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Alternaria eichhorniae, a biological control agent for waterhyacinth: mycoherbicidal formulation and physiological and ultrastructural host responses

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Abstract

The bioherbicidal efficacy of different alginate formulations of *Alternaria eichhorniae* 5 (isolate Ae5), a virulent Egyptian isolate, was compared on waterhyacinth (*Eichhornia crassipes*). The fungus was formulated as 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). Maximum DS, but not DI, was obtained with the alginate pellets of mycelium plus culture filtrate. Alginate formulations supplemented with the hydrophilic polymer were more effective in promoting disease. Physiological changes associated with the treated waterhyacinth plants were determined 3, 6 and 9 days after treatment. Waterhyacinth plants treated with alginate pellets of mycelium plus culture filtrate of Ae5 had the lowest levels of pigments, carbohydrates and relative water content. Infection of waterhyacinth with Ae5 led to a significant increase in total phenols of leaves as compared to control. Penetration of waterhyacinth leaves by the fungus occurred only through the stomata, and the invading hyphae were located in the intercellular spaces of leaf tissues. Cytological changes noted in infected cells included changes in chloroplast, nucleus and mitochondria. Invagination of the plasma membrane, particularly at plasmodesmata was also noticed in infected cells. The associations between the infection process, the physiological disorder and the ultrastructure of infected leaves are discussed.

Introduction

Eichhornia crassipes (Mart.) Solms is the most troublesome aquatic weed in Egypt and one of the world's worst aquatic weeds (Holm et al., 1977). Mechanical and chemical control methods are the most commonly used management tools but are not completely satisfactory. Despite successes with biological and integrated methods of control of aquatic weeds in the United States (Charudattan, 1986), biological control has not yet been used on a large scale in Egypt and waterhyacinth still continues to cause serious problems in Egypt and in many other countries. *Alternaria eichhorniae* Nag Raj and Ponnappa was first reported as a potential biocontrol agent for waterhyacinth in 1970 in India (Nag Raj and Ponnappa, 1970); the fungus was recorded on this host from various other regions in India (Charudattan, 1973), Bangladesh (Badur-ud-Din, 1978), Indonesia (Mangoendihardjo et al., 1977), and Thailand (Rakvidhyasastra et al., 1978), and in 1987 in Egypt (Shabana, 1992; Shabana et al., 1995c). Shabana et al. (1994, 1995a, 1995b) have demonstrated the safety, efficacy, and feasibility of using an isolate of *A. eichhorniae* (Ae5) as a mycoherbicide for waterhyacinth in Egypt. They reported that none of 97 economically important, non-target plant species and cultivars evaluated in host range trials was susceptible to Ae5 and found that the efficacy of Ae5 for controlling waterhyacinth increased with the number of applications of the formulation. Four applications at

10-day intervals produced 93% disease (leaf necrosis and blight) and 81% decrease in fresh weight under greenhouse conditions two months after application.

The purpose of this investigation was to evaluate the efficacy of three mycoherbicide formulations of Ae5and to elucidate the infection process and cytological changes induced by Ae5 in waterhyacinth leaves by using scanning electron microscopy. So far, no ultrastructural studies have been reported concerning the infection process and subsequent tissue colonization of waterhyacinth by *A. eichhorniae*.

Materials and methods

Host plant. Waterhyacinth plants (20–30 cm height with a leaf surface area of 20–40 cm^2) were collected from a naturally infested irrigation canal near the Mansoura University campus, El-Mansoura, Egypt during the summer of 1993. They were trimmed of any necrotic or senescent tissue, and maintained for 2 weeks in a 1 m^2 concrete pool containing tap water. Individual plants were transferred a day before inoculation to 15 cm deep, 12 cm diam plastic pots containing about 700 ml tap water.

Mycoherbicide production. Ae5, a virulent Egyptian isolate of *A. eichhorniae*, was grown on fresh potato dextrose broth supplemented with 10% glycerol for 15 days at 28 °C in the dark. The mycelium was harvested and rinsed with sterile distilled water and divided into two equal parts. Three inoculum preparations were made as follows: the first preparation was made by blending one-half of the harvested mycelium with distilled water at 1:1 (w/v) for 10 s in a blender. The blended mycelial suspension was diluted 1:4 (v/v) with 1.33% (w/v) sodium alginate (BDH Chemicals Ltd., Poole, England) in distilled water. The second preparation was made by blending the other half of the mycelial mat with the culture filtrate at 1:1 (w/v) for 10 s in a blender. The mycelial-filtrate suspension was diluted 1:4 (v/v) with 1.33% (w/v) sodium alginate in distilled water. The third preparation was made by stirring the culture filtrate only with 1.33% (w/v) sodium alginate in distilled water at 1:4 (v/v). Each preparation was dripped into 0.25 M $CaCl_2$ to form gel beads of 3–4 mm diam. The beads were harvested over sieves, rinsed with distilled water, and spread on a carton sheet to be air-dried with electric fans. Alginate pellets, prepared similarly but without the fungus and/or its culture filtrate, were used for the control treatments.

Evaluation of the alginate formulations with or without a hydrophilic gel for weed control efficacy. The formulations of Ae5, namely, mycelium-alginate, mycelium-filtrate-alginate, and filtrate-alginate were applied to waterhyacinth plants in the greenhouse. Twenty four individual clusters of 3- to 5-leaved waterhyacinth plants were placed in plastic pots containing 700 ml of tap water. The leaves (laminae) and bulbous petioles (pseudolaminae) of 12 plants were pre-wetted with tap water to facilitate adherence of Evergreen 500 (polyacrylamide; Chemie Linz AG, St. Peter-Strasse 25, P.O.Box 296, A-4021 Linz, Austria), a hydrophilic polymer (0.25 g $plant^{-1}$), and/or the alginate pellets dusted onto the plants. Three replicate plants were sprinkled with each pelletized formulation (0.5 g $plant^{-1}$) and resprayed with tap water using a hand-operated, low-pressure sprayer to provide high humidity. Three plants sprayed with tap water and dusted with Evergreen 500 followed by fungus-free alginate pellets served as control #1. The other twelve plants were sprayed with tap water to facilitate adherence of pelletized formulations but were not dusted with the gel. Three replicate plants from this group were sprinkled with each pelletized formulation (0.5 g $plant^{-1}$) and sprayed again with tap water. Three plants sprayed with plain water and sprinkled with fungus-free alginate pellets served as control #2. After inoculation, all plants were covered with clear polyethylene bags for 48 h to maintain high humidity and kept in a greenhouse (30 $\pm$ 3 °C, about 49% relative humidity and approximately 300 $\mu E/m^2/s$ light at midday) at the University of Mansoura campus, El-Mansoura, Egypt. Plants were arranged on the benches in a completely randomized design. Disease incidence (DI) and disease severity (DS) were recorded 3, 6, and 9 days after inoculation. DI, the presence or absence of disease, was determined as a percentage of number of leaves on the plant that exhibited disease symptoms and/or necrotic damage. DS, the amount of disease and/or phytotoxin damage, was rated by comparing actual damage to a pictorial disease scale of 0 to 9, where 0 = healthy and 9 = 90% diseased (Freeman and Charudattan, 1984). DS was determined for each leaf and values were summed and averaged to derive DS for a whole plant. The experiment was repeated twice and the data were statistically analyzed with the Statistical Analysis System (SAS Institute, 1988). All comparisons first were subjected to analysis of variance (ANOVA) and significant dif-

ferences among treatment means were determined with the LS means test. Arcsine square-root (angular) transformation was applied to the DI observations to induce homogeneity of variance among treatments. Regression analysis was also used to determine relationships among treatment means.

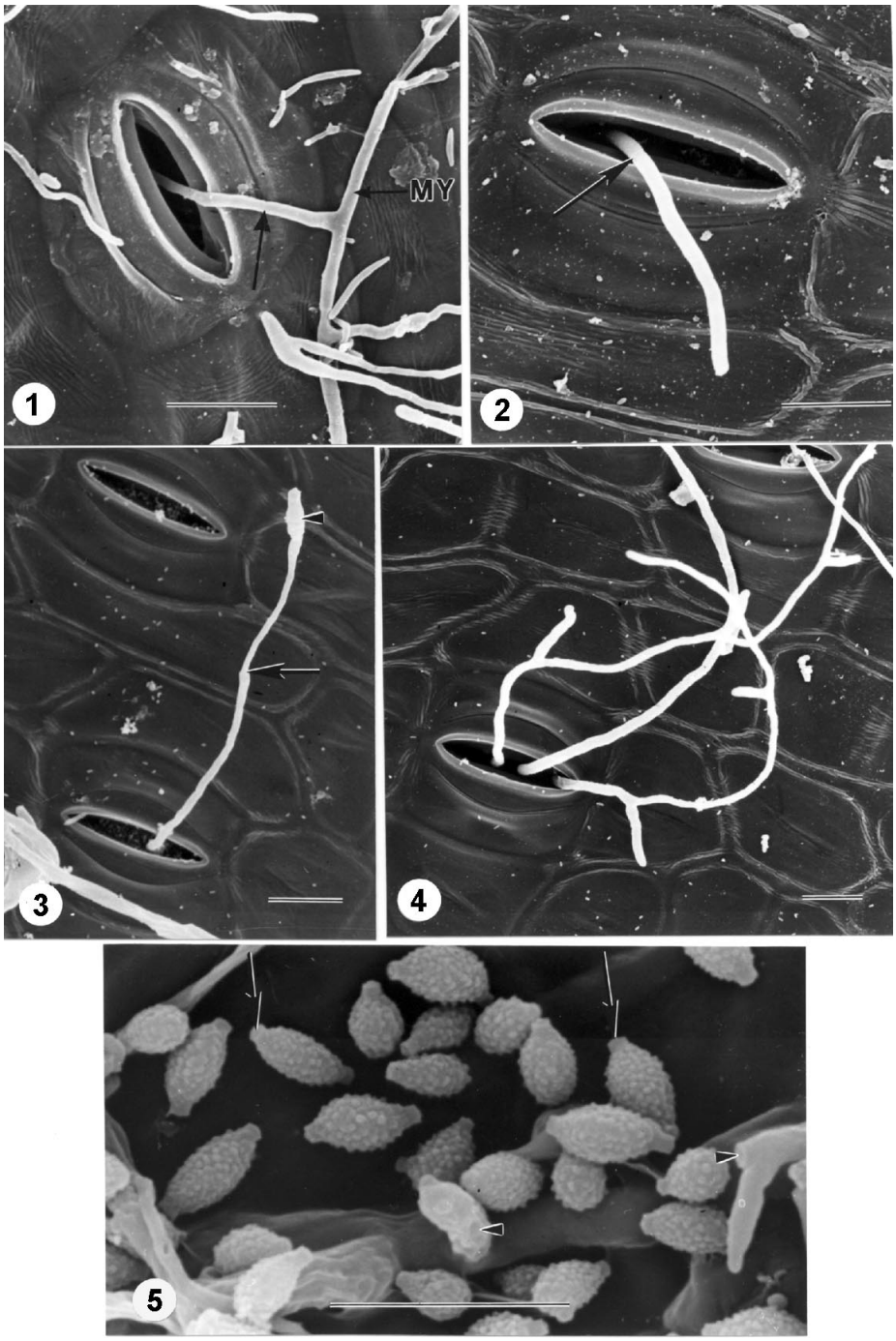
Electron microscopy. Waterhyacinth leaves treated with alginate formulation of mycelium plus culture filtrate were examined by scanning (SEM) and transmission electron microscopy (TEM). Three days after inoculation, leaf segments from areas adjoining the lesions and from areas 2–4 cm distant from lesions were removed and prepared for electron microscopy. Leaf segments from healthy control plants corresponding to approximately the same locations as those from diseased leaves were removed and similarly prepared for electron microscopy. The method adopted from Mercer and Birbeck (1972) and modified by Baka and Aldesuquy (1992) was used for SEM. Leaf segments were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0, washed in the same buffer, and postfixed in 1% OsO_4. Leaf segments were dehydrated in a graded acetone series, dried, and coated with gold. Samples were then examined and photographed using a JEOL JSM-6400 scanning electron microscope. Leaf pieces from infected and healthy leaves were processed for TEM according to the method of Hayat (1989) and modified by Baka and Aldesuquy (1992). Leaf pieces (1.0 mm^2) were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0, washed in the same buffer, postfixed in 1% OsO_4, dehydrated in a graded series of ethanol, and embedded in Spurr's resin. Ultrathin sections were cut using a Reichert ultramicrotome, stained with 2.0% uranyl acetate followed by lead citrate. Sections were viewed and photographed using a JEOL 100-S transmission electron microscope.

Physiological studies. Plant pigments, carbohydrates, relative water content (RWC), and total phenols of treated and control leaves were determined 3, 6, and 9 days after treatment with the three Ae5 formulations without Evergreen 500. At each period, six leaves (one leaf from each plant, replicated) were randomly selected for determination of physiological parameters. Plant pigments, Chlorophyll *a*, *b* and carotenoids were determined according to spectrophotometric methods recommended by Wellburn and Lichtenthaler (1984). Reducing sugars, sucrose, polysaccharides, and total carbohydrates from healthy and treated leaves were determined as described by Younis et al. (1969). The relative water content (RWC) of both healthy and treated leaves was determined according to the technique described by Weatherly (1950). The saturated water deficit (SWD), the amount of water required to reach the saturation point (100%), was then calculated and expressed as a percentage. Total phenols were extracted from healthy and treated leaves and estimated by the method of Ribereau-Gayon (1972). Data on plant pigments, carbohydrates and total phenols were statistically analyzed with the Statistical Analysis System (SAS Institute, 1988). All comparisons first were subjected to analysis of variance (ANOVA) and significant differences among treatment means were determined with an LS means test. Data on RWC and SWD were transformed to arcsine square roots to induce homogeneity of variance among treatments and then subjected to ANOVA.

Results

Evaluation of the alginate formulations. Disease did not develop on non-inoculated control plants (Table 1); there was therefore no cross-contamination of control plants from inoculated plants during the experiment. The formulation type (treatment) and the gel had significant effects ($P = 0.0001$) on both DI and DS. The DI and DS on inoculated plants increased significantly with time after inoculation ($P \leq 0.01$ and $P = 0.0001$, respectively). The highest levels of DS, but not DI, were obtained from mycelium-filtrate-alginate formulation supplemented with Evergreen 500 ($P = 0.0001$), particularly at days 6 and 9 after inoculation. The least effective formulation in terms of DS was the filtrate-alginate formulation (Table 1). There was no relationship between DI and DS since disease can occur on all leaves of the plant (DI = 100%) although its severity may be low (low DS).

Scanning and transmission electron microscopy. The morphological characteristics of Ae5 on waterhyacinth leaf are shown in Figures 1–5. The mycelium spread on the leaf surface and branched before entering through stomata (Figure 1). After colonization within leaf tissue, the conidiophores emerged from the stomata singly or in clusters (Figures 2–4). The conidiophores were septate and branched (Figure 4). The conidia were oblong and characterized by rough surfaces and one or two projections at one or both ends, possibly the positions of their attachment to the conidiophore and a second conidium along a chain (Figure 5). TEM

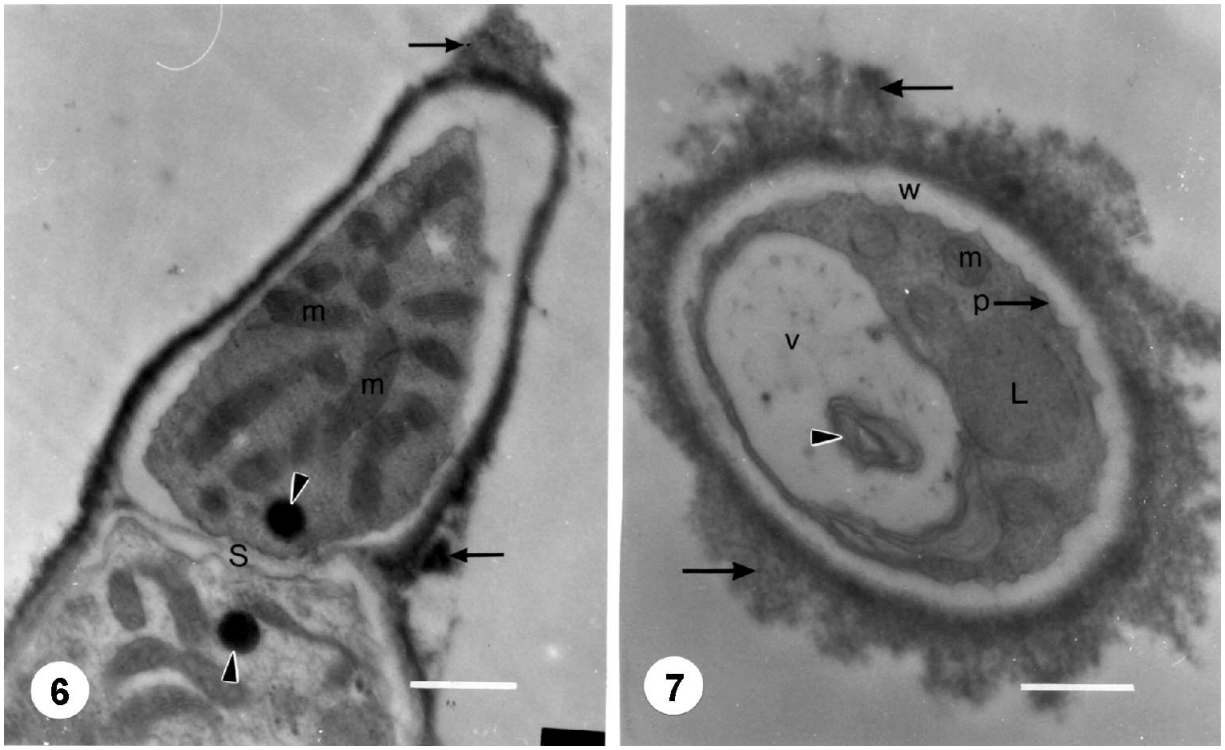


Figures 1–5. Scanning electron micrographs of a waterhyacinth leaf treated with alginate formulation of mycelium plus culture filtrate of *Alternaria eichhorniae* 5, Ae5. (1) Mycelial branch (arrow) arising from mycelium (MY) entering the leaf tissue through a stoma. (2) Growing conidiophore emerging from a stoma. Note the septum (arrow). (3) Mature conidiophore emerging from a stoma. Note the septum (arrow) and the formation of a spore (arrowhead). (4) Three branched conidiophores emerging from one stoma. (5) Numerous conidia on leaf surface of waterhyacinth. Note the rough surface of spores, their projections (arrows), and the spore scars (arrowheads) on conidiophores. Scale bars = 10 μm.

Table 1. Disease incidence (DI) and disease severity (DS) caused by different mycoherbicidal components of *Alternaria eichhorniae* 5 in presence or absence of a gelling agent

Treatment[a]	Gel[b]	Days after treatment					
		3		6		9	
		DI	DS	DI	DS	DI	DS
Control	–	0.00 c[c]	0.00 d	0.00 e	0.00 e	0.00 d	0.00 f
(healthy)	+	0.00 c	0.00 d	0.00 e	0.00 e	0.00 d	0.00 f
Mycelium	–	83.33 ab	1.33 c	83.33 bc	3.33 b	91.67 ab	4.67 c
	+	91.67 a	1.67 bc	100.00 a	3.67 b	100.00 a	5.33 bc
Filtrate	–	0.00 c	0.00 d	33.33 d	1.00 d	41.67 c	1.33 e
	+	70.00 b	1.00 c	73.90 c	2.00 c	85.00 b	2.67 d
Mycelium+	–	67.23 b	2.33 ab	73.90 c	3.67 b	93.33 ab	5.67 b
filtrate	+	75.00 b	2.67 a	91.67 ab	4.67 a	100.00 a	6.67 a

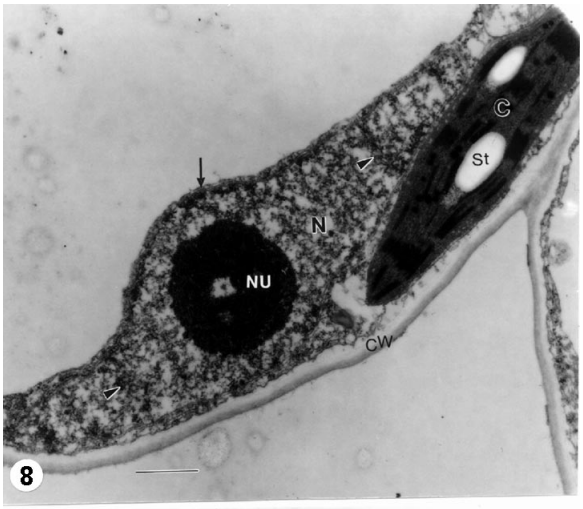
[a] See text for details.
[b] + = Evergreen 500 was dusted on the prewetted plants (0.25 g plant^{-1}) immediately before sprinkling the pelletized formulation; – = no Evergreen 500 was dusted.
[c] Values within a column followed by the same letter(s) are not significantly different according to LS means test ($P \leq 0.02$ for DI and 0.008 for DS).

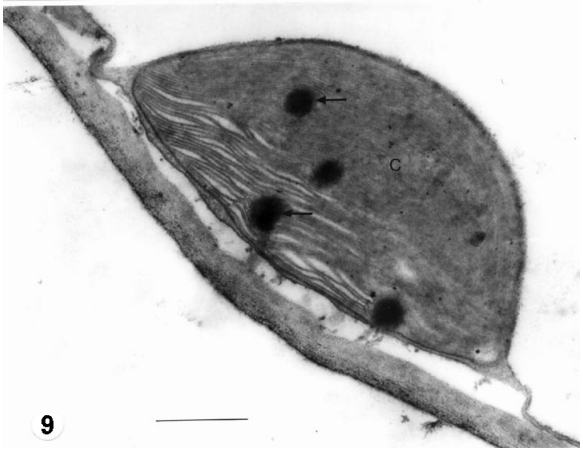


Figures 6–7. Transmission electron micrographs of a hyphae of *Alternaria eichhorniae* 5, Ae5, located in the intercellular space of a waterhyacinth leaf treated with alginate formulation of mycelium plus culture filtrate of Ae5. (6) A longitudinal section, the mycelium contains numerous mitochondria (m), septum (s), and two electron-dense bodies (arrowheads) at both sides of the septum. A mucilaginous matrix borders the mycelial tip (arrows). (7) A transverse section, showing electron-lucent fungal wall (w), fungal plasmalemma (p), mitochondria (m), vacuole (v), a large lipid body (L) and membranous structure (arrowhead). A mucilaginous matrix surrounds the mycelium (arrows). Scale bars = 0.5 μm.

examination revealed that the mycelium was located in the intercellular spaces of leaf tissues. As typical for mycelium of Deuteromycetes, the mycelium of Ae5 was septated and had numerous mitochondria, vacuoles, lipid bodies, and membranous structures (Figures 6, 7). The most striking feature was the presence of electron-dense materials surrounding the mycelium which may act as a cement for the attachment of the mycelium to the host cell wall (Figures 6, 7).

The ultrastructure of the cells from non-inoculated healthy leaves revealed the presence of normal organelles such as the chloroplast, nucleus, and mitochondria. The chloroplast was characterized by a well-organized membrane system of grana and intergranal lamellae, a well-defined envelope, large starch grains, and very few small plastoglobuli (Figure 8). Infection of waterhyacinth leaves by Ae5 caused major changes in the ultrastructure of chloroplasts, nuclei and mitochondria. The chloroplast was lens-shaped (Figure 9) or spherical (Figure 10). The disorganization of the membrane system of the chloroplast, the breakdown of the chloroplast envelope, an increase in the number and size of plastoglobuli and the disappearance of starch grains, are also indicative of infection (Figures 9, 10). The nucleus became spherical, its envelope degenerated and the heterochromatin was concentrated at the periphery of nucleus (Figure 11). The mitochondria from infected cells had swollen cristae and a disorganized envelope (Figure 12). The precipitation of electron-dense substances at mitochondrial envelopes was a characteristic feature of infection (Figure 13). The appearance of large lipid bodies in the cytoplasm and the disturbance of the wall-lining plasma membrane were conspicuous after infection (Figure 14). In addition, the infection caused an invagination of the plasma membrane, often at the plasmodesmata (Figure 15). The invagination of the plasma membrane could possibly be a maker for membrane modification as a result of infection.

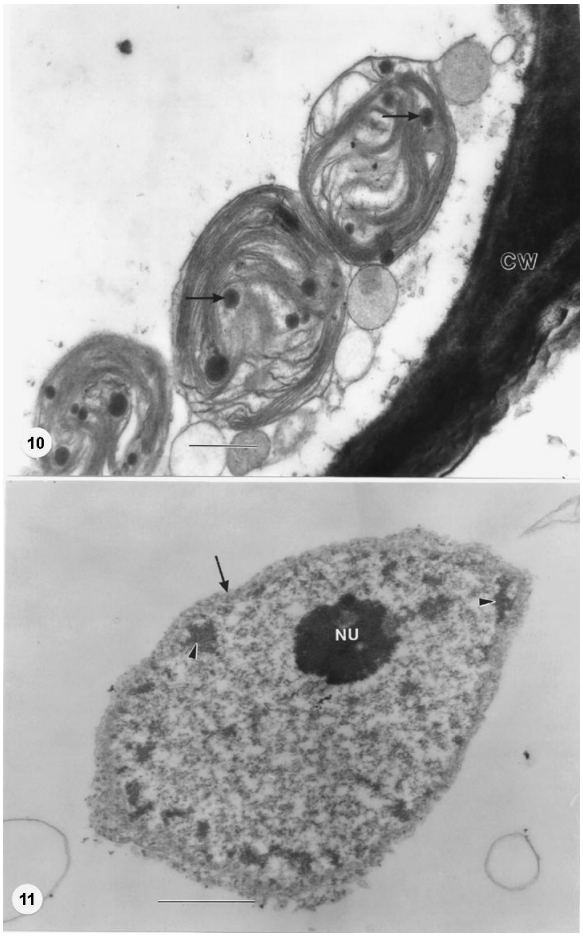




Figures 8–9. Transmission electron micrograph of a part of a cell from non-inoculated healthy (8) and inoculated (9) leaves of waterhyacinth with alginate formulation of mycelium plus culture filtrate of *Alternaria eichhorniae* 5, Ae5. (8) Normal nucleus (N) with nucleolus (NU). The nucleus is enclosed by a nuclear envelope (arrow). The heterochromatin (arrowheads) is distributed randomly within the nucleus. An elongated chloroplast (c) with well-organized membrane system of grana (g), starch grains (St), and cell wall (CW) can be seen. (9) An ellipsoidal chloroplast (c) with disorganized membrane system and plastoglobuli (arrows). Scale bars = 0.5 μm.

Physiological aspects: pigment content. The mycelium plus culture filtrate formulation had the greatest effect in lowering the level of total photosynthetic pigments followed by the formulation of mycelium alone and there was no significant difference between them after 6 and 9 days post-inoculation (Table 2). The treatment with the least effect was culture filtrate alone; it was not significantly different from the formulation of the mycelium alone at day 9 post-inoculation. The total pigments content of the infected leaves decreased linearly as the disease development progressed; thus, a rapid decline, especially in chlorophyll *a* and *b*, occurred over the time of experiment. In the non-inoculated healthy leaves, the reduction of pigment content over time was not seen.

Carbohydrate content. Each formulation caused a significant decrease (P = 0.0001) in the total carbohydrates [soluble sugars (reducing sugars and sucrose) and insoluble sugars (polysaccharides)] in inoculated waterhyacinth leaves compared with the healthy con-



Figures 10–11. Electron micrograph of part of a cell from a waterhyacinth leaf treated with an alginate formulation of mycelium plus culture filtrate of *Alternaria eichhorniae* 5, Ae5. (10) Shows spherical degenerated chloroplasts with plastoglobuli (arrows) and cell wall (CW). Scale bar = 0.5 μm. (11) A spherical nucleus in a cell from an infected leaf showing heterochromatin (arrowheads) concentrated at the periphery of the nucleus, nucleolus (NU), and degenerated nuclear envelope (arrow). Scale bar = 1 μm.

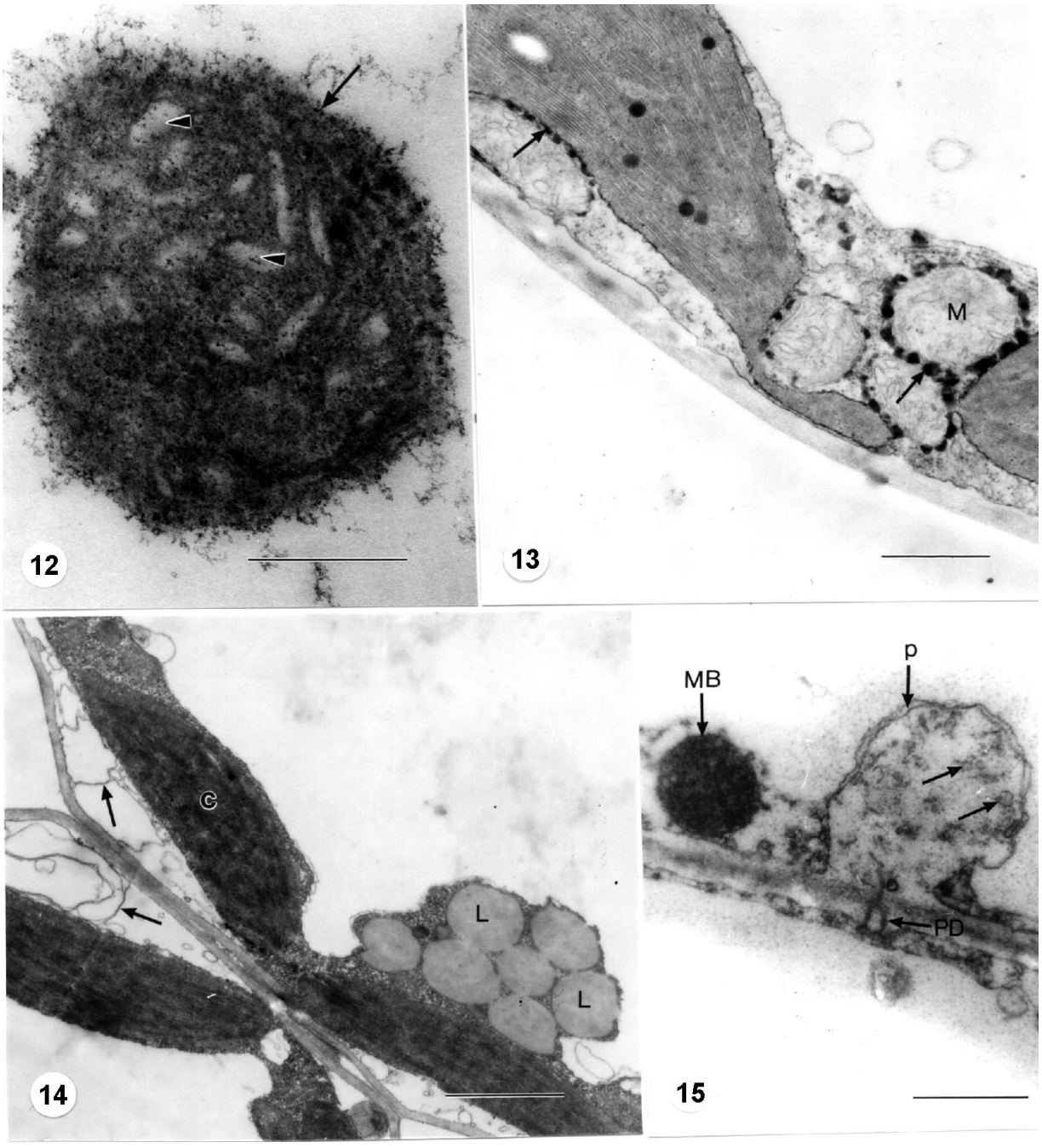
Table 2. Pigments content in healthy and treated leaves of waterhyacinth (values expressed as mg g^{-1} fresh wt.)

Treatment[a]	Days after inoculation	Chl. *a*[b]	Chl. *b*[c]	Carotenoides	Total pigments
Control (healthy)		0.70 a[d]	0.56 a	0.09 a	1.35 a
Mycelium	3	0.58 b	0.43 b	0.04 c	1.05 c
Filtrate		0.64 ab	0.49 b	0.06 b	1.19 b
Mycelium+ filtrate		0.37 c	0.35 c	0.03 c	0.75 d
Control (healthy)		0.67 a	0.54 a	0.06 a	1.27 a
Mycelium	6	0.24 c	0.28 bc	0.04 b	0.56 c
Filtrate		0.38 b	0.30 b	0.04 b	0.72 b
Mycelium+ filtrate		0.23 c	0.22 c	0.03 b	0.48 c
Control (healthy)		0.66 a	0.50 a	0.07 a	1.23a
Mycelium	9	0.22 b	0.19 b	0.04 b	0.45 bc
Filtrate		0.25 b	0.21 b	0.05 b	0.51 b
Mycelium+ filtrate		0.18 b	0.18 b	0.02 c	0.38 c

[a] See text for details.
[b] Chl. *a* = chlorophyll *a*.
[c] Chl. *b* = chlorophyll *b*.
[d] Values within a column for each day followed by the same letter(s) are not significantly different according to LS means test (for days 3, 6, and 9: $P \leq 0.007$, 0.002, and 0.0001, respectively, for chl. *a*; $P \leq 0.05$, 0.03, and 0.0001, respectively, for chl. *b*; $P \leq 0.007$ for days 3, 6, and 9 for carotenoids; and $P \leq 0.01$, 0.005, and 0.04, respectively, for total pigments).

trols (Table 3). It is also obvious that the soluble sugars (reducing sugars and sucrose) decreased with the progress of the experiment both in healthy and treated plants. The polysaccharides increased in infected leaves but were mostly stable in healthy control leaves over time. The leaves which were treated with mycelium plus culture filtrate formulation significantly had the greatest level of decline in polysaccharides and total carbohydrates than those treated with formulations of mycelium or filtrate alone. However, there was no significant difference between the formulations of mycelium plus culture filtrate and culture filtrate alone with regard to declining levels of soluble sugars in the treated leaves particularly after 6 and 9 days of treatment. The mycelium alone was the second most effective formulation in reducing the levels of polysaccharides but was the third most effective among the formulations in causing the decline of soluble sugars, particularly at days 6 and 9 post-inoculation.

Relative water content (RWC) and saturation water deficit (SWD). The type of formulation (treatment) significantly influenced the levels of RWC and SWD (P = 0.0007 and 0.0001, respectively) of the treated waterhyacinth leaves. The formulation of mycelium plus culture filtrate caused a significant decrease ($P \leq 0.05$) in the RWC of inoculated leaves relative to non-inoculated control leaves after only 6 and 9 days of inoculation (Table 4); however, there was no significant difference between this formulation and the for-



Figures 12–15. Electron micrographs of a part of a cell from a waterhyacinth leaf treated with an alginate formulation of mycelium plus culture filtrate of *Alternaria eichhorniae* 5, Ae5. (12) A single degenerated mitochondrion from an infected cell showing swollen cristae (arrowheads) and disorganized mitochondrial envelope (arrow). Scale bar = 0.25 μm. (13) Shows electron-dense material (arrows) deposited on mitochondrial envelopes. Chloroplast tail (T) and mitochondria (M) can also be seen. Scale bar = 0.5 μm. (14) Shows disorganized plasma membranes (arrows), degenerated chloroplasts (c) and lipid drops (L) inside the cytoplasm. Scale bar = 1.0 μm. (15) Shows an invaginated plasma membrane (p) at the plasmodesmata (PD), lomasome-like vesicles (arrows), membranous fragments (arrowheads), and microbody (MB). Scale bar = 0.5 μm.

Table 3. Carbohydrates content in healthy and treated leaves of waterhyacinth (values expressed as mg g^{-1} dry wt.)

Treatment[a]	Days after inoculation	Reducing sugars	Sucrose	Polysaccharides	Total carbohydrates
Control (healthy)		0.45 a[b]	2.42 b	8.83 a	11.70 a
Mycelium	3	0.38 b	2.10 c	4.18 c	6.66 c
Filtrate		0.21 c	2.66 a	4.89 b	7.76 b
Mycelium+ filtrate		0.22 c	2.01 d	3.18 d	5.41 d
Control (healthy)		0.42 a	2.21 a	8.75 a	11.38
Mycelium	6	0.31 b	2.03 b	4.97 c	7.31 b
Filtrate		0.17 c	1.33 c	5.27 b	6.77 c
Mycelium+ filtrate		0.15 c	1.27 c	3.78 d	5.20 d
Control (healthy)		0.40 a	2.18 a	8.84 a	11.42 a
Mycelium		0.24 b	1.78 b	5.35 c	7.37 b
Filtrate	9	0.15 c	1.16 c	6.17 b	7.48 b
Mycelium+ filtrate		0.12 c	1.11 c	4.63 d	5.86 c

[a] See text for details.
[b] Values within a column for each day followed by the same letter(s) are not significantly different according to LS means test (for days 3, 6, and 9: $P \leq 0.03$, 0.002, and 0.008, respectively, for reducing sugars; $P = 0.05$, 0.0003, and 0.0001, respectively, for sucrose; and $P = 0.0001$ for polysaccharides and total carbohydrates).

Table 4. Relative water content (RWC) and saturation water deficit (SWD) in healthy and treated leaves of waterhyacinth

Treatment[a]	Days after inoculation					
	3		6		9	
	RWC (%)	SWD (%)	RWC (%)	SWD (%)	RWC (%)	SWD (%)
Control (healthy)	92.0 a[b]	8.0 c	92.5 a	7.5 c	92.9 a	7.1 c
Mycelium	89.0 a	11.0 b	88.7 a	11.3 b	83.9 bc	16.1 b
Filtrate	91.0 a	9.0 bc	90.2 a	9.8 b	85.2 ab	14.8 b
Mycelium + filtrate	85.2 a	14.8 a	80.6 b	19.4 a	73.1 c	26.9 a

[a] See text for details.
[b] Values within a column for each day followed by the same letter(s) are not significantly different according to LS means test (for days 3, 6, and 9: $P \leq 0.66$, 0.03, and 0.05, respectively, for RWC and $P = 0.02$, 0.04, and 0.0001, respectively, for SWD).

mulation of mycelium alone at day 9 post-inoculation. In contrast, a significant increase in the SWD in the infected/damaged waterhyacinth leaves was obtained by using the three formulations relative to controls. However, there was no significant difference between the level of SWD in the control leaves and those leaves treated with the formulation of culture filtrate alone at day 3 after treatment. The inoculated plants with the mycelium plus culture filtrate formulation showed the highest level of water deficit, whereas the lowest level of water deficit was observed in the healthy control plants. The water deficit in plants inoculated with the formulation of mycelium alone was not significantly different from that in plants treated with the formulation of culture filtrate alone (Table 4). It was also observed that SWD was significantly higher ($P = 0.0001$) at day 9 compared to days 3 and 6 after inoculation. However, there was no significant difference between the level of SWD at days 3 and 6 after treatment. Thus, there appeared to be a positive correlation between the amount of disease and/or damage expressed on the treated plants and the degree of SWD.

Phenols content. Treatment of waterhyacinth leaves with all formulations led to a highly significant increase ($P = 0.0001$) in the total phenol content compared to the healthy control plants at day 9 after inoculation (Table 5). Plants treated with the formulation of culture filtrate alone had the highest level of phenols followed by those inoculated with the formulation of mycelium alone; however, there was no significant difference between them at day 9 after inoculation. The plants inoculated with the mycelium plus culture filtrate formulation had lower levels of phenolic compounds than those treated with any of the other formulations. Generally, the concentration of total phenols increased as time progressed.

Discussion

Formulation and application methods are of paramount importance in determining the effectiveness of pathogens as mycoherbicides (Boyette and Templeton, 1981). Adding substances that increase the efficacy of the biocontrol agent, e.g., polymers that extend leaf-wetness period, amendment with pathogen-produced exocellular phytotoxins, or other plant-stress inducing factors, is desirable. Modification of the process for formulating the biocontrol agent (Shabana et

Table 5. Phenolic compounds content of healthy and treated leaves of waterhyacinth (μg g^{-1} dry wt.)

Treatment[a]	Days after inoculation		
	3	6	9
Control (healthy)	350 d[b]	440 d	501 c
Mycelium	550 b	589 b	1210 a
Filtrate	690 a	610 a	1220 a
Mycelium + filtrate	520 c	510 c	1050 b

[a] See text for details.
[b] Values within a column for each day followed by the same letter(s) are not significantly different according to LS means test (for days 3, 6, and 9: $P \leq 0.0002$, 0.005, and 0.0001, respectively).

al., 1995b; Walker, 1981) produced environmentally benign, biodegradable pellets relatively uniform in size. 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. The procedure of drying the pellets immediately after pelletization could simplify the large-scale production of the bioherbicide. The four-stage application that we used in this study (1: wetting waterhyacinth plants with water, 2: dusting the wetted plants with Evergreen 500 as a sticker and maintaining humidity for prolonged periods, 3: sprinkling the pelletized formulation to increase adherence on the plant and take advantage of a long period of high humidity, and 4: re-wetting the plants with extra water to provide high humidity) was successful and produced complete death of the weed 4 weeks after application with the mycelium-filtrate-alginate formulation. The increase in the severity of disease on plants treated with Evergreen 500 plus the mycelium-filtrate-alginate formulation in this study may be due to the fungus being aided by both of the gels, sodium alginate in the pelletized formulation and Evergreen 500 which was applied on the plants immediately before inoculation, and possibly by the phytotoxins produced by the fungus in the cultural medium (Nag Raj and Ponnappa, 1970; Charudattan and Rao 1982; Robeson et al., 1984). Shabana (1992) pointed out that applying the fungal formulation with a hydrophilic gel promoted disease since the gel holds water for the extended periods required to promote infection; he also found that a higher concentration of the partially purified culture extract (10%) of Ae5 was required for symptom expression on waterhyacinth leaves suggesting a role of toxins in disease development. Additionally, Shabana (1992) suggested the possibility that many toxins were produced by this fungus that might have involvement in the pathogenicity of the organism. Results of the present investigation showed that the most effective formulation, in terms of causing DS, was the mycelium filtrate followed by mycelium alone and the least effective to be the formulation of culture filtrate alone. This result may be logical since in the formulation of mycelium filtrate, the fungus was supported with more phytotoxins, those in the culture filtrate which assist in disease initiation and the toxins that the fungus excretes during the infection process, whereas the formulation of the mycelium alone would only benefit from toxins excreted during pathogenesis. The formulation of culture filtrate alone caused the lowest DS because of the absence of the living agent and, presumably, the absence of higher concentrations of toxins which would produce more disease.

Infection with Ae5 led to disorganization of the chloroplast membrane system, breakdown of the chloroplast envelope, increase in number and size of plastoglobuli in chloroplasts, and disappearance of starch grains. These results agree in part with the findings of Martyn et al. (1983a) who reported an increase of plastoglubuli in chloroplasts in waterhyacinth leaves infected with *Acremonium zonatum*. The function of plastoglobuli is not fully understood, but they are believed to be reservoirs of excess lipids (Greenwood et al., 1963) and may be products of senescence since they increase in both size and number during ageing (Baka and Aldesuquy, 1991). The significance of increased numbers of plastoglobuli in infected leaves is not known (Martyn et al., 1983a). The disappearance of starch from chloroplasts due to infection with Ae5 coincided with the observation of Martyn et al. (1983a) on waterhyacinth infected with *A. zonatum* and also with many observations on different infected hosts (Baka, 1987). Decrease in starch is common in many foliar diseases (Wheeler, 1975).

In the current study, the invagination of plasma membranes apparent in waterhyacinth plants infected with Ae5 may have been caused by toxins produced by the pathogen; the invagination was followed by the development of membranous fragments and lomasome-like vesicles which might originate from the invaginated plasma membrane. Plasma membranes are necessary for the maintenance of ionic and metabolic gradients essential for growth, development, movements, and signal transduction. Therefore, even slight alteration in these membranes may cause dramatic cellular disorder (Langsdorf et al., 1991). Our observations were similar to those of Park et al. (1977) on sus-

ceptible apple leaves infected with *Alternaria mali* and Langsdorf et al. (1991) on tomato leaves treated with alternaric acid. Alterations in the physiological characteristics of the plasma membranes near plasmodesmata were detected in waterhyacinth plants infected with Ae5 in the present study; these may cause changes in membrane permeability which could induce leakage of electrolytes. Such modifications may be attributable to toxin effects, as explained by Kohmoto et al. (1993) who reported similar results with host-specific *Alternaria alternata* toxins on susceptible host plants, and by Langsdorf et al. (1991) on tomato cells treated with alternaric acid.

As a foliar pathogen, *A. eichhorniae* affects most of the plant's photosynthetically active tissues; thus, there was a positive correlation between the DS and chlorophyll level in the infected plants. This result would explain why plants inoculated with the formulations of mycelium plus culture filtrate and mycelium alone of Ae5 had the lowest level of chlorophyll. Pathogenic fungi may diminish the rate of photosynthesis in infected leaves by affecting either the chloroplasts or chlorophyll content directly, or through the enzymes concerned with photosynthesis (Aldesuquy and Baka, 1991). In the present investigation, the observed interference with plastid development in infected leaves resulting in deformed plastids and a decrease in chlorophyll content may be explained by an inhibition, caused by fungal toxins, of photophosphorylation in the terminal stages of ATP synthesis, as suggested by Arntzen (1972). The significant decrease in chlorophyll *a* and *b* and carotenoids of the infected leaves of waterhyacinth compared to the controls is in agreement with many studies which report that fungal pathogens cause a reduction in chlorophyll concentration to a great extent (Aldesuquy and Baka, 1991; Scholes and Farrar, 1985). The reduction in chlorophyll content in the inoculated waterhyacinth plants in this investigation coincided with the observed ultrastructural changes in chloroplasts; similar findings have been reported by Baka (1987) in other hosts.

Infection by pathogenic fungi may lead to substantial changes in the carbohydrate content of infected plants which may reflect an alteration in the different metabolic processes that are favourable or unfavourable for fungal development (Aldesuquy and Baka, 1991; Hawang and Heitefuss, 1986). Treatment with Ae5 formulations led to a decrease in the levels of soluble and insoluble carbohydrates in infected/damaged waterhyacinth leaves compared to healthy ones, and was probably due to an increase in the respiration of treated plants (Daly et al., 1961), and/or an increase in dehydrogenase and pentose cycle enzyme activities (Cutter, 1951). Depletion of starch in the chloroplast could be attributed to sporulation (Baka and Aldesuquy, 1992; Sziraki et al., 1984) or a decrease in photosynthetic efficiency.

A significant increase in SWD was obtained from waterhyacinth leaves treated with the three formulations only at days 6 and 9 after inoculation compared to the healthy controls. This increase may be caused by the pathogen altering the nutrient, mineral, photosynthetic and hormonal relationsof the plant.

Phenolic compounds have unlimited potential in accounting for the many differences that occur in disease resistance (Martyn et al., 1983a, 1983b; Nicholson, 1992). Matern and Kneusel (1988) have proposed that the defensive strategy of plants exists in two stages. The first is assumed to involve the rapid accumulation of phenols at the infection site, which function to slow (or even halt) the growth of the pathogen. The second would involve the activation of specific defences such as the synthesis of phytoalexins or other stress-related substances. Our results confirm this view since a spectacular increase of phenols followed the treatment of waterhyacinth leaves with the Ae5 formulations compared to the healthy leaves. Additionally, studies on phenolic inhibitors in non-infected waterhyacinth plants were conducted by Singh and Srivastava (1983) who reported the existence of *p*-cumaric, chlorogenic, vanillic, ferulic, protocatechuic, resorcylic, and *p*-hydroxybenzoic acids in healthy leaves of waterhyacinth. This, in general, interprets our observation of phenolic compounds existing in the non-inoculated control plants. Our results also showed that phenolic levels in the inoculated waterhyacinth leaves with the formulations of mycelium alone and mycelium plus culture filtrate of Ae5 were significantly less than those in leaves treated with the formulation of culture filtrate alone. This result may provide evidence that the fungus Ae5 has its own means of confronting the defence of the plant by suppressing the production of phenolic compounds in the host so extending the longevity of disease.

Finally, it can be concluded that for best levels of bioherbicidal efficacy and weed stress, the alginate formulation of mycelium plus culture filtrate of Ae5 should be employed along with a hydrophilic polymer to waterhyacinth plants. The gel prevents dehydration and provides moisture for the germinating inoculum while the phytotoxic fractions serve as disease promoting factors. This formulation can be useful in manag-

ing waterhyacinth in Egypt and probably also in other countries.

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