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Fungal pathogens as potential mycoherbicides to control water hyacinth (*Eichhornia crassipes*)

K.R. Aneja

Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana 136 119, India
Corresponding author Email: anejakr@yahoo.ca; anejakr@gmail.com

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ABSTRACT

Water hyacinth (*Eichhornia crassipes*), a free-floating aquatic weed and native of Amazon River, is one of the fastest growing plants whose seeds can remains viable for more than 28 years in the mud. Controlling methods for water hyacinth include physical, chemical and biological, but biological using fungal pathogens and/or insects or both as a consortium is effective and eco-friendly. The world of fungi provides a fascinating and almost endless source of biological diversity, which is a rich source for exploitation. Studies conducted on fungal diversity of water hyacinth yielded 21 fungal pathogens, of these two: *Fusarium chlamydosporum* and *Bipolaris sorokiniana* recorded for the first time on this weed globally and 7 fungal pathogens [*Alternaria alternata* isolate-1 and isolate-2 (AL-14), *Cercospora rodmanii, Phoma sorghina, Epicoccum nigrum, Acremonium* sp., *Alternaria* sp. and *Stemphylium* sp.] identified as new records for the country. Biocontrol studies conducted on seven fungal pathogens showed maximum biocontrol efficacy in *Cercospora rodmanii* (95.3%), followed by *Alternaria eichhorniae* (80%) and *A. alternata* (45-72%), revealing all the desired characteristics, such as: can be easily cultured and maintained on natural host, host -specific, easily disseminated and can be mass produced in a short span of time. Moreover, the phylloplane microflora is not antagonistic to these pathogens, thus the biocontrol efficacy would not be affected by the surface microflora. They have the potential to be developed as a mycoherbicide/s either alone or as a consortium to manage water hyacinth worldwide either used alone or in combination with insects and or herbicide.

Keywords: Water hyacinth, Bioherbicides, *Alternaria alternata, A. eichhorniae, Fusarium chlamydosporum, Epicoccum nigrum; Cercospora rodmanii*

INTRODUCTION

Water hyacinth [*Eichhornia crassipes* (Mart.) Solms] is commonly called blue devil, Bengal terror, Florida devil, sumudra-sokh, million dollar weed, and jal kumbhi. It is a floating aquatic weed and native of Amazon River, Brazil (South America). It is one of the fastest growing plants which primarily reproduces from runners or stolons. Each plant of water hyacinth can produce thousands of seeds each year which can remain viable for more than 28 years (Sullivan and Wood, 2012). McLean (1922) suggested 1888 or 1889 as the possible year of its entry into India. It has recently been reported that water hyacinth is a vigorous grower that can double their mat size within one to two weeks (Dickinson and Royer, 2014). It is estimated that 20-40 per cent of the total utilizable water is currently infested by this weed in our country. Water hyacinth causes water loss through evapo-transpiration which is more significant than indigenous weeds. This weed causes many problems in canals, ponds, lakes, rivers such as blocking of canals and causing floods, reduction of water quality, oxygen depletion, increased evapotranspiration rate, reduction in fish production and aquatic crops (lotus, chestnut), the beauty of ponds, and increase in human diseases caused by mosquitoes (Rosenthal *et al.,* 1984; Aneja and Singh, 1992; DWSR, 2009; Gupta and Yadav, 2020).

Biological control, the intentional use of biological control agents (BCAs), particularly fungal pathogens, to reduce the vigorous growth, density and impact of weeds, offers a tremendous opportunity to provide agriculture, forestry and aquatic ecosystems with effective tools for abundant productivity while minimizing impact on human's health and environment through an eco-friendly strategy to be used in integrated weed management systems. Bioherbicides, a subset of biopesticides, are the formulations based on the live microorganisms e.g. fungi, bacteria and viruses, used to manage/control weeds. The world of fungi provides a fascinating and almost endless source of biological diversity, which is rich source for exploitation (Manocharachary *et al.,* 2014). In a majority of cases, the bioherbicide interestingly includes a fungal biocontrol agent (Aneja *et al.,* 2017), hence the term bioherbicide is interchangeably used to mycoherbicide. In addition to the BCAs, they are formulated in various ways to improve their delivery and effectiveness that enhance their ability to tolerate adverse environmental conditions, especially moisture, temperature and ultraviolet radiation which have inhibitory effects on fungal germination, growth and host infection (Aneja, 1998, 1999; Aneja *et al.,* 2017).

Biological control of water hyacinth has been suggested as the most efficient method (Bennett and Zwolfer, 1968; Bates and Hentges, 1976; Conway, 1976; Charudattan, 1986, 1987, 2001; Jayanth, 1988; Gupta and Yadav, 2020). Biological control of this weed using weevils (*Neochetina* spp.) and fungal biocontrol agents is under investigation all over the world as it is considered to be the cheapest, environmentally safe as and poses no threat to non-target organisms, environment and biodiversity (Charudattan, 1984; Gopal, 1987; Sushilkumar, 2004; DWSR, 2009). Although water hyacinth entered into India about a century ago (McLean, 1922; Biswas and Calder, 1954), a regular survey of pathogenic fungi on this weed was under taken only after 1960 (Nag Raj, 1965; Ponnappa, 1970; Nag Raj and Ponnappa, 1967, 1970; Charudattan, 1984, 2001; Freeman and Charudattan, 1984; Aneja and Singh, 1989; Aneja and Srinivas, 1991; Aneja, 1999; Aneja *et al.,* 2017).

This paper deals with the biodiversity of fungi associated with water hyacinth and their potential to be used as biocontrol agents for developing mycoherbicides, an eco-friendly strategy currently being used around the world.

MATERIALS AND METHODS

In the years 1985-2014, surveys for natural enemies of water hyacinth were conducted in Haryana, Punjab, Western Uttar Pradesh, Chandigarh and Delhi. Diseased leaves were collected in sterilized polythene bags and brought to the laboratory for study of symptoms, isolation, identification, pathogenicity test and evaluation of the virulent fungal pathogens for their biocontrol potential. The diseased specimens and live cultures of the fungal pathogens identified were sent to the International Mycological Institute, U.K. for the confirmation of the identification and record.

Isolation of fungal pathogens

The diseased leaves were washed thoroughly in running tap water to remove soil particles adherent to the leaves. The infected portions of the leaves were cut into 1-1.5 cm fragments, surface disinfected in 0.1% $HgCl_2$ for 20-60 seconds and then rinsed in sterile distilled water six to seven times. These fragments were transferred to potato dextrose agar (PDA) and water hyacinth dextrose agar (WHDA) plates supplemented with streptomycin sulphate and incubated at 28±2°C. The constituents of the WHDA medium are as follows:

Water hyacinth leaves	- 200.0 g
Dextrose	- 15.0 g
Agar-agar	- 20.0 g
Distilled water	- 1000 ml.

Fresh water hyacinth leaves (200 g) were washed in running tap water. These were boiled for 15-20 min in 500 ml distilled water and filtered through cheese cloth for the collection of leaves extract. The rest of the procedure was similar to the PDA preparation (Aneja, 2023).

Pathogenicity test and evaluation for biocontrol potential

Pathogenicity and biocontrol efficacy test of the chosen fungal isolates were determined in *in vitro* and *in vivo* (Aneja, 2023).

***In vitro* pathogenicity test:** young, medium sized and mature healthy leaves of water hyacinth were used for inoculations. They were washed with sterilized water and wiped with a cotton swab dipped in 70% ethyl alcohol. Some of the leaves, before inoculation, were injured on abaxial surface by pricking with a flamed needle. Mycelial discs, taken from 5-day-old colony, were placed on injured and uninjured portions and covered with sterile moist cotton. The inoculated leaves were kept in sterilized trays, covered with polythene sheets and incubated at 28 ± 2°C. Regular checks for the appearance of symptoms were made after 3 days of incubation.

Biocontrol efficacy test: water hyacinth plants were grown in 16 pits, each pit of 0-5 x 0.5 x 0.5 m dimensions. Conidial and mycelial suspensions of the pathogen were prepared from 7-day-old cultures grown on WHDA medium. Spraying of inoculum was done in eight pits, four of which were covered with polythene sheets to maintain relative humidity near saturation and stop the interaction of insects, and the other four were left uncovered to provide natural environmental conditions. Eight pits served as uninoculated controls; four pits were covered with polythene sheets while the remaining four left uncovered. Observations were made one month after inoculation.

Antagonism studies

Dual culture technique (Aneja, 2023) was used to test the antagonism among the phylloplane microflora and fungal biocontrol agents, and between the fungal biocontrol agents.

RESULTS AND DISCUSSION

During the surveys conducted for fungal pathogens in various parts of Haryana, Punjab, Western part of U.P, Chandigarh and Delhi, a total of 21 fungal pathogens were identified on water hyacinth (**Table 1**). Of these, seven fungal pathogens [*Alternaria alternata* isolate-1 and isolate-2 (AL-14), *Cercospora rodmanii, Phoma sorghina, Epicoccum nigrum, Acremonium* sp., *Alternaria* sp. and *Stemphylium* sp.] were reported as new disease records on water hyacinth from India and 2 fungal pathogens namely *Fusarium chlamydosporum* and *Biopolaris sorokiniana* reported for the first time on this host globally. Five fungal pathogens: *A. alternata, A. eichhorniae, F. chlamydosporum, E. nigrum,* and *C. rodmanii* were evaluated for their biocontrol efficacy against water hyacinth, in especially designed experimental pits at the Botanical garden, Kurukshetra, with the aim to search for potential biocontrol agents which could be developed as mycoherbicides in the near future.

Table 1: Diversity of fungal pathogens associated with water hyacinth at Kurukshetra

S. No	Fungal pathogen	Symptoms
1 **	*Acremonium* sp.	Leaf spot
2 **	*Alternaria alternata* *A. alternata (AL-14)*	Left spot Leaf spot and leaf blotches
3	*Alternaria eichhorniae*	Leaf spot and severe leaf blight
4	*Alternaria* sp.	Leaf spot
5 *	*Bipolaris sorokiniana*	Leaf spot
6	*Cephalosporium eichhorniae (Acremonium zonatum)*	Stem and root rot
7	*Cercospora piaropi*	Left spot and leaf necrosis
8 **	*Cercospora rodmanii*	Leaf spot
9	*Curvularia lunata*	Leaf spot
10	*Cylindrocladium scoparium var. brasiliensis*	Leaf spot
11 **	*Epicoccum nigrum*	Leaf spot
12 *	*Fusarium chlamydosporum*	Leaf spot
13	*Fusarium equiseti*	Left spot
14	*Fusarium solani*	Reddish brown leaf spot
15 **	*Fusarium* sp.	Leaf decay
16	*Helminthosporium bicolor (Cochliobolus bicolor)*	Leaf spot
17	*Marasmiellus inoderma*	Foliar blight
18	*Myrothecium roridum*	Leaf spot
19 **	*Phoma sorghina*	Leaf spot
20	*Rhizoctonia solani*	Foliar blight
21 **	*Stemphylium* sp.	Leaf spot

* Reported for the first time on this weed globally.
** Reported for the first time on this weed from India.

***Alternaria alternata* – Water hyacinth system**

A severe leaf spot disease (**Fig. 1a**) showing lesions of variable size ranging from minute, dark brown lesions to large irregular patches in various aquatic bodies of Kurukshetra were observed. On the basis of symptomology, cultural and morphological characteristics of the conidia (**Fig. 1b**), the fungus was identified as *Alternaria alternata* (Fr.) Keissler and confirmed by the C.A.B. International Mycological Institute, England. It is a new disease reported for the first time on the host (diseased specimen and culture deposited in the IMI, UK (IMI No. 303589). Pathogenicity test and Koch's postulates were confirmed. The disease severity was found 45 percent on small leaves, 62 percent on medium leaves and 72% on mature leaves of water hyacinth post one month inoculation in the experimental pits which were left uncovered, the disease severity was found to be lesser on three types of leaves in the covered pits (Aneja and Singh, 1989). The pathogen has been found to have all the features suggested by Freeman (1977) that makes it a desirable candidate as biological control agent of this weed, such as: it can be easily disseminated and self-maintaining, can be easily cultivated on the nutrient medium containing natural host as a carbon source and mass produced easily in a short time, host-specific and capable of limiting populations without eliminating the host. Abbas *et al.* (1995) reported that the AAL toxin produced by *A. alternata* is known to play an important role in the pathogenesis of the blight disease of water hyacinth. This toxin is an effective herbicide at low concentration against a number of weeds including water hyacinth. Thus, there is a potential *A. alternata* and/or the AAL toxin to be developed as a mycoherbicide.

***Alternaria eichhorniae* – Water hyacinth system**

Another leaf spot disease severely affecting water hyacinth plants was observed in various ponds of Kurukshetra and Ambala. The leaves and petioles had very large characteristics necrotic spots with dark centres surrounded by brownish black margins (**Fig.1c**). On Potato dextrose agar, the fungus produced red colored phytotoxic compound and characteristic long-beaked, large-sized, muriform, pigmented conidia (**Fig.1d**). Based on its morphology and cultural characteristics, the pathogen was identified as *Alternaria eichhorniae*. The pathogenicity test and Koch's postulates were confirmed. This pathogen had been isolated first of all from South India in 1970 and had been suggested as a biocontrol agent for water hyacinth (Nag Raj and Ponnappa, 1970). Our studies carried out for its potential to be used as a bioherbicide, showed infection up to 80 per cent. The older leaves were found to be more susceptible than the young leaves. The development of the disease was found to be more rapid at 25°C. Host-pathogen relationship studies revealed that the penetration of water hyacinth leaves by the fungus occurred only through the stomata, and the invading hyphae were located in the intercellular spaces of the leaf tissues. *In vitro* studies conducted on ultraviolet irradiation/treatment on *A. eichhorniae* revealed that six days exposer of 12 hour UV (354 nm) and 12 hour dark-alternating cycle has stimulating effect on both sporulation and virulence of the pathogen. Our studies are in conformity with the studies carried out in Sudan who reported *A. eichhorniae* to cause 100% killing of the weed when used in combination with *Neochetina bruchi* and *N. eichhorniae* (El Tayeb and Bashir, 1992). The fungus is known to produce two nonspecific toxins (e.g., bostrycin and 4-deoxybostrycin) in the red pigmented culture filterate which are phytotoxic to the leaves of water hyacinth (Charudattan and Rao,1982). This fungus has been found to possess all the desirable features of a potential myco-herbicide.

***Fusarium chlamydosporum* – Water hyacinth system**

During the field survey conducted, 3rd leaf spot disease showing elliptical to irregular patches on water hyacinth, was observed (**Fig. 1e**). Isolation made from the diseased leaves yielded a fungus showing hyaline, cylindrical macroconidia having transverse septa produced in sporodochia but lacking microconidia (**Fig. 1f**). Abundant chlamydospores are formed singly, in chains or clusters, and hence the fungus identified as *Fusarium chlamydosporum*. The culture and disease specimens have been deposited at IMI, U.K. (IMI No. 333323). This is a new disease reported for the first time on the host from the globe (Aneja *et al.,*1990). The pathogenicity test and Koch's postulates were confirmed. Green house and laboratory experiments showed that the large size leaves exhibit more infection compared to the small and medium size leaves. Sporulation of the fungus was found to be better on potato sucrose agar than potato dextrose agar and PDA+Y. Evaluation work conducted in covered and uncovered pits revealed better potential in the former, infection ranging from 25 to 54%.

***Epicoccum nigrum* – Water hyacinth system**

Another leaf spot disease on water hyacinth plants (**Fig. 1g**) showing leaf spots leading to compact zonations initiating from the top of the leaves and spreading backwards was reported from Rajpura. The pathogen was identified as *Epicoccum nigrum*, a dematiaceous hyphomycetous fungus characterized by dark brown, globose to subglobose, rough walled, muriform (having both transverse and longitudinal septa) developing from short lateral branches (conidiogenous cells) (**Fig. 1h**). The disease on water hyacinth has been reported for the first time in India (Aneja *et al.,* 1990). The culture and specimen have been deposited at the IMI, UK (IMI No. 33324) Koch's postulates were confirmed. Water hyacinth dextrose agar was found to be best medium for its culturing than that of PDA, PSA and CDA. Biocontrol efficacy in experimental pits ranged between 20 and 50 percent and was found to be better in uncovered pits than covered pits (Aneja and Srinivas, 1991). *E. nigrum* is a weak pathogen and has been reported on several hosts from India, including *Sorghum vulgare* and *Zea mays*, hence extensive host range studies are needed before considering it as a biocontrol agent for water hyacinth.

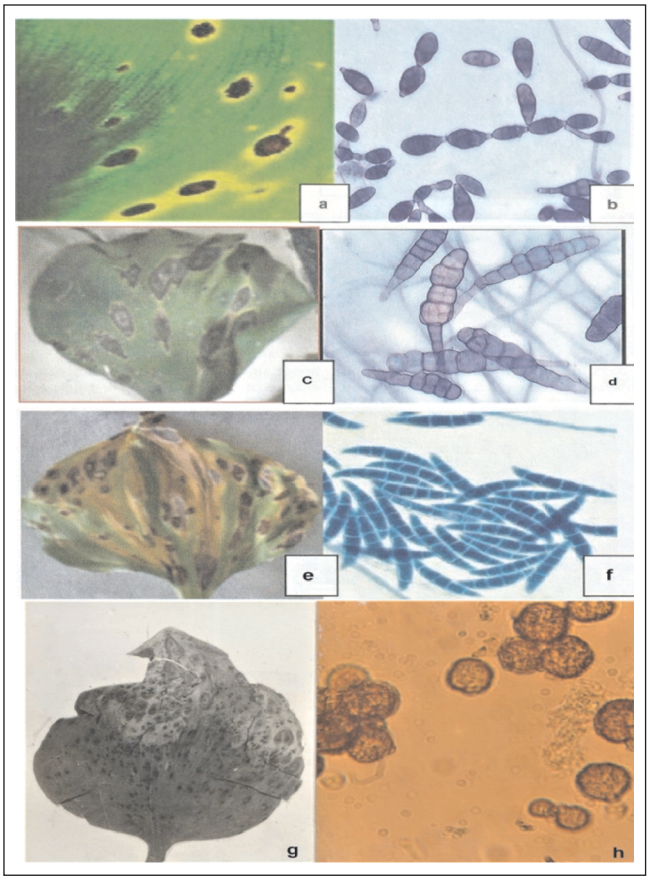

Fig. 1: Disease symptoms and cultural characteristics of four fungal pathogens. **a,b** *Alternaria alternata*. **c,d** *Alternaria eichhorniae*. **e,f** *Fusarium chlamydosporum*. **g,h** *Epicoccum nigrum*

***Cercospora rodmanii* – Water hyacinth system**

During the surveys, another leaf spot disease severely affecting the water hyacinth was observed from several ponds in Kurukshetra, Ambala and Rajpura. The infected leaves have punctuate to circular, dark spots with their tips necrotic and chlorosis of lamina (leaf) and petiole prominent (**Fig. 2**). On isolation, a coelomycetous fungus was identified *Cercospora rodmanii* based on its characteristics dark brown conidiophores arising in fascicles of 3-9 bearing hyaline, truncate-based, acicular and multicellular conidia (**Fig.2**). Interestingly, on flooding the mycelial disks in sterilized water by the newly devised method of Aneja and Mehrotra (1978), *Asteromella* (**Fig. 2**) producing a pycnidium was identified. Pathogenicity test and Koch's postulates were confirmed. The identification of the fungus was confirmed by the IMI, UK (IMI No. 329783). This fungus was first of its occurrence in India and the 2nd in the world. The naming of the species is based on its first-time occurrence on water hyacinth from the Rodman reservoir in Florida, USA (Aneja and Srinivas, 1990).

Evaluation of *C. rodmanii* for its biocontrol potential to control the weed revealed 95% infection of leaves in experimental pits. Host range studies conducted on this pathogen showed that it did not infect any other tested plants expect water hyacinth. Studies conducted on antagonism between the phylloplane microflora of water hyacinth and *C. rodmanii* revealed that none of this fungal and bacterial isolates are antagonistic to the BCA indicating that phylloplane flora would not affect its biocontrol efficacy. Potato dextrose yeast extract agar was found the best culture medium for the growth and sporulation of the fungus followed by water hyacinth dextrose agar and PDA. Our studies conducted on *Neochetina eichhorniae*, a weevil, alone and in consortia with *C. rodmanii* resulted in enhancement of biocontrol efficacy of the fungus. Our results are in conformity with Charudattan (1984) and Freeman and Charudattan (1984) who suggested *C. rodmanii* as a potential biocontrol agent and to be developed as a bioherbicide for controlling this weed in the USA. Based on these findings, Abbott laboratories, USA developed an experimental formulation of *C. rodmanii*-ABG-5003, that consisted of mycelium and conidia of the fungus. It was applied as a wettable powder for controlling this weed. The notable advantage of this fungus is that it was compatible with various chemical herbicides and insects for controlling water hyacinth (Conway *et al.,* 1978; Charudattan, 1986, 2001). *C. rodmanii* has the potential to be developed as a mycoherbicide for controlling water hyacinth in India.

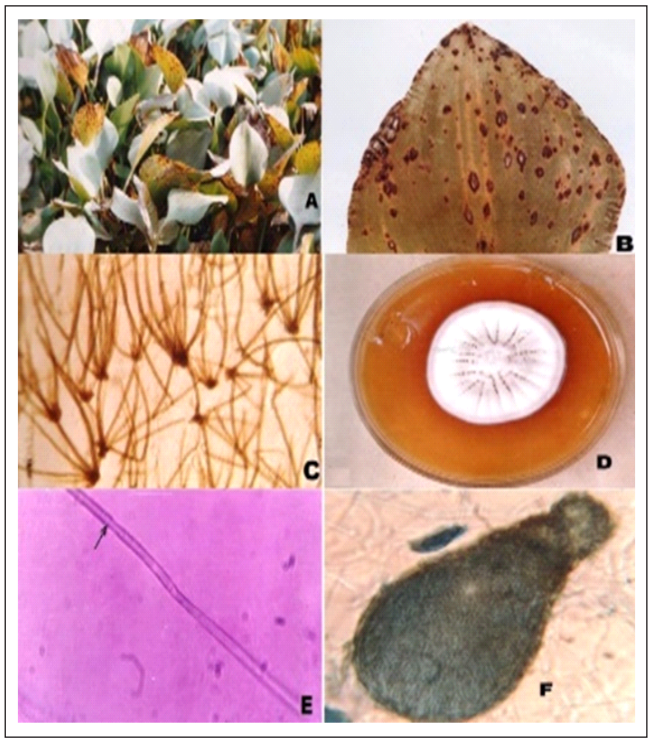

Fig. 2: *Cercospora rodmanii* on water hyacinth. **A.** Disease severity in the field **B.** Leaf spots **C.** Conidiophores arising in clusters from the leaf **D.** Colony on PDAY medium **E.** Attachment of a conidium on a conidiophore **F.** Pycnidium of *Asteromella* produced under *in vitro* conditions when mycelial disks were flooded in sterilized water.

***Phoma sorghina* – Water hyacinth system**

A leaf spot disease of water hyacinth caused by a fungus showing infections on leaves and petioles characterized by large irregular blotches (**Fig. 3A**) was observed at Kurukshetra. On isolation, dark, globose to irregular shaped pycnidia producing ellipsoid, single-celled conidia (**Fig. 3B**), sometimes producing chlamydospores were observed. The fungus was identified *Phoma sorghina*, a member of *Coelomycetes* (now classified in *Ascomycota*). The culture and disease specimens have been deposited at IMI, UK (IMI No. 333325). Koch's postulates were confirmed. *P. sorghina* on water hyacinth is a new record for India (Aneja *et al.,* 1990). It is a weak pathogen having a wide host range, hence cannot be a suitable biocontrol agent for this weed.

***Stemphylium* sp. – Water hyacinth system**

Populations of water hyacinth plants were found infected in various parts of Haryana (Kurukshetra, Ambala, Kaithal) by a leaf spot disease. Symptoms on the leaves are small, round to elliptical to irregular spots, coalescing to form irregular blotches (**Fig. 3C**). A fungus producing brown, small conidiogenous cells with swollen tip, each bearing a solitary, muriform, dark, globose to ellipsoid conidium with 2-5 transverse septa and 1-4 longitudinal septa (**Fig. 3D**), was identified an unknown species of *Stemphylium*, a member of dematiaceous *Hyphomycetes*, classified in *Ascomycota*. Koch's postulates were proved. It is recorded for the first time on this host.

***Alternaria* sp. – Water hyacinth system**

Heavy infestations of water hyacinth showing leaf spots (**Fig. 3E**) were recorded from Kurukshetra, Ambala (Haryana), Rajpura (Punjab), Chandigarh, Shamli (U.P) and Delhi. The symptoms ranged from oval to elliptical leaf spots, abundant on mature leaves and petioles. A fungus was isolated showing characteristics beaked muriform black conidia with 2-3 transvers septa and 1-2 longitudinal septa (**Fig. 3F**), morphologically different from *Alternaria alternata* and *A. Eichhorniae* (Aneja *et al.,* 2014). Koch's postulates were proved. The diseases specimens and live culture have been deposited at IMI, UK (IMI No. 341106). It is a new disease record on this host.

***Acremonium* sp. – Water hyacinth system**

A leaf spot disease characterized by zonate, oval to irregular, marked with light brown rings, mainly produced on mature leaves (**Fig. 3G**). In localized patches on stagnant water hyacinth plants was observed in a village Narkatari (Kurukshetra). On isolation, the fungus produced unicellular, hyaline to slightly pigmented conidia from the small conidiogenous cells, characteristics of the genus *Acremonium* (**Fig.3H**). The culture and diseased specimen have been deposited at IMI, UK (IMI No. 341105). Koch's postulates were confirmed. The pathogen has been found to be different from *Acremonium zonatum*, reported earlier on this host, hence is a new record for this weed.

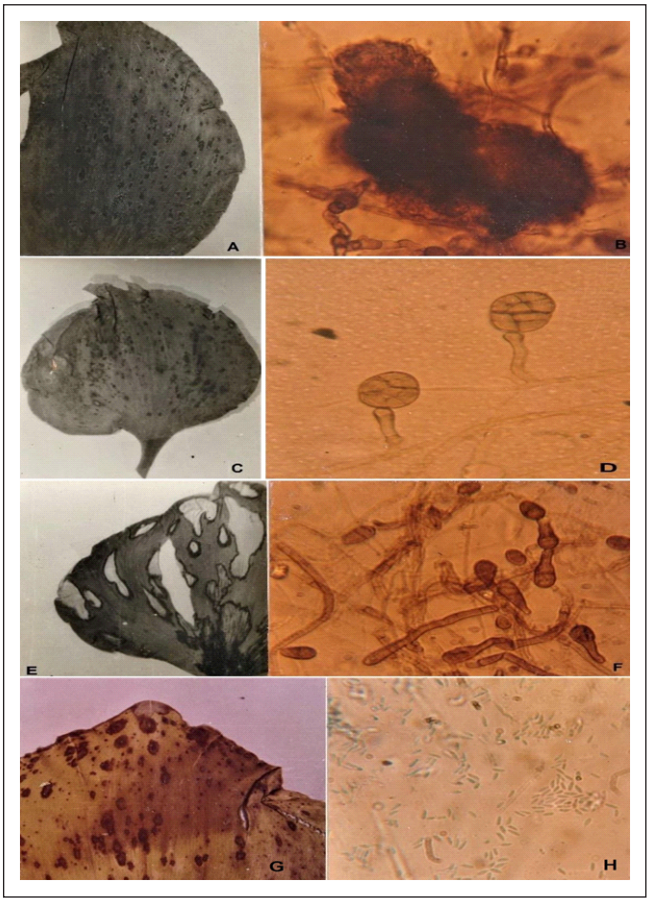

Fig. 3: Symptoms and cultural characteristics of four fungal pathogens on water hyacinth. **A,B** *Phoma sorghina*. **C,D** *Stemphylium* sp. **E,F** *Alternaria* sp. **G,H** *Acremonium* sp.

Mycoherbicides research to control weeds began in the 1940s. The earlier experiments simply involved moving indigenous fungal pathogens between populations of target weeds. For example, the fungus *Fusarium oxysporum* used against prickly pear or pear cactus (*Opuntia ficus-indica*) into Hawaii, before the release of the moth *Cactoblastis cactorum*. The level of activity increased tremendously in the early 1970s that resulted to the development of two mycoherbicides: DeVine^{R} in 1981, a formulation of *Phytophthora palmivora* to control milkweed vine in the USA, and Collego^{TM} (later called Lockdown^{TM}), a formulation of *Colletotrichum gloeosporioide*s f. sp. *aeschynomene* to control Northern jointvetch in the United States. Over 22 bioherbicides have been developed around the globe (Aneja *et al.,* 2017). The use of mycoherbicides-weed control formulation based on fungal pathogens, is the only safe, cost-effective, and environmentally sustainable method of control. The studies carried out at Kurukshetra reveal the potential to develop a mycoherbicide based on a consortium of fungi:

Alternaria eichhorniae, A. alternata and *C. rodmanii*, to be used alone or in combination with the weevils *Neochetina eichhorniae*. Biological control of water hyacinth using a fungus *C. rodmanii* (formulation called ABG-5003) in Florida, USA (Conway *et al.,* 1978; Charudattan, 1984, 2001) and weevils (*Neochetina* spp.) is already in use in the three States of America: Louisiana, Texas and Florida (Gupta and Yadav, 2020). It has been found that the weevils also carries the microbes involving their roles in limiting the growth of water hyacinth plants (Charudattan, 1986, 2001; Jimenez, 2014). *N. eichhorniae* is also being successfully used to control this weed in an integrated management approach since 2003 in a pond of village Junmani by DWSR, Jabalpur, MP (India) (Sushilkumar, 2004; DWSR, 2009). However, the main limitation on the use of biological predation on water hyacinth is the life cycle of weevils, which is 90 days (Sanders *et al.,* 2014). Hence the necessity of using mycoherbicide with the weevils, in place of herbicide (e.g., 2, 4-D glyphosate and paraquat) for the successful control/management of water hyacinth in India and other countries of the world as an eco-friendly strategy.

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