CHAPTER 2:

SURVEY FOR COLLECTION OF DISEASED LEAVES AND ISOLATION, PURIFICATION AND IDENTIFICATION OF FUNGAL STRAINS FROM WATERHYACINTH

2. SURVEY FOR COLLECTION OF DISEASED LEAVES AND ISOLATION, PURIFICATION AND IDENTIFICATION OF FUNGAL STRAINS FROM WATERHYACINTH

2.1. Introduction

The negative impact of the infestation of waterhyacinth has drawn the need for controlling the invasiveness of the weed by several manual, mechanical, chemical, biocontrol or integrated means. These methods implemented to control unrestricted spread of the weed have produced mixed results (Charudattan et al. 1996). The hazardous short-term effects of use of chemicals (Dagno et al. 2012) for weed control and laborious mechanical control have led on to a myriad of ongoing research on trying to control it through various means including biocontrol (Sushilkumar 2011). Biocontrol agents including arthropods and plant pathogens have been released around the world to control the weed in most economical and sustainable manner (Ray and Hill 2013, Firehun et al. 2015) and arthropod agents being very limited, the use of plethora of phytopathogens have proved to be very promising, in order to control this macrophyte.

Studies show that together with other biotic and abiotic stress factors, pathogens can cause significant reduction in waterhyacinth biomass, especially following natural outbreaks of arthropods or pathogens (Ray and Hill 2012). Several studies on isolation, identification and biocontrol potential of the fungi have been tested in various infested areas worldwide (Freeman et al. 1981, Naseema and Balakrishnan 2001, Daddy et al. 2003, Praveena and Naseema 2006, Okunowo et al. 2008, Ray 2008). However, the continual survey and lookout of new species can reduce the stress on limited species and broaden the list for agents against this weed, apart from some of the most commonly used microbial biocontrol agents like various species of *Alternaria, Acremonium, Fusarium*, etc (Ray and Hill 2012a). Pathogens, which are also native or endemic, are comparatively found to be more potent and ideal for the development of biocontrol agents (Cuda et al. 2008). Damage by pathogenic fungi to waterhyacinth plants (De Jong and De Voogd 2003) leave the water surface clear of the weed. So, an increase in the array of such pathogens only adds to the hope of better and effective control. Hence, an initiative has been undertaken to enhance the

biodiversity of phytopathogens from waterhyacinth in India, to control the macrophyte, with better efficacy.

Further proper identification of the phytopathogens was deemed necessary, to understand their uniqueness, their functional and structural importance and forthcoming characteristics. Although several fungal species can be easily differentiated morphologically based on some of the notable diagnostic characteristics' marks (Humber 1997), but for detailed and identification till the species level molecular techniques are essential (Dutta et al. 2015, Firehun et al. 2017). Introduction of digital images of the microscopic view of the fungi gives more accuracy than the hand-drawn version of microscopic images (using camera-lucida), which were used previously. With this introduction of digital imaging, procedures involving the development of slides, needs to be done with more accuracy and tactfully, ensuring collection of spores or hyphae of fungi from particular growing region of the plates, etc. Using proper dye for visualization of the microorganism and using the microscope with right aperture, focal length and light/ electron to be passed also hence becomes of utmost importance.

To understand with precision, where morphological identification using taxonomic analysis, slowly fell short, use of molecular techniques took its place (Borman et al. 2008), based on PCR amplification of the conserved regions of the fungal genome and sequencing of the resultant PCR products (Haynes at al. 1996, Makimura et al. 1994, Sandhu et al. 1995). Further to the precautions maintained for morphological identification, identification using molecular techniques also required upholding of some safety measures, like making contaminant-free culture, choosing the right primers, cross-checking the purity of the sample with proper assays and/or use of proper bioinformatics tools to analyze and also hint at the relatedness among them.

The present chapter reports six years study during which we surveyed water bodies in and around Kolkata, India. Interestingly waterhyacinth was first introduced into the country in 1889 as an ornamental plant in the AJC Bose Botanical Garden (then Royal Botanical Garden), Kolkata (Gopal 1987). We isolated and identified the indigenous fungal pathogens associated with the weed and subjected them to biocontrol potential and host range studies (which will be covered in the **Chapter 3**, following this).

2.2. Materials and methods

2.2.1. *Site of collection*

Periodical surveys were conducted by visiting various waterhyacinth infested sites. The waterhyacinth leaves bearing damage or disease symptoms, suspected to be from fungal phytopathogens, were collected from various infested sites in and around Kolkata (22.5726° N, 88.3639° E) and some areas of Santiniketan (Coordinates: 23.6700° N, 87.7200° E) during the period of 2014 to 2019 covering wide geographical areas of the states with different climatic conditions. The sites were then mapped using DIVA-GIS software version 7.5.

Disease symptoms bearing plant and leaf were wrapped in layers of dry paper toweling, kept in paper bags and brought to the laboratory, as specimens, for isolation and pathogenicity testing. This helped to absorb the moisture content to prevent secondary microbial growth.

2.2.2. *Isolation and purification of pathogens*

2.2.2.1. **Isolation of pathogens**

The diseased, symptom-bearing, leaves were washed vigorously under running tap water to remove unwanted soil particles, brought along from the field of collection. The potential fungal agents were isolated by transferring disease marks (in forms of necrosis and/or chlorosis) on the leaves to prepared media plates. Approximately 2 square mm cross-sectional segments of the diseased leaves and petiole were sliced superficially along the margins of diseased lesions and surface is decontaminated by sequential immersion in 70 % ethyl alcohol. This helps to improve the sodium hypochlorite (NaOCl) penetration, as about 1.0 % sodium hypochlorite (v/v) helps to eliminate superficial contaminating propagules and, then at the end, three times in sterile distilled water to remove the traces of the disinfectants used (Ray and Hill, 2012). The medium for the isolations was Potato Dextrose Agar (PDA) (Merck) and supplemented with chloramphenicol (10 % w/v), to reduce bacterial contamination, in Petri dishes (**Annexure I-1.2**), and incubated at 27 °C, under sterile conditions. Isolations were also made on Rose Bengal Chloramphenicol agar (Merck) or waterhyacinth extract dextrose agar (WhDA) plates (**Annexure I-1.2**).

2.2.2.2. **Culturing and subculturing**

Streak-plate and subculturing techniques (Agarwal and Hasija 1986) were used for purification of the fungal pathogens from waterhyacinth. The edges around the growing zones of fungal colonies were isolated and transferred to PDA or malt yeast extract agar (MEA) (Merck) plates, as per sporulation. Fungi were cultures repitatively until pure cultures were obtained. Cultures with cross-contamination with other fungus were sub-cultured and purified. This process was continued until monoculture of the particular fungus was obtained per plate.

2.2.2.3. **Maintenance of isolates**

After purification the fungal cultures of isolates were marked with a lab number (like WHK-) and multiplied on PDA plates. The inoculated slants were incubated in the BOD at 27 ± 10 °C. Routinely transfer was done into fresh slants, to ensure their survivability and change of media, to thrive on.

PDA-prepared slants were used for short-term maintenance of the stock cultures of the isolates and the slants were supplemented with 10 % WhDA and MEA media and then refrigerated and stored at 4–6 °C. The Petri plates or slants were tightly sealed with Parafilm (Merck) to ensure contamination-free storage.

Apart from normal refrigeration, for long-term maintenance, the following procedures were undergone:

- Freezing at low temperature range from -20 to -80 °C, where the cultures grown on agar slant in screw-capped test tubes were placed directly in -20 or -80 °C refrigerator (Carmichael 1956). Some slow to non-sporulating fungi were scraped from media plates, placed in sterile cryotubes, containing 10 % glycerol in water was refrigerated at -80 °C (Nakasone et al. 2004).

- Liquid Nitrogen: Living cells are hugely affected by thawing and freezing, for that storing in liquid nitrogen is a great option. For non-sporulating fungi or fungi with mycelial growths, fungi transferred (with the help of sterile inoculating loop) in a 2 ml sterile screw-capped polypropylene vials, filled with 0.5-1.0 ml sterile 10 % glycerol, were placed directly in the -170 °C vapor phase of liquid nitrogen tank (Nakasone et al. 2004). The frozen marked preparations were later retrieved from

the freezer and quickly thawed in 37 °C water-bath. The thawed agar plugs were transferred in appropriate media plates and then used after 2-7 days.

To retrieve a culture in usable condition from refrigeration, a part of it is pricked and they are placed on growing media and left to grow in the BOD incubator at 27 ± 10 °C

2.2.3. *Identification of fungal strains*

The fungi were initially identified on the basis of the visual cues like their growth (from plate macroscopic and microscopic) characteristics (Barnett 1960, Ellis 1971, 1976), conidial measurement, sporulation and pigmentation production on growth media (Domsch et al. 2007, Gilman 1959, Holliday 1993, Robinson 2011, Watanabe 2010). But morphological characters can confirm only till the genus level. For more specific identity, molecular characterization is necessary. The isolates showing higher potential against the host weed, waterhyacinth (evaluated on the basis of the initial pathogenicity trials) was molecularly characterized using 18S rDNA molecular technique and the fungi with the best result, its conidia and spores were observed under scanning electron microscope (SEM) for further confirmation.

2.2.3.1. **Growth estimation and morphological identification**

Growth, of the fungal cultures, was measured as a mean diameter increment of the growth-covered zone, after 14 days of incubation at around 25 °C on the respective agar media in Petri-plates (of diameter 100 mm). The macroscopic features, like colony colour, reverse-plate colony colour, texture of the mycelium, adds a further insight on to the categorizing of the fungi. Further presence and the colouration of several metabolites (enzymes, secondary metabolites, etc.) production on the growing Petri-plates (Homolka 2013) or other characteristics helps morphologically to estimate the fungi to the macroscopic extent.

Understanding the phytopathogens to macroscopic extent cannot reach the concluding statement, until microscopic features (like hyphal branching, conidia structures, presence or absence of clamp connections or hyphal vacuolization, etc.) are understood. Hence, the fungal strain isolated from infected waterhyacinth, was morphologically identified by slide cellophane (Butler and Mann, 1959) and slide culture technique (Harris 1986) with lactophenol as mounting medium and observed

under Zeiss Axio Scope.A1 Microscope for morphological identification of the genus using pictorial book references (Agarwal and Hasija 1986, Ainsworth et al. 1973, Barnett 1960, Barnett and Hunter 1972, Holliday 1993, Robinson 2011, Watanabe 2010). The conidial surfaces obtained from cultures were later analyzed for scanning electron microscopy (SEM) following Melo and Faull's method (Melo and Faull, 2004).

Slide cellophane technique: The sticky portion of the cellophane tape is made to come in contact where the fungus is growing and sporulating sparsely, since satisfactory slides can seldom be made from crowded, dense areas of growth. Sufficient pressure is applied to pick up the fructifications without detaching spores from sporophores. The pressure to apply will vary with different fungi. Then the cellophane tape bearing the fungi is placed slowly along the slide, with the sticky end facing upwards. Few drops of lactophenol as a mounting medium is added after which a clean cover glass is placed. Finally, the slide is observed under a light microscopy using either high dry or oil immersion object. Observation with the dissecting microscope is helpful in observing and identifying of fungi.

Slide culture technique: Aseptically, with a pair of forceps, a sheet of sterile filter paper was placed in a Petri dish followed by placing a sterile U-shaped glass rod on the filter paper. (Rod was sterilized by flaming, and then held by forceps). Enough sterile water (about 4 ml) was poured on filter paper to completely moisten it. With forceps, a sterile slide was then placed on the U-shaped rod. With a sterilized scalpel a 5 mm square block of the medium from the plate of Sabouraud's agar or PDA medium was cut and placed carefully aseptically to the centerof the slide. Once placed, spores or mycelial fragments of the fungus, to be examined, were inoculated on the agar media. Aseptically, a sterile cover glass on the upper surface of the agar cube was then slowly placed. The cover on the Petri dish is placed and the entire set-up incubated at room temperature for 48 hours. 48 hours after incubation, the slide was examined under low power. If growth occurred there would be growth of hyphae and production of spores. If growth is inadequate and spores are not evident, the fungi are allowed to grow for another 24–48 hours before making the stained slides. A drop of lactophenol cotton blue stain was then placed on a clean microscope slide. Once grown, the cover glass from the slide culture was removed and the block of agar discarded. A drop of 95 % ethanol was added to the hyphae on the cover glass. On evaporation, the cover glass,

with mold side down, was placed on the drop of lactophenol cotton blue stain on the slide and examined under a microscope.

Scanning electron microscopy (SEM) technique: The 5 to 7 days old fungal culture on PDA plates grown at around 27 °C were used. About 5 x 10 mm of the culture growing in the aforesaid culture were taken and primarily fixed with 2.5 % glutaraldehyde solution overnight at 4 °C refrigeration. Next morning, there were washed with 0.1 M sodium phosphate buffer solution (pH 7.2) (**Annexure I-1.1**) thrice, 20 min each. There were post-fixed with buffer 1.0 % OsO_4 for 2 hours (Melo and Faull 2004). The samples after which were dehydrated in an ethanol series upgradation (30 %, 50 %, 70 % and 95 %), dipped for 20 min in each alcohol dilution and 45 min for the final absolute alcohol (Sharma et al. 2017). Then samples were dried in liquid carbon dioxide. The samples were then mounted on silver stub and gold-sprayed by cathodic spraying. Morphology of the fungi were observed on Zeiss EVO 50 scanning electron microscope, operating at 20.00 kV.

2.2.3.2. **Molecular identification**

Ensuring further confirmation, molecular identifying technique using 18S rDNA technique were implemented, to analyze the sequences and then with the help of some bioinformatics tools deciphering the fungi till the species level.

DNA isolation

For DNA isolation, Erlenmeyer flasks containing 150 ml of potato dextrose broth (PDB) (Himedia) was inoculated from the margin of 8-days-old PDA cultures for the fungal isolate and incubated at 28 ^{o}C and 100 rpm. After 5 to 6 days of growth, the contents of the flasks were filtered through sterile cheesecloth and rinsed three times with sterile, cold deionised water. Then the fresh mycelium was frozen in liquid nitrogen and ground to a fine powder, and genomic DNA was extracted by the method of Flores et al. (1997). The DNA solution of each isolate was diluted to 50 ng/mL and kept at -20 ^{o}C until further use.

Quantification and quality assessment of DNA

The DNA stock was quantified using Nanodrop spectrophotometer at 260 and 280 nm, where the concentration and purity of the DNA was checked under the UV

absorbance. The purity was judged on the optical density (OD) ration at 260:280 nm. 1.8-2.0 is considered to be the best range for the purity of DNA. Concentration was calculated using the following formula,

Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor

To check the quality and purity of the DNA, agarose gel electrophoresis was performed. Agarose 0.8 % (w/v) in 0.5X TAE (pH 8.0) buffer (Sambrook and Russel, 2001) was used for submarine gel electrophoresis. Ethidium bromide (1 %) was added at 10 µl /100 ml. The wells were charged with 5 µl of DNA preparations mixed with 1 µl gel loading dye. Electrophoresis was carried out at 80 V for 30 min at room temperature. DNA was visualized under UV using UV transilluminator. The DNA was further used for Polymerase Chain reaction (PCR).

PCR amplification, visualization and purification

18S RNA gene fragment was amplified by PCR from genomic DNA using 18S gene universal primers: 1F and 4R

1F CTGGTGCCAGCAGCCGCGGGYAA

4R CKRAGGGCATYACWGACCTGTTAT

Amplified PCR product was then purified using Xcelgen Gel extraction kit, to remove contaminants. To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2 % agarose gel containing ethidium bromide (1 % solution at 10 µl/100 ml) at constant 5 V/cm for 30 min in 0.5 X TAE buffer. The amplified product was visualized as a single compact band, of expected size (approx. 850 bp), under UV light and documented by gel documentation system (Bio-Rad).

Sequencing of purified DNA

The concentration of the purified DNA was determined and subjected to automated DNA sequencing on ABI 3730xI Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturer's instructions. After cycling, the extension products were purified and mixed well in 10 µl of Hi-Di formamide. The contents were mixed on

shaker for 30 min at 300 x g. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95 ^{o}C for 5 min, snap chilled and loaded into autosampler of the instrument.

Sequence and phylogenetic analysis

Consensus sequence, of 18S gene in SSU region, generated from forward and reverse sequence data using aligner software, were compared with those deposited in the National Centre for Biotechnology Information (NCBI) site using the Basic Local Alignment Search Tool for nucleotides (BLAST) with the nr database of NCBI GenBank database. The closest matches were used to identify the major group of fungi to which each sequence belonged.

After BLAST, based on maximum identity score, first fifteen sequences were selected and aligned using multiple alignment software program CLUSTALW (Thompson et al. 1994). After alignment with CLUSTALW from the MEGA6 software package (Tamura et al. 2007), phylogenetic reconstruction of the dataset was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree, inferred from 1000 replicates, was taken to represent the evolutionary history of the taxa analyzed. Branches, corresponding to partitions reproduced in less than 50 % bootstrap replicates (BP), were collapsed. The percentage of replicate trees with the associated taxa, clustered together in the bootstrap test (1000 replicates), were denominated next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA6.

2.2.4. *Frequency of occurrence of fungal isolates*

The frequency of appearance of fungal species, isolated from various infested sites of waterhyacinth, were counted with respect to the other genera. The frequency percentage of each genus was calculated in terms of the total number of fungal isolates via this calculation (Ray and Hill 2013a):

$$Frequency\ (\%) = \frac{Number\ of\ isolates\ in\ a\ genus\ \times 100}{Total\ number\ of\ isolates}$$

Statistical analysis

The experimental data were analysed using software SPSS version 25. The frequency fungal isolates data were analyzed using Chi square test.

2.3. Results

2.3.1. *Site of collection and collection of diseased leaves*

During the course of field visits at regular frequent intervals, samples of several, potent fungal pathogen-infected, waterhyacinth leaves were collected from the field, covering several places in and around Kolkata and some places of Shantiniketan, West Bengal, India (**Table 2.1, Figure 2.1**). Diseased lesions showing leaf spots, necrotic spots, blights and rots of any shapes and sizes and dieback symptoms (**Figure 2.2**) were collected.

TABLE 2.1: Waterhyacinth infested sites surveyed from 2014–2019 for the collection of the fungi

SL NO	SITE	LATITUDE (° N)	LONGITUDE (° E)
1	Kandua	22.574627	88.182184
2	Santragachi	22.581372	88.282796
3	Nischinda, Bally	22.655332	88.323845
4	10 No. Rajabagan	22.652371	88.370132
5	Notunpara, Baruipur	22.371033	88.442811
6	Surjopurhat, Baruipur	22.301128	88.464596
7	Dakshin Barasat, S-24 Pgs	22.225514	88.445575
8	Botanical Garden, Shibpur	22.558687	88.291121
9	Rajchandrapur, Bally	22.655029	88.317249
10	Par Dankuni, Hoogly	22.697702	88.296937
11	Uluberia	22.471215	88.094221
12	Bikrampur	22.457071	88.396385
13	Kacharpatty, Santiniketan	23.660825	87.702449
14	Suripukur, Santiniketan	23.66152	87.687244

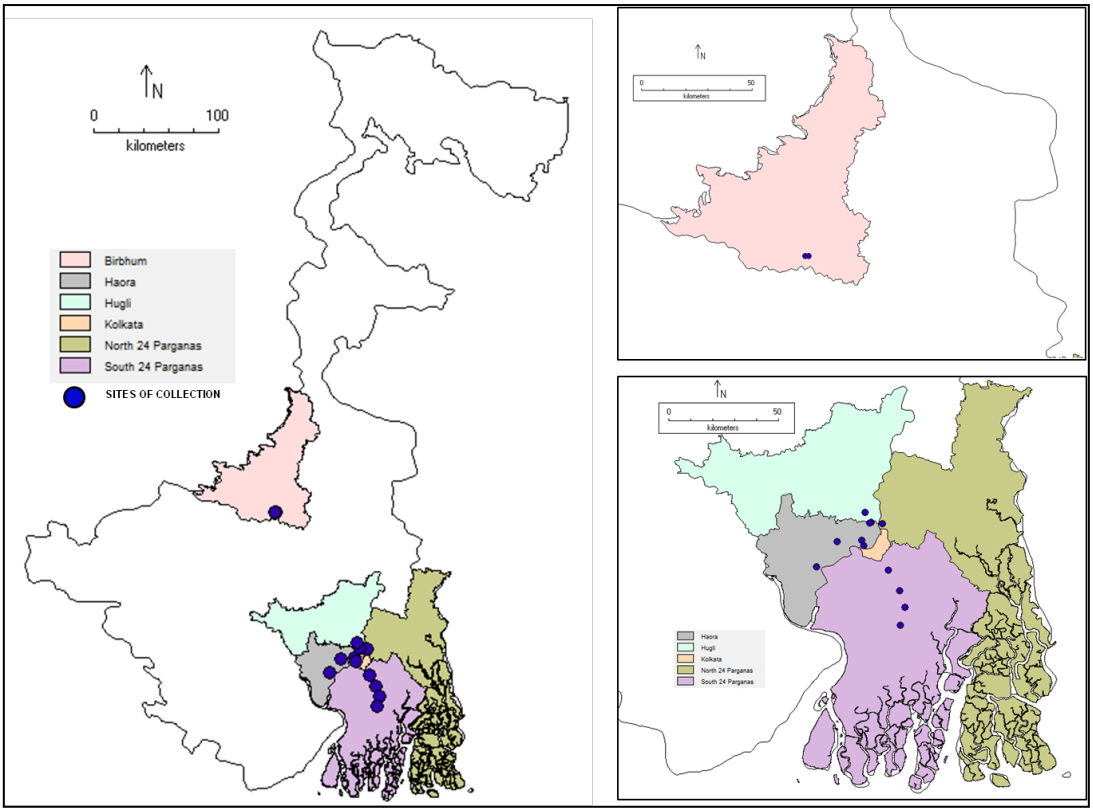
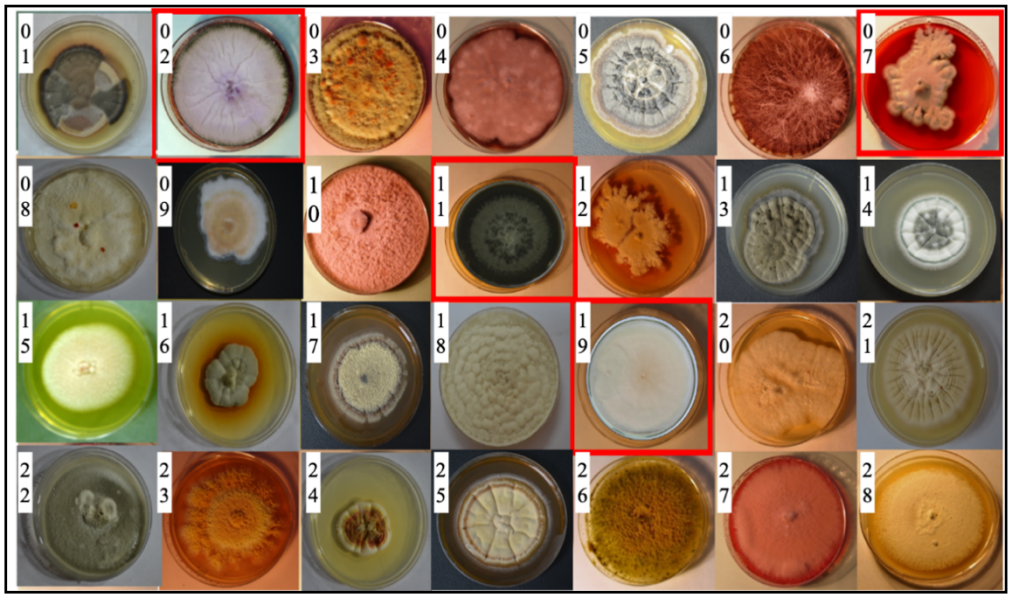


Figure 2.1: Sites of collection of infected waterhyacinth leaves in and around Kolkata and Birbhum

Figure 2.2: Different kinds of lesions and symptoms of infection on waterhyacinth leaves

2.3.2. *Isolation, purification of fungal strains from waterhyacinth*

Following culturing and sub-culturing techniques, pure monoculture forms of several fungi were cultured (**Figure 2.3**). 152 isolates, belonging to more than 25 genera (**Table in Figure 2.3**) were purified from diseased and infected plant parts. Many were not considered further because they either failed to grow, were contaminated, or belonged to the non-pathogenic and/or non-sporulating sterile group. Apart from that, some rapid growing isolates on PDA plates, mostly those of *Aspergillus, Penicillium* and *Rhizopus*, were also not further considered. The rate of growth helped to categorize the phytopathogen on the basis of sterile, fast-growing fungi, usually as a result of secondary infection of the plant leaves.



Sl no.	Isolate no.	Fungal isolate
01	WHK-28	*Curvularia* sp.
02	WHK-59	*Fusarium oxysporum* sp. *lycopersici*
03	WHK-99	*Fusarium* sp.
04	WHK-46	*Alternaria* sp.
05	WHK-138	*Pleospora herbarum* (Pers.) Rabenh.
06	WHK-97	*Pleospora herbarum* (Pers.) Rabenh.
07	WHK-3	*Alternaria alternata* (Fr.) Keissler
08	WHK-106	*Fusarium oxysporum f* sp. *cumini* strain F11
09	WHK-104	*Arthrinium hispanicum* Larrondo & Calvo
10	WHK-42	*Alternaria alternata* (Fr.) Keissler
11	WHK-37	*Paradendryphiella salina* (G.K. Sutherland) Woudenberg & Crous
12	WHK-141	*Alternaria alternata* (Fr.) Keissler
13	WHK-152	*Stemphylium* sp.
14	Sterile fungi	*Pencillium* sp.

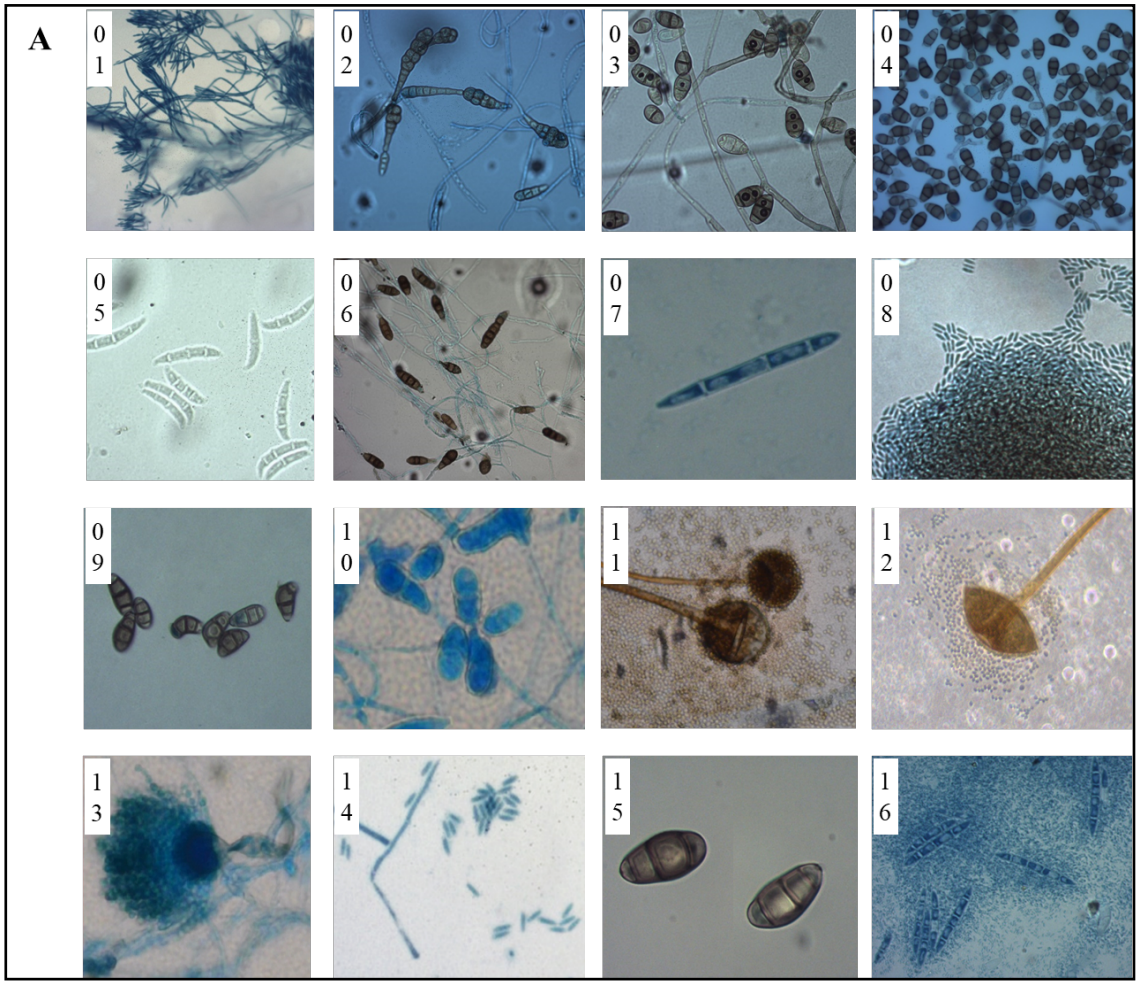
Sl no.	Isolate no.	Fungal isolate
15	WHK-17	*Acremonium* sp.
16	WHK-38	*Alternaria alternata* (Fr.) Keissler
17	WHK-88	*Fusarium* sp.
18	WHK-30	*Myrothecium* sp.
19	WHK-74	*Fusarium verticillioides* (Sacc.) Nirenberg
20	WHK-89	*Cercospora* sp.
21	Sterile fungi	*Aspergillus* sp.
22	WHK-142	*Nigrospora* sp.
23	WHK-19	*Trichothecium* sp.
24	WHK-96#	*Setosphaeria rostrata* K.J. Leonard
25	WHK-20	*Rhizoctonia* sp.
26	WHK-23	*Cochliobolus geniculatus* R.R. Nelson
27	WHK-43	*Alternaria alternata* (Fr.) Keissler
28	WHK-12	*Alternaria japonica* Yoshii 1941

Figure 2.3: Pure cultures of some fungal flora isolated from waterhyacinth with their tabulated legend

2.3.3. *Identification of the fungi*

The fungal colony growth characteristics based on their growth rate, colony plate and reverse plate colony characteristics, metabolite production on plates (**Figure 2.4**), along with microscopic view (be it under compound microscope or scanning electron microscope) of the conidial structure, hyphal branching (**Figure 2.5 A, B** and **C**) and other fine details of the fungi helps to morphologically categorize many fungal specimens to their closely related genera, if not to their species level.

Figure 2.4: Release of red-coloured pigment (metabolite) in the growing plates, along the rim of the growing fungi- *Altenaria* sp.



Sl. no.	Isolate no.	Fungal isolate
01	WHK-30	*Myrothecium* sp.
02	WHK-03	*Alternaria alternata* (Fr.) Keissler
03	WHK-37	*Paradendryphiella salina* (G.K. Sutherland) Woudenberg & Crous
04	WHK-46	*Alternaria* sp.
05	WHK-74	*Fusarium verticillioides* (Sacc.) Nirenberg
06	WHK-97	*Pleospora herbarum* (Pers.) Rabenh.
07	WHK-59	*Fusarium oxysporum* sp. *lycopersici*
08	WHK-106	*Fusarium oxysporum f* sp. *cumini* strain F11
09	WHK-28	*Curvularia* sp.
10	WHK-19	*Trichothecium* sp.
11	Sterile fungi	*Aspergillus* sp.
12	Sterile fungi	*Aspergillus* sp.
13	Sterile fungi	*Aspergillus* sp.
14	WHK-17	*Acremonium* sp.
15	WHK-23	*Cochliobolus geniculatus* R.R. Nelson
16	WHK-88	*Fusarium* sp.

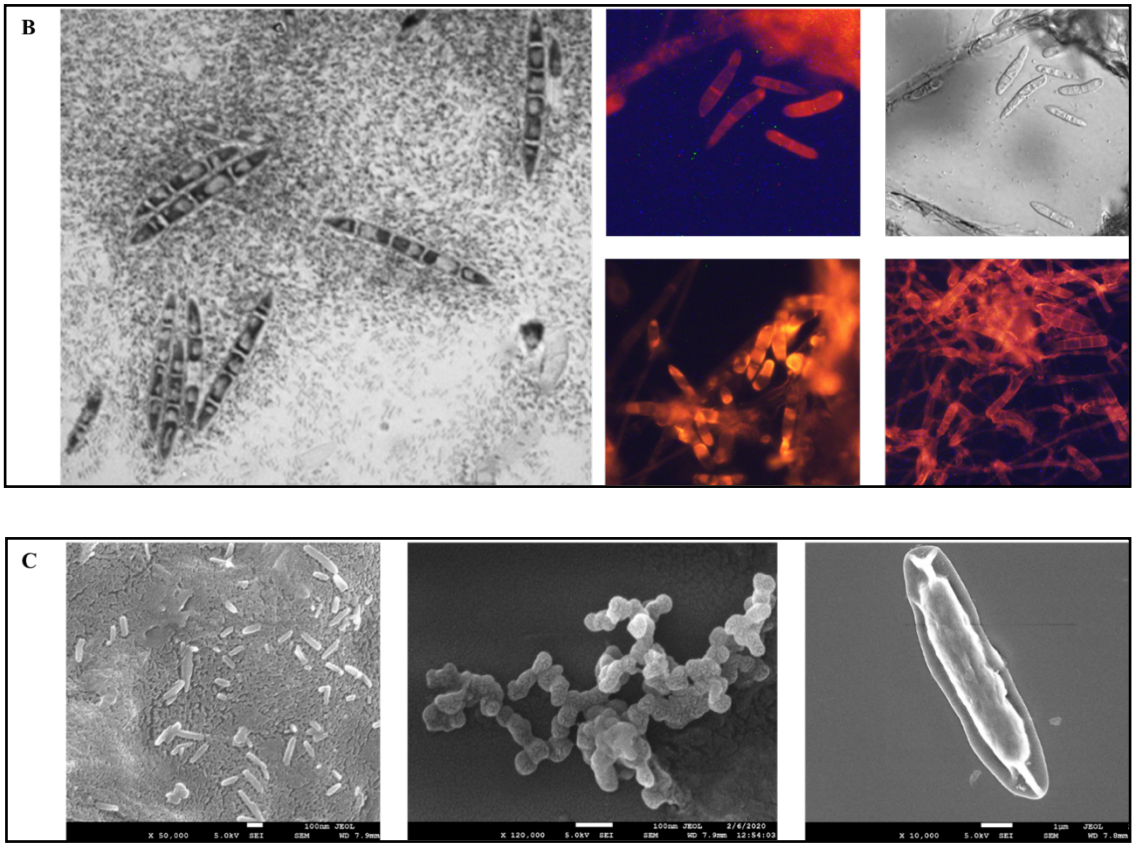


Figure 2.5 A: Microscopic images of various fungi collected from waterhyacinth infested leaves, observed under compound microscope, after lactophenol and glycerol staining. The respective fungi have been highlighted in the table following it. **B**: A particular fungus (here strain WHK-59), undergoing microscopic image revelation, for proper focusing on the spores. The enlarged image gives a view of the fungi under compound microscope (in grayscale); other images, are after staining with Congo-red, where the stain highlights just the spores and contour of the spores and its density. **C**: Images of fungi of WHK-59 strain, under scanning electron microscope.

Morphological and molecular analysis grouped the phytopathogens into 17 genera (**Table in Figure 2.3**). Among the plethora of fungal strains isolated, many phytopathogens, were previously reported from waterhyacinth, but *A. hispanicum*, *C. geniculatus*, *F. oxysporum* sp. *lycopersici* 4287, *F. oxysporum* f sp. *cumini* strain F11, *F. ventricosum*, *F. verticillioides and S. rostrata* are reported for the first time from the survey.

The 20 most potent phytopathogenic fungi (discussed in the upcoming **Chapter 3**), were further identified and characterized by using 18S rDNA molecular techniques.

The DNA bands of the isolates were around 900 bp (**Figure 2.6**), with A260/A280 within 1.8 to 2.0, in a nanodrop, confirmed its purity. Nucleotide BLAST of the consensus sequences identified the isolates with about 99 % precision level. The indistinguishable morphological and phenotypic differences were cleared with DNA sequencing data analysis.

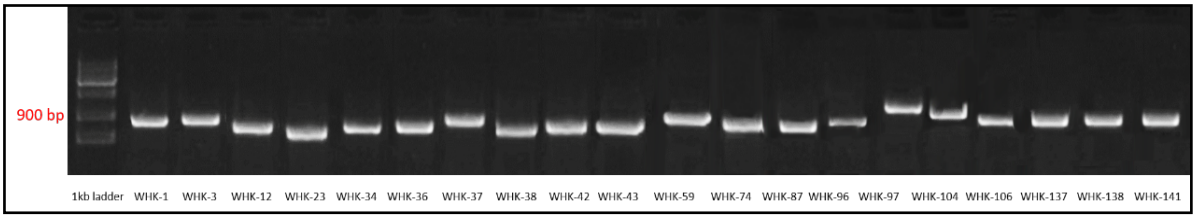


Figure 2.6: Validation of PCR using 18S rDNA gene universal fungal primers 1F-4R for the 20 potent fungal isolates. A 1kb DNA ladder was used as marker. Amplicon size is around 900 bp.

1F CTGGTGCCAGCAGCCGCGGYAA

4R CKRAGGGCATYACWGACCTGTTAT

The phylogenetic tree of the 20 potent isolates (**Figure 2.7**) broadly divided into two main clades. The most evolved clade includes *F. verticilloides* 7600 (WHK-74 and WHK-87), *F. oxysporum* sp. *lycopersici* 4287 (WHK-59), *F. oxysporum* f. sp. *cumini* strain (WHK-106) and *Arthrinium hispanicum* strain IMI 326877 (WHK-104). Another clade consists of mostly consists of the different strains of *Alternaria* sps. - *A. japonica* (WHK-12), *A. alternata* strain HA4087 (WHK-34; WHK-42; WHK-141), *A. alternata* strain FC007 (WHK-3), *A. alternata* strain S-f6 (WHK-43), *A. alternata* ATCC 28329 (WHK-38) and its close related species *Ulocladium botrytis* (WHK-36), *P. salina* (WHK-37), *Cochliobolus geniculatus* (WHK-1, WHK-23), *Pleospora herbarum* (WHK-97, WHK-137, WHK-138) and *Setosphaeria rostrata* (WHK-96).

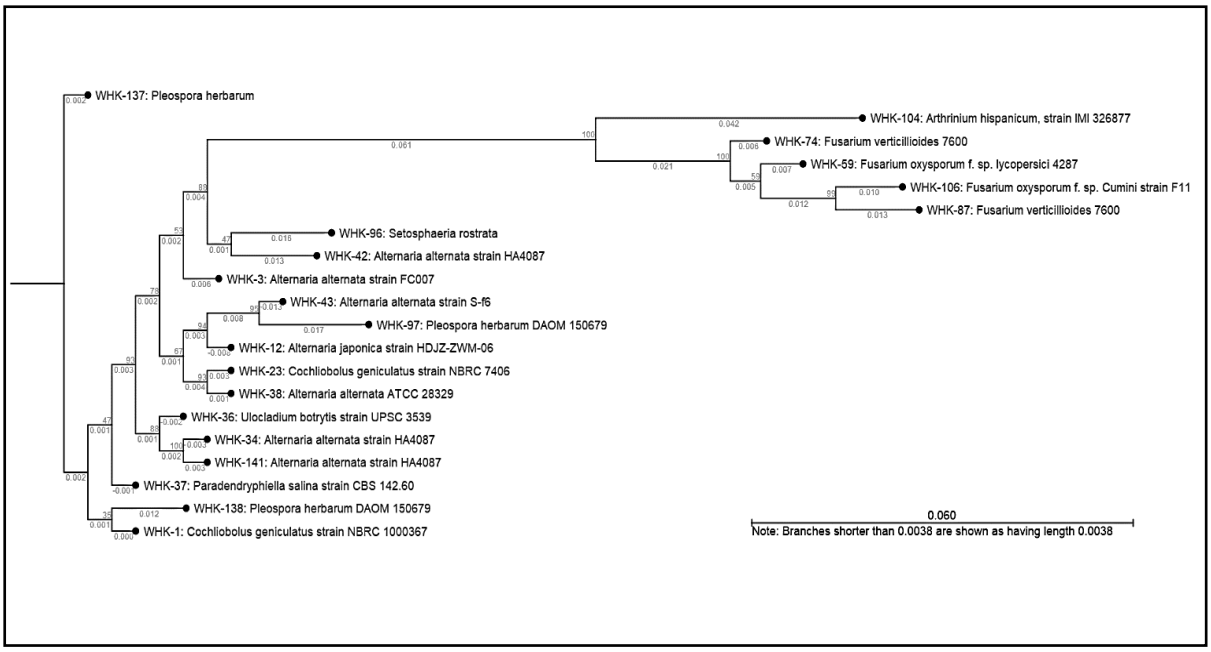


Figure 2.7: Phylogenetic analysis and relatedness of the potent fungi against the biocontrol of waterhyacinth

2.3.4. *Frequency of fungal isolates*

Fusarium Link (8.45 %), followed by *Alternaria* Nees (6.34 %) were the most common genus, found among the potent phytopathogens, isolated from the infected waterhyacinth. Among the 12 isolates of *Fusarium* Link., *F. oxysporum* appeared mostly with 2.11 % rate of occurrence followed by *F. verticilloides* (1.41 %), *F. roseum*, and *F. ventricosum* both with 0.7 % of occurrence. On the other hand, *A. alternata* was prevalent among the *Alternaria* sps. with 4.23 % rate of occurrence (**Figure 2.8**). *Curvularia* sp. and *Clodosporium* sp. followed with 4.23 % and 3.52 % rate of occurrence respectively.

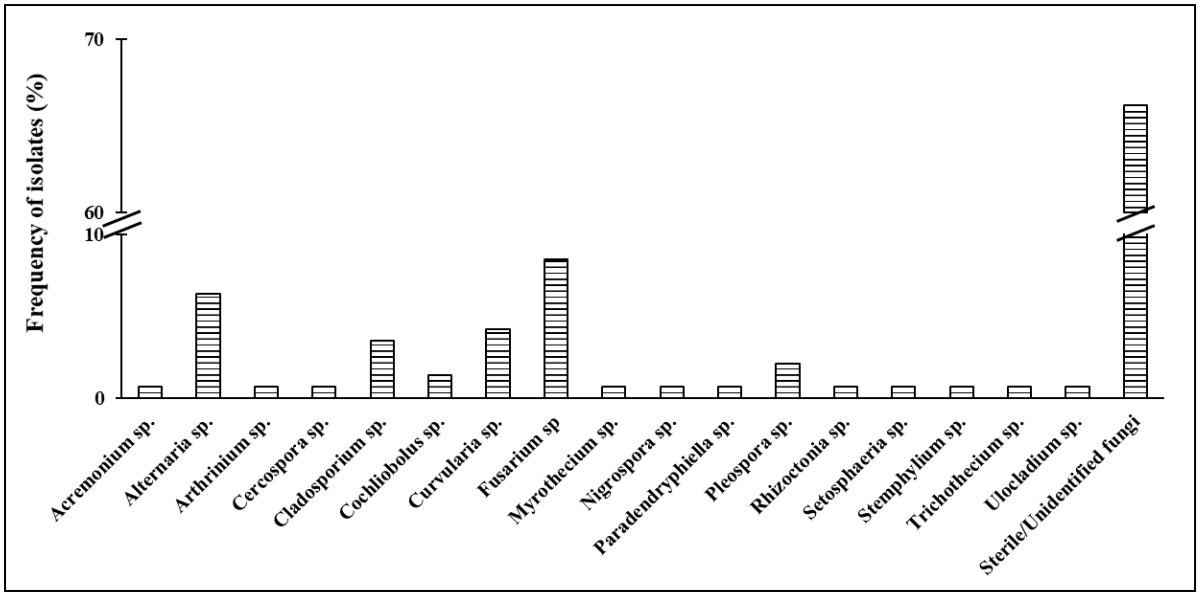


Figure 2.8: Frequency of occurrence of different fungal genera isolated from infected waterhyacinth from various regions in and out of Kolkata and some parts of Birbhum from 2014–2019

2.4. Discussion

Periodical surveys have highlighted the fact that a rich diversity of fungal pathogens is associated with waterhyacinth in and around Kolkata, India. Many of these fungal pathogens possibly share a common association with those documented earlier from the Amazon River Basin, the place of origin of the weed, (Evans and Reeder 2001) and other countries invaded by waterhyacinth (Ray and Hill 2013, Firehun et al. 2017). Several pathogens possibly co-evolved with waterhyacinth and globally spread with the weed itself. Several reports (Ray and Hill 2012, Freeman et al. 1974, Evans 1987, Shabana et al. 1995a, Firehun et al. 2017) have hinted at the association of a rich mycobiota with waterhyacinth, worldwide. With the invasive infestation of the weed, extensive periodical surveys were conducted to broaden the array of fungal pathogens, because many species are limited to certain geographical distribution (Evans and Reeder 2001).

Evolution in microscopy techniques, with use of electrons to scan the surface of fungal species, have helped in revealing finer details to 3D view of species' morphological characteristics of the fungal species. But based on the conventional phenotypic identification of species, a huge quality of time and labor has proved to be unstable

and as well as subjected to variety of changes, based on their culture medium (Borman et al. 2008), which has eventually paved the path for utilization of molecular tools for identification of species, which are faster with high rates of accuracy. Even with higher accuracy rates and concluding on finer distinction of the closely related species, such approaches have long been held up due to the presence of potent PCR inhibitors in fungal cultures, and complications, intrinsic in breaking fungal cell walls (Tang et al. 1993).

If identification was a key step to understand and predict some characteristics of the fungi, maintaining the cultures were also equally a challenging job. Lyophilization techniques, were highly ignores for the inevitable and irreversible structural damages that were provided by the formation of ice-crystals. Another significant way of managing cultures was submitting cultures to some organization (like National Fungal Culture Collection of India or NFCCI) that keeps a copy of your sample for future record and accession.

Characterization and phylogenetic analysis of the mating-type loci in the asexual ascomycetes of *Ulocladium* have drawn close relatedness with *Alternaria, Cochliobolus, Stemphylium* (Geng et al. 2014). Even the clade to which *P. herbarum, S. rostrata* and *P. salina* belongs is closely associated with the big family of *Alternaria* spp. (Woudenberg et al. 2013, Kusai et al. 2016).

The survey mainly showed prevalence of *Fusarium* spp., followed by *Alternaria* spp., which is contrary to many survey reports conducted worldwide, where the widespread of the latter is much higher (Ray and Hill 2012, Firehun et al. 2017). However, most of the fungal taxa are likely to be opportunistic pathogens that may have possibly jumped hosts from surrounding plants and are unlikely to be a specialist to waterhyacinth, resulting in the collection of a huge number of sterile and unfertilized fungi.

2.5. Conclusion

The anthropogenic spread of, one of the top ten globally recognised invasive weed, waterhyainth, have left an enormous negative impact on the socio-economical aspect. Without disrupting the environmental balance and also pacing up with the high infestation rate of this weed, use of biocontrol agents like, phytopathogens from the infection of the host, against them have been highly acknowledged. This have led to

the excavation of a variety of indigenous and potent agents. With the discovery of array of phytopathogens, more details of their pathogenicity need to be further analyzed for the proper evaluation and establishment as potential biocontrol agents, which will be further investigated upon in the upcoming chapters.