed in control plants.

5.8 Partial characterization of the bioactive residue

Phytochemical characterization of the culture filtrate and lead extracts was carried out to assess the nature of the compound in the spent broth and bioactive fraction of chloroform and hexane. The hexane fraction exhibited the presence of alkaloids and terpenoids which are possibly responsible for higher phytotoxic activity when compared to chloroform fraction. The chloroform fraction also indicated the presence of

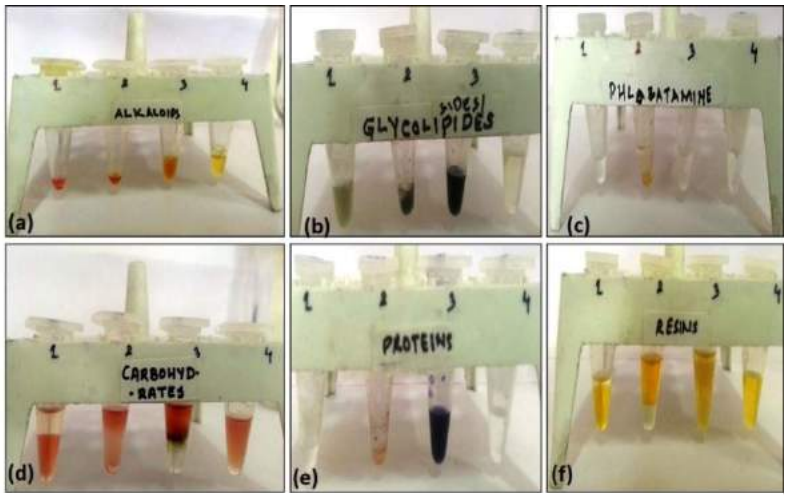

Fig. 5.16 Phytochemical analysis of culture filtrate, hexane, chloroform and un-inoculated broth (control) of #19 BJSSL. (a) alkaloids (b) glycolipids (c) phlobatamine (d) carbohydrates(e) proteins (f)rResins (terpenes)

terpenoids and alkaloids however, the presence of the compounds was in a lower concentration as compared to the hexane fraction (Table 5.4; Fig 5.16)

Table 5.4 Phytochemical characterization of the culture filtrate, lead solvent fractions of #19BJSSL and its comparison with control

Class of Compound	Culture filtrate	Chloroform	Hexane	Control (Un-inoculated broth)
Alkaloid	(+++)	(+)	(+++)	(-)
Steroids	(-)	(-)	(-)	(-)
Terpenoids	(++)	(+)	(++)	(-)
Saponins	(++)	(-)	(-)	(-)
Flavonoids	(-)	(-)	(-)	(-)
Resins	(-)	(-)	(-)	(-)
Tannins	(-)	(-)	(-)	(-)
Anthroquinone	(-)	(-)	(-)	(-)
Phlobatamine	(-)	(-)	(-)	(-)
Carbohydrates	(+++)	(-)	(-)	(+++)
Proteins	(+++)	(-)	(-)	(-)
Esters	(-)	(-)	(-)	(-)

5.9 Food poison assay

For development of a formulation the effect of phytotoxic hexane fraction was tested on the growth of #19BJSSL. Of the different concentrations tested, hexane fraction at a concentration of 4 µg/ml and 5 µg/ml drastically reduced the growth rate and colony size of the fungus. The

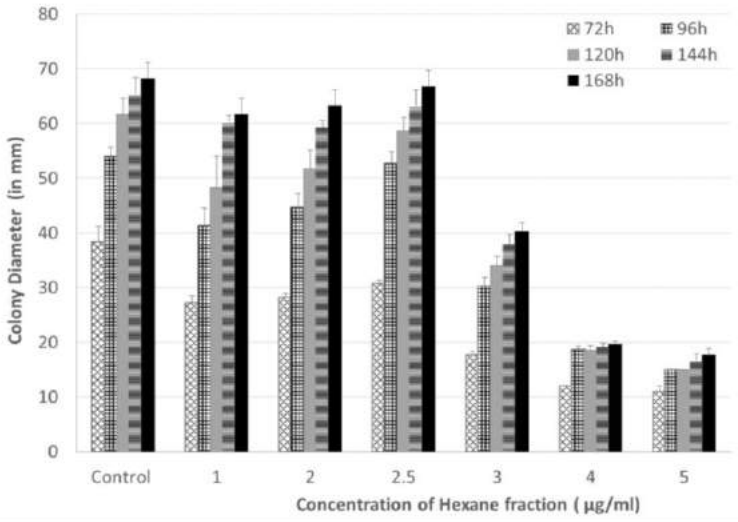

Fig 5.17 Food poison assay to determine compatible concentration of hexane fraction of#19BJSSL for development of formulation with the spores. All values are Mean ± SD of triplicate values. Means with different alphabets are significantly different by Tukey's post hoc analysis at $p<0.05$.

colony size of #19BJSSL was reduced by 67.7% and 74.1% at concentration at 4 µg/ml and 5 µg/ml respectively. This was further confirmed by one way repeated measures ANOVA (F (6, 35) =3.247, p=0.03. Further Dunnet's multiple comparison test also confirmed this observation and indicated a non-significant change in the colony diameter when hexane fraction was incorporated into the growth medium between concentrations of 1 µg/ml to 2.5 µg/ml. However, 3 µg/ml concentration of the hexane fraction affected the growth of #19BJSSL and reduced the colony size by 41.4% at 168 h (Table 5.5; Fig 5.17-Fig 5.18). However, as the maximum tolerable limit was 2.5 µg/ml which does not induce any significant reduction in the colony size/ growth, it was taken up for the preparation of bioherbicidal population by mixing with spores of #19BJSSL.

Table 5.5 Inhibition of the colony size (diameter in mm) when compared to control for finding suitable concentration for formulation development

Concentration (µg/ml)	**Inhibition in colony size (%)**				
	72 h	**96 h**	**120 h**	**144 h**	**168h**
1	28.7	24	21.7	7.657	9.7
2	26.1	17	16.2	9.188	7.3
2.5	**19.8**	**2.4**	**4.86**	**3.063**	**2.3**
3	53.8	44	44.9	41.81	41
4	68.7	65	70	70.6	71
5	71.3	26	75.7	74.43	74

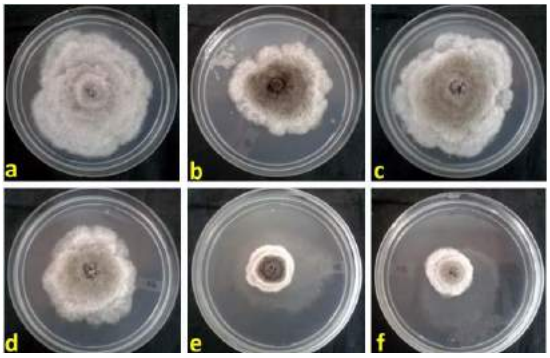

Fig 5.18 Effect of hexane fraction on growth of #19BJSSL for development of a suitable formulation. (a) Control, (b) 2 µg/ml (c) 2.5 µg/ml (d) 3 µg/ml, (d) 4 µg/ml, (e) 5 µg/ml

5.10 Evaluation of bioherbicidal formulation by whole plant hydroponics assay

Efficacy of bioherbicidal formulation and only spore suspension of #19BJSSL were evaluated for their mycoherbicidal potential to control *Eichhornia crassipes* by whole plant hydroponics

assay. It was observed that the pathogenic process immediately started in the case of bioherbicidal formulation and by 24h, 13% infection/damage occurred in the plants. This process was delayed when only spores of #19BJSSL were used. The mortality/damage of the whole plants were three folds higher in case of bioherbicidal formulation as compared to spore suspension only at 72hpt. After 192 hpt (8d) the spore suspension only induced ~84.4% mortality while the bioherbicidal formulation exhibited 100% mortality. Infact 100% mortality of the plants was achieved much earlier at 168 hpt. Further, two way ANOVA analysis F (7, 70) = 46.34, p< 0.0001 also revealed significant effect of time as well as treatment (formulation/ spores) on the disease development in test plants. The AUDPC value of the bioherbicidal formulation was much higher 11172 ± 447.5 as compared to spores i.e. 7547.2± 603.8 (Fig 5.19-Fig 20).

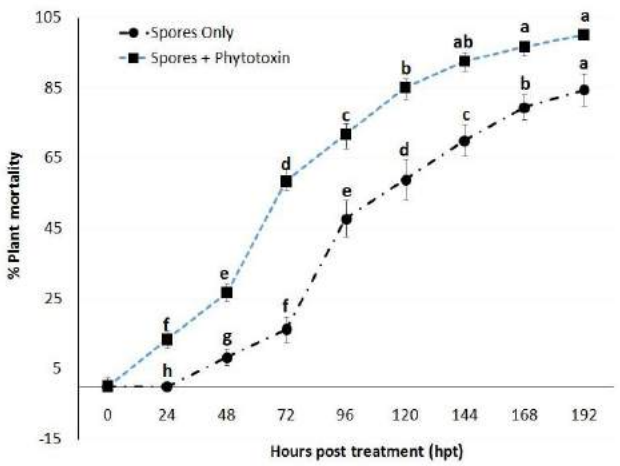

Fig 5.19 Plant mortality/ damage caused by spores and spore + phytotoxin formulation of #19 BJSSL in ehole plant bioassay. All values are Mean ± SD of triplicate values. Means with different alphabets are significantly different by Tukey's post hoc analysis at p<0.05

Fig 5.20 Comparison of mortality of *Eichhornia crassipes* at different treatments after 144 hpt. (a) control (b) spores of #19BJSSL ($1x10^6$ spores/ml) (c) hexane fraction of culture filtrate #19BJSSL (d) bioherbicidal formulation (Spores ($1x10^6$ spores/ml + 2.5µg/ml hexane fraction residue)

5.11 Host specificity of # 19BJSSL

As #19BJSSL exhibited potential for use as a mycoherbicide in a formulation with its own phytotoxin for possible application to control water hyacinth infesting Harike wetland, it was imperative to test its host range on non-target plants i.e. crops, since the water from Harike is extensively used for irrigation of agricultural fields in parts of Punjab and Rajasthan. Spore suspension (1x 10^6 spores/ml) did not induce any pathogenesis in terms of lesions, necrosis, chlorosis or wilting in Wheat (*Triticum aestivum*), *Brassica campestris*, *Solanum tuberosum*, *Brassica oleracea*, and *Solanum melongena* in the pot assays when compared to their respective control (Fig 5.21). This indicated that #19BJSSL (*Diplodia mutila*) is a host specific isolate which infects *Eichhornia crassipes*.

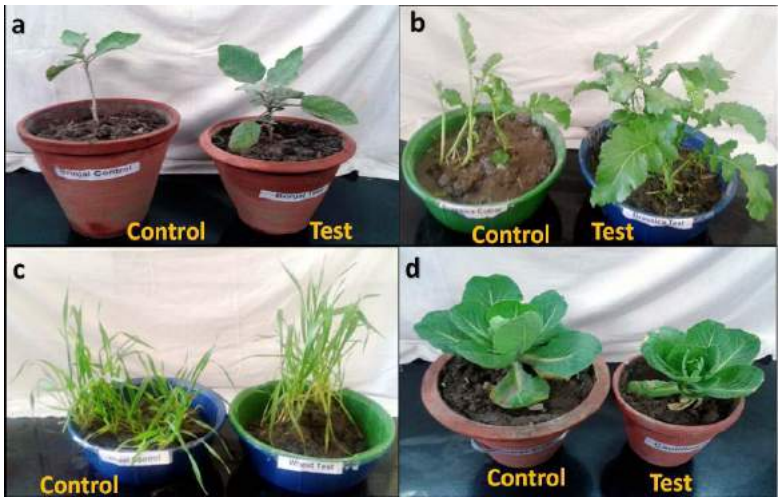

Fig 5.21 Testing of spores of #19BJSSL on non-target plants (a) *Solanum melongena* (Eggplant) (b) *Brassica campestris* (Mustard) (c) *Triticum aestivum* (Wheat) (d) *Brassica oleracea* (Cauliflower). No pathogenesis or phytotoxic damage was observed till one month

5.12 Influence on water quality subsequent to mycoherbicidal treatment

The electrical conductivity of the water treated with the bioherbicidal formulation was significantly enhanced when compared to control. Similarly, TDS also doubled where the plants were killed using bioherbicide. However, a non-significant change in TSS was recorded (Fig 5.22a,b). Bioherbicidal control of water did not change pH but made the water body anoxygenic. The evaporation rate reduced by ~7% after *Eichhornia* was killed due to bioherbicidal treatment. The COD of the water in bioherbicidal treated tub drastically enhanced

and ~2.5 times increased COD was observed as compared to control. Similarly, there was approximately five times increase in the BOD values in the treated sample compared to the control (Fig 5.22c). The total coliforms in the treated samples increased by three times approximately when compared with the untreated (control). A similar rise was also seen in the number of fecal coliforms in the treated samples as compared to control.

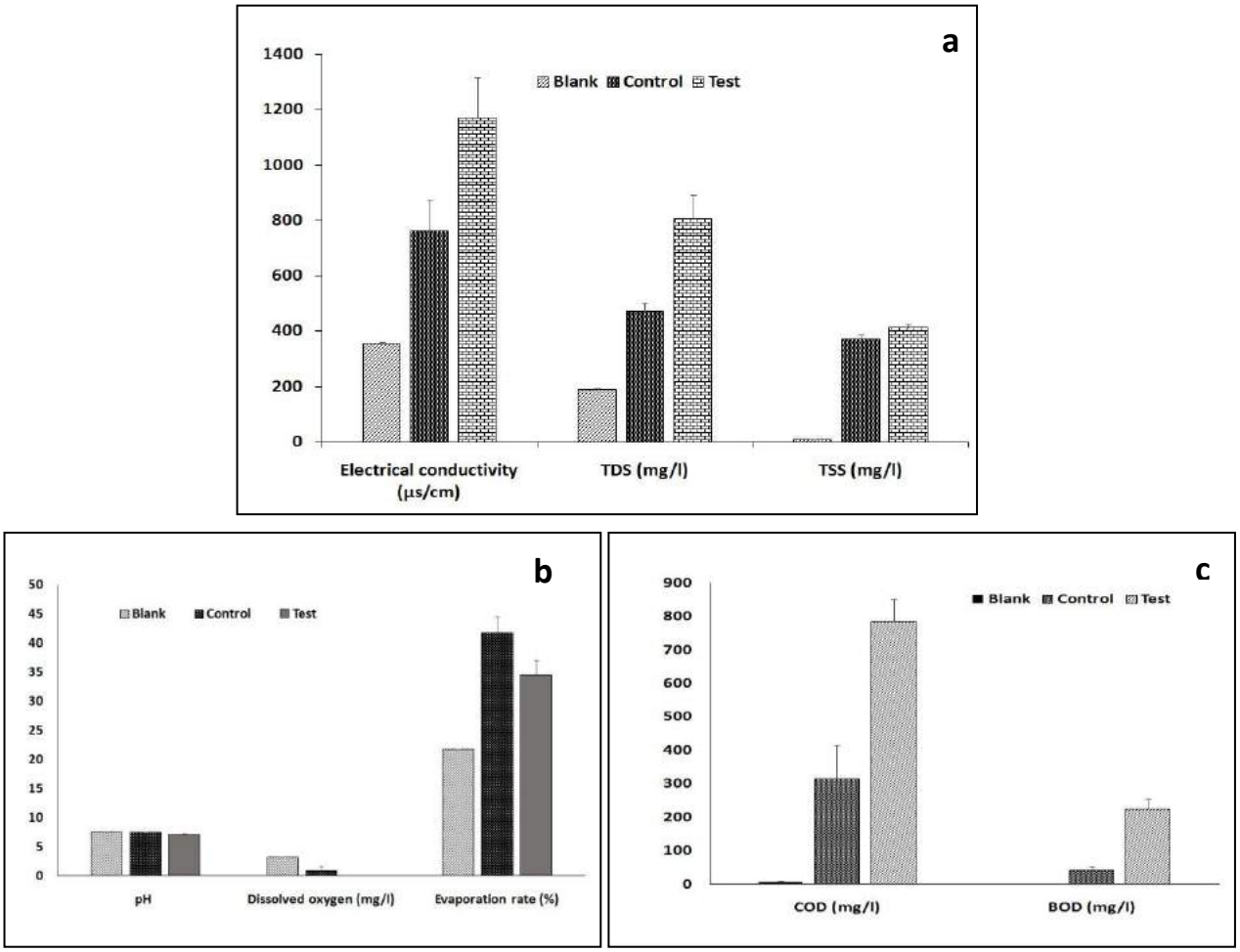

Fig 5.22 Changes in physic-chemical parameters of water after mycoherbicidal treatment. (a) Electrical conductivity, TDS and TSS, (b) pH, DO and evaporation rate, (c) BOD and COD

Chapter 6

Discussion

6. DISCUSSION

Invasive plant species are having severe ecological as well as economic impacts. They generally are defined as “alien species whose establishment and spread threatens ecosystems, habitats or species with economic and environmental harm” (McNeely et al., 2001). Globalization is largely responsible for their movement across continents. Today, *Eichhornia crassipes*, a free floating water plant native of South America has been recognized as a globally invasive species predominantly found in tropical and sub-tropical water bodies in over 50 countries across five continents (Julien et al., 2001; Villamagna and Murphy, 2010). This plant has a significant impact on water quality, ecological communities, recreation, evapotranspiration, agriculture and fisheries (Harley, 1990; Villamagna and Murphy, 2010).

Harike is one of the largest man-made wetland, which came into existence in 1952 during conservation of water resources from river Sutlej and Beas. It has been declared as a Ramsar Site in 1990 by IUCN, thereby making it internationally important and a priority zone for a variety of flora and fauna (Moza and Mishra, 2008). The wetland is also a source of water for irrigation, and human consumption in six districts of Punjab as well as Rajasthan.

Due to invasion and infestation of *Eichhornia crassipes*, the wetland is facing a severe deterioration in terms of water loss, water quality, apart from threat to biodiversity. Mechanical methods have been previously attempted here to remove this weed and rejuvenate the water body, however, it has proven to be cost intensive, time consuming as well as leading to the resurgence of the weed more vigorously. Chemical herbicides despite exhibiting a good control and check on *E. crassipes* cannot be used in Harike due to their residual toxicity, and side effects on the non-target organisms. As the water from the wetland is being used for the

irrigation, chemical herbicides if used pose a serious threat of bio-magnification. Hence the only plausible option for the control of *Eichhornia crassipes* is biological control.

Biological control of weeds using microorganisms is a well-established and defined strategy which has two distinct approaches- classical and augmentative or innundative approach. In the present work, innundative biological control using plant pathogens was attempted for management of *Eichhornia crassipes* infesting Harike wetland. In this study, the pathogenic mycoflora associated with water hyacinth growing in the Harike wetland was explored, checked for their pathogenic potential using different bioassays and further developed it into a formulation for possible use as a mycoherbicidal formulation. This approach represent a method to reduce dependence on synthetic herbicides and mechanical methods, reduce weed seed bank populations and potentially reduce cost of the weed control in crop production, rangeland restoration, forestry and aquatic systems (Kennedy and Stubs, 2007; Bailey et al., 2010).

Alternaria species exhibited highest occurrence of isolates obtained from the diseases plant samples of water hyacinth from Harike which was followed by *Fusarium* sp. The present study also encountered some new isolates like *Phaeoacremonium* sp., *Acremonium* sp. and *Botryosphaeria* sp. which have not been reported so far (Singh et al., 2016 a,b). In India, mycoflora of water hyacinth inhabiting wetlands have not been explored so far, however, there are reports of host specific isolates from water hyacinth infesting other water bodies like ponds, tanks, rivers which have been tested for their possible use as mycoherbicides. *Myrothecium roridum* var. *eichhorniae* was first reported by Ponnappa (1970) as a host specific variety. *Alternaria alternata* (Fr.) Keissler was also reported by Nag Raj and Ponnappa (1970).

Cercospora rodmanii was also isolated from leaves of water hyacinth at Kurukshetra (Haryana) (Aneja and Srinivas, 1990). The original isolate of *Cercospora rodmanii* was commercialized as a mycoherbicide ABG 5003. Babu et al., (2002) also reported the potential of *Alternaria alternata* for the possible development into a mycoherbicide for checking the spread of water hyacinth. During the survey of waterways in southern Kerala, two potential fungal pathogens isolated from water hyacinth were identified as *Myrothecium advena* and *Fusarium pallidoroseum* (Cooke) which caused more than 50 % damage to the water hyacinth (Praveena and Naseema, 2004). However, the predominant pathogens isolated during the survey comprised of *Curvularia lunata*, *Colletotichum gloeosporioides*, *F. pallidoroseum*, *F. monoliforme* and *F. oxysporum*. While surveying the pathogenic mycoflora of water hyacinth in Niger river, Dagno et al., (2012) also reported highest incidence of *Curvularia* sp. followed by *Fusarium sp*. However, in the present study we could not identify around 25% of cultures due to non-induction of reproductive structures.

Further, in the preliminary screening using *in vitro* detached leaves, only 9 isolates exhibited a significant leaf area damage of which two isolates #8BJSSL and #19BJSSL exhibited highest leaf area damage and disease development in terms of AUDPC. Similar criterion was used by Dagno et al., (2012) to identify three isolates viz. *Fusarium* sp., *Cadophora* sp. and *Alternaria* sp. for their prospective evaluation as a mycoherbicide for management of water hyacinth in Mali, Africa. However, in the detached leaf assay, the host defense mechanisms do not function hence the pathogenic interaction between the host and the fungus cannot be accurately interpreted and therefore it becomes imperative to assess them on whole plants. Hence, assessment of pathogenic potential of #8BJSSL and #19BJSSL was done using hydroponic

based whole plant assays. The pathogenic potential of #19BJSSL in the hydroponic whole plant assay in terms of plant mortality appeared to be significantly higher as compared to #8BJSSL which probably is attributed to some secondary mechanism of the fungus to overcome the inherent defense mechanisms of water hyacinth. #8BJSSL morphologically appeared to be *Phaeoacremonium* sp. while #19BJSSL was morphologically similar to *Botryosphaeria* species. Both of these species appeared to be novel as there are no reports of their occurrence or pathogenesis on *Eichhornia crassipes*. The isolates exhibiting significant pathogenesis of water hyacinth in the whole plant bioassay comprised of *Acremonium zonatum*, *Alternaria eichhorniae*, *Bipolaris hawaiiensis*, *Fusarium oxysporum* and *Myrothecium roridum* (Ray and Hill, 2012). Thus, we used molecular tools to correlate with our morphological studies and correctly identify them. The molecular analysis confirmed #8BJSSL to be *Phaeoacremonium italicum* (Singh et al., 2016a) and #19BJSSL as *Diplodia mutila* (Singh et al., 2016b). *Phaeoacremonium* species have been predominantly associated with diseased woody plants and extensively studies regarding two prominent disease of grapevines Petri disease and esca (Gramaje et al., 2015). *Phaeoacremonium italicum* however, has been reported from *Olea europea* and *Vitis vinifera* (Raimondo et al., 2014; Carlucci et al., 2015). However, the present study is the first report of *Phaeoacremonium* sp. on non- woody plant as a pathogen (Singh et al., 2016a). The Botryosphaeriaceae family encompasses a range of morphologically diverse fungi which exist as opportunistic pathogens, endophytes or saprobes which predominantly attack the woody hosts (Slippers and Wingfield, 2007). Botryosphaeriaceae family of fungi comprises of anamorphic and teleomorphic states of *Lasiodiplodia*, *Diplodia*, *Botryosphaeria*, *Fusicoccum*, *Dothiorella* and *Sphaeropsis* species. *Diplodia* species have been found to be

associated with different disease symptoms such as canker, gummosis, fruitrot, dieback and twig blight (Lazzizera et al., 2008; Phillips et al., 2012; Abdollahzadeh et al., 2013). Similarly, some species of *Diplodia* namely *D. corticola*, *D. mutila*, *D. pinea* and *D. seriata* have been reported as well-known pathogens of woody plants (Phillips et al., 2012). Recently, *D. corticola* has been reported to cause stem cankers in *Quercs rubra* (Martin et al., 2016); *D. mutila* causing branch cankers in bristlecone Fir in California (Sims et al., 2016); *D. bulgarica* has been reported as a new pathogen on apple trees in Iran and Bulgaria (Abdollahzadeh, 2015); *D. seriata* causing dieback and cankers in *Prunus laurocerasus* (Quaglia et al., 2014). The diseases caused by *Diplodia* species have been associated with the onset of the stress factors and therefore it become imperative to isolate, identify and understand ecology and pathogenicity of fungi under diverse existence. More recently, *Diplodia* species have been reported to exist as an endophyte in mangroves from South Africa (Osorio et al., 2016). Thus, in the present study, *Diplodia mutila* #19BJSSL has been identified as a pathogen on aquatic weed, *E. crassipes* for the very first time, which is non-woody in nature. Here also, it exhibits a die-back symptom as in woody plants.

As #19BJSSL-*Diplodia mutila* was exhibiting higher pathogenic potential based on AUDPC as well as plant mortality, it was imperative to understand the possible mechanism of pathogenesis. It is well known that culture filtrate of some plant pathogenic fungi can produce disease like symptoms due to small molecular weight compounds which are referred as phytotoxins (Wenzel, 1985; Daub, 1986; Buiatti and Ingram, 1991; Crino, 1997; Svabova and Lebeda, 2005). Crude culture filtrate allows easy bioassay and screening of phytotoxic effects on the plants, leaves, and cut shoots. Culture filtrates can also be used for management of

weeds (Javaid and Adrees, 2009). Hence, in the present study also, the culture filtrate of #19BJSSL-*Diplodia mutila* and #8BJSSL-*Phaeoacremonium italicum* have been evaluated for their phytotoxic action on water hyacinth using *in vitro* leaf puncher assay. *Diplodia mutila* #19BJSSL exhibited a higher phytotoxic potential when compared to #8BJSSL. *Diplodia mutila* from cypress and other oak species have been found to produce phytotoxic metabolites (Evidente et al., 1997).

Further, the phytotoxicity of culture filtrate of #19BJSSL-*Diplodia mutila* was higher as compared to *Phaeoacremonium italicum* (#8BJSSL) therefore; it was subjected to liquid-liquid extraction using organic solvents of different polarities in order to obtain solvent extracts which are then subjected to phytotoxicity assays. Hence, we also tested four solvent extracts obtained from culture filtrate of #19BJSSL by *in vitro* leaf puncture assay in which, the maximum activity resided in the hexane fraction followed by chloroform fraction. Organic extracts from cultures of *D. quercina*, a pathogen responsible for a disease oak anthracnose were tested by *in vitro* leaf puncture assay to obtain the phytotoxins (Maddau et al., 2009). Ethyl acetate fraction of *Phoma herbarum* FGCC no. 75 exhibited best phytotoxic activity when compared to other solvent extracts by the detached leaf bioassay (Vikrant et al., 2006).

Subsequently, the phytotoxic action of #19BJSSL was to ascertain on the whole plants of *Eichhornia crassipes*, so the culture filtrate as well as hexane and chloroform extract residues were tested. The maximum phytotoxicity was induced by culture filtrate of #19BJSSL followed by hexane and chloroform extract respectively. This suggested that there exists a synergistic action of multiple compounds which induced higher phytotoxicity by the culture filtrate in the whole plant assay. Similarly, petroleum ether fraction of culture filtrate of *Fusarium oxysporum*

f.sp. *ciceris* exhibited the maximum phytotoxicity followed by chloroform fraction. However, hexane fraction of the culture broth did not exhibit any phytotoxic activity (Khan et al., 2004). Another mycoherbicidal candidate *Colletotrichum dematium* FGCC#20 for the control and management of congress grass (*Parthenium hysterophorus*) was found to induce phytotoxicity when its culture filtrate was tested by seedling and detached leaf assays. However, the phytotoxicity resided in the ethyl acetate fraction (Singh et al., 2010). *Diplodia* species have also been reported to be producers of different classes of phytotoxic metabolites for inducing pathogenesis. Diplopyrone (tetrahydropyranpyran-2-one) was isolated from *Diplodia mutila*, a fungal pathogen of cork oak (Evidente et al., 2003). Afritoxinones A and B and dihydrofuropyran-2-ones produced by *Diplodia africana* have been evaluated on host and non-host plants (Evidente et al., 2012). Hence, we carried out the phytochemical studies of culture filtrate, hexane and chloroform fractions obtained from the culture filtrates. The phytochemical study indicates the presence of alkaloids and terpenoids which are generally lipophilic present in the hexane extract as well as culture filtrate in the highest concentration. Phytochemical analysis of culture filtrate and ethyl acetate extract of *Penicillium* sp. isolated as an endophyte from *Centella asiatica* revealed the presence of phenols and flavonoids. They also indicated the presence of alkaloids (Devi et al., 2012). Similarly, ethyl acetate extract of endophytic fungi isolated from *Cissus quadrangularis* also exhibited the presence of alkaloids (Chathurdevi and Gowrie, 2015). Hydroxymellein derivatives, isosclerone and tyrosol are lipophilic phytotoxins produced by *Neofusicoccum parvum* (Evidente et al., 2010).

The use of mycoherbicides as alternative to chemical herbicides has been widely accepted for over three decades now, however, their environmental window of efficacy as

compared to synthetic chemicals is quite poor. This necessitates the enhancement in efficacy of mycoherbicides under varied environmental conditions. The different methods incorporated to enhance the mycoherbicidal potential is by extending the dew period, improving the spore dispersal mechanism and enhancing the virulence (Saxena and Pandey, 2009). Surfactants like Tween-80 and Silwett-77 have been used as with BCA for development of effective mycoherbicidal formulations. Formulations help in increasing the efficiency of application as well as efficacy of BCA (Evans and Reader, 2001).

The idea of developing a formulation is to weaken the biochemical or physical defense mechanisms of the host plant for enabling the BCA to gain easy access or enhance the aggressiveness of the BCA to overcome environmental vagaries which restrict the process of pathogenesis at times. Herbicide Imazaquin was formulated with spores of *Alternaria zinnia* Pape ex M.B. Ellis increased the efficacy of the fungus to control *Xanthium occidentale* and restricted the plant to revive as it interfered in the process of protein synthesis (Auld et al., 1997). Similarly, *Fusarium solani* (Mart.) App. & Wr. f. sp. *cucurbitae* combined with trifluralin controlled *Cucurbita texana* (Scheele) Gray (Texas Gourd) better than single treatments with the fungus or the herbicide, exhibiting a synergistic activity (Weidemann and Templeton, 1988). However, the inherent drawback with this approach is the residual toxicity of the chemical herbicides apart from the product becoming cost intensive. Hence, combining crude phytotoxins which play an immense role in the process of pathogenesis or weakening the defense mechanisms of plants appeared to be more lucrative. Culture filtrates having admixture of three phytotoxins have been found to improve the bioherbicidal potential of *Ascochyta caulina* for the biological control of *Chenopodium album* in terms of speed in disease

onset and severity of the disease (Zonno et al., 1998; Netland et al., 2001; Vurro et al., 2001). Hence, we evaluated the compatibility of hexane fraction of the culture filtrate with the spores of #19BJSSL-*Diplodia mutila* using food poison assay. This technique is generally adopted to evaluate the anti-fungal activity especially of essential oils (Singh et al., 2004; Saxena and Pandey, 2009). We found that a concentration of 2.5 µg/ml of hexane fraction was most compatible on growth of #19BJSSL and therefore this was taken up for developing the formulation by mixing with the spores ($1x10^6$ spores/ml) which was subsequently tested by hydroponics whole plant assay. When compared to only spores and toxin, the formulation comprising of hexane fraction exhibited better bioherbicidal activity both in terms of disease onset as well as intensity of the disease or disease severity. Similar results have been reported in the case of *Ascochyta caulina* wherein the biological control efficacy of the fungus was enhanced by 30% (Vurro et al., 2001). Saxena and Pandey, (2009) also reported significant improvement in the mycoherbicidal activity of *Alternaria alternata* for the biological control of *Lantana camara* in terms of onset of disease and disease severity when spores were formulated with crude phytotoxins. Further, as the futuristic application of this formulation has to been in a wetland which is also serving the purpose of providing water for irrigation in the states of Punjab and Rajasthan, it was imperative to evaluate its host specificity. The spores of #19BJSSL-*Diplodia mutila* did not exhibit any pathogenesis on predominant crops of the region tested viz. wheat, brinjal, mustard and cauliflower and duckweed. Hence, it is expected that the spores of #19BJSSL-*Diplodia mutila* would not affect the economic crop plants in the region. Host range of *Alternaria alternata*, a potential biological control agent for water hyacinth was tested on 29 plant species and was found to be safe i.e. did not induce any pathogenicity except on water

lettuce (*Pistiastratiotes*) (Babu et al., 2002). Similar results were reported with *Cercospora piaropi*, which is also a potential biological control agent of *Eichhornia crassipes* (Jimenez and Lopez, 2001). It was also found that the water quality of the treated tub deteriorated as compared to those untreated with the mycoherbicidal formulation however, it significantly reduced the evapotranspiration rate thus preventing water loss. This is attributed to death of *Eichhornia crassipes* which enhanced the electrical conductivity, TDS of the water apart from drastically enhancing the COD and BOD. The high bacteriological count in treated water was attributed to the deterioration of dead biomass of water hyacinth which supported luxuriant bacterial growth which led to increase in total coliforms and fecal coliforms.

Thus, the present study establishes that #19BJSSL (*Diplodia mutila*) spores and its crude phytotoxins as a formulation could be effectively used for management of water hyacinth in Harike wetland, however this strategy has to be integrated with the mechanical means for removal of the dead biomass to rejuvenate the water body.

Chapter 7

Conclusion

7. CONCLUSIONS

Thus, from the present study, we can conclude that, using the bioherbicidal formulation of #19BJSS-*Diplodia mutila* and its phytotoxins, we can control the spread and infestation of water hyacinth in Harike wetland. Further, a check on water quality of the wetland can be improved by integrating the biological control method with the mechanical means i.e. as soon as the hyacinth dies due to bioherbicidal formulation; it should be mechanically removed so as to rejuvenate the water body. Thus, biological control using #19BJSSL formulation appears to be a plausible alternative for the recovery of Harike Wetland.

Chapter 8

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Diplodia mutila as a new pathogen on water hyacinth *Eichhornia crassipes* in Harike wetland

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ABSTRACT

The present publication reports the mycobiota associated with *Eichhornia crassipes* (C Mart.) Solms (Pontederiaceae) infesting the Harike Wetland at Punjab in India and their potential application as a biological control agent based on their pathogenicity to the host plant. Of the 30 fungal isolates recovered from the infested samples of *E. crassipes* only one isolate #19BJSS caused 98% damage or infestation to the leaves during the *in vitro leaf* bioassay while it exhibited 100% plant death during the whole plant bioassay after 168 hours post inoculation (hpi). Morphological studies revealed it to be a member of *Botryosphaeriaceae* family which was further confirmed by Internal Transcribed Spacers (ITS) phylogenetic studies wherein the isolate #19BJSS clustered with *Diplodia mutila*. *Botryosphaeria* species have been predominantly associated with canker and die back diseases of woody plants. This is the first report of occurrence of *Diplodia mutila* #19BJSS as a pathogen of *E. crassipes* and for its potential as a biological control agent for the management of water hyacinth.

MS History: 01.05.2016 (Received)-28.08.2016 (Revised)- 11.10.2016 (Accepted)

Key words: *Eichhornia crassipes*; ITS-rDNA; mycoherbicides; plant pathogen; Botryosphaeriaceae.

Citation: Singh, B., Saxena, S., Meshram, V. and Kumar, M**.**. 2016. *Diplodia mutila* as a new pathogen on water hyacinth *Eichhornia crassipes* in Harike wetland. *Journal of Biopesticides*, 9 (2): 180-188.

INTRODUCTION

Eichhornia crassipes (C. Mart) Solms, (Pontederiaceae) commonly known as water hyacinth is an aquatic plant world-wide. This plant has been designated as among top 100 invasive species by International Union of Conservation of Nature (IUCN) (Tellez *et al.,* 2008). The rapid growth and proliferation rate of water hyacinth is a global concern since it poses serious environmental and conservation problems having socioeconomic repercussions. It has been recognized as the top ten world's worst weed that has invaded Europe, Africa, Asia and North America (Ray and Hill, 2016).

Water hyacinth in India has become a serious problem in Harikewetland, a Ramsar site created in 1953 due to construction of a barrage at the confluence of the rivers Sutlej and Beas in Punjab, India (Singh *et al*., 2016). Harike wetland is of both national and international importance since it is inhabited by diverse flora and fauna of which many have been red listed (Singh *et al*., 2016). Apart from 400 species of avifauna existing in this area there is a huge concentration of migratory birds in this wetland and hence it declared as a bird sanctuary (Tiwana *et al.*, 2008). Another important aspect of Harike wetland is being an important source of water for irrigation and drinking through its feeder canals in the states of Punjab and Rajasthan apart from ground water recharging. Presently, approximately 40% of this wetland has been infested by water hyacinth, which causes water loss due to excessive evapotranspiration rate apart from several waterborne disease downstream (ICID report 2002; Matt McDonald Report, 2015). Thus there is a need to revive this water body by suppressing the growth of water hyacinth.

Chemical methods of controlling *Eichhornia* in Harike wetland is not recommended as the water from its feeder canals is being used for drinking as well as irrigation. Mechanical removal has been carried out several times but this is successful only for a short duration since water hyacinth resurges (Ndimele *et al.*, 2011). Biological control of the weeds

using microorganisms is being sought as a cost effective, more benign and effective strategy to control pests. Of the scores of fungal pathogens which attack water hyacinth some promising ones which have the potential to be used as a biological control agent *Cercospora piaropi, Alternaria eichhorniae, Alternaria alternata, Myrothecium roridum* and *Rhizoctonia solani*. Some of these have been tested under controlled conditions and have reportedly reduced the biomass of water hyacinth (Martyn and Freeman 1978; Charudattan *et al,*. 1985; Shabana *et al*., 1995). The innundative or bioherbicidal approach is a well-establishedapproach and many mycoherbicides have been developed and commercialized since mass production of the biological control agent is easily achievable using fermentation process (Saxena, 2015). Hence fungal pathogens offer opportunities to be developed as bioherbicides for the management of *Eichhornia crassipes*.

Fungi associated with *Eichhornia crassipes* have been studies in the Amazon basin and has been prospected for its possible use as mycoherbicide (Evans and Reeder, 2001) and using insects (Coetzee *et al*, 2011). However, very limited information exists on the mycobiota associated with *Eichhornia crassipes* for its prospective use as a biological control agent in Indian aquatic bodies. Nag Raj and Ponnappa (1970) first reported the blight of water hyacinth caused by *Alternaria eichhorniae*. Subsequently an isolate of *Alternaria alternata* (Fr.) Keissler from Haryana (Aneja and Singh, 1989), Tamil nadu and Kerala (MohanBabu *et al.*, 2003) have been tested for the potential as a mycoherbicide to control *Eichhornia crassipes*. *Fusarium pallidoroseum* and *Myrothecium advena* have also been tested for their prospective use as a biocontrol agent of water hyacinth in Kerala (Praveena and Naseema, 2004). More recently mycoherbicidal potential of indigenous isolate of *Alternaria japonica* and *Phaeoacremonium italicum* has been evaluated for the control of water hyacinth (Dutta *et al*., 2015; Singh *et al*., 2016)

Thus the aim of this study was to explore the pathogenic mycobiota associated with *E. crassipes* and identify a suitable pathogen which could be developed into a mycoherbicide for possibly checking the spread and growth of water hyacinth in Harike wetland.

MATERIALS AND METHODS

Collection and isolation of the fungal pathogens

Infected plant samples (stolons, leaves and swollen bases) exhibiting necrotic spots and chlorotic symptoms were collected from the Harike wetland, Punjab during rainy and early winter seasons in the year 2012. These were stored in sterile polyethylene bags and transported to the laboratory, stored at 4ºC and processed within 24 h. Surface debris of the plant parts were removed by washing them under running tap water for 15 min. Subsequently they were swapped dry using sterile blotting paper. Leaf pieces were subsequently washed with 1% sodium hypochlorite for 1 to 2 min followed 30% ethanol for 1 min and then finally rinsed with sterile water thrice and air dried in laminar air flow. The infected portions were then dissected into 1×1 cm segments using a sterile blade. These surface sterilized plant segments were further cut into 2 to 4 mm segments and inoculated on Potato Dextrose Agar (PDA) medium (initial pH 4.5, HiMedia) supplemented with streptomycin (1mg/mL). Maximum of eight segments were inoculated per plate and incubated at 26±1°C for 7-days. The obtained fungal isolates were then sub-cultured over PDA plate as pure cultures and subsequently stored on a PDA slants containing 15% glycerol. These were tentatively identified using standard mycological keys given by Barnett and Hunter (1998).

Inoculum production

Fungal spores were harvested by flooding 10 d old culture of each isolate grown on water agar at 26 ± 2 ºC with sterile distilled water. Subsequently the spore concentration

was adjusted to 1 x 10^6 spores /mL using a hemocytometer and then mixed with 0.05% of Tween 20 (Sigma Aldrich).

***In vitro* detached leaf pathogenicity assay**

Healthy *Eichhornia* leaves were sprayed with spore concentration of 1x10^6 spores/mL using a hand sprayer. Three leaves were used for per fungal isolate were used in the assay. These were then placed in an aseptic moist chamber prepared in 90 mm petri dishes and kept in a growth chamber with controlled conditions of 26 ± 2°C; 75 ± 15% relative humidity and 15 h (7350 lx) illumination for a period of one week. Leaves were visually examined every 24 h and percentage of symptomatic area of the leaves were recorded. All the tests were performed in triplicates (Saxena and Pandey, 2009). The disease progression during the *in vitro* detached leaf bioassay was established by determining the Area under Disease Progressive Curve (AUDPC) value of each isolate was calculated by the formula:

$$\text{AUDPC} = \sum_{i}^{n-1}\left[\left(y_i + y_{i+2}\right)/_2\right)(t_{i+1}-t_i)]$$

where

y_i is the assessment of the disease (percentage, proportion, ordinal score etc.) at the *i*th observation,

t_i is time (in days, hours etc.) at the *i*th observation, and

n is the total number of observations.

Whole plant bioassay for pathogenicity

The best isolate exhibiting highest pathogenicity in the în vitro detached leaf bioassay was taken up for further evaluation of pathogenicity on whole plants. Briefly 4 to 6 week old plants of *E. crassipes* were grown in tubs with illumination of 12 h daily for a period of one week for acclimatization under laboratory conditions. These were then sprayed with fungal inoculum of a spore concentration of 1x 10^6 spores/mL. Ten replicates were used in the experimental as well as in the control set. The control set were received only sterile distilled water (SDW). Plants were observed daily for disease severity (Saxena and Pandey, 2009). The experiment was repeated thrice.

Molecular identification of the selected fungus

The genomic DNA of the selected fungal isolate (#19BJSS) was extracted using Wizard® Genomic DNA purification kit (Promega, USA). For the DNA isolation, 2 to 3 mycelial discs (10 mm) of 4 to 5 d old selected fungal culture were scooped out and crushed to very fine powder in mortar and pestle by using liquid nitrogen. The powder was immediately transferred to the 2.0 mL micro centrifuge tube and the further extraction was carried out as per the instructions of the kit manufacturer. The internal transcribed spacer (ITS) regions of the nuclear rDNA was amplified using ITS1 (5' TCCGTA GGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.,* 1990) using MyCycler™ (Bio-Rad). The amplification was carried out in 25µl reaction mixture containing 25ng of extracted genomic DNA, 0.8 µM of each primer (ITS1 and ITS4), 0.2mM of dNTP, 1.5 mM $MgCl_2$, 1.5U of Taq DNA Polymerase in 10 X Taq buffer. The Thermal cycling conditions consisted of initial denaturation at 96°C for 5 min followed by 39 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s followed by final extension at 72°C for 5 min. The amplicons were purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA). Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

Sequencing and Phylogenetic analysis

Purified products (amplicons) were sent for direct sequencing to Chromus Biotech, Bangalore. The Final sequence was obtained by assembling the obtained sequences using Sequencher ver.5 (www.genecodes.com). Sequences obtained in the present work were submitted in the NCBI database with Accession No. HG738871. The final sequence was subjected for BLAST similarity search against the database

Table 1. List of isolates and their accession number used in the phylogenetic analysis of #19BJSS (GenBank Accession HG 738871)

Name	Isolate number	Accession number*	Host	Place
Diplodia mutila	A24-AA-2	KP026317	*Amelanchir annifolia*	Canada
Diplodia mutila	1476	KM580526	*Vitis vinifera*	Chile
Diplodia mutila	4D33	KF778779	*Juglans regia*	USA
Diplodia mutila	KER-U-DMAPR1	KF535901	*Prunus armenica*	Iran
Diplodia mutila	BEI36	KT954166	*Vitis vinifera*	France
Diplodia seriata	DA221	KC960900	Pear	China
Diplodia seriata	KER-U-DSGG2	KC535906	*Prunus domestica*	Iran
Diplodia scrobiculata	CBS109944	DQ458899	*Pinus greggi*	Mexico
Diplodia mutila	BSDP1	KC789072	Date Palm	Iran
S. sapinea	CBS 393.84	DQ458895	*Pinus nigra*	Netherlands
S. sapinea	WA0000019144	JX981458	*Lycopodium sp.*	Poland
B. rhodina	CSS	GU226856	*Vitis vinifera*	China
B. rhodina	3-5	GU323603	-	China
B.eucalyptorum	MUCC387	DQ131571	*Eucalyptus sp.*	Australia
B.eucalypticola	MUCC388	DQ131571	*Eucalyptus globus*	Australia
A.niger	BGD22	HM107005	*Rhizophora stylosa*	China

maintained by NCBI to ascertain the homology with closely related organisms (Table 1). The sequences showing highest similarity (over 98%) for the locus were initially selected and included in the phylogenetic analysis and aligned with the respective sequence (HG738871) obtained in the present work by using ClustalW. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). All positions containing gaps and missing data were eliminated. Clade stability was assessed by bootstrap analysis with 1000 bootstrap replicates.

RESULTS AND DISCUSSIONS

Isolation of Pathogenic fungi

The diseased leaves and stolons of *Eichhornia crassipes* collected from the Harike wetland possessed leaf spots, necrotic flecks, leaf blights and led to isolation of 30 isolates which comprised 11 genera (Table 2). The frequency of occurrence of *Alternaria* sp. is 26.7% followed by *Fusarium* with 20%, *Penicillium* and *Aspergillus* species with 13.3% and *Botryosphaeria* species with 6.7 %. The other fungi which were isolated during the sampling were *Acremonium* sp., *Nigrospora* sp., *Curvularia* sp., *Trichoderma* sp., *Didymella* sp. and *Artheminium*sp.

In vitro detached leaf pathogenicity assay

All the fungal isolates recovered from the diseased samples of *E. crassipes* were tentatively identified and tested using an *în vitro* detached leaf bioassay. Out of the 30 isolates tested, only *Diplodia* species #19BJSS exhibited 98% kill after 7 days post inoculation. The symptoms of the disease started appearing after 48 hpi and 60% kill was observed by 96 hpi. The symptoms include chlorosis of leaves as well as black necrotic spots leading to necrosis and death of the leaves by the end of the assay (Fig 1). The other isolates

Table 2. Fungal isolates inhabiting *Eichhornia crassipes* and related *în vitro* pathogenicity test on detached leaves

Culture code	Tentative identification	Leaf area damaged (%) after different hours post inoculation (hpi)					
		48	72	96	120	144	168
#1 BJSS	*Alternaria* sp.	10 ± 0^{d}	18 ± 2^{c}	23 ± 2^{bc}	30 ± 4^{b}	38 ± 2^{a}	43 ± 2^{a}
#2 BJSS	*Aspergillus* sp.	0^{c}	0^{c}	8 ± 2^{b}	10 ± 0^{ab}	12 ± 2^{ab}	13 ± 2^{a}
#3 BJSS	*Fusarium* sp.	0^{d}	10 ± 0^{c}	15 ± 0^{b}	22 ± 2^{a}	23 ± 2^{a}	25 ± 0^{a}
#4 BJSS	*Fusarium* sp.	0^{c}	5 ± 0^{bc}	12 ± 2^{b}	20 ± 4^{a}	23 ± 2^{a}	23 ± 2^{a}
#5 BJSS	*Alternaria* sp.	17 ± 2^{de}	32 ± 8^{cd}	50 ± 11^{bc}	70 ± 11^{ab}	82 ± 8^{a}	87 ± 2^{a}
#7 BJSS	*Botryosphaeria* sp.	20 ± 4^{d}	40 ± 4^{c}	61 ± 3^{b}	80 ± 0^{a}	83 ± 2^{a}	88 ± 2^{a}
#9 BJSS	*Fusarium* sp.	15 ± 2^{e}	28 ± 2^{d}	45 ± 4^{c}	55 ± 4^{b}	67 ± 2^{a}	73 ± 2^{a}
#10 BJSS	*Fusarium* sp.	35 ± 4^{e}	45 ± 0^{d}	55 ± 4^{c}	63 ± 2^{bc}	68 ± 2^{ab}	73 ± 2^{a}
#11 BJSS	*Acremonium* sp.	10 ± 4^{ef}	22 ± 8^{de}	37 ± 10^{cd}	48 ± 5^{bc}	62 ± 2^{ab}	68 ± 2^{a}
#12 BJSS	*Nigrospora* sp.	0^{c}	0^{c}	5 ± 0^{b}	5 ± 0^{b}	8 ± 2^{a}	10 ± 0^{a}
#14 BJSS	*Alternaria* sp.	22 ± 2^{e}	32 ± 2^{d}	43 ± 2^{c}	52 ± 2^{b}	63 ± 2^{a}	68 ± 2^{a}
#15 BJSS	*Penicillium* sp.	0^{d}	7 ± 2^{cd}	17 ± 2^{bc}	27 ± 6^{b}	40 ± 4^{a}	50 ± 4^{a}
#17 BJSS	*Penicillium* sp.	0^{f}	28 ± 2^{e}	42 ± 2^{d}	54 ± 3^{c}	63 ± 2^{b}	74 ± 3^{a}
#19 BJSS	*Diplodia* sp.	26 ± 3^{e}	40 ± 4^{d}	60 ± 4^{c}	82 ± 5^{b}	93 ± 6^{ab}	98 ± 2^{a}
#20 BJSS	*Alternaria* sp.	0^{e}	10 ± 4^{d}	20 ± 4^{c}	27 ± 2^{bc}	33 ± 2^{b}	43 ± 2^{a}
#21 BJSS	*Alternaria* sp.	0	0	0	0	0	0
#23 BJSS	*Aspergillus* sp.	0	0	0	0	0	0
#27 BJSS	*Fusarium* sp.	0	0	0	0	0	0
#29 BJSS	*Fusarium* sp.	0	0	0	0	0	0
#34 BJSS	*Curvularia* sp.	0	0	0	0	0	0
#38 BJSS	*Artheminium* sp.	0^{d}	0^{d}	0^{d}	13 ± 2^{c}	23 ± 2^{b}	32 ± 2^{a}
#42 BJSS	*Alternaria* sp.	0^{d}	0^{d}	0^{d}	5 ± 0^{c}	13 ± 2^{b}	22 ± 2^{a}
#44 BJSS	*Penicillium* sp.	0	0	0	5	5	10
#52 BJSS	*Didymella* sp.	0	0	0	0	0	0
#54 BJSS	*Trichoderma* sp.	0	0	0	0	0	0
#57 BJSS	*Alternaria* sp.	0^{d}	0^{d}	15 ± 0^{c}	20 ± 4^{c}	28 ± 2^{b}	37 ± 2^{a}
#14 BJSSS	*Aspergillus* sp.	0	0	0	0	0	0
#16 BJSSS	*Penicillium* sp.	0	0	0	0	0	0
#17 BJSSS	*Alternaria* sp.	0^{e}	0^{e}	5 ± 0^{d}	15 ± 0^{c}	22 ± 2^{b}	38 ± 2^{a}
#19 BJSSS	*Aspergillus* sp.	0	0	0	0	0	0

*Mean values along with their Standard error (±) are given in the table. Mean values represented by same alphabets in each row are not significantly different by Tukey's post hoc test at $p<0.05$

which also exhibited pathogenic potential were #7BJSS *Botryosphaeria* species followed by # 5 BJSS *Alternaria* species respectively with 88% and 87%, respectively (Table 2). Pathogenic potential of the different isolates tested were significantly different by one way ANOVA at $p<0.05$: $F(7, 232) = 11.13$, $p \leq 0.0001$. #19BJSS *Diplodia* species has not been reported as a pathogen or a biological

control agent of *Eichhornia crassipes*. Higher AUDPC of # 19BJSS also indicated that it possesses a higher pathogenic potential as compared to #7BJSS and # 5 BJSS (Fig.2). Hence #19BJSS *Diplodia* species was further selected to assess its potential as an innundative biological control using whole plant bioassay.

Fig.1. *în vitro* detached leaf assay of spores of #19BJSS (*Diplodia mutila*) 168 hpi.

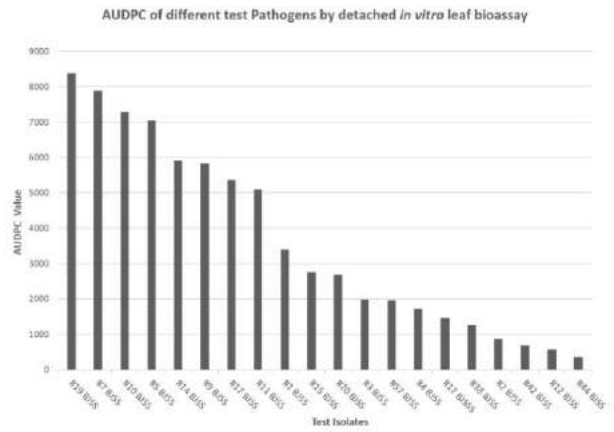

Fig.2. AUDPC of different test pathogens by *în vitro* detached leaf bioassay.

Whole plant bioassay for pathogenicity

In the whole plant bioassay the disease onset began after 2 d after inoculation. 50% damage of the whole plants was observed at the 4th day while above 90% death by 6thday after spore application (Figs.3, 4). The plants eventually died on the 7th day. There was not a significant difference in AUDPC observed in the *in vitro* leaf assay and whole plant bioassay with values of 8380 and 7842 respectively indicating its pathogenic potential.

Molecular identification of #19 BJSS

The Phylogenetic relationship of the isolate under study was carried out by sequencing the ITS region of the isolate. The sequence, thus obtained was showing 98% sequence similarity with *Sphaeropsis sp, Diplodia* sp. and *Botryosphaeriacae* sp. Hence, the present study undertaken the 17 sequences comprising *Diplodia mutila, Diplodia seriata, S. sapinea, B. rhodina* and *B. eucalyptorum.* The Neighbor-Joining tree was divided into three clades viz. Clade I, II and III. Clade I was further sectioned into two subclades- Sub-clade I which clustered isolate under study (#19BJSS) along with six strains of *Diplodia mutila* with high bootstrap support. Sub- clade II grouped strains of *D. seriata* and *S. sapinea.* However, *B. rhodina* and *B. eucalyptorum* formed their clades namely clade II and III respectively. *Aspergillus niger* was chosen as out-group to root the tree (Fig.5).

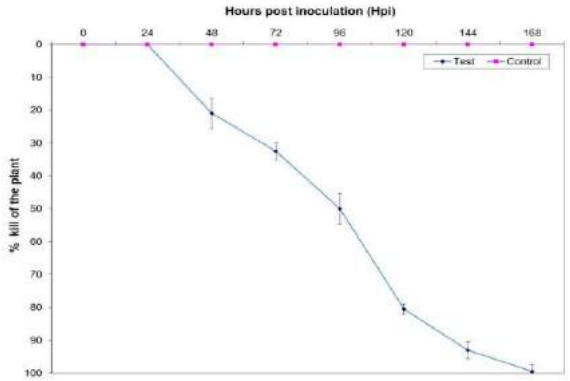

Fig.3. Disease progress caused by spores spray (1x10^6 spores/mL) of #19BJSS (*Diplodia mutila*) during whole plant bioassay.

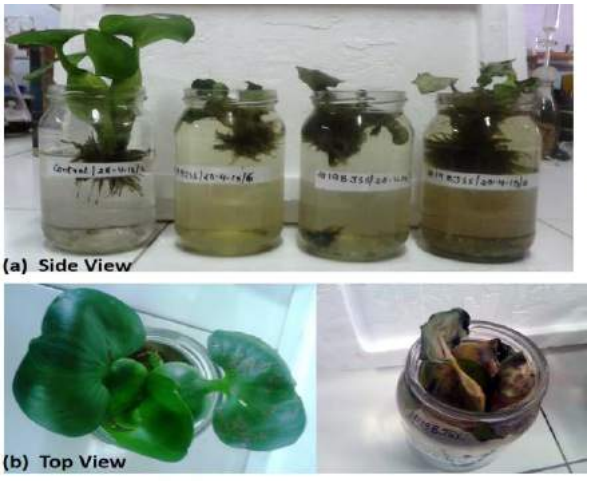

Fig. 4. Whole plant bioassay exhibiting the kill caused by spore suspension (1 X 10^6 spores/ mL) of # 19BJSS (*Diplodia mutila*).

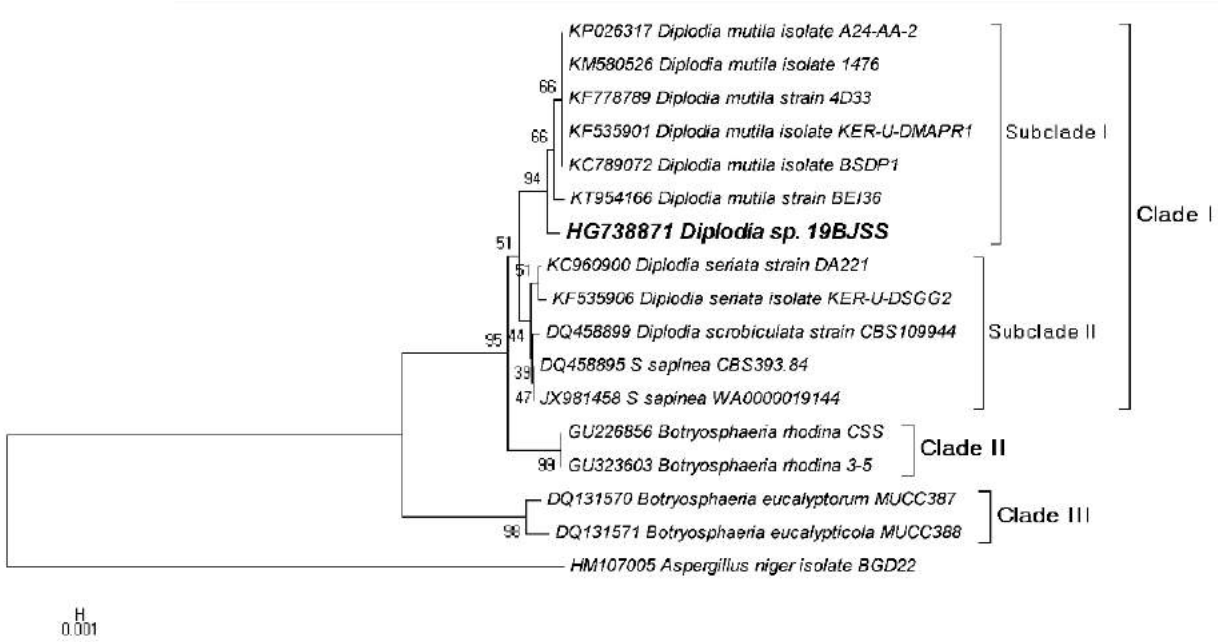

Fig. 5. Phylogenetic placement of #19 BJSS.

The Botryosphaeriaceae family encompasses a range of morphologically diverse fungi which exist has opportunistic pathogens, endophytes or saprobes which predominantly attack the woody hosts (Slippers and Wingfield, 2007). Botryosphaeriaceae comprises of anamorphic and teleomorphic states of *Lasiodiplodia*, *Diplodia*, *Botryosphaeria*, *Fusicoccum*, *Dothiorella* and *Sphaeropsis* species. *Diplodia* species have been found to be associated with different disease symptoms such as canker, gummosis, fruit rot, dieback and twig blight (Lazzizera e*t al.*, 2008; Phillips *et al.*, 2012; Abdollahzadeh *et al.*, 2013). Similarly some species *of Diplodia* namely *D. corticola*, *D. mutila*, *D. pinea* and *D. seriata* have been reported as well-known pathogens of woody plants (Phillips *et al.*, 2012). Recently *D. corticola* has been reported to cause stem cankers in *Quercus rubra* (Martin *et al.*, 2016); *D. mutila* causing branch cankers in bristlecone Fir in California (Sims *et al.*, 2016); *D. bulgarica* has been reported as a new pathogen on Apple trees in Iran and Bulgaria (Abdollahzadeh, 2015); *D. seriata* causing dieback and cankers in *Prunus laurocerasus* (Quaglia *et.al*, 2014). The diseases caused by *Diplodia* species have been associated with the onset of the stress factors and therefore it become imperative to isolate, identify and understand ecology and pathogenicity of fungi under diverse existence. More recently *Diplodia* species have been reported to exist as an endophyte in mangroves from South Africa (Osoria *et al.*, 2016). In the present publication we have identified *Diplodia mutila* as a pathogen for the very first time on aquatic weed *E. crassipes* which is non-woody in nature. ITS sequencing was primarily adopted to identify the pathogen which has been a method to report novel as well as known species of *Diplodia*. The pathogenicity of *D. mutila* was confirmed by establishing the Koch's postulates exhibiting characteristic symptoms of dieback. Further establishment of it safety on non–target plants and animals is essential for its possible use as a biological control agent for the management of water hyacinth.

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Mycobiology

Research Article

Mycoherbicidal Potential of *Phaeoacremonium italicum*, A New Pathogen of *Eichhornia crassipes* Infesting Harike Wetland, India

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Abstract Mycoherbicides are exclusive biotechnology products which offer a non-chemical solution to control noxious weeds on the land as well as aquatic in systems, viz a viz saving environment from hazardous impact of synthetic chemicals. The present paper highlights the mycobiota associated with *Eichhornia crassipes* infesting Harike wetland area of Punjab and evaluation of their pathogenic potential for futuristic application as a mycoherbicide. Of the 20 isolates tested by leaf detached assay and whole plant bioassays, only one isolate (#8 BJSSL) caused 100% damage to *E. crassipes*. Further, the culture filtrate of this isolate also exhibited a similar damage to the leaves in an *in vitro* detached leaf assay. The potential isolate was identified as *Phaeoacremonium italicum* using classical and modern molecular methods. This is the first report of *P. italicum* as a pathogen of *E. crassipes* and of its potential use as a biological control agent for the management of water hyacinth.

Keywords β-Tubulin, Fungi, Mycoherbicides, Plant pathogen, Ramasar site, Weeds

Weeds are attacked by fungi, bacteria and viruses. Fungi, predominantly are responsible for an array of weed diseases and thus possess potential to be developed as mycoherbicides/mycoweedicides based on their pathogenic potential. The main advantage of using mycoherbicides/mycoweedicides is reduction in use of chemical weedicides which pose a severe threat to human health and environment [1]. Fungi attacking weeds are diverse assemblage of species which markedly differ in their morphology, physiology, and pathogenicity.

Water hyacinth [*Eichhornia crassipes* Mart. Solms (Pontederiaceae)] is an invasive and noxious aquatic weed posing serious economic, social and environmental problems in India and other tropical and subtropical regions of the world [2]. *E. crassipes* currently holds the status of one of the world's worst aquatic weed [3, 4]. Water hyacinth continues to be a serious problem in Harike wetland, a Ramsar site created in 1953 by construction of a barrage at the confluence of rivers Sutlej and Beas in Punjab, India. Harike wetland is a premium water source for irrigation and drinking through its feeder canals in the states of Punjab and Rajasthan, India. Apart from this, it is also inhabited by a rare flora and fauna of which many have been designated as threatened species. It has been declared as a bird sanctuary since a huge concentration of migratory birds apart from 400 species of avifauna exists in the wetland area. Approximately, 40% of Harike is infested by water hyacinth, which leads to water loss due to excessive evapotranspiration and causing water borne diseases downstream. Hence, there is an urgent need to rejuvenate this water body by suppressing the growth of water hyacinth. Since, this surface water is not only a major drinking water source but also used for irrigation. The chemical methods of controlling water hyacinth are not recommended as they cause adverse effects and often lead to residual toxicity. Mechanical operations have been tried and are successful for short duration as resurgence of water hyacinth occurs.

Biological control using plant pathogens in recent years has proven to be a cost effective, environmentally safe solution to control weeds. Of the several fungal pathogens reported to attack water hyacinth, some prominent ones are *Acremonium zonatum*, *Cercospora piaropi*, *Myrothecium roridum*, *Rhizoctonia solani*, *Uredo eichhorniae*, *Helminthosporium/Bipolaris* spp.,

Mycobiology 2016 June, **44**(2): 85-92
http://dx.doi.org/10.5941/MYCO.2016.44.2.85
pISSN 1229-8093 • eISSN 2092-9323

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Received February 9, 2016
Revised March 4, 2016
Accepted April 16, 2016

and *Alternaria eichhorniae*. Controlled studies have indicated that *C. piaropi*, *Alternaria eichhorniae*, *Alternaria alternata*, and *Acremonium zonatum* induce biomass reduction during biocontrol of water hyacinth [5-8]. Thus, it is proven that these fungal pathogens could be successfully used as classical or innundative biological control agents for water hyacinth.

There exists very limited information on mycobiota associated with *E. crassipes* for prospective use as a mycoherbicide from India. The first report of *Alternaria eichhorniae* causing blight of water hyacinth was given by Nag Raj and Ponnappa [9], followed by *Alternaria alternata* (Fr.) Keissler from Haryana [10], *Alternaria alternata* from Tamil Nadu and Kerala [11], *Fusarium pallidoroseum* and *Myrothecium advena* Sacc. from Kerala [12].

As innundative biological control advocates the isolation and selection of natural enemy from the same geographical area where the weed is infesting, the present investigation was undertaken to explore the pathogenic mycoflora associated with *E. crassipes* infesting in Harike wetland for their possible use as mycoherbicides.

MATERIALS AND METHODS

Sample collection and isolation of pathogenic fungi. Infected plant parts (stolons and leaves) with necrotic spots were collected from the Harike wetland area, Taran Taran Sahib, Punjab, India in June 2011. The plant parts were kept in sterile bags, brought to the laboratory, and processed within 24 hr. Plant parts were washed under running tap water for 15 min to remove the surface debris. The infected portions were then dissected into 1 × 1 cm segments using a sterile blade. Infected plant parts were subsequently washed with 1% sodium hypochlorite for 1~2 min followed 30% ethanol for 1 min and then finally rinsed with sterile water thrice and air dried in laminar air flow. These surface sterilized plant segments were further cut into 2~4 mm segments and inoculated on potato dextrose agar (PDA) medium (initial pH 5.5; HiMedia, Mumbai, India) supplemented with streptomycin (1 mg/mL; HiMedia). Maximum of eight segments were inoculated per Petri dish and incubated at 26 ± 1°C for 7 days. The obtained fungal isolates were then sub-cultured over PDA plate as pure cultures and subsequently stored on PDA slants containing 15% glycerol [13]. These were tentatively identified using standard mycological keys given by Barnett and Hunter [14].

Frequency of fungal isolates. The fungal isolates obtained from the diseased water hyacinth located at different sites in Harike wetland were counted based on their frequency of occurrence. The percentage of occurrence was expressed using the formula:

Frequency (%) = Number of isolates of a genus × 100/Total number of isolates.

Inoculum production and pathogenicity test on detached leaves. For each of twenty fungal isolates, spores were harvested by flooding the 7-day-old colonies with sterile distilled water grown on PDA at 28 ± 2°C. Thereafter spore concentration was adjusted to 1×10^6 spores/mL using a hemocytometer and then mixed with 0.05% of Tween 20 (Sigma Aldrich, St. Louis, MO, USA). These were then sprayed over healthy *Eichhornia* leaves using a hand atomizer (Borosil, Mumbai, India) which were then placed in an aseptic moist chamber prepared in 90-mm Petri dishes (Tarsons, Kolkata, India) and incubated at 28 ± 2°C for 7 days under 12 hr of photoperiod (7350 lx). Leaves were visually examined every 24 hr and percentage of symptomatic area of the leaves were recorded. All the tests were performed in triplicates [15]. The disease progression in the *in vitro* detached leaf bioassay was established by determining the area under disease progressive curve (AUDPC) value of each isolate using the formula

$$AUDPC = \Sigma_i^{n-1}[(y_i + y_{i+2})_2/(t_{i+1} - t_i)]$$

, where 'y_i' is the assessment of the disease (percentage, proportion, ordinal score, etc.) at the ith observation, 't_i' is time (in days, hours, etc.) at the ith observation, and 'n' is the total number of observations. The isolate exhibiting maximum pathogenecity in detached leaf assay was selected for testing.

Pathogenicity tests on the whole plants. Four-to six-week old plants having 4~8 leaves were grown in tubs with illumination of 12 hr daily for 1 wk for acclimatization under laboratory conditions. These were then sprayed with fungal inoculum of a spore concentration to 1×10^6 spores/mL. Ten replicates were used in the experimental as well as in the control set. The control set received only sterile distilled water. Plants were observed daily for the disease severity according to Chiang *et al.* [16] until all plants died. The experiment was repeated thrice. The disease progression was determined by calculating the AUDPC values as mentioned in the previous section.

Production of the culture filtrate. The isolate, #8 BJSSL which exhibited highest pathogenicity in the *in vitro* detached leaf bioassay was grown in liquid culture to assess the phytotoxic potential of the culture filtrate. A 5-mm mycelial plug of the 7-day-old fungal culture was inoculated into 100 mL of pre-sterilized Richard's broth (HiMedia) in 250 mL Erlenmeyer flasks (Schott Duran, Mainz, Germany). These were incubated in an orbital shaker (Eppendrof, Chennai, India) at 28 ± 2°C, 120 rpm for 15 days. The mycelium was separated from the liquid medium initially with a sterile muslin cloth followed by Whatmann No. 4 filter paper and finally through 0.22 µm nitrocellulose membrane (Whatmann, GE health care and Life Sciences, Piscataway, NJ, USA) to make it cell free [17].

Detached leaf bioassay of the culture filtrates. Healthy leaves of water hyacinth was sprayed with cell free culture

filtrate (1 mL) of #8 BJSSL and incubated at 28 ± 2°C for 7 days with 12 hr of light and dark conditions as used previously for testing the pathogenic potential using spores. The phytotoxicity was recorded as percentage of leaf area damaged. All the tests were performed in triplicate [15].

Morphotaxonomy of the selected fungal pathogen. The selected fungal isolate (#8 BJSSL) was grown over PDA, malt extract agar (MEA), and corn meal agar (CMA) medium (HiMedia) [18, 19]. The plates were incubated at 28 ± 2°C for 7~10 days with alternate cycle of light and dark for 12 hr. Colony morphology, growth pattern and appearance were noted. For studying microscopic characters, the mycelial mass was picked from the fine tip of the pre-sterilized needle and teased over the glass slide. The fungal mass was mounted using lacto phenol cotton blue dye (HiMedia). The microscopic characters were then observed using Nikon Stereozoom microscope (SMZ 745T; Nikon, Gurgaon, India) coupled with NIS element D 3.2 software and Nikon Eclipse Compound microscope (E100). Microscopic measurements were done using stage and ocular scale and confirmed by Image J software with at least 30 observations per structure.

Phylogenetic analysis of the selected fungal pathogen. Fungal genomic DNA isolation was carried out by using Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). For genomic DNA isolation, 0.5 g of the mycelial mass was scrapped from the 10-day-old culture and crushed into very fine powder in mortar and pestle using liquid nitrogen. The powder was transferred into micro-centrifuge tube and DNA isolation was further carried out by using Wizard Genomic DNA purification kit (Promega). The β-tubulin gene sequence was amplified using bena-T1 (5′ AACATGCGTGAGATTGTAAGT 3′), bena-T22 (5′ TCT-GGATGTTGTTGGGAATCC 3′) primer pair. Amplification was performed in a 25 μL reaction mixture volume consisting of 25 ng of extracted genomic DNA, 0.8 μM of each primer pair, 2.5 mM of dNTPs, 1.5 mM $MgCl_2$, 1.5 U of *Taq* DNA polymerase. The thermal cycling parameters was 96°C for 5 min followed by 35 cycles of 95°C for 1 min, 58°C for 1.30 min, 72°C for 1.30 min followed by final extension at 72°C for 7 min [18, 19]. The amplified product (≈ 800 bp) was purified using Wizard SV gel and PCR clean up system kit (Promega) and the purified products were sequenced at Xcleris Labs (Ahmadabad, Gujarat, India).

Sequence assembly, alignment, and phylogenetic analysis. The chromatograms obtained following sequencing were manually edited and checked for its purity using Sequencher ver. 5 (http://www.genecodes.com). The final consensus sequence was submitted in GenBank under accession number KP696755. The final sequence of #8 BJSSL was then subjected for BLAST similarity search in the NCBI database to ascertain the homology with closely related organisms. The sequences showing highest similarity for each locus were selected and aligned with the respective sequences obtained in the present work by using CLUSTALW.

Table 1. Fungal isolates inhabiting *Eichhornia crassipes* and their pathogenicity in *in vitro* detached leaf bioassay

Sample No.	Culture code	Tentative identification	% Leaf area damage[a] after different hours post inoculation (hpi)[b]						
			0	24	48	72	96	120	144
1	#3 BJSSL	*Fusarium* sp.	0	0	0	11 ± 1	16 ± 2	24 ± 1.7	26.7 ± 0.6
2	#4 BJSSL	*Fusarium* sp.	0	0	0	6 ± 1	11 ± 1	11 ± 1.2	12 ± 2
3	#5 BJSSL	*Alternaria* sp.	0	0	23 ± 3	52 ± 3	73 ± 3	83 ± 2.9	90 ± 2.9
4	#6 BJSSL	Zygomycetes	0	0	7 ± 3	22 ± 6	38 ± 10	47 ± 2.9	50 ± 2.9
5	#7 BJSSL	*Botryosphaeria* sp.	0	0	22 ± 3	42 ± 3	85 ± 9	92 ± 2.9	95.3 ± 0.6
6	#8 BJSSL	*Phaeoacremonim* sp.	0	17 ± 3	28 ± 5	57 ± 6	87 ± 3	96 ± 1.7	100 ± 0
7	#9 BJSSL	*Fusarium* sp.	0	0	12 ± 3	33 ± 3	68 ± 3	70 ± 0	71.7 ± 2.9
8	#10 BJSSL	*Fusarium* sp.	0	0	37 ± 6	46 ± 1	57 ± 3	72 ± 2.9	83.3 ± 2.9
9	#11 BJSSL	*Acremonium* sp.	0	0	12 ± 3	32 ± 3	52 ± 3	73 ± 2.9	81.7 ± 2.9
10	#12 BJSSL	*Nigrospora* sp.	0	0	0	0	5 ± 0	5 ± 0	6.67 ± 2.9
11	#14 BJSSL	*Alternaria* sp.	0	0	23 ± 3	37 ± 3	50 ± 5	57 ± 2.9	83.3 ± 2.9
12	#15 BJSSL	*Penicillium* sp.	0	0	7 ± 3	18 ± 3	33 ± 3	42 ± 2.9	48.3 ± 2.9
13	#17 BJSSL	*Penicillium* sp.	0	0	32 ± 3	44 ± 3	60 ± 3	72 ± 2.5	81.7 ± 2.9
14	#20 BJSSL	*Alternaria* sp.	0	0	7 ± 3	22 ± 3	38 ± 3	43 ± 2.9	45.3 ± 0.6
15	#34 BJSSL	*Curvularia* sp.	0	0	0	0	0	0	0
16	#54 BJSSL	*Trichoderma* sp.	0	0	0	0	0	0	0
17	#57 BJSSL	*Aspergillus* sp.	0	0	0	0	0	0	0
18	#72 BJSSL	Zygomycetes	0	0	8 ± 3	17 ± 3	23 ± 3	32 ± 2.9	33.3 ± 2.9
19	#8 BJSSS	Zygomycetes	0	0	0	0	0	0	0
20	#14 BJSSS	*Aspergillus* sp.	0	0	0	0	0	0	0
21	Control	No organism	0	0	0	0	0	0	0

[a]Mean values along with their standard error (±) are given in the table.
[b]Spore concentration = 1×10^6 spores/mL.

Evolutionary relationship of the selected isolate based on β-tubulin gene was inferred using the maximum parsimony (MP) method. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (20 replicates). The tree was drawn to scale, with branch lengths calculated using the average pathway method and were in the units of the number of changes over the whole sequence. The confidence interval of internal nodes was assessed by employing bootstrap analysis (1,000 replicates). Gaps and missing characters were excluded from the study [20-22].

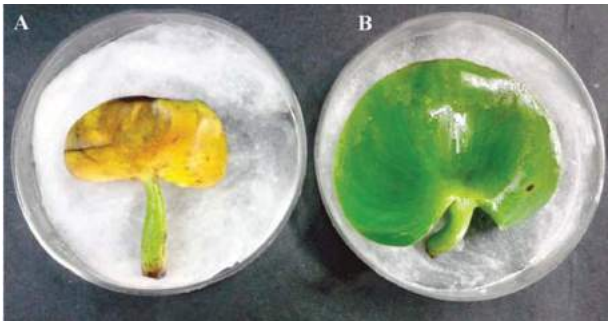

Fig. 1. *In vitro* detached leaf assay of spores of *Phaeoacremonium italicum* (#8 BJSSL) after 144 hpi. A, Test leaf showing damage; B, Control leaf.

RESULTS

Isolation of pathogenic fungi. Twenty fungal isolates were obtained from diseased leaves and stolons of *Eichhornia crassipes* collected from Harike wetland. These isolates comprised of 11 different genera (Table 1). The frequency of occurrence of *Fusarium* is 20% (04), followed by *Alternaria* with 15% (03) and *Penicillium* and *Aspergillus* species with 10% (02 isolates each). The other fungi which have been isolated are *Botryosphaeria* sp., *Pheoacremonium* sp., *Acremonium* sp., *Nigrospora* sp., *Curvularia* and *Trichoderma* sp.

Pathogenicity test on detached leaves. All the fungi recovered from diseased samples of *E. crassipes* were tested using *in vitro* detached leaf bioassay. Out of 20 isolates tested, only *Phaeoacremonium* species (#8 BJSSL) exhibited 100% kill after 144 hr post inoculation (hpi). The development of symptoms started after 24 hr and became prominent after 48 hr. The symptoms included chlorosis of the leaves and appearance of black and brown spots in due course of time due to necrosis (Fig. 1). The other isolates exhibiting pathogenic potential were *Botryosphaeria* species (#7 BJSSL) and *Alternaria* species (#5 BJSSL) which were exhibiting 95.3% and 90% killing of the leaves after 6 days (Table 1). Further higher AUDPC also indicated #8 BJSSL to be the most pathogenic isolate followed by #7 BJSSL and #5 BJSSL (Figs. 1 and 2). To date, *Phaeoacremonium* species has yet not been reported as a pathogen or a possible biological control agent on *Eichhornia crassipes*. Hence, *Phaeoacremonium* species #8 BJSSL was further selected to test its potential as an innundative biocontrol agent using *in vitro* whole plant bioassay.

Whole plant bioassay. In the whole plant bioassay, the disease onset began after 48 hpi. Fifty percent damage of

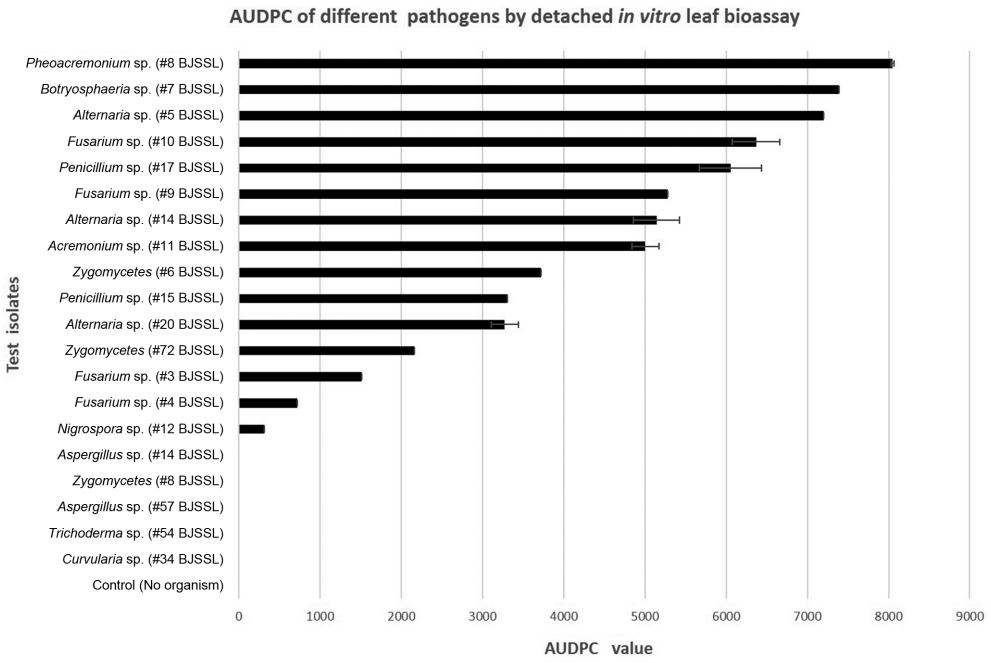

Fig. 2. Area under disease progressive curve (AUDPC) of different test pathogens by *in vitro* detached leaf bioassay.

the whole plants was observed at around 84 hpi and above 90% death by 144 hpi (Figs. 3 and 4). A significant difference was observed in AUDPC between the *in vitro* leaf assay and whole plant assay with values of 8,049.6 ± 14.6 and 7,842 ± 411.8, respectively. To further potentiate the pathogenic efficacy and process of disease development phytotoxic activity of the secondary metabolites of the test, isolate #8 BJSSL was again tested by *in vitro* detached leaf bioassay.

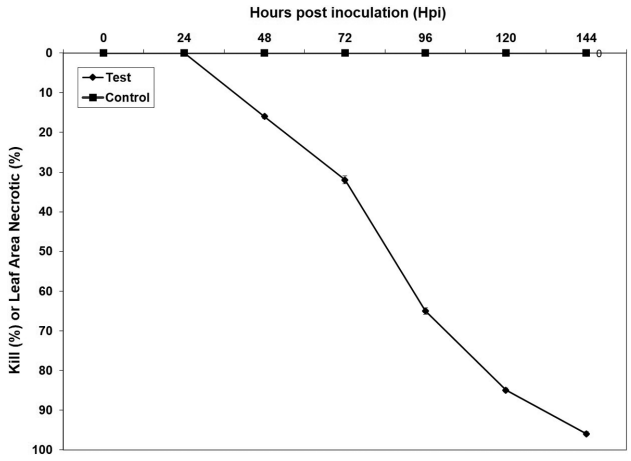

Fig. 3. Disease progress caused by spores spray (1×10^6 spores/mL) of *Phaeoacremonium italicum* (#8 BJSSL) during whole plant bioassay.

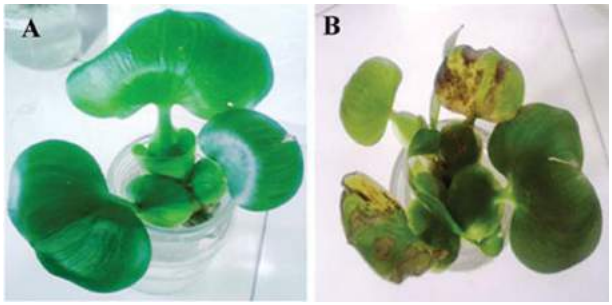

Fig. 4. Whole plant bioassay exhibiting the kill caused by spore suspension (1×10^6 spores/mL) of *Phaeoacremonium italicum* (#8 BJSSL). A, Control plant with no pathogenic symptoms; B, Test plant showing pathogenic symptoms developed post-inoculation.

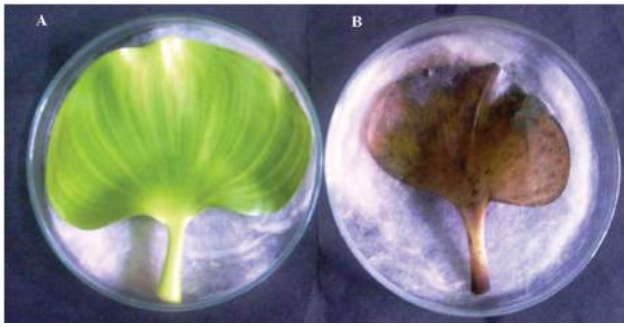

Fig. 5. *In vitro* detached leaf assay of culture filtrate of *Phaeoacremonium italicum* (#8 BJSSL) after 144 hpi. A,Test leaf showing damage; B, Control leaf.

***In vitro* detached leaf assay of culture filtrate of #8 BJSSL.** The *in vitro* leaf assay using cell free culture filtrate of #8 BJSSL also exhibited prominent phytotoxic effect. The symptoms appeared after 48 hpi and rapidly damaged the leaf causing chlorosis and necrosis at 96 hpi and complete death of leaves by 144 hpi (Fig. 5). The AUDPC values of *in vitro* leaf assay of spores and culture filtrate were quite similar viz. 8,049.6 ± 14.6 and 8,780 ± 320 suggesting that they could be mixed together to develop a potential formulation.

Morphotaxomony of #8 BJSSL. Colonies over PDA medium were moderately growing reaching a radius of

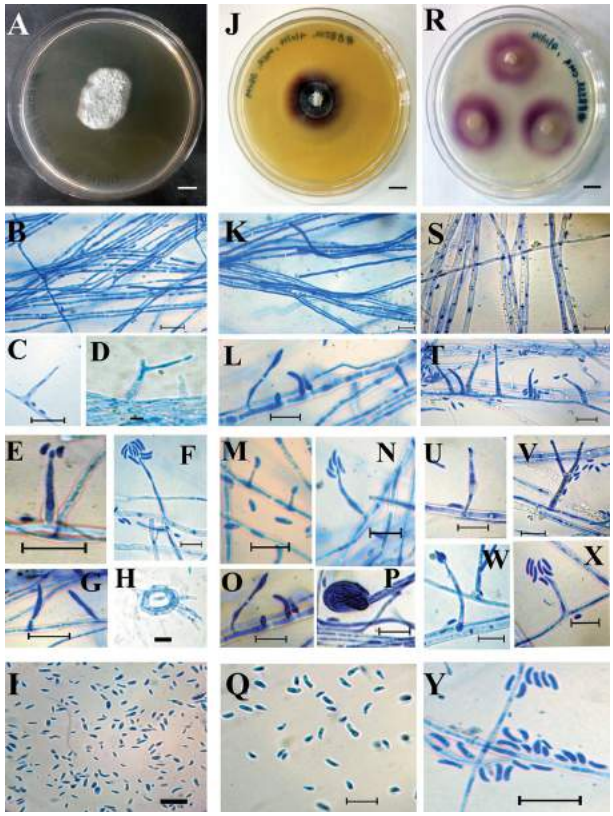

Fig. 6. Morphological and microscopic structures of *Phaeoacremonium italicum* (#8 BJSSL). A, J, R: Fourteen-day-old colonies at 28°C on potato dextrose agar (PDA) (A), malt extract agar (MEA) (J), and corn meal agar (CMA) (R) (scale bar = 10 mm). B~I, Structures on the surface of PDA: B, Hyphae over PDA medium; C, G, Unbranched conidiophore; D, Branched conidiophore; E, F, Adelophialides with conidia; H, Hyphal coils; I, Conidia; K~Q, Microscopic characters on produced over MEA; K, Hyphae; L, O, Unbranched conidiophores; M, N, Adelophialides with conidia; P, Hyphal coil; Q, Conidia; S~Y, Features produced over CMA; S, Hyphae; T, U, Unbranched conidia; V, Branched conidiophore; W~X, Adelophialides with conidia; Y, Conidia (scale bars = 10 μm).

12 ± 1.2 mm in 14 days at 28°C. Colonies were white in color, reverse vinaceous, flat, cottony at the centre and powdery with felty margins (Fig. 6A). Hyphae was found singly or in bundle of 6~7 (Fig. 6B). Conidiophores borne on the aerial mycelium were usually single and unbranched having an inflated bottom and tapering towards the edge (Fig. 6C~6G). Certain hyphal coils were also observed (Fig. 6H). The dimension of the conidia were (3.78) 6.22 (10.15) × (1.52) 2.03 (3.23) µm and were allantiod to obovate in shape (Fig. 6I).

Over MEA medium colonies reach a radius of 39.67 ± 1.15 mm after 14 days at 28°C. Colonies were olive grey to vinaceous colored, flat and felty margined (Fig. 6J). Mycelium consisting of branched septate hyphae measuring (0.99) 1.64 ± 0.37 (2.64) µm that occur singly or as bundle of 6~8 (Fig. 6K). The dimensions of conidiophore was [(3.35) 7.43 ± 2.48 (12.08)] × [(1) 1.54 ± 0.42 (2.89)] µm which were borne on the aerial mycelium and were usually single, unbranched, cylindrical, slightly inflated with narrow collarate (Fig. 6L~6O). Adelophialides were terminal, monophialadic, smooth and collarate (Fig. 6M and 6N). Several hyphal coils were also observed (Fig. G). Conidia [(2.48) 3.72 ± 0.69 (5.09)] × [(0.93) 1.14 ± 0.73 (1.87)] were single celled, allantoid to oblong-ellipsoidal in shape (Fig. 6Q).

Colonies on CMA medium were moderately growing (41.33 ± 2.08 mm), vinaceous colored, flat, with a felty margin (Fig. 6R). Mycelium consisting of long cylindrical and septate hyphae measuring (1.2) 2.22 ± 0.43 (2.96) µm that occured singly or as synmeta of 5~6 (Fig. 6S). The conidiophore measured [(6.96) 17.43 ± 4.67 (27.83)] × [(1.03) 1.75 ± 0.41 (2.17)] µm which arised from the aerial mycelium or the submerged hyphae, usually single, long, branched, slightly inflated at the bottom and collarate at the edge (Fig. 6T~6V). Adelophialides were terminal and collarate (Fig. 6W and 6X) conidia. The conidia measured [(2.29) 3.8 ± 0.91 (6.94)] × [(0.76) 1.11 ± 0.22 (1.52)] µm and were single celled and allantoid in shape (Fig. 6Y). Thus based on the above features, the isolate was identified as *Pheoacremonium italicum* sp.

Molecular identification of #8 BJSSL. The MP tree based on β-tubulin region resolved into 4 different clades of *Pheoacremonium* sp. Clade I comprised of eight strains of *P. italicum* and #8 BJSSL. Clade II grouped three strains of *P. alvesii*, Clade III grouped *P. ruberigenum* species and Clade IV clustered three strains of *P. scolyti*. The tree was rooted with *Lasiodiplodia gonubiensis*. Based on the phylogenetic tree of β-tubulin the correct speciation of the selected isolated #8 BJSS was confirmed as *P. italicum* (Fig. 7).

DISCUSSION

Over the years, several management strategies such as chemical, physical, and biological, etc. have been adopted to control water hyacinth. However, strategies like chemical treatment and physical removal have failed because of their hazardous environment effects and large-scale implementation problem. This calls for an alternative organic approach to clean up the water bodies. The biological method including usage of microorganism or their metabolites has attracted

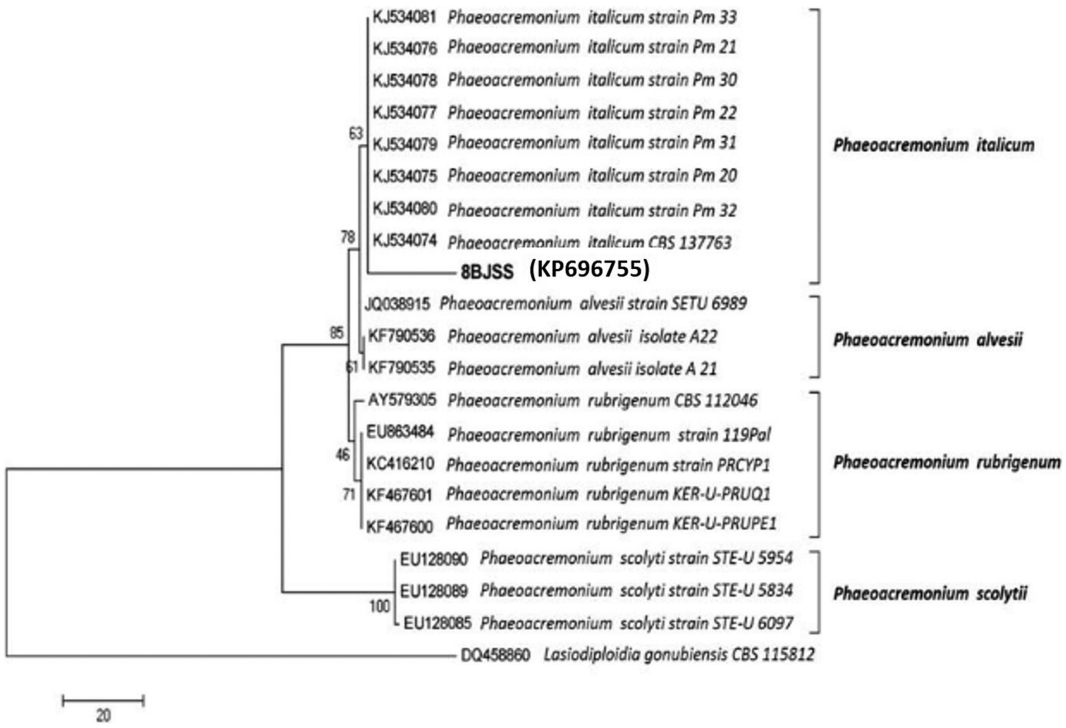

Fig. 7. Phylogenetic placement of #8 BJSSL as *Phaeoacremonium italicum*.

researchers to exploit them as a novel bio-resource for controlling this obnoxious weed [23]. The current study focuses on isolation and exploration of pathogenic mycobiota of water hyacinth to control them. During the study, 20 pathogenic fungal isolates belonging to 11 different genera were isolated. *Fusarium* was the most dominant fungal colonizer followed by *Aspergillus* and *Alternaria* sp. which are amongst the most common isolated plant pathogens. Fungal pathogens like *Acremonium*, *Alternaria*, *Fusarium*, *Aspergillus*, and *Penicillium* sp. have been previously reported from water hyacinth [8, 13]. Praveena and Naseema [12] documented isolation of 21 fungal isolates occurring on water hyacinth in Kerala which includes *Alternaria* sp., *Aspergillus* sp., *Curvularia* sp., *Fusarium* sp., *Nigrospora* sp. Shaker [24] isolated 20 fungal species from water hyacinth inhabiting in middle and south of Iraq of which *Alternaria alternata*, *Acremonium* sp., *Cladsporium herbarum*, and *Fusarium* sp. were the most frequently isolated species. During the study, fungal species like *Botryosphaeria* and *Phaeoacremonim* sp. have been recovered which were never reported earlier from water hyacinth to best of our knowledge. In the screening studies through *in vitro* detached leaf assay, we have found that spore suspension of *Phaeoacremonim* sp. (#8 BJSSL) exhibited maximum leaf damaging property causing 100% killing in 144 hpi. Similarly, *Botryosphaeria* sp. (#7 BJSSL) and *Alternaria* sp. (#5 BJSSL) also caused 96% and 90% killing of the leaves after 144 hpi. Further, spore suspension of #8 BJSSL also exhibited similar activity in whole plant bioassay. El-Morsy [13] screened 22 fungal isolates for their ability to infect water hyacinth out of which *Alternaria alternata*, *Drechslera hawaiiensis*, and *Ulocladium atrum* showed 79%, 78%, and 70% tissue death after four weeks post inoculation. Fungal species like *Myrothecium advena* and *Fusarium pallidoroseum* caused over 50% infection of the weed [12]. Shaker [24] found that *Alternaria alternata*, *Acremonium* sp., and *Phoma eupyrena* were the most aggressive fungal isolate to water hyacinth causing 91.66%, 83%, and 75% damage in pathogenicity test. During our study, the development of pathogenic symptom was assessed using AUDPC. The AUDPC analysis provides the rate of disease severity during a time course as a single unit and therefore is considered as an appropriate measure in studying the disease development in the plants. When tested on whole plants of water hyacinth, *P. italicum* (#8 BJSSL) was causing dieback symptoms leading to the death of the plant. A significant difference in the AUDPC values of the *in vitro* leaf assay and the whole plant assay indicated the role of inherent plant mechanisms which resist the process of disease development. This could probably be due to development of physiological or genetic resistance mechanisms which delays the onset of disease development. Production of phytoalexins in response to pathogens is also a mechanism of resisting disease development [25-27]. In an attempt to understand the role of phytotoxins produced by *P. italicum*, the culture filtrate of #8 BJSSL was also tested. This could be used in development of suitable mycoherbicidal formulations. Further, based on classical and molecular tools, the isolate #8 BJSSL has been identified as *P. italicum*. Similar morphological structures were produced by *Phaeoacremonium* species associated with olive wilt [19]. This is the first record of its occurrence as a pathogen on *Eichhornia crassipes* from Harike wetland, Punjab, India as well as a possible biocontrol agent. Considering the importance of water hyacinth as a problematic weed, *P. italicum* (#8 BJSSL) may have potential benefits also for biological control, when its host specificity and pathogenicity has been tested at physiological as well as genetic levels in plant and animal systems.

ACKNOWLEDGEMENTS

The authors are thankful to Head, Department of Biotechnology, Thapar University, Patiala, Punjab, India for providing the necessary infrastructure to carry out the research work. Vineet Meshram is thankful to University Grants Commission (UGC), Govt. of India for Rajiv Gandhi National fellowship (F.16–1866(SC)/2010/SA–III).

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