Evaluation of microfungi for the biological control of water hyacinth in Egypt

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Twenty-two fungal species were isolated from surface sterilized water hyacinth parts with pronounced blight syndrome. The most common species were *Alternaria alternata*, *Drechslera hawaiiensis* and *Ulocladium atrum.* The patterns of fungal abundance were influenced positively by changes in conductivity (EC) and negatively by pH and temperature (TE). As a result of a pathogenicity test, and on the basis of disease severity, the pathogenic species were divided into mildly, low moderately, high moderately and severely damaging species. Of these species only *Al. alternata,* (associated with 79% tissue death), *D. hawaiiensis* (78%) and *U. atrum* (70%) showed high disease severity. Because *Al. alternata*, was reported as a plurivorus species with several pathotypes, *D. hawaiiensis* and *U. atrum* were selected for further study. Formulation and daily spraying of water permitted conidial germination and infection by these species in the field. Both treatments gave similar results. Disease incidence (DI) and disease severity (DS) increased with increasing incubation period up to 30 days of incubation. Simultaneously, the chlorophyll content decreased in the infected leaves compared to healthy ones. Both DI and DS decreased after 30 days incubation indicating that it is not efficient to use *D. hawaiiensis* and *U. atrum* either separately or in a mixed formulation as biocontrol agents.

Key words: *Drechslera*, invasive species, pathogens, *Ulocladium*, weed control.

Introduction

Eichhornia crassipes (Mart.) Solms-Laubach (*Pontederiaceae*, water hyacinth) is a free-floating aquatic plant, native to the Amazon Basin in South America. It has become widespread and is considered to be the worst aquatic weed through out the tropical and subtropical regions of the world (Center, 1994; Wright and Purcell, 1995). Water hyacinths were most likely introduced into Egypt in the 1890's, as ornamentals. The explosive growth rate of the plant and its ability to infest a wide range of freshwater habitats have created

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enormous environmental and economic problems. Water hyacinth has spread throughout Africa causing widespread problems to millions of users of water bodies and water resources, and this is especially in severe in Egypt. Several projects have therefore been set up all over the world to investigate controlling water hyacinth (Beshir and Bennett, 1985; Westerdahl and Getsinger, 1988; Charudattan, 1990; Helsel, 1997). Of the control methods available, biological control methods provide favourable results in terms of environmental sustainability and costs. Biological control using plant pathogens has been shown to be highly effective against water hyacinth under experimental conditions (Shabana, 1997a,b).

Several highly virulent fungal parasites are known to cause diseases of water hyacinth (Charudattan, 1990). Among the known pathogens are *Acremonium zonatum, Alternaria alternata, Al. eichhorniae, Bipolaris* spp*., Fusarium chlamydosporum, Helminthosporium* spp*., Cercospora rodmanii, Myrothecium roridum, Rhizoctonia solani* and *Uredo eichhorniae* (Charudattan, 1990; Aneja *et al*., 1993). These fungi are easy and inexpensive to produce and therefore have potential for development as bioherbicides. Of these fungi *Alternaria eichhorniae*, *Cercospora rodmanii* and *Fusarium chlamydosporum* have been studied to a significant extent (Charudattan, 1990; Aneja *et al*., 1993; Shabana, 1997a,b). Other virulent pathogens have not been fully evaluated

The aims of this investigation were to isolate, screen and rank fungi infecting water hyacinth plants on the basis of their pathogenicity and ability to cause damage, and to test the potential of highly pathogenic species as biocontrol agents.

Materials and methods

Habitat

The study areas were located in Deltaic region of Egypt specifically in Damietta Province, between Damietta and New Damietta (Kafr El-Bateikh) where several irrigation canals infested with *Eichhornia crassipes* have been surveyed for the presence of pathogenic fungi. Water hyacinth plants completely covered the water surface in association with *Pistia staratoites*. Water hyacinth plants in the main irrigation canal were badly infected with fungi and showed different symptoms. The symptoms initially appeared as small necrotic spots and became leaf blight that covered the entire leaf. The symptoms occurred over all plant parts, on the stolons, swollen bases and leaf

blades. Leaf blight was also observed on *Pistia staratoites.* On the canal bank, chronic blisters were also recognized on leaves and spikes of *Cyperus* spp.

Sampling procedures and isolation methods

For isolation of pathogenic species, infected parts (stolons, swollen bases and leaf blades) were collected from Hallawa irrigation canal (1), New Damietta irrigation canal (2) and Kafr El-Bateikh irrigation canal (3) (about 1 km apart), in clean plastic bags, brought to the laboratory, and then stored in a refrigerator at 4 C, and processed within 48 hours. Stored plant parts were scrubbed under running water to remove surface debris, dissected into small segments; approximately 1 × 1 cm, and surface-sterilized by sequential immersion in 5% hypochlorite for 1-2 minutes followed by 75% ethanol for 30 seconds. Surface sterilised segments (4 segments/plate) were selected, plated out on water agar amended with 0.5 gm streptomycin, 2000 units penicillin G per plate and 0.3 g/L Rose Bengal. Ten plates were used for each plant part. The plates were then incubated at 27 C for 7-15 days. Developing hyphal tips and/or spores were picked up aseptically and inoculated on to potato-dextrose agar (PDA). Developing colonies were then picked up and subcultured on PDA. Approximately 200 fungal isolates were made. The isolates are stored and maintained in slants of PDA, in an incubator at the Department of Botany, Mansoura University. Based up on their frequency of occurrence, the isolated species were classified as very frequent (> 20%) frequent (10-20%) and infrequent (< 10%) as adopted by Tan and Leong (1989).

Classification of fungi

Isolates were identified using various agar media to promote sporulation. Non-sporulating strains were grouped within Class *Agonomycetes* (sterile fungi) of anamorphic fungi according to similarities in colony morphology and production of chlamydospores or sclerotia (Lacap *et al*., 2003).

Water characteristics

Conductivity and pH of water were determined with a YSI ® Conductance Meter Model and 35 micro-computer pH-meter 6209 (JENCO). Temperature measurements were made *in situ* with a mercury-filled thermometer. This characteristics and given in Table 1.

Table 1. Water parameters that were determined in three irrigation canals about 1 km apart.

Parameters	Sites*		
	1	2	3
pH	7.4	7.4	7.3
Temperature C	32	33	32
Conductivity mmhose	14.0	12.7	13.8

* 1. Hallawa irrigation canal; 2. New Damietta irrigation canal; 3. Kafr EL-Bateikh irrigation canal.

Statistical analysis

Canoco: a Fortran program version 2.1 (Ter Braak, 1988) was used to relate fungal population structure to changes in water variably. Canoco is particular efficient for ordination of sparse data sets (data containing many zero values compared to the number of non zero values). The theory of applying ordination to this type of data is described in Ter Braak (1986). This multivariate technique embraces canonical correspondence analysis (CCA) that has been used here. The data are arranged on two axes. The numerical importance of an axis is judged by looking at its Eigen values. The Eigen values are a measure of separation of the species distributions along the ordination axis (Ter Braak, 1987). They are always between zero and one. Higher Eigen values indicate important ordination axis.

Pathogenecity test

Healthy water hyacinth plants were collected from natural infestations in New Damietta and maintained in a sterilized greenhouse. For the inoculation procedure, plants were kept in plastic dishes (12 cm × 9 cm) filled with water, 3 plants per dish. A 2-mm plug of each culture was grown out on a PDA broth for 10 days under aseptic conditions and incubated on a laboratory bench at 26 C ± 2. Mycelium and, if present, spores were harvested, rinsed with sterile distilled water and blended aseptically with distilled water (1:1 w/v). The resulted mycelium suspension was diluted to give 1×10^6 propagule/ml. The leaves and swollen bases of the plants were wounded manually by removing the cuticle layer with an empty pen cover and painted with Tween 80. For comparison of pathogenicity, each suspension was then liberally applied to the surface of water hyacinth plants using a hand-sprayer. Results were recorded two and four weeks after spraying.

Field experiments

Moisture application

Fungal suspensions of 1×10^6 propagule/ml in 1% Tween 80 were sprayed manually, using hand sprayer, on to the surface of water hyacinth plants. Dishes containing three plants per dish were set up and used in the field. To ensure enough there was moisture for conidial germination, water was sprayed on to the plants in the first three days of application; 3 times in the first day (morning, midday and night) two times on the second day (midday and night), and once on the third day (midday). A control experiment (the application of water without lacking fungus) was carried out simultaneously.

Formulation

Inocula of *Drechslera hawaiiensis* and *Ulocladium atrum* were developed as above. The blended fungal mycelium suspension was diluted 1:4 (v/v) with 1.33% (w/v) sodium alginate in distilled water. This mixture was dripped into 0.25 mol $CaCl_2$ to form gel beads of 3-5mm. The beads were then sifted and air-dried to yield pellets. The dried pellets were ground and the resulting powder contained about 1×10^6 propagule/g. Alginate pellets were also prepared for the control experiment. The fungal suspension emulsion was composed of 0.5 g (1% w/v) mycelium-alginate-powder, 75 ml (15% v/v) corn oil, 15 ml (4% v/v) of an emulsifier soybean lecithin and 500 ml (80%) water (Shabana, 1996). For comparison of the pathogenicity, separate and mixed fungus suspensions were then liberally applied to the surface of a water hyacinth plants, three plants per dish set up in the field using a hand-sprayer. A control experiment was carried out simultaneously using formulation without fungal propagules.

Plants were rated for disease symptoms including leaf spots, leaf lesions, and leaf death after 15, 30 and 60 days. The impact of the pathogens was determined by counting the number of leaves infected per total number of leaves present (disease incidence, DI) and subsequently by assessing the type of damage (disease severity, DS). DS was determined for each leaf on a scale of 0 to 9, where 0 = healthy, and 9 = 100% diseased (Freeman and Charudattan, 1984). Values for individual leaves were summed and averaged to derive DS for a whole plant. Finally, isolates were categorized into five groups: "N", isolates that did not cause any significant damage or infection; "Mild", isolates that caused less than 25% damage of the leaf area; "Low Moderate" isolates caused 26-50% damage of the leaf area; "High Moderate",

isolates that damaged 51-75% of the leaf area and "Severe", fungi that cause greater than 75% damage of the leaf area.

Chlorophyll determination

To determine the effects of the pathogens on plant metabolism: Ten-4 mm-diam disks were excised from leaves with a cork borer at infection points. The chlorophyll content was then determined using Sequoia-Turner spectrophotometer Model 340 (Wellburn and Lichtenthaler, 1984).

Results

Biodiversity

Twenty-two fungal species were isolated and identified (Table 2). They were predominantly anamorphic taxa. The majority of anamorphic fungi were hyphomycetes (19 spp. = 161 colonies) and the minority are *Agonomycetes* (1 sp = 2 colonies). Zygomycota (1 sp = 3 colonies) and Ascomycota (1 sp = 3 colonies) were also represented (Table 2). Of these fungi, *Drechslera hawaiiensis* (29.6% of colonies), *Alternaria alternata* (26%) and *Ulocladium atrum* (26%) were very frequent species. The highest count of these fungi (87 colonies) was detected from the infected leave blades and the lowest one was from the stolon.

Water parameters

The relative importance of the determined water parameters to the distribution of microfungi is illustrated in CCA biplot (Fig. 1) where water parameters and microfungi are arranged on the basis of their scores on two axes. The Eigen values of the first two axis used are 0.161 and 0.129. Species and environmental variables are highly significantly correlated (0.926 and 0.924) (Table 3). Where pH and temperature (TE) are negatively correlated with the first CCA axis, conductivity (EC) correlated positively (Fig. 1). Likewise, species on the second axis are negatively correlated with pH and EC and positively with TE. Species at the edges of the axes are usually uncorrelated with any variables while species and total counts in the centre of the biplot are presumably highly correlated or uncorrelated.

Table 2. List of fungi isolated from different parts of shoot system of *Eichhornia crassipe*s, from irrigation canals at Kafr EL-Bateikh, Damietta Egypt.

Localities	**1**			**2**			**3**				
Fungal pathogens	**Stolon**	**Swollen leaf base**	**Leaf blade**	**Stolon**	**Swollen leaf base**	**Leaf blade**	**Stolon**	**Swollen leaf base**	**Leaf blade**	**Total count**	**Frequency occurrence**
Acremonium strictum Gams			1						1	2	7.4
Acremonium charticola (Lindau) Gams						1				1	7.4
Alternaria alternata (Fres.) Keissler	5	10	12	5	3	2		3		40	26
Aspergillus carneus (Van Tiegh) Bloohwitz			1						1	2	7.4
A. niger Van Tiegh		1			1			1		3	11
A. sulphureus Fres.								1		1	7.4
Cladosporium cladosporioides (Fres.) de Varies			1			1			1	3	11
Drechslera australiensis (Bugnicourt) Subram. & Jain *ex*. Ellis		2	6			2		1		11	14.8
D. halodes (Drechsler) Subram. & Jain		2	4		1	2		2		11	18.5
D. hawaiiensis (Bugnicourt) Subram.	3	5	7	1	3	6		6	7	38	29.6
Fusarium semitectum Berk & Rav.									1	1	3.7
Fusarium sp.						1				1	7.4
Penicillium chrysogenum Thom	1									1	7.4
P. purpurogenum Stoll.			2							2	3.7
Phoma sp.			2				1		1	4	11
Rhizoctonia solani			2			3		2		7	11
Scopulariopsis brevicaulis (Sacc.) Bain.								1		1	7.4
Stachybotrys chartarum (Ehrenb. ex Link)			1			1				2	7.4
Sterile Mycelia			1			1				2	11
Ulocladium atrum Preuss	2	5	8		2		3	2	6	28	26
Verticillium sp.						1				1	3.7
Yeast				1						1	3.7
Mucor circinelloides		1			2					3	7.4
Chaetomium sp.	1				2					3	7.4
Total Count	12	26	48	7	14	21	4	19	18	169	

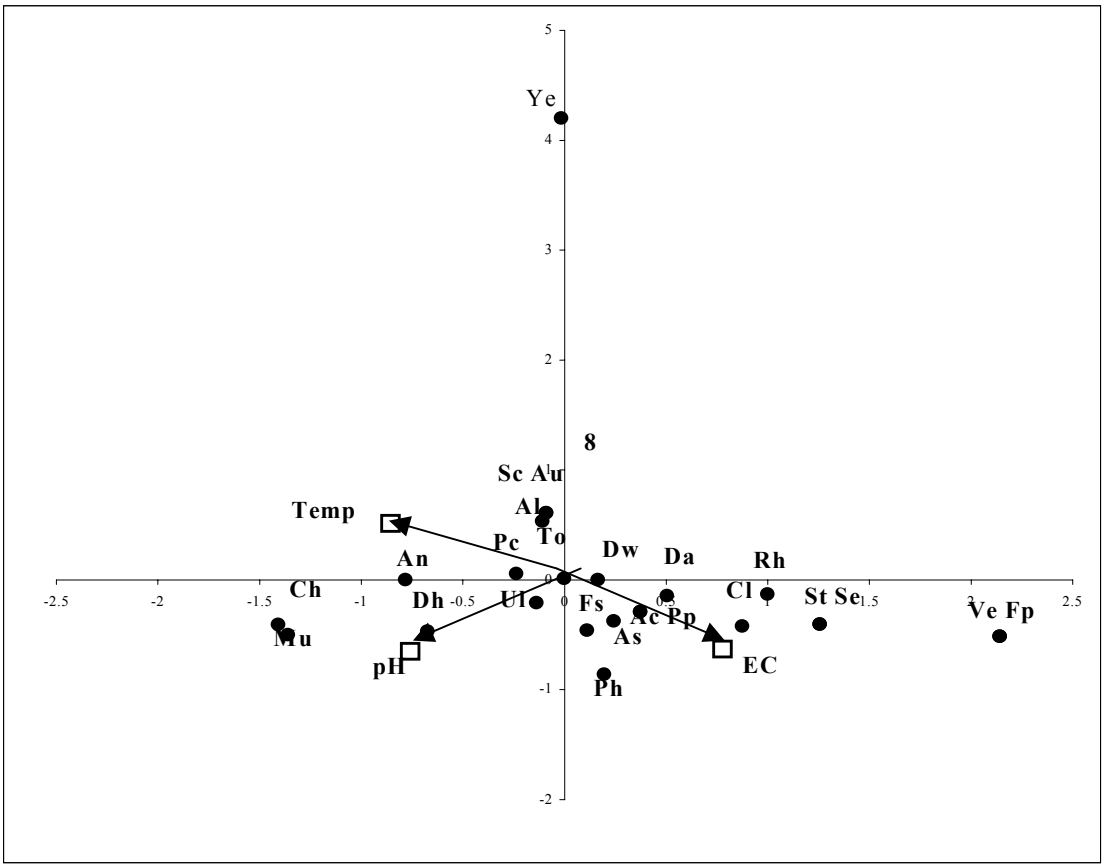

Fig. 1. Ordination diagram based on canonical correspondence analysis of the water hyacinth fungi (•) with respect to three environmental variables (□)*;* pH; Temp, Temperature; EC, Conductivity; As*, Acremonium strictum;* Al*, Alternaria alternata,* A*n, A. niger;* Au*, A. sulphureus;* Cl*, Cladosporium cladosporoides;* Da*, Drechslera australiensis;* Dh*, D. halodes;* Dw*, D. hawaiiensis;* Fs*, Fusarium semitectum;* Fp*, Fusarium* sp., *Pc, Penicillium chrysogenum;* Pp*, P. purpurogenum;* Ph*, Phoma* sp.; Rh*, Rhizoctonia solani,* Sc*, Scopulariopsis brevicaulis;* St*, Stachybotrys chartarum;* Se*, Sterile Mycelia;* Ul*, Ulocladium atrum;* Ve*, Verticillium* sp.; Ye*,* Yeast*;* Mu*, Mucor circinelloides;* Ch*, Chaetomium* sp.

Pathogenicity

Twenty-two species were tested for their ability to infect water hyacinth plants *in vitro*. Table 4 illustrates that only six species were able to infect the plant and produce disease symptoms. Disease started as small necrotic spots and developed into a leaf blight that entirely covered the whole leaf after a maximum of four weeks from incubation. The six species are *Al. alternata,*

Drechslera australiensis, D. halodes, D. hawaiiensis, Rhizoctonia solani and *Ulocladium atrum*. Fig. 2 illustrates that these fungi were most frequently isolated from leaf blades from original samples. Of them, *Drechslera hawaiiensis* had a highest occurence on the blades (20 colonies) and swollen bases (14 colonies). *Alternaria alternata* had its highest occurrence on swollen bases (16 colonies) and blades (14 colonies). The other species may be able to infect the plant and act as normal endophytes (Table 2).

Table 3. General statistical analysis out put of Cannoco program. Species water parmeters correlation.

	Axis 1	**Axis 2**		**Species-environment Correlation**		**Inflation factor**
				Axis 1	**Axis 2**	
Eigen values	0.161	0.129				
Species-environment correlations	0.926	0.924				
Cumulative percentage variance						
of species data	48.2	65.4				
of species-environment relation:	62.1	100				
Sum of all unconstrained Eigen values			0.752			
Sum of all canonical Eigen values			0.201			
pH				-0.703	-0.601	1.1106
Temperature				-0.793	0.477	1.1106
Conductivity				0.719	-0.582	0

Eigen values: are measures of separation of the species distributions along the ordination axis.

On the basis of disease incidence (DI) and severity (DS) the pathogenic species were divided into mildly, low moderately, high moderately and severely infectious species (Table 4). Of these species only *Al. alternata*, *D. hawaiiensis* and *U. atrum* showed high disease severity. These fungi were associated with a high percentage of tissue death after 4-weeks incubation (79%, 78% and 70% respectively).

Formulation and moisture application

Tables 5 and 6 illustrate that in both experiments the disease severity increased gradually between 15 and 30 days after inoculation and that this was accompanied with a decrease in chlorophyll content in diseased leaves as compared to healthy ones (Fig. 3). The *Drechslera hawaiiensis* formulation produced lower chlorophyll content than *U. atrum* and the mixed culture (*D. hawaiiensis* + *U. atrum*) formulation (Figs. 3, 4). Similar results were obtained when cultures were not formulated and tested plants were sprayed with water

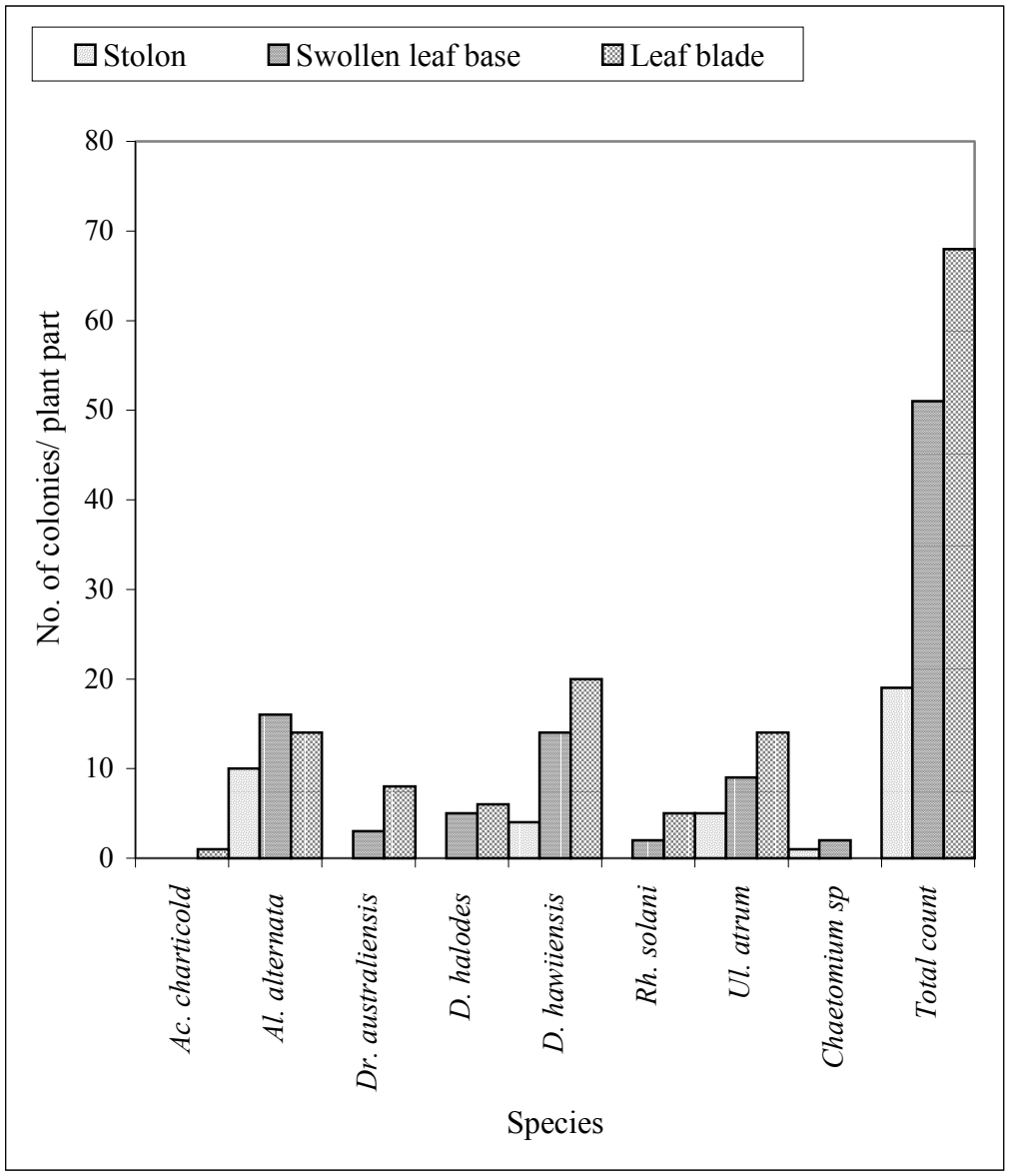

Fig. 2. List of pathogenic fungi isolated from shoot system of *Eichhornia crassipes*, Damietta Egypt.

instead of formulation (Fig. 4, Tables 5, 6). Conversely, after 60 days the disease severity and disease incidence decreased in both experiments accompanied with the production of newly formed leaves.

Table 4. Pathogenecity test (PT) of isolated fungi and testing of positive species (+) as a biocontrol agent.

Species	PT	Incubation days			
		14		28	
		DI	DS	DI	DS
Acremonium strictum	N				
Acremonium charticola	N				
Alternaria alternata	+	65	30	70	79
Aspergillus carneus	N				
A. niger	N				
A. sulphureus	N				
Cladosporium cladosporoides	N				
Drechslera australiensis	+	45	26	65	50
D. halodes	+	20	25	25	30
D. hawaiiensis	+	60	28	70	78
Fusarium semitectum	N				
Fusarium sp.	N				
Mucor circinelloides	N				
Penicillium chrysogenum	N				
P. purpurogenum	N				
Phoma sp.	N				
Rhizoctonia solani	+	15	10	20	15
Scopulariopsis brevicaulis	N				
Stachybotrys chartarum	N				
Sterile Mycelia	N				
Ulocladium atrum	+	50	28	65	70
Verticillium sp.	N				
Yeast	N				

DI, disease incidence; DS, disease severity; "N", no significant damage or infection; "Mild", < 25% of infection; "Low Moderate" 26-50% of infection; "High Moderate", 51-75% of infection and "Severe", > 75% of infection.

Table 5. Influence of fungal suspension accompnying with spraying water on the growth of water hyacinth in field condition.

Pathogencity index	Incubation days					
	15		30		60	
Species	DI	DS	DI	DS	DI	DS
Drechslera hawaiiensis	33	65	40	70	35	60
Ulocladium atrum	28	50	33	54	30	50
D. hawaiiensis + Ulocladium atrum	27	50	33	55	30	50

DI, disease incidence; DS, disease severity.

Discussion

Biodiversity

Twenty-two species were isolated from different plant parts. These fungi have previously been detected from a wide geographical area (Domsch *et al*., 1980). The most common species were *Alternaria alternata*, *Drechslera hawaiiensis* and *Ulocladium atrum*. These are widely distributed taxa, common non-specific saprobes. *Alternaria alternata* has been described as a pathogen of water hyacinth in Bangladesh (Bardur-ud-Din, 1978), Australia (Galbraith and Hayward, 1984), India (Aneja and Singh, 1989) and Egypt (Elwakil *et al*., 1989; Shabana *et al.,* 1995). These facultative pathogens have been also isolated from soil and aquatic habitats (Ellis, 1971; EL-Morsy, 1999, 2000; EL-Morsy *et al*., 2000). *Drechslera hawaiiensis* and *Ulocladium atrum* also behave analogously (Ellis, 1971 and 1976) but have not previously been isolated as water hyacinth pathogens. The other non-pathogenic species were considered as normal endophytes that have been isolated previously from several terrestrial habitats (Domsch *et al*., 1980; Moubasher *et al*., 1985; Khallil *et al*., 1991; EL-Morsy, 1999, 2000; EL-Morsy *et al*., 2000).

Table 6. Pattern of effect of fungal formulation on the growth of water hyacinth in field condition.

Pathogencity index	**Incubation days**					
	12		**24**		**60**	
Fungal formulation	**DI**	**DS**	**DI**	**DS**	**DI**	**DS**
Drechslera hawaiiensis	35	70	40	75	38	72
Ulocladium atrum	30	50	33	54	30	52
D. hawaiiensis + *Ulocladium atrum*	30	70	33	55	30	50

DI, disease incidence; DS, disease severity.

Canonical correspondence analysis (CCA)

CCA analysis has been used to display the interrelationships between the environment and specificity with hosts in previous studies (EL-Morsy, 1999; EL-Morsy *et al*., 2000). The CCA biplot (Fig. 2) reflects the effect of water parameters on the distribution of fungi. The microfungi alignment is shown on two axes. The length of the arrows indicates the importance of the factors (longer arrows = more important) (Ter Braak, 1988). However, all variables measured here have produced arrows of equal length, thus, they are

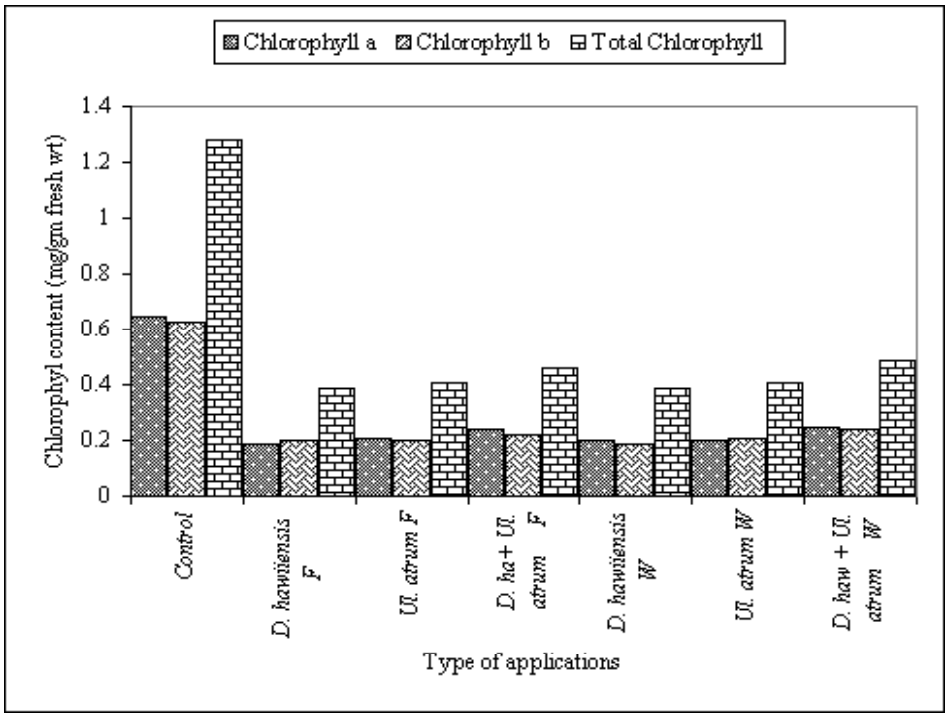

Fig. 3. Chlorophyll content of healthy and treated leaves of water hyacinth after 12, 24 and 60 days of infection. F= Formulation; W = Water application.

of similar importance to fungal distribution. In fact, the patterns of fungal abundance were influenced positively by changes in EC and negatively by pH and TE. Indeed, most fungi have several pH optima, hence, it was believed that pH would not be a decisive factor (Lund, 1934) in fungal distribution. On the contrary, Tyler (1989) reported that any appreciable changes in pH may affect the availability of nutrients and thus govern species diversity. Species at the centre of the diagram are possibly strongly influenced by EC, pH and TE or uncorrelated with any variable. The same conclusion has been stated earlier in studies by Filipelo *et al*. (1997), EL-Morsy (1999) and EL-Morsy *et al*. (2000).

Pathogenicity

As a result of pathogenicity tests and on the basis of disease severity, *Al. alternata* (79% of tissue dead), *D. hawaiiensis* (78%) and *U. atrum* (70%) were found to be the most destructive species. *Alternaria alternata* has a worldwide distribution and has been isolated from almost all habitats (Ellis, 1971; Farr *et al.,* 1989). This fungus has been fully evaluated as a non-efficient biocontrol

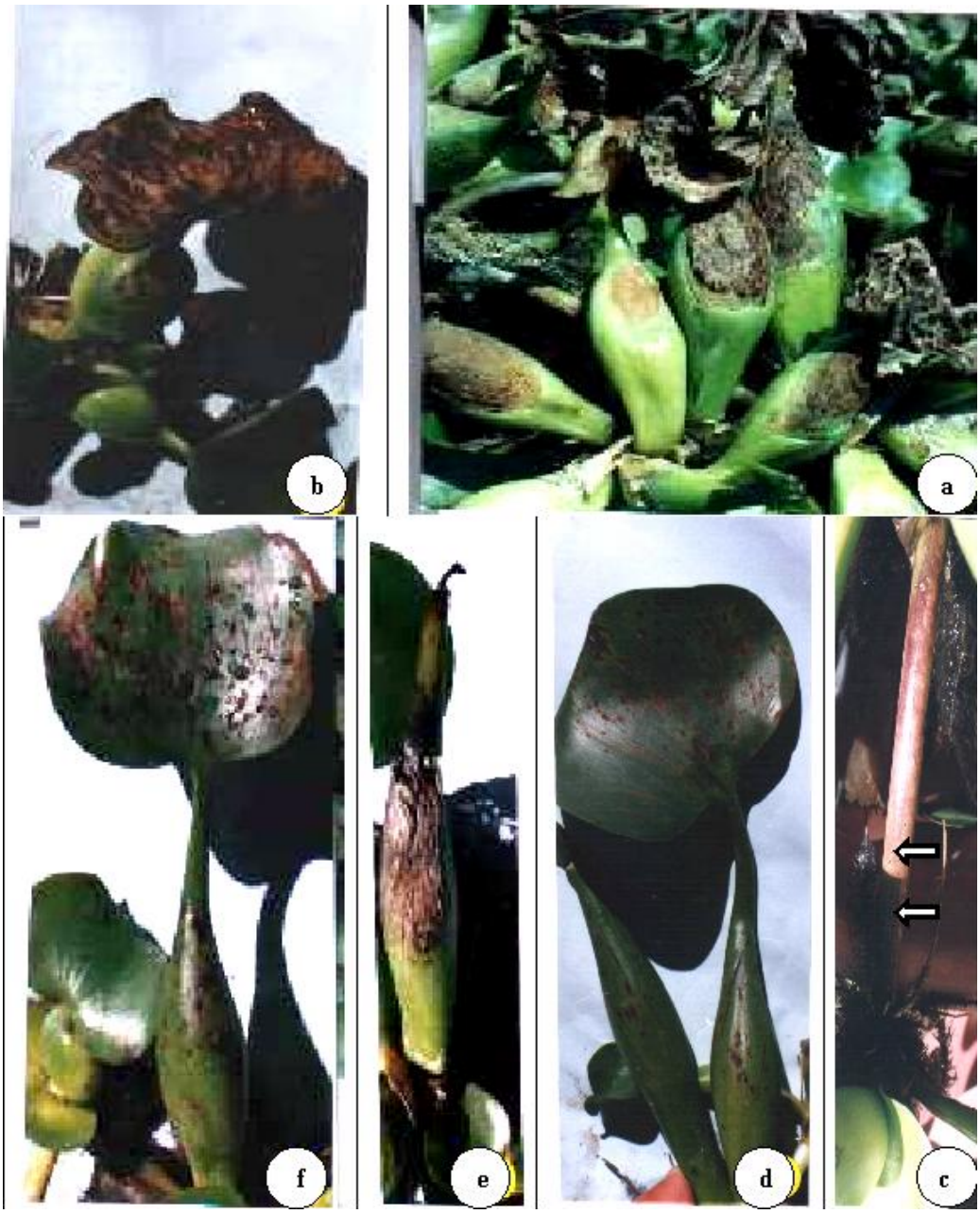

Fig. 4 a-f. Various blight symptoms on water hyacinth plant. **a.** Naturally infected plants showing blight symptoms on leaves parts. **b-c.** Blight symptoms after infection with *Ulocladium atrum*. **d-e.** Blight symptoms after infection with *Drechslera hawaiiensis*. **f.** Symptoms after infection with mixed inoculums of *U. atrum* and *D. hawaiiensis*. Note infected stolon in c (arrowed).

agent (Bardur-ud-Din, 1978; Aneja and Singh, 1989). *Drechslera hawaiiensis* was first isolated from rice grains and then from soil, plants, and textiles and from other substrata from several countries (Ellis, 1971). *Ulocladium atrum* also has a worldwide distribution and has been isolated from seeds, stems and leaves of several plants and soil (Ellis, 1976). These latter taxa have not previously isolated as pathogenic species of water hyacinth. Therefore, as compared to *Al. alternata* which is a plurivorus species with several pathotypes including water hyacinth (Ellis, 1971; Domsch *et al.*, 1980; Farr *et al.,* 1989), *D. hawaiiensis* and *U. atrum* needed to be tested for their pathogenicity in case they had potential as bioherbicides.

Formulation and moisture application

Formulation and daily spraying of water permitted conidial germination and infection of target plants in the field. The degree of DI and DS increased with increasing incubation period and gave maximum severity after 30 days of incubation. This occurred with a decrease in chlorophyll content. Similarly, Shabana (1997a,b) stated that there is a negative correlation between the DS and chlorophyll content in the infected leaves. After 30 days, both DI and DS decreased and this was attributed to the increase in the number of newly formed leaves.

In conclusion, there was little differences in DI and DS between the two treatments (propagule in formulation vs. propagule in water) in the field. *Drechslera hawaiiensis* and *Ulocladium atrum* are not efficient biocontrol agents, despite their severe effects on the plants, as infection by the fungus was not accompanied with complete death after 60 days incubation.

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