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Biological Control of Some Serious Weeds in Dakahlia District. II. Mycoherbicial Production and Physiological Host Responses

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Four pathogenic fungal isolates belonging to different genera including *Alternaria*, *Fusarium* and *Curvularia* were isolated from selected diseased weeds growing in the fields in Dakahalia district. The inoculum of these pathogenic fungi specific to weeds were cultured, standardized and formulated as alginate pellets containing mycelium plus culture filtrate. These mycoherbicides were evaluated for disease severity (DS). Maximum DS was obtained with the alginate pellets of mycelium filtrate *Fusarium solani*. Physiological changes of the treated weed were determined 5 and 10 days after treatments. As compared to the healthy weeds, all mycoherbicide formulations significantly decreased the amount of photosynthetic pigments and subsequently soluble and insoluble sugars in the infected weeds. The mycoherbicide formulation of *F. solani* had the greatest effect on lowering to the abovementioned amount in the leaves of *Chenopodium murale*. Generally, treatment of weed leaves with the specific mycoherbicide led to a highly significant increase in total phenol content when compared to the healthy control weed. *C. murale* infected with the mycoherbicide formulation of *F. solani* had higher levels of phenolic compounds than those other treated weeds particularly after 10 days of inoculation.

KEYWORD: Carbohydrates, Disease severity, Mycoherbicide, Phenolic compounds, Weeds

Weeds in Dakahlia district are a major problem in agriculture. Most economic plants are lost every year due to damage done by weeds to various agricultural crops. Mechanical and chemical control methods, which are most commonly used, are not completely satisfactory (Sonawane and Ambekar, 1999). Biological control of weeds using plant pathogens is a practical and environmentally sound method of weed management. A variety of herbaceous, woody, climbing, aquatic, and parasitic weeds have been shown to be capable of being controlled by plant pathogens (Charudattan, 1991). Many examples of weed control with pathogens exist, such as the control of hamakua pamakani weed (*Ageratina riparia* by the *Entyloma compositarum*; Trujillo *et al.*, 1988), milkweed vine or strangler vine (*Morrenia odorata* by DeVine [*Phytopthora palmivora*]; Kenney, 1968), musk thistle (*Carduus nutans* by *Puccinia carduorum*; Baudoin *et al.*, 1993), sicklepod (*Cassia obtusifolia* by *Alternaria cassiae*; Walker and Riley, 1982; Charudattan *et al.*, 1986), silverleaf nightshade (*Solanum elaeagnifolium* by the nematode *Orrina phyllobia*; Parker, 1991), skeletonweed (*Chondrilla* juncea by *Puccinia chondrillina*; Supkoff *et al.*, 1988), yellow nutsedge (*Cyperus esculentus* by *Puccinia canaliculata*; Phatak *et al.*, 1987), and wild persimmon (*Diospyros virginiana* by *Cephalosporium diospyri*; Griffith, 1970).

Biological control with plant pathogens is an effective, safe, selective and practical means of weed management that has gained considerable importance (Charudattan, 1986; Flint and Thomson, 2000; Pemberton and Strong, 2000; Bouda *et al.*, 2001). Despite considerable success with biological and integrated control methods in the world (Daniel *et al.*, 1973; TeBeest, 1991; Harman *et al.*, 1996; Alec and De Clerck-Floate, 1999), biological control means have not yet been used on the large scale in Egypt and weeds still creates serious problems in Egypt and many other countries (Shabana *et al.*, 1997; Stewart *et al.*, 2000). Harman *et al.* (1996) reported that a number of arthropod species exotic to weeds of New Zealand can be used as biological control. Furthermore, Alec and De Clerck-Floate (1999) concluded that *Omphalapion hookeri* (Kirby) can be used as a biological control agent for chamomile, *Matricaria perforata* Merat of the Canadian prairies by reducing the weeds reproductive output. A significant reduction in weed biomass was due to application of herbicides and hand-weeding once in comparison with the treatments on unwedded control was reported (Sonawance and Ambekar, 1999). Finally, Flint and Thomson (2000) studied the seasonal infection of the weed dyer's woad in New Zealand by a *Puccinia* sp. Rust used for biocontrol .

Alternaria eichhorniae Nag Raje was first reported as a biocontrol agent for waterhyacinth in 1970 in India (Nag Raj and Ponnappa, 1970) and in 1987 in Egypt (Shabana, 1970). In Egypt, Shabana *et al.* (1997) tested the efficacy of mycoherbicide of *A. eichhorniae* against waterhyacinth. Who concluded that the alginate formulation of mycelium plus culture filtrate of this fungus should be employed along with a hydrophilic polymer to waterhya-

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cinth plants. This formulation can be useful in managing waterhyacinth in Egypt and also probably in other countries. In this connection, waterhyacinth plants treated with aliginate pellets of mycelium plus culture filtrate of *A. eichhorniae* (isolate 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 (Shabana *et al.*, 1997; 2001). Cytological changes noted in infected cells included changes in chloroplast, nucleus and mitochondria.

The purpose of this investigation was conducted to evaluate the efficacy of some prepared mycoherbicide formulations of different specific pathogenic fungi on its weeds under field conditions. The physiological changes (Phtosynthetic pigments, carbohydrates and total phenolic compounds) as result of this infection were also appraised.

Materials and Methods

Sampling. Some serious weeds growing in the field in Dakahalia district were collected during the winter of 2001. They were transferred to the laboratory and then preserved in sterilized plastic bags for further study.

Isolation of pathogen. The infected organs for each weed were washed with tap water and then rinsed with sterile distilled water. Small fragments of diseased area were surface sterilized by 10% Clorox for 6 min, and then washed in sterilized water several times. These fragments were incubated at 28°C for 6~10 days on potato dextrose agar. After incubation period, fungi were purified and maintained at 4°C for the identification.

Identification of isolated fungi. In this study, the pathogenic fungi specific to selected weeds were identified according to the keys of Ellis (1976) and Carmichael *et al.* (1980). These fungi were used for mass production (Table 1).

Mycoherbicide production. Each fungus was grown on fresh potato dextrose broth medium (PDB) for 7 days at 28°C in the dark. The mycelium was harvested and rinsed with sterile distilled water. Inoculum preparation (half of mycelium mat with the culture filtrate preparation) was made by blending of mycelium with fungal culture filtrate 1 : 1 (W/V) for 10 S in a blender. The blended filtrate- mycelium suspension was diluted 1 : 4 (V/V) with 1.33% sodium alginate in distilled water. Alginate-fungal suspension was dripped into 0.25 M calcium chloride to form gel beads of 3~4 mm diameter. The beads were harvested onto sieves, rinsed with distilled water and spread on a carton sheet to be air dried by using electric fans and then maintained at 4°C until used. Alginate pellets, prepared similarly but without the fungus were used for the control treatments.

Application of mycoherbicide. Under field conditions, the leaves of each weed were pre-wetted with tap water to facilitate adherence of pelletized formulation of specific pathogen. Four replicate plants from each weed were sprinkled with pelletized fungal formulation (0.5 g/plant) and sprayed again with tap water. After incubation, all plants were covered with clear polyethylene bags for 48 h to maintain high humidity in a natural condition. Four replicates of the plants from each treatment were harvested at 5 and 10 days after inoculation.

Disease severity (DS). The presence or absence of disease was determined as a percentage of number of leaves on the weed that exhibited disease symptoms and/or necrotic damage. DS, the amount of disease and/or pathotoxin damage was rated by comparing actual damage to a pictorial disease acale of 0 to 9 where 0 = healthy and 9 = 90% disease (Freeman and Charudattan, 1984). Control plants were sprayed with fungus free alginate pellets.

Physiological studies

Plant pigments. Chlorophyll a, b and carotenoids were determined according to spectrophotometers methods recommended by Wellburn and Lichtenthaler (1984).

Carbohydrates. The procedure of extraction, clarification and measurements of reducing sugars, sucrose and polysaccharides from healthy and infected leaves were determined as described by Abood (1990).

Total phenols. Total phenols were extracted from healthy and treated leaves and estimated by the method of Ribereau-Gayon (1972).

All data were subjected to analysis of variance (ANOVA) Significant differences among treatments means were determined with Duncan multiple test at $P = 0.05$.

Table 1. List of specific pathogen isolated from weeds in Dakahlia district and disease symptoms

Pathogen	Principal weed	Associated plant	Disease symptoms
Alternaria fasciculata	*Echinochloa colonum*	Citrus	Foliar blight
A. macrospora	*Cuscuton pedicellata*	Clover	Leaf-spot
Fusarium solani	*Chenopodium murale*	Tomato	Vascular wilt and chlorosis
Curvularia lunata	*Vicia sativa*	Green bean	Chlorosis

Table 2. Disease severity (DS) caused by different mycoherbicides of weeds collected from Dakahlia district

Weed	Mycoherbicide treatment	Disease severity (%)	
		Days after inoculation	
		5	10
Echinochloa colonum	Healthy	0.0 b*	0.0 b
	Infected	35.0 a	43.0 a
Cuscuton pedicellata	Healthy	0.0 b	0.0 b
	Infected	60.8 a	65.7 a
Chenopodium murale	Healthy	0.0 b	0.0 b
	Infected	70.5 a	95.3 a
Vicia sativa	Healthy	0.0 b	0.0 b
	Infected	67.0 a	86.2 a

*Values within a column for each weed followed by the same letters are not significantly different (Duncan multiple rang test at $P = 0.05$).

Results and Discussion

Disease severity (DS). DS was determined for each leaf and values were summed and averaged to derive DS for a whole plant (Table 2). The increase in the severity of disease on plants treated with mycelium-filtrate aliginate formulation of pathogen in this study could be due to the fungus being aided by sodium alginate which applied on the plants immediately before inoculation as well as by the pathotoxins produced by the fungus in the cultural medium. Our data agree with those of Robeson *et al.* (1984), Charudattan, 1986 and Shabana *et al.* (1997) who found that the most effective formulation causing disease severity was the mycelium filtrate followed by mycelium alone and the least effective to be the formulation of culture filtrate alone. In this connection, sodium alginate is commonly used in many food products (Connick, 1979) and any residues in plants or water would not be toxic to nontarget organisms. The procedure of drying the pellets immediately after pelletization could simplify the large-scale production of the bioherbicide. Results of the present investigation showed that the most effective formulation in terms of DS was the mycelium-filtrate of *Fusarium solani* on the *Chenopodium murale* weed. This finding is quite logical since with the formulation of culture-filtrate, the fungus might be supported with more amount of pathotoxins in culture filtrate which might to assist in disease initiation toxins along with pathotoxins of fungus produced during infection process.

Pigments content. The data presented in Table 3 clearly elucidated that there was a massive decrease in the amount of photosynthetic pigments in infected leaf of weeds, as result of mycoherbicide application, when compared with the healthy plants. The mycoherbicide formulation of *F. solani* had the greatest effect in lowering the amount of photosynthetic pigments of *C. murale* leaves. However, there was no significant difference between infected and healthy *E. colonum* weed.

Thus, there is a negative correlation between the DS and chlorophyll level in the infected plants (Tables 2 and 3). The pathogenic fungi may diminish the rate of photosynthesis in the infected leaves by affecting either the chloroplasts or chlorophyll content directly or through the enzymes concerned with photosynthesis (Aldesuquy and Baka, 1992). Arntzen (1972) suggested that the decrease of chlorophyll in infected weed might be explained by an inhibition caused by the fungal toxins of photophosphorylation at the terminal steps of ATP synthesis. The significant decrease in chlorophyll **a** and **b** and carotenoids of the infected leaves of weeds compared to the control healthy plants was in agreement with many studies which reported that the fungal pathogens cause a reduction in chlorophyll concentration to a great extent (Aldesuquy and Baka, 1992). The reduction in chlorophyll content in the inoculated weed plants in this investigation coincided with the ultrastructural changes of chloroplasts of infected

Table 3. Content of pigments in healthy and infected leaves of weeds from Dakahlia district

Weed	Mycoherb. treatment	Amount of pigments (μg/g fresh weight)					
		Days after inoculation					
		5			10		
		Chl. a	Chl. b	Carotenoids	Chl. a	Chl. b	Carotenoids
Echinochloa colonum	Healthy	530 a*	449 a	80 a	568 a	451 a	71 a
	Infected	510 a	449 a	75 a	499 a	410 a	62 a
Cuscuton pedicellata	Healthy	670 a	530 a	133 a	660 a	522 a	129 a
	Infected	580 a	415 a	118 a	543 b	349 b	100 a
Chenopodium murale	Healthy	750 a	660 a	210 a	741 a	652 a	210 a
	Infected	410 b	395 b	187 a	318 b	301 b	153 b
Vicia sativa	Healthy	680 a	550 a	120 a	660 a	500 a	105 a
	Infected	370 b	340 b	085 a	290 b	291 b	065 b

*Values within a column for each weed followed by the same letters are not significantly different (Duncan multiple rang test at $P = 0.05$).

Table 4. Content of carbohydrates in healthy and infected leaves of weeds in from Dakahlia district

Weed	Mycoherb. treatment	Content of carbohydrates (μg/g fresh weight)					
		Days after inoculation					
		5			10		
		Reducing sugars	Sucrose	Polysaccharides	Reducing sugars	Sucrose	Polysaccharides
Echinochloa colonum	Healthy	0.43 a*	2.00 a	7.62 a	0.35 a	1.87 a	8.30 a
	Infected	0.33 a	1.88 b	6.95 b	0.24 a	1.48 a	6.28 a
Cuscuton pedicellata	Healthy	0.55 a	4.20 a	9.02 a	0.42 a	3.90 a	10.08 a
	Infected	0.43 a	3.50 a	7.82 a	0.31 b	2.88 b	07.05 a
Chenopodium murale	Healthy	0.68 a	4.45 a	9.83 a	0.61 a	4.09 a	11.19 a
	Infected	0.41 b	3.91 b	5.18 b	0.30 b	2.88 b	04.95 b
Vicia sativa	Healthy	0.60 a	3.95 a	9.71 a	0.55 a	3.20 a	10.22 a
	Infected	0.40 b	2.66 b	5.89 a	0.31 b	2.05 b	03.18 b

*Values within a column for each weed followed by the same letters are not significantly different (Duncan multiple range test at $P = 0.05$).

plants of waterhyacinth (Shabana *et al.*, 1997).

Carbohydrate content. It is obvious from Table 4 that the four mycoherbicide formulations significantly caused a marked decrease, to some extent, in soluble sugars (r-educing sugars and sucrose) and insoluble sugars (poly-saccharides) in the infected weeds compared with the healthy controls. It is also obvious that the soluble sugars decreased with the progress of the experiment both in healthy and treated plants. Unlikely, the polysaccharide increased in infected leaves but were mostly stable in healthy control leaves over time. The leaves which were treated with mycoherbicide of *F. solani* had the greatest level of decline in soluble and insoluble sugers than those treated with other mycoherbicides. However, there was no significant difference in these contents between healthy and infected *E. colonum* particularly after 10 days of inoc-ulation. Carbohydrates, principally sugars and starch, are the most abundant organic constituents of plants serving as important sources of energy enabling plants to survive through periods of disease stress, nutrient depletion, drought, etc. Infection by pathogenic fungi may lead to substantial changes in the carbohydrate content of infected plants which may reflect the alteration in the different metabolic processes favorable or unfavorable for fungal develop-ment (Aldesuquy and Baka, 1992). Our results showed that the treatment with different mycoherbicides led to a decrease in the level of soluble and insoluble carbohy-drates in weed leaves as compared with healthy ones. This decrease in infected/damaged tissues was probably due to the increase in respiration of treated plants (Daly *et al.*, 1961), and/or increase in dehydrogenase and pentose cycle enzyme activities (Cutter, 1951). Depletion of starch in the chloroplast could be attributed to sporulation which has been reported by Baka and Aldesuquy (1992) or likely to be due to the decrease in photosynthetic effi-ciency.

Phenol content. Generally, the treatment of weed leaves with specific mycoherbicide led to a highly significant increase ($P = 0.05$) in total phenol content when com-pared to the healthy control plants (Table 5). However, no significant difference was observed in these contents between healthy and infected *Echinochloa colonum* weed at 5 and 10 days after inoculation. On the other hand, *Chenopodium murale* infected with the mycoherbicide formulation of *F. solani* had higher levels of phenolic compounds than those other treated weeds particularly after 10 days of inoculation.

Generally, the concentration of total phenols increased as time progressed. Many authors indicated that phenolic compounds might have unlimited potential in accounting for the many differences that occurred in plant response to disease (Nicholson, 1992 and Martyn *et al.*, 1983). In this connection, 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

Table 5. Phenolic compounds of healty and infected leaves of weeds in Dakahlia district

Weed	Mycoherbicide treatment	Phenolic compounds (μg/g dry weight)	
		Days after inoculation	
		5	10
Echinochloa colonum	Healthy	690 a*	725 a
	Infected	715 a	793 a
Cuscuton pedicellata	Healthy	538 a	612 a
	Infected	621 b	689 b
Chenopodium murale	Healthy	370 a	420 a
	Infected	495 b	1205 b
Vicia sativa	Healthy	460 a	551 a
	Infected	558 b	1210 b

*Values within a column for each weed followed by the same letters are not significantly different (Duncan multiple range test at $P = 0.05$).

phenols at the infection site, which function to slow 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 conviction since a spectacular increase of phenols followed the treatment of weed leaves with the pathogens formulation as compared to the healthy leaves. Additionally, studies on phenolic inhibitors in noninfected weed plants were conducted by Singh and Srivastava (1983). They reported the existence of p-cumaric, chlorogenic, vanillic, ferulic, protocatechuic, resorcylic, and p-hydroxybenzoic acids in healthy leaves of weeds. This, in general, interprets our observation of being phenolic compounds existing in the noninoculated control plants.

As compared to healthy plants for each treatment, results of the present investigation showed that the phenols contents in the inoculated *Chenopodium murale* weed leaves with the formulations of mycelium plus culture filtrate of pathogen (*F. solani*) were greatly higher than those in leaves of weed treated with other mycoherbicides.

The results reported here permit the conclusion that the biological control with fungal pathogens (Mycoherbicides) offers a feasible alternative and supplement to chemical weed control. Economic and public demands for affordable and safe herbicides are expected to increase in this field. With our increasing knowledge of mycoherbicides has come a better understanding of the limitations facing this field. These include efficacy, extreme level of host specificity, incompatibility with chemical pesticides, the need to produce spores as inocula, technical difficulties in product development, competition from chemical herbicides, economic based on market size, and potential building of weeds resistant to the microbial herbicide. Further researches for the application of these mycoherbicides as a biological control of weeds should be focused on the previous aspects.

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