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Fungi occurring on waterhyacinth (*Eichhornia crassipes* [Martius] Solms-Laubach) in Niger River in Mali and their evaluation as mycoherbicides

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ABSTRACT

We recovered 116 fungal isolates in 7 genera from waterhyacinth plants having pronounced blight symptoms collected in Mali. Isolation frequency of the genera was *Curvularia* (60.32%), *Fusarium* (42.92%), *Alternaria (*11.6%), *Coniothyrium* (11.6%), *Phoma* (3.48%), *Stemphylium* (3.48%), and *Cadophora* (1.16%). On the basis of *in vivo* pathogenicity tests in which the diseased leaf area percentage and disease severity were visually estimated using a disease severity index, three isolates, *Fusarium* sp. Mln799, *Cadophora* sp. Mln715, and *Alternaria* sp. Mlb684 caused severe disease. These were later identified as *Gibberella sacchari* Summerell & J.F. Leslie, *Cadophora malorum* (Kidd & Beaumont) W. Grams, and *Alternaria* sp. respectively. This is the first report to highlight *C. malorum* as a candidate biocontrol agent against waterhyacinth. Neither *C. malorum* Mln715 nor *Alternaria* sp. Mlb684 in host specificity tests showed any pathogenicity toward 17 crop plants of economic importance in Mali.

Key Words: biocontrol agents, mycoherbicide, native fungi, waterhyacinth.

INTRODUCTION

Waterhyacinth (*Eichhornia crassipes* [Martius], Solms-Laubach; *Pontederiaceae*) is a free-floating aquatic weed, native to the Amazon Basin in South America (Morsy 2004) distributed across the tropics and subtropics between 39°N and 39°S (Téllez et al. 2008). Man has clearly been the main agent responsible for spreading this species around the world, as its entry into Africa, Asia, Australia, and North America coincided with the arrival of the vessels of the first explorers or with historically documented human activities (Shabana 1997).

Waterhyacinth is considered the world's worst aquatic weed (Lata and Dubey 2010). As in many tropical and subtropical regions worldwide, waterhyacinth creates serious agricultural and navigation problems in District of Bamako in Mali (Figure 1). The plant not only affects irrigation, water flow, water use, and navigation, it also poses a health risk by enabling the breeding of mosquitoes, bilharzias, and other human parasites (Adebayo and Uyi 2010). Water quality is affected as well, by the increasing accumulation of detritus in the water (Morsy 2004). Fishing can be affected because of the competitive advantage given to trash fish species in weed-infested waters. In many instances, fish are killed when oxygen levels are depleted through plant respiration and decomposition of senescent vegetation. The waterhyacinth infestation is particularly severe in the Delta of Niger and in all irrigation systems of the Office of Niger (ON) according to Dembélé (1994). Several billion dollars are spent each year by the Office du Niger and Energie du Mali to control this weed in the Niger River (Dagno et al. 2007).

Chemical, physical, and biological means are used to control waterhyacinth infestations. In Mali, chemical (2-4, D) and mechanical control methods have been used since 1997 to manage this weed (Dembélé and Diarra 1997); however, these methods provide only a temporary management solution. Chemical methods can be dangerous for humans as well as animals because people drink river water in Mali and use it to prepare food. For this reason, and in a sustainable management perspective, an integrated approach seems necessary. In recent years, attention has focused on biological control, which could provide a cost-effective, environmentally safe solution to the waterhyacinth problem. The biological control of weeds by means of plant pathogens has gained acceptance as a practical, safe, and environmentally beneficial weed management method applicable to agro-ecosystems (Charudattan 2005, Boyette et al. 2007). Most emphasis has been on fungal pathogens as biocontrol agents (Vincent 2001, Shabana 2005). Several highly virulent fungi are known to cause diseases of waterhyacinth, the best known being *Acremonium zonatum* (Sawada) W. Gams, *Alternaria alternata (*Fr.) Keissler, *A. eichhorniae* Nag Raj & Ponnappa, *Bipolaris* spp., *Fusarium chlamydosporum* Wollenw & Reinking, *Helminthosporium* spp., *Cercospora piaropi* Tharp, *Myrothecium roridum* Tode ex fr., *Rhizoctonia solani* Kühn, and *Uredo eichhorniae* Gonz.-Frag. & Cif. (Charudattan 2001, Morsy 2004, Naseema et al. 2004). Among these, *A. eichhorniae*, *C. piaropi*, *A. alternata*, and *F. chlamydosporum* have been studied most extensively (Babu et al. 2002, Shabana 2005).

The aim of this study was to survey and identify indigenous phytopathogenic fungal isolates of waterhyacinth in Mali, with a view to developing them as bioherbicides. We report

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Figure 1. Niger River infested by waterhyacinth in District of Bamako.

on the occurrence, pathogenicity, and host specificity of the significant fungal waterhyacinth pathogens found in 2006 and 2007 in Mali.

MATERIALS AND METHODS

Habitat

The study areas were located in the District of Bamako, Segou, and Niono regions with GPS coordinates 12° 40'N, 7° 59'W; 13° 26'N, 6° 15'W; and 14° 15'N, 5° 59'W, respectively. The Region of Niono is the main area for irrigated rice culture and vegetable and fruit productions. Areas of Bamako, Segou, and Niono have different climates. Indeed, Bamako (South of Mali) has five rainy months with 70% relative humidity (RH), Segou (Middle of Mali) has four rainy months with 60% RH, and Niono (Nord-East of Mali) has three rainy months with 55% RH (yearly averages). All of these regions are infested with waterhyacinth.

Sampling procedure, isolation, and fungal identification

From 2006 to 2007, 1000 samples consisting of infected parts of waterhyacinth (petiole and leaf) were collected from the River Niger in the District of Bamako (Sites 1, 2, and 3, about 3 km apart), Sebougou Lake in Segou (Site 4), and the evacuation canal of wastewater from households (a reservoir for irrigation) in Niono (Site 5). Samples were transported to the laboratory in clean plastic bags and stored at 4 C until examined.

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 96% ethanol for 30 sec, 14% hypochlorite for 30 sec, and then rinsed in sterile water for 1 min. Surface-

sterilized segments (4 segments per plate) were plated on potato dextrose agar (PDA, Merck, Darmstadt, Germany) supplemented with 0.5 mg streptomycin. Three plates were used for petioles and leaves. The plates were incubated at 25 C for 5 to 7 d. Fungi that developed on the plant pieces were isolated, and pure cultures were obtained by the single-spore or hyphal-tip technique, depending on the type of fungal isolate.

Identification of the different fungal genera was based on morphological characteristics of each growing microbial colony as described by Lacap et al. (2003) and Ainsworth et al. (1973). According to Martínez and Charudattan (1998), fungi having rapid growth and sporulation on PDA plates in 24-48 hours were excluded from our fungal collection. They demonstrated some distinctive characteristics of saprophytes. So, isolates in the genera *Penicillium*, *Aspergillus*, and *Trichoderma* were excluded from further consideration after their initial isolation. The retained fungal isolates were identified by the Industrial Fungal & Yeast collection (BCCM/MUCL-Louvain-la-Neuve, Belgium).

Based on their frequency of occurrence, the isolated fungal genera were classified as very frequent (>20%), frequent (10-20%), or infrequent (<10%), as reported by Morsy (2004). All fungal isolates were stored at the Plant Pathology Unit of Gembloux Agro-Bio Tech, University of Liege-Wallonia, Belgium.

Environmental factors

Water samples were collected from the waterhyacinth-infested sites where diseased plants were collected. Water mineral levels and pH were determined at each site along with temperature and RH. Water samples were analysed by the National Laboratory Water of Mali (LNE). Temperature and RH measurements were performed *in situ* with a thermohygrometer (Thermohygrometer HT5B, W-TECH, 38300 RUY-MONTCEAU, France).

Pathogenicity tests

During experiments, the initial conidial inoculum was taken from petri dish cultures on PDA medium, preserved at 4 C for no more than 6 months and then subcultured at 25 C on different culture media before use. Healthy waterhyacinth plants were collected from natural infestations in the River Niger in Mali and maintained in a greenhouse at Gembloux Agro-Bio-Tech, University of Liege.

In a preliminary screen, the fungal pathogens were tested for their effects on 8 cm^2 leaf fragments cut from healthy, 15- to 20-day-old healthy waterhyacinth plants. Each fragment was placed on the surface of a petri plate containing agar supplemented with furfurylaminopurine-6 (1 mg mL^{-1}). Spore suspensions (20 mL) were prepared from the fungal isolates (2 × 106 spores mL^{-1} in 5% Tween® 20) and manually sprayed onto a set of nine leaf fragments. After inoculation, the petri plates were immediately sealed with Parafilm to prevent water loss and incubated at 25 C with a 16 h photoperiod for 4 weeks. After a preliminary screen to detect the most virulent isolates, the pathogenicity of selected isolates was evaluated by inoculating a set of healthy waterhyacinth plants maintained in 15 L plastic pots under greenhouse conditions. The nine most virulent isolates were selected for a second screening on whole, 15- to 20-day-old healthy waterhyacinth plants. Fourteen-day-old cultures of the fungal isolates were used in the test. Three leaves of each plant were inoculated by spraying with a 20 mL spore suspensions (5×10^6 spores mL^{-1} in 5% Tween® 20). Inoculated plants were covered with plastic for 72 h and then maintained in greenhouse were the environmental parameters are 85 ± 5% RH at 25 C with a 16 h photoperiod. The relative humidity of room was maintained by a humidificator (Humidificator DELONGHI UH800E, MANUTAN, 95506 GONESSE, France). The plants were rated for disease symptoms 6 weeks after incubation. The experiment was repeated three times.

Leaf fragments and plants were rated for disease symptoms including leaf spots, leaf lesions, and leaf death after 4 and 6 weeks incubation, respectively. The impact of the pathogens was determined 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 as described by Morsy 2004. Values for individual leaves were summed and averaged to derive DS for a whole plant. Finally, isolates were categorized into four groups according to diseased leaf area: Mild, <25%; Low Moderate, 26-50%; High Moderate, 51-75%; and Severe, >75%.

Data were subjected to one-way ANOVA performed with SAS software 9.1 (SAS Institute, Cary, NC, USA). When effects were significant, Duncan's multiple range tests was employed for mean separation.

Host specificity tests

The host specificity of the selected fungi also was tested in a greenhouse. In addition to three ecotypes of waterhyacinth (Ecotypes from District of Bamako, Segou and Niono) and the invasive aquatic fern (*Salvinia molesta* Mitchell), the test included, several food crop plant species chosen for their economic and ecological importance as described by Martinez and Gutierrez (2001). Nonaquatic plants were grown in pots filled with aseptic commercialized soil free of any fungi. For aquatic plants, the soil contained in 8 L plastic pots was replaced by 15 L plastic pots with water. Each pot contained one plant. The experiment was replicated three times. The waterhyacinth ecotypes and the aquatic fern were inoculated by spraying, whereas the fungus was inoculated by watering with a suspension containing 5×10^6 spores mL^{-1} to simulate irrigation conditions. The plants were monitored for disease symptoms every week for 4 weeks.

RESULTS AND DISCUSSION

Biodiversity

To our knowledge, this is the first extensive survey of pathogens infecting waterhyacinth in Mali. Our survey included all sections of the River Niger and all irrigation areas where waterhyacinth is reported as a serious problem. Waterhyacinth plants in the main irrigation canal were found to be infected by various fungi and displayed a wide variety of symptoms. The symptoms initially appeared as small necrotic

spots and developed into a leaf blight spreading over the leaf surface and the petiole. The infecting fungi (116 isolates of filamentous fungi of the phylum *Ascomycetes*) were identified as belonging to seven known genera (Table 1) on the basis of their morphological characteristics and the arrangement and structure of their conidia.

The genera *Curvularia* (60.32% of the isolates) and *Fusarium* (42.92%) were classified as very frequent; *Alternaria (*11.6%) and *Coniothyrium* (11.6%) as frequent; and *Phoma* (3.48%), *Stemphylium* (3.48%), and *Cadophora* (1.16%) as infrequent. We obtained 67 isolates from infected petioles and 49 from leaves. *Curvularia*, *Fusarium*, and *Stemphylium* appeared widely distributed over all infested areas of Mali, whereas *Alternaria* was found only in Bamako, and *Cadophora* and *Phoma* only in Niono.

Previous reports on the mycobiota of waterhyacinth indicate that the genera *Fusarium*, *Curvularia*, and *Alternaria* are frequently isolated from this weed (Evans and Reeder 2001, Praveena and Naseema 2004), and that *Alternaria* and *Fusarium* are particularly common (Babu et al. 2003, Morsy 2004, Praveena and Naseema 2004).

Environmental factors

According to Laboratoire National des Eaux (2010), temperature, RH, pH, and mineral levels recorded for the areas infested with waterhyacinth were recorded in Table 2. Téllez et al. (2008) reported that the number of daughter plants of this weed is greatest at 25-40° C and an RH of 15-75%. According to Mandryk and Wein (2006), pH must be between 6 and 8, with the plant showing maximum growth at pH 7. Temperature and pH ranges recorded at the infested sites in Mali (26-35 C and pH 6.72-7.79) are thus nearly optimal for the growth and vegetative reproduction of waterhyacinth. Factors associated with water pollution (e.g., wastewater from households, industries, and agriculture) most likely also contribute to high waterhyacinth infestations in Mali.

Pathogenicity testing of fungal isolates

In the preliminary screen, all 116 fungal isolates were tested *in vitro* on leaf fragments of waterhyacinth plants. All fungal isolates were able to infect the plant and produce some disease symptoms (Table 3). Disease started as small necrotic spots and developed into a leaf blight that tended to spread over the leaf; however, only nine pathogens produced symptoms more than 50% of the total foliar area by the end of the 4-week incubation period: Mls214 (*Curvularia* sp.), Mln285 (*Curvularia* sp.), Mln286 (*Fusarium* sp.), Mlb603 (*Fusarium* sp.), Mlb633 (*Curvularia* sp.), Mlb682 (*Alternaria* sp.), Mlb684 (*Alternaria* sp.), Mln715 (*Cadophora* sp.), and Mln799 (*Fusarium* sp.).

Disease severity values were calculated over the 4-week period for all 116 isolates (Table 3). The DS values at the end of the experiment for the nine most damaging pathogens were 50, 51, and 50% for *Curvularia* sp. isolates Mls214, Mln285, and Mlb633, respectively; 52, 50, and 70% for *Fusarium* sp. isolates Mln286, Mlb603, and Mln799, respectively; 55 and 69% for 281 *Alternaria* sp. isolates Mlb682 and Mlb684, respectively; and 72% for *Cadophora* sp. isolate Mln715.

The second screening was carried out *in vivo* using the nine fungal isolates displaying the highest antagonistic activ-

Table 1. List of fungal genera isolated from disease hyacinth plants collected in infested areas in Mali.

	Bamako		Localities Segou Part of plant		Niono			
Fungi	Petiole	Leaf	Petiole	Leaf	Petiole	Leaf	Total count	Frequency of occurence
Fusarium sp.	11	13		1	6	6	37	42.92
Curvularia sp.	19	7	3	1	11	11	52	60.32
Coniothyrium sp.	5	2	1			2	10	11.60
Alternaria sp.	5	5					10	11.60
Phoma sp.					2	1	3	3.48
Stemphylium sp.	1				2		3	3.48
Cadophora sp.					1		1	1.16
Total	41	27	4	2	22	20	116	

Table 2. Environmental parameters of areas infested by waterhyacinth in Mali.

	Site*				
Parameter	S1	S2	S3	S4	S5
Water pH	7.79	7.20	6.72	6.95	7.12
Air relative humidity (RH)	65	60	60	55	55
Air temperature	26	28	27	30 3	5
Na^+ mg L^{-1}	305	175	105	98 1	50
Ammoniacal N mgl^{-1}	18.50	35.20	10.40	5.32	41.90
NO_3- mg L^{-1}	12.80	10.20	13	8.70	11.30

*S1: Reservoir lake of the hydroelectric dam of Sotuba (Distict of Bamako); S2: waste water canal of Badalabougou (District of Bamako); S3: canal of the Marietou Hotel (District of Bamako); S4: Sebougou Lake (Segou) in the central delta of River Niger; S5: wastewater canal in Niono.

Table 3. Pathogenicity of fungi collected from waterhyacinth in Mali (tests applied to detached leaves of wa terhyacinth).

Isolate number	Genus	Waterhyacinth response DS
Mlb436	*Fusarium* sp.	70
Mlb78	*Fusarium* sp.	52
Mlb455	*Fusarium* sp.	50
Mln776	*Fusarium* sp.	49
Mlb11	*Fusarium* sp.	43
Mlb22	*Fusarium* sp.	42
Mlb324	*Fusarium* sp.	42
Mlb498	*Fusarium* sp.	41
Mlb310	*Fusarium* sp.	39
Mln799	*Fusarium* sp.	38
Mln286	*Fusarium* sp.	38
Mlb603	*Fusarium* sp.	36
Mln745	*Fusarium* sp.	36
Mln959	*Fusarium* sp.	33
Mlb127	*Fusarium* sp.	33
Mlb411	*Fusarium* sp.	33
Mlb115	*Fusarium* sp.	32
Mlb697	*Fusarium* sp.	32
Mlb167	*Fusarium* sp.	31
Mlb114	*Fusarium* sp.	31
Mlb340	*Fusarium* sp.	30
Mln906	*Fusarium* sp.	29
Mlb523	*Fusarium* sp.	29
Mlb326	*Fusarium* sp.	28
Mln977	*Fusarium* sp.	27
Mln700	*Fusarium* sp.	27
Mln885	*Fusarium* sp.	26
Mlb130	*Fusarium* sp.	23
Mln236	*Fusarium* sp.	21
Mlb335	*Fusarium* sp.	20
Mlb139	*Fusarium* sp.	20
Mls200	*Fusarium* sp.	18
Mlb639	*Fusarium* sp.	16
Mlb355	*Fusarium* sp.	16
Mln890	*Fusarium* sp.	16
Mlb101	*Fusarium* sp.	15
Mln277	*Fusarium* sp.	12
Mln285	*Curvularia* sp.	51
Mls214	*Curvularia* sp.	50
Mlb633	*Curvularia* sp.	50
Mlb332	*Curvularia* sp.	45
Mlb109	*Curvularia* sp.	38
Mlb179	*Curvularia* sp.	33
Mlb180	*Curvularia* sp.	33
Mlb55	*Curvularia* sp.	32
Mlb178	*Curvularia* sp.	32
Mlb501	*Curvularia* sp.	31
Mlb344	*Curvularia* sp.	29
Mln279	*Curvularia* sp.	27
Mln960	*Curvularia* sp.	27
Mln963	*Curvularia* sp.	27
Mlb500	*Curvularia* sp.	25
Mls205	*Curvularia* sp.	24
Mlb304	*Curvularia* sp.	24
Mls215	*Curvularia* sp.	23
Mlb301	*Curvularia* sp.	23
Mln917	*Curvularia* sp.	23

DS = Disease severity. Mild, <25% of diseased leaf area; Low Moderate, 26-50% of diseased leaf area; High Moderate, 51-75% of diseased leaf area; and Severe, >75% of diseased leaf area after 6-week test period.

Table 3. (Continued) Pathogenicity of fungi collected from waterhyacinth in Mali (tests applied to detached leaves of wa terhyacinth).

Isolate number	Genus	Waterhyacinth response DS
Mlb40	*Curvularia* sp.	22
Mlb170	*Curvularia* sp.	22
Mln825	*Curvularia* sp.	22
Mlb171	*Curvularia* sp.	21
Mln830	*Curvularia* sp.	21
Mln835	*Curvularia* sp.	21
Mln898	*Curvularia* sp.	21
Mln970	*Curvularia* sp.	21
Mln709	*Curvularia* sp.	20
Mln838	*Curvularia* sp.	20
Mln893	*Curvularia* sp.	20
Mln896	*Curvularia* sp.	20
Mln926	*Curvularia* sp.	20
Mln892	*Curvularia* sp.	19
Mln913	*Curvularia* sp.	19
Mlb52	*Curvularia* sp.	18
Mln887	*Curvularia* sp.	18
Mlb70	*Curvularia* sp.	16
Mln762	*Curvularia* sp.	16
Mlb141	*Curvularia* sp.	15
Mlb152	*Curvularia* sp.	15
Mlb10	*Curvularia* sp	14
Mlb66	*Curvularia* sp.	14
Mlb69	*Curvularia* sp.	14
Mln241	*Curvularia* sp.	14
Mlb322	*Curvularia* sp.	14
Mlb24	*Curvularia* sp.	13
Mlb432	*Curvularia* sp.	13
Mln281	*Curvularia* sp.	12
Mlb35	*Curvularia* sp.	11
Mlb526	*Coniothyrium* sp.	47
Mln757	*Coniothyrium* sp.	21
Mlb360	*Coniothyrium* sp.	19
Mln749	*Coniothyrium* sp.	19
Mls210	*Coniothyrium* sp.	18
Mlb440	*Coniothyrium* sp.	18
Mlb486	*Coniothyrium* sp.	16
Mlb187	*Coniothyrium* sp.	15
Mlb627	*Coniothyrium* sp.	15
Mlb349	*Coniothyrium* sp.	14
Mlb338	*Coniothyrium* sp.	12
Mlb39	*Coniothyrium* sp.	6
Mlb684	*Alternaria* sp.	69
Mlb682	*Alternaria* sp.	55
Mlb632	*Alternaria* sp.	51
Mlb568	*Alternaria* sp.	49
Mlb513	*Alternaria* sp.	48
Mlb129	*Alternaria* sp.	48
Mlb406	*Alternaria* sp.	47
Mlb305	*Alternaria* sp.	47
Mlb517	*Alternaria* sp.	45
Mlb104	*Alternaria* sp.	42
Mln930	*Phoma* sp.	48
Mln292	*Phoma* sp.	47
Mln808	*Phoma* sp.	36
Mln988	*Stemphylium* sp.	18

DS = Disease severity. Mild, <25% of diseased leaf area; Low Moderate, 26-50% of diseased leaf area; High Moderate, 51-75% of diseased leaf area; and Severe, >75% of diseased leaf area after 6-week test period.

Table 3. (Continued) Pathogenicity of fungi collected from waterhyacinth in Mali (tests applied to detached leaves of wa terhyacinth).

Isolate number	Genus	Waterhyacinth response DS
Mln992	*Stemphylium* sp.	18
Mlb641	*Stemphylium* sp.	17
Mln715	*Cadophora* sp.	72

DS = Disease severity. Mild, <25% of diseased leaf area; Low Moderate, 26-50% of diseased leaf area; High Moderate, 51-75% of diseased leaf area; and Severe, >75% of diseased leaf area after 6-week test period.

ity *in vitro*. Among them, only isolates Mln799 (*Fusarium* sp.), Mln715 (*Cadophora* sp.), and Mlb684 (*Alternaria* sp.) showed a DS of at least 70% after a 6-week incubation (Figure 2). According to Morsy (2004), disease incidence is considered High Moderate if DS = 51-75% and Severe if DS > 75% of leaf area covered by disease (Figure 3).

For isolates Mls214 (*Curvularia* sp.), Mlb602 (*Fusarium* sp.), and Mlb632 (*Fusarium* sp), DS ranged from 40 to 47%, suggesting they are not strictly pathogenic and could be saprotrophs. Several strains of *Curvularia*, *Fusarium*, and *Alternaria* are reported to be biotrophic fungi, living within the host without seriously altering its physiology (Evans and Reeder 2001).

Alternaria and *Fusarium* are ubiquitous fungal genera and have been isolated from almost all habitats infested with waterhyacinth worldwide (Evans and Reeder 2001, Martinez and Lopez 2001). These genera include many species or strains that may be pathogenic toward several crops (Morsy et al. 2000, Babu et al. 2003). They also include some strains that seem specifically pathogenic toward waterhyacinth. Among them, *A. eichhorniae*, *A. alternata*, and *F. pallidoroseum* (Cooke) Sacc. have been reported as promising biological control agents against waterhyacinth in Egypt and India (Aneja and Singh 1989, Shabana 1997, Morsy 2004, Naseema et al. 2004).

This is the first report to mention the genus *Cadophora* on waterhyacinth. This genus, however, is recognized as pathogenic towards corn, rice, apple, and pear (Frisullo 2002). The present results highlight *Fusarium* sp. isolate Mln799, *Cadophora* sp. isolate Mln715, and *Alternaria* sp. isolate Mlb684 as potential bioherbicides for use in controlling waterhyacinth in Mali. These isolates were later identified as *Gibberella sacchari*

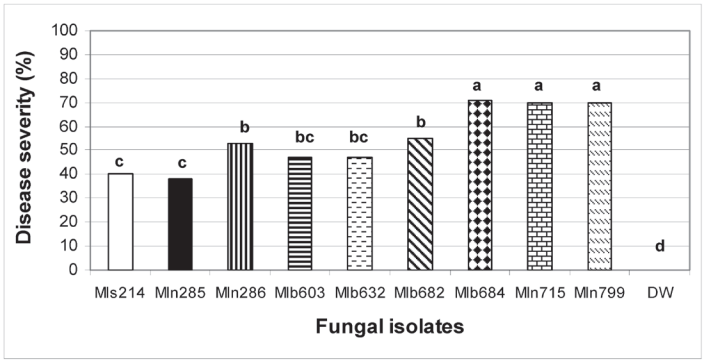

Figure 2. Disease severity (DS) recorded on waterhyacinth plants 42 days after incubation with nine fungal isolates (Mls214 = *Curvularia* sp., Mln285 = *Curvularia* sp., Mln286 = *Fusarium* sp., Mlb603 = *Fusarium* sp., Mlb632 = *Fusarium* sp., Mlb682 = *Fusarium* sp., Mlb684 = *Alternaria* sp., Mln715 = *Cadophora* sp., and Mln799 = *Fusarium* sp.). Duncan's multiple range tests was used to separate means at $P < 0.05$. Treatments having the same letters are not significantly different. DW: Sterlised water with no added pathogen

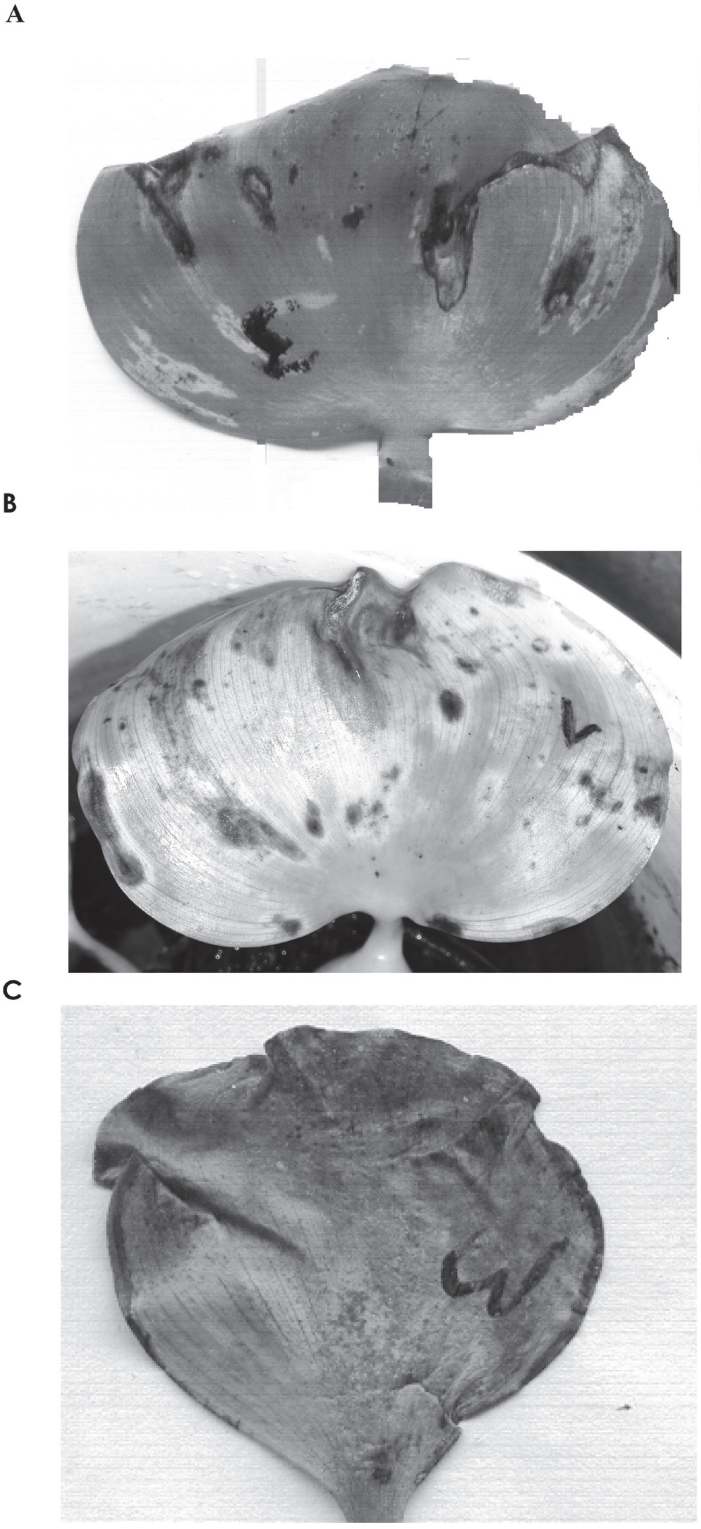

Figure 3. Leaf spots and blight symptoms on waterhyacinth plants after infection with (A) isolate Mln799 (*Fusarium* sp.); (B) isolate Mln715 (*Cadophora* sp.); and (C) isolate Mlb684 (*Alternaria* sp.).

Summerell & J.F. Leslie, *Cadophora malorum* (Kidd & Beaumont) W. Grams, and *Alternaria* sp. respectively by Industrial Fungal & Yeast collection (BCCM/MUCL - Louvain-la-Neuve, Belgium). Because of its growth at 37° C (human body temperature), isolate Mln799 (*G. sacchari*) was eliminated from our collection and from further planned investigations.

Host range

Host range testing protocols using nontarget plants have been developed to assess the safety of pathogens as biological agents (Babu et al. 2002). Among the fungi recorded to date as pathogens of waterhyacinth, some of *Alternaria*, notably *A. eichhorniae* and *A. alternata*, have been proposed as possible promising biocontrol agents against this weed (Shabana 1997, Morsy 2004). Yet, Babu et al. (2003) report that carrots, sunflowers, and beans are susceptible to *A. eichhorniae*, and similar results have been reported for *A. alternata* (Morsy 2004). This led us to test the host ranges of *C. malorum* isolate Mln715 and *Alternaria* sp. isolate Mlb684.

When these strains were delivered via water supplied to 19 crop-plants and weeds belonging to 15 different families, results indicate that 4 weeks after application of either isolate, waterhyacinth (all three ecotypes) and *Salvinia* showed lesions on their leaves, whereas none of the food crop plants showed any symptoms of disease (Table 4).

Our inoculation method could affect this outcome: in field use the mycoherbicide will be directly sprayed on the waterhyacinth and *Salvinia*. Based on our results, these fungal isolates would not be expected to affect economically important plants in Mali and should thus be viewed as potential candidates for managing waterhyacinth infestations.

Table 4. Host specificity of isolates Mlb684 (*Alternaria* sp.) and Mln715 (*Cadophora* malorum) (+ = symptoms were observed).

		Rating for	
Plant family	Common name	Mlb684	Mln715
Rosaceae	Pear	–	–
	Apple	–	–
Poaceae	Rice	–	–
	Sorghum	–	–
	Corn	–	–
Amaranthaceae	Beet	–	–
	Amaranth	–	–
Apiaceae	Carrot	–	–
Caricaceae	Papaya	–	–
Liliaceae	Onion	–	–
Brassicaceae	Turnip	–	–
Fabaceae	Bean	–	–
Asteraceae	Sunflower	–	–
Malvaceae	Sorrel of Guinea	–	–
Piperaceae	Pepper	–	–
Apiaceae	Celery	–	–
Solanaceae	Tomato	–	–
Pontederiaceae	Waterhyacinth	–	–
	Ecotype 1	+	+
	Ecotype 2	+	+
	Ecotype 3	+	+
Salviniaceae	Aquatic fern	+	+

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Influence of biological control damage on efficacy of penoxsulam and two other aquatic herbicides on waterhyacinth

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ABSTRACT

Populations of waterhyacinth (*Eichhornia crassipes* [Mart.] Solms.) in the southeastern United States have been reduced through separate biological and chemical control efforts. However, damaging populations are still widespread, and integrated approaches are needed. In this study, the effect of combined application of penoxsulam (2-(2,2-difluoroethoxy)-6-(trifluoromethyl)-N-(5,8-dimethoxy[1,2,4]triazolo-[1,5c] pyrimidin-2-yl)-benzenesulfonamide) and biological control (BC) agents (*Neochetina* spp. waterhyacinth weevils and a conidial suspension of the fungus *Cercospora piaropi* Tharp) on mortality and colony growth was examined in outdoor tanks. BC alone caused damage but did not kill shoots. Combined BC and penoxsulam application reduced time to death by 1 to 2 weeks compared to penoxsulam alone in subsurface applications at 5 to 10 µg ai L^{-1} or in foliar applications at 0.035 kg ai ha^{-1}. At 10 µg ai L^{-1}, this combination also caused a net decline in shoot density. Penoxsulam and BC killed plants 3 weeks earlier than did triclopyr (3,5,6,-trichloro-2-pyrinyloxy-acetic acid, triethylamine salt; 0.42 kg ae ha^{-1}) and BC, or glyphosate (N-[phosphonomethyl]glycine, isopropylamine salt; 0.56 kg ae ha^{-1}) and BC in a winter test. Triclopyr with or without BC killed plants 3 weeks earlier than the other two herbicides in a summer test. The efficacy of penoxsulam was positively influenced by biological control damage, indicating that damage surveys prior to application may permit dose reduction to achieve efficacy.

Key Words: *Eichhornia crassipes*, fungus, glyphosate, integrated control, *Neochetina*, triclopyr.

INTRODUCTION

Chemical control of aquatic weeds can provide benefits for water users and wildlife by protecting and conserving water resources, but applications must be managed to minimize risks and costs (Richardson 2008, Netherland 2009). Aquatic weeds have also been targeted with biological control (BC), a strategy considered successful against waterhyacinth (*Eichhornia crassipes* [Mart.] Solms., Pontederiaceae; Julien 2001). Pantropical releases of two leaf- and shoot crown-feeding weevils (*Neochetina* spp.) (Coleoptera: Curculionidae) and other agents have decreased waterhyacinth density (Julien 2001, Center et al. 2002), increased availability of water (Martínez Jiménez and Gómez Balandra 2007) for human use and native plants, and saved human livelihoods dependent on water navigation and fishing in Africa and southeast Asia (Van Driesche et al. 2010). BC of waterhyacinth in the United States has primarily involved two introduced leaf-feeding weevils (*Neochetina bruchi* Hustache and *Neochetina eichhorniae* Warner; Coleoptera: Curculionidae; Center et al. 2002). Additive interactions can occur between *Neochetina* spp. and two fungal pathogens that are native or adventive in the United States (*Cercospora piaropi* Tharp and *Acremonium zonatum* [Saw.] Gams; Charudattan 1986, 2001, Moran 2005, Martínez Jiménez and Gómez Balandra 2007). Pathogens such as these can be applied to plants (Auld et al. 2003, Hallett 2005) or may interact with the weevils in natural infections. However, climate (Julien 2001), plant nutrient availability (Room et al. 1989, Wheeler and Center 1996, Center and Dray 2010), and low host plant density (Wilson et al. 2006) can all limit establishment, dispersal, and efficacy of BC agents of waterhyacinth and other aquatic weeds, as can the use of other control methods which limit host plant availability to agents (Center et al. 1999, Moran 2004, Schooler et al. 2007).

Recent research (Hill and Coetzee 2008, Richardson 2008, Santos et al. 2009) and regulatory debates (EPA 2010) have highlighted the importance of integrated management for

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aquatic weeds. In general, little information is available on combined arthropod-herbicide strategies for aquatic weed control (Ainsworth 2003). However, increased damage and reduction in plant biomass and/or survival has been demonstrated in waterhyacinth (Charudattan 1986), Eurasian watermilfoil (*Myriophyllum spicatum* L; Nelson and Shearer 2008) and hydrilla (*Hydrilla verticillata* [L. f.] Royle; Nelson and Shearer 2009) using reduced doses of herbicides combined with BC agents (insects and/or plant pathogens) compared to insects alone. On waterhyacinth, insect populations may benefit from lush regrowth following lethal herbicide application (Center et al. 1999, Center and Dray 2010). Sublethal application may improve plant quality for herbivores (Wright and Bourne 1990, Haag and Habeck 1991) and/or slow plant growth, allowing more time for insects to develop (Jidhav et al. 2008). Toxicity through direct contact can occur; several products applied to *Neochetina* spp. cause 10 to 15% mortality (Patnaik et al. 1987), possibly due to lipophilic adjuvants (Haag 1986). However, BC agents are usually not indirectly affected by feeding on live, herbicide-weakened terrestrial weeds (Tipping 1991, Lindgren et al. 1999, Boydston 2004, Williams et al. 2004), and direct toxicity is minimal or absent (Lindgren et al. 1998, Rees and Fay 1989). Insect damage can enhance herbicide efficacy against terrestrial weeds (Lym and Nelson 2002, Boydston 2004, Williams et al. 2004, Metzger et al. 2008), but little is known about the influence of sublethal BC damage on aquatic herbicide efficacy.

Chemical control of waterhyacinth is still often necessary in the southeastern United States and California (Center et al. 2002, Spencer and Ksander 2004). Many chemical tools are available (Gettys 2009, Netherland 2009), but selection of an herbicide and dose is restricted by label regulations, costs, and requirements imposed by water users. Damage caused by BC could reduce the effective dose of herbicide needed. The objective of this study was to determine the impact of damage by *Neochetina* spp. weevils and a fungal pathogen (*Cercospora pioropi*) on the efficacy of penoxsulam applied to waterhyacinth at rates at or below label recommendations, in comparison with the combined efficacy of triclopyr or glyphosate and BC damage.

MATERIALS AND METHODS

Plant, insect and fungal culturing and application

Waterhyacinth was collected from a field population near Monte Alto, Hidalgo County, Texas (26°24.796N, 97°57.549W) and cultured for 2 months in a 1000 L cattle tank (0.8 m water depth). Colonies for experiments were established with shoots (taken from the 1000 L tank) in 400 L plastic tanks (5 to 10 shoots per tank, tank area = 0.94 m^2, 0.6 m depth), caged with 1.0 mm mesh screening supported by PVC pipe to a height of 0.5 m above the top of the tank. Culturing and experimental tanks were filled with treated municipal water supplemented with 70 mg L^{-1} ammonium, 12 mg L^{-1} nitrate, 34 mg L^{-1} phosphate, 83 mg L^{-1} potassium, and 1 to 3 mg L^{-1} micronutrients. Blue dye[2] (0.01% v/v) was added once at tank initiation time to inhibit algal growth. The water was aerated continuously with aquarium pumps and refertilized with nitrogen once per month. For experiments, plants were allowed 1 week to acclimate in the 400 L tanks.

The two *Neochetina* waterhyacinth weevil species (~70% *Neochetina bruchi* Hustache, 30% *Neochetina eichhorniae* Warner) were collected from multiple local field sites 1 day before each experiment, or were isolated from plants in a 1000 L tank maintained as above inside a 3.0 by 3.7 by 2.6 m cage made of 13-mesh cm^{-1} saran plastic screening. For applications, 25 weevils were surface-sterilized with 0.9% benzalkonium chloride, rinsed in sterile water, and immediately released into each tank involving BC treatment. Weevils were not sexed, but were collected from mature, healthy plants and were thus expected to show a near-1:1 sex ratio (Center and Dray 1992).

The fungus *Cercospora piaropi* Tharp was isolated from infected leaves collected from local field infestations and cultured on solid potato dextrose agar (PDA; 19.5 g L^{-1}) with 5 g L^{-1} yeast extract. Two week-old cultures were used for inoculations. Spores were suspended in a formulation containing 0.05% w/v Kelgin® LV humectant[3], 0.003% v/v IVOD® citrus oil[4], and 0.2% v/v Tween® 80 surfactant[5], and the concentration was adjusted to 1×10^6 spores mL^{-1}.

The objective of the study was to examine the impact of BC by the two weevils and *C. piaropi* in combined infestations and infections, as occur in the field. Tanks were infested with weevils (25 per tank) and sprayed with *C. piaropi* (5 mL suspension per tank) 1 day prior to herbicide applications. The effectiveness of the BC treatment to cause damage was examined using data from the July 2009 test of effects of BC on efficacy of three herbicides (see 'Interactions of herbicide and biological control', below). The number of scars made by waterhyacinth weevils on the youngest unfurled leaf lamina were counted 1 and 2 weeks after treatment (WAT), as was percent coverage of the lamina of the oldest live leaf with necrotic spotting indicative of *C. piaropi* infection. Data were ranked (ties = mean) and analyzed with Kruskal-Wallis analyses of variance (SAS PROC GLIMMIX; SAS Institute 2004) and Tukey least-square mean separation.

Herbicides and application methods

Three herbicides were evaluated in this study. Penoxsulam[6] is recommended for control of floating aquatic weeds at doses ranging from 25 to 75 µg ai L^{-1} for water subsurface application (done with a pipette in this study), or at rates of 0.024 to 0.096 kg ai ha^{-1} for foliar applications (SePRO 2009a). Triclopyr[7] is recommended for foliar application to waterhyacinth (SePRO 2009b) at rates of 1.8 to 6.8 kg ae ha^{-1}, and the isopropylamine salt of glyphosate[8] is recommended for foliar application (Nufarm 2008) at rates of 3.8 to 4.6 kg ae ha^{-1}. All foliar applications included 0.25% v:v methylated seed oil surfactant[9] and were made using a hand-held household pressurized sprayer[10] at a spray volume of 230 L ha^{-1}. Herbicides were applied between 9 and 10 AM in full sun. Daily temperature averaged 29.6 ± 0.7 C during the 2008-2009 summer experiments and 17.2 ± 1.2 C in one test run under winter conditions (Dec 2008). To determine a sublethal subsurface dose of penoxsulam, this compound was applied to the water in a preliminary 42-day experiment without BC in May 2008 at concentrations of 0 to 50 µg ai L^{-1} (3 replicates per concentration, replicate = 1 tank).

Interactions of herbicide and biological control

Four experiments examined the effect of BC on penoxsulam efficacy. In the first experiment, beginning in July 2008 and lasting 42 days, penoxsulam was applied to the water to achieve a dose of 5 or 10 µg ai L^{-1}, with or without addition of *Neochetina* spp. weevils and *C. piaropi* (4 reps), while four tanks were left untreated (no control method). In an experiment started in August 2008 and lasting 56 days, penoxsulam was applied as a foliar spray at a rate of .0175 or 0.035 kg ai ha^{-1} (4 reps) with and without BC, while four tanks were left untreated.

In two additional foliar spray experiments (started in December 2008, winter 2008 test, and July 2009, summer 2009 test), penoxsulam (0.035 kg ai ha^{-1}), triclopyr (0.42 kg ae ha^{-1}), or glyphosate (0.56 kg ae ha^{-1} isopropylamine salt) was applied. The winter 2008 test involved four treatments (penoxsulam + BC, triclopyr + BC, glyphosate + BC, and BC alone, 4 replicate tanks per treatment), and lasted 75 days. The inclusion of other controls (e.g., herbicides alone, and no treatment) was limited in this winter test by plant and weevil availability. The summer 2009 test involved eight treatments (3 reps per treatment): an untreated control, BC alone, penoxsulam, triclopyr, and glyphosate alone, and each herbicide + BC. This experiment lasted 70 days.

Data collection

Six to eight plants per tank were tagged on the youngest unfurled leaf, and tags were moved as needed to avoid loss from dying leaves. The living or dead status of tagged plants was checked at least weekly. A plant was scored as dead if all leaf blades and petioles were brown to black and flaccid and no buds or regrowth were present on the central shoot crown. In some cases the tagged plant could not be found, presumably due to sinkage, and was scored as dead. Total shoots per tank was determined weekly. Untreated and BC-only colonies became overcrowded, and excess shoots were removed every 2 weeks (to achieve a density of approximately 70 shoots per tank). Removed shoots were dried in an uncooled greenhouse (temperatures reaching 50 C. At the end of each experiment, all living and dead shoots were removed, combined with previously removed shoots, if any, and oven-dried at 70 C for 72 h for determination of total dry biomass (DW).

Data analysis

In all four tests (two with penoxsulam alone, two involving all three herbicides), the median number of days after treatment (DAT; day 0 = day of biological control agent application) at which death of tagged plants occurred was determined for each tank, and these medians were used in a survival analysis with PROC LIFETEST in SAS (SAS Institute 2004) and Kaplan-Meier product-limit estimates to determine a survival probability curve for each treatment. These curves were then compared with a Wilcoxon χ^2 test. For simplicity, survival is presented here as median days to plant death for each treatment. For the winter 2008 and summer 2009 tests involving multiple herbicides, efficacy was also compared using Kruskal-Wallis analyses of variance on ranked percentages of tagged plants that were dead 6 WAT in each tank. For all experiments, the change in shoot density over the timeframe of each trial and final DW were compared among treatments using ANOVAs. Normality and homogeneity of variance assumptions were verified with Wilk's lambda in SAS PROC UNIVARIATE and Levene's test in SAS PROC GLM, respectively.

For the summer 2009 test involving BC alone, each of the three herbicides alone, penoxsulam + BC, triclopyr + BC and glyphosate + BC, final DW and final shoot density was converted to percent control for each replicate tank using the average value for these two variables across the three no-treatment tanks. The expected additive effect of BC and herbicide control combined was then calculated for each of the three combined treatments as in Colby (1967), using average percent control values for BC or herbicide alone. Differences in observed versus expected control were examined with sign tests to determine significance of positive values (indicative of synergism) or negative values (indicative of antagonism) from zero (lack of significance is indicative of additive or non-interactive effects).

RESULTS AND DISCUSSION

Damage caused by biological control

Neochetina spp. weevils and *C. piaropi* fungus applied to the plants caused damage within 1 week in all tests, either with or without herbicide application. In representative data from the summer 2009 test, youngest unfurled leaves had 7.7 ± 1.4 scars caused by waterhyacinth weevils 1 WAT (mean ± SE across all four treatments involving BC), and 40% necrosis (22 to 58%, 95% lower-upper confidence intervals across all four treatments involving BC) on oldest unfurled leaves caused by infection by *C. piaropi* (Figure 1). Comparisons of ranked data showed differences in damage between weevil- and fungal-exposed plants and non-BC tanks (for scarring, $F_{7, 16} = 22.4$, $P < 0.001$; for necrosis, $F_{7, 16} = 17.3$, $P < 0.001$; Figure 1), but none among the four treatments involving BC (BC alone, penoxsualm + BC, triclopyr + BC, and glyphosate + BC), indicating that herbicide application did not negatively affect the ability of the BC agents to cause damage. By 2 WAT, a new youngest unfurled leaf had emerged, and the prior oldest live leaf had died. New youngest unfurled leaves observed 2 WAT had significantly more damage (17.4 ± 3.0 scars per leaf, averaged across all four treatments involving BC) than did plants in treatments lacking BC (0.14 ± 0.10; $F_{1,21} = 82.6$, $P < 0.001$), while live oldest leaves in tanks subjected to any of the four treatments involving BC had almost no fungal necrosis (4%, 1 to 7% confidence interval), indicative of transient effects of fungal inoculation.

Penoxsulam efficacy and biological control

Application of penoxsulam between 10 and 50 µg ai L^{-1} with no BC in a preliminary 42-day test led to plant death after a median of 37 days at 10 µg ai L^{-1}, 29 days at 20 and 25 µg ai L^{-1}, and 20 days at 50 µg ai L^{-1} (data not shown). Survival analysis showed a significant difference in survival probability among these doses (Wilcoxon Chi-square, $\chi^2 = 21.6$, P =

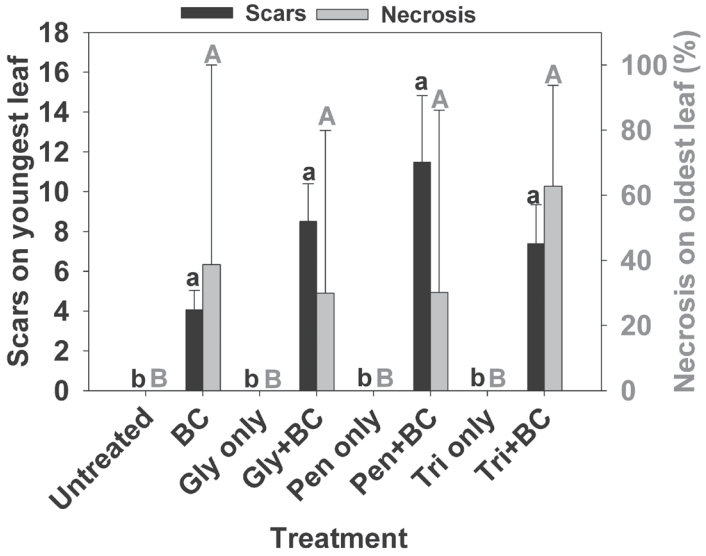

Figure 1. Scars (mean ± SE) made by *Neochetina* spp. weevils on youngest unfurled leaves (left axis) and percent necrosis (mean + upper 95% confidence interval) on oldest live leaves (right axis) caused by the fungus *C. piaropi* one week after application of biocontrol agents and/or herbicides onto plants in 1 m^2 tanks (n = 3). Bars with the same lowercase (scars) or uppercase (necrosis) letter are not significantly different in Tukey mean comparisons of ranked data ($\alpha = 0.05$). Abbreviations: BC = biological control, application of 25 weevils and 5×10^6 *C. piaropi* fungal spores per tank; Gly = glyphosate, 0.56 kg ae ha^{-1} isopropylamine salt; Pen = penoxsulam, 0.035 kg ai ha^{-1}; Tri = triclopyr, 0.42 kg ae ha^{-1} triethylamine salt.

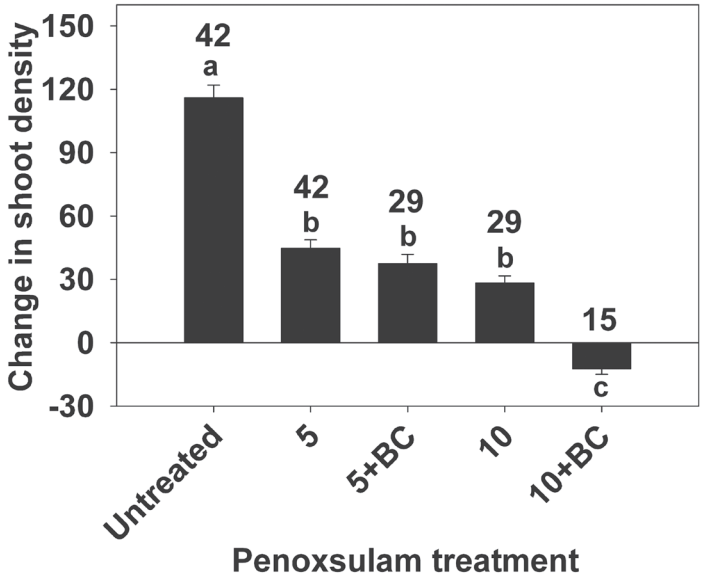

Figure 2. Change (mean ± SE) in waterhyacinth shoot density (m^{-2}) in a 42-day test in 1 m^2 tanks treated with subsurface penoxsulam at 5 or 10 µg ai L^{-1} with or without application of biological control (+BC; 25 waterhyacinth weevils and 5×10^6 *C. piaropi* fungal spores per tank; n = 4). Bars with the same lowercase letter are not significantly different in Tukey mean comparisons of ranked data ($\alpha = 0.05$). Numbers above bars give median days to death of marked plants.

0.002). The results are consistent with the high below-label (to 2 µg ai L^{-1}) efficacy demonstrated by penoxsulam against waterhyacinth in previous studies (Richardson and Gardner 2007).

After subsurface application of penoxsulam in July 2008, survival probability functions varied among treatments (χ^2 = 26.5, P < 0.001). Controls (no BC or herbicide) and plants treated with 5 µg ai L^{-1} penoxsulam without BC survived the duration of the test (42 days). Median days to plant death were similar for plants at 5 µg ai L^{-1} + BC and 10 µg ai L^{-1} without BC (29 days), while plants exposed to 10 µg ai L^{-1} penoxsulam + BC died 2 weeks earlier (after 15 days) than did plants exposed to penoxsulam at this same dose without BC (Figure 2). Despite the death of many marked shoots, net live shoot density per tank increased due to production of daughter plants, except in tanks treated with 10 µg ai L^{-1} + BC ($F_{4,15}$ = 121, P < 0.001; Figure 2). Final DW (combined dead and live material) was reduced 50 to 56% by penoxsulam at 5 µg ai L^{-1}, but this reduction occurred in tanks treated with either penoxsulam alone (166 ± 21 g) or penoxsulam + BC(200 ± 42 g) in comparison to control tanks (385 ± 30 g; $F_{2,9}$ = 13.3, P = 0.002) (data not shown).

Foliar application of 0.0175 or 0.035 kg ai ha^{-1} penoxsulam in the test started in August 2008 led to plant death less than 6 WAT with or without BC, but survival varied by treatment (χ^2 = 23.1, P < 0.001). At 0.035 kg ai ha^{-1}, penoxsulam + BC treatment decreased time to death compared to penoxsulam alone by almost 2 weeks (17 vs. 30 days; Figure 3). Penoxsulam reduced new shoot gain ($F_{4,15}$ = 12.1, P < 0.001), but changes in shoot density did not differ between tanks treated with penoxsulam + BC and herbicide alone (Figure 3). Final tank DW did not vary significantly among treatments ($F_{4,15}$ = 2.8, P = 0.06; data not shown), although 35 g ai ha^{-1} penoxsulam +

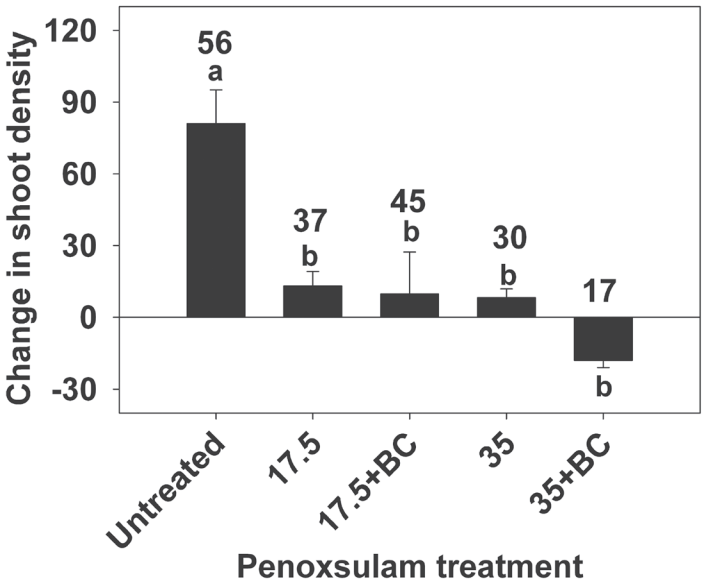

Figure 3. Change (mean ± SE) in shoot density (m^{-2}) in a 56-day test in 1 m^2 tanks treated with foliar penoxsulam at rates of 0.0175 or 0.035 kg ai ha^{-1} with or without application of biological control (+BC; 25 waterhyacinth weevils and 5×10^6 *C. piaropi* fungal spores per tank; n = 4). Bars with the same lowercase letter are not significantly different in Tukey mean comparisons of ranked data ($\alpha = 0.05$). Numbers above bars give median days to death of marked plants.

BC caused an 87% reduction in DW (35.0 ± 4.1 g) compared to the control tanks (267.4 ± 32.2 g), whereas penoxsulam alone at this dose reduced DW by 52% (127.7 ± 34.4).

Comparative efficacy of three herbicides and biological control

In the winter (December) 2008 comparison of the efficacy of sublethal doses of three herbicides in the presence of BC, survival varied among treatments ($\chi^2 = 14.9$, $P = 0.002$). Plants treated with penoxsulam + BC died at least 19 days earlier than plants treated with triclopyr + BC or glyphosate + BC (Table 1). In comparisons of ranked percent mortality 6 WAT, penoxsulam + BC (88%), and triclopyr + BC (28%) differed from glyphosate + BC (3%) and BC alone (0%; $F_{3,12} = 24.0$, $P < 0.001$). Increases in shoot density were lower in colonies treated with penoxsulam + BC and triclopyr + BC than in BC-only tanks ($F_{3,12} = 17.8$, $P < 0.001$), and combined penoxsulam + BC caused a net decline in shoot density (Table 1). Final DW did not vary among treatments ($F_{3,12} = 2.9$, $P = 0.08$; Table 1), probably because of the inclusion of dead biomass in the dried samples.

The comparative efficacy of the three herbicides differed in the summer (July) 2009 test from the winter 2008 test. Survival of marked plants varied significantly ($\chi^2 = 14.9$, $P = 0.002$), but triclopyr, rather than penoxsulam had the shortest median kill time, 21 DAT in tanks subjected to triclopyr + BC or triclopyr alone (Table 2). Only triclopyr without BC differed in percent mortality of marked shoots 6 WAT (95%) from untreated colonies (0%; $F_{7,16} = 3.0$, $P = 0.035$; triclopyr and BC: 67% mortality). Consistent with the August 2008 test, foliar penoxsulam + BC treatment killed marked plants in the summer 2009 2 weeks faster than did penoxsulam alone (Table 2). Median time to death was actually 1 week longer in tanks subjected to glyphosate + BC relative to glyphosate alone, but percent mortality 6 WAT was similar (39 and 44%, respectively). In a tank study in South Africa (Jadhav et al. 2008), a 2-fold more concentrated dose of glyphosate (isopropylamine salt, 1.1 kg ae ha^{-1}) stopped plant growth but did not kill plants; 2.2 kg ae ha^{-1} was required for mortality. In the summer 2009 test, colonies treated with any of the three herbicides, either alone or + BC, gained no more than 18% of the shoots gained by colonies subjected to BC alone or no treatment ($F_{7,16} = 17.6$, $P < 0.001$; Table 2), as herbicides outperformed BC alone, but there were no differences among herbicides, and no enhancement by BC of the negative effect of herbicides alone on shoot gain. Final DW was reduced by herbicides compared to untreated control colonies ($F_{7,16} = 7.6$, $P < 0.001$), but the combination of BC and herbicide never reduced biomass more than herbicide alone (Table 2). Shoot gain and final biomass were virtually identical in the winter 2008 and summer 2009 tests in colonies subjected to BC only (Tables 1 and 2).

Final shoot density and final DW from the summer 2009 test were converted to percentages of control values and used to determine expected additive control effects of herbicides and BC (Colby 1967). Differences in observed minus expected values for each herbicide with BC did not differ from zero in signed rank tests ($P \geq 0.25$), indicating that biological and chemical control effects were additive or noninteractive.

Conclusions for integrated control

BC did not have additive effects on the efficacy of triclopyr or glyphosate, but waterhyacinth shoot death was achieved 2 weeks more rapidly after penoxsulam application in the presence of *Neochetina* spp. weevil and *C. piaropi* fungal lesion damage than in the absence of damage. The summer 2008 subsurface application results suggest that colonies treated with 10 µg ai L^{-1} penoxsulam and BC were unable to replace dying plants, with a similar nonsignificant trend for foliar applications in the test involving penoxsulam in August 2008, and specifically for penoxsulam in the winter 2008 test. The mechanism(s) driving the positive penoxsulam + BC interaction is/are uncertain but could relate to more rapid penetration of herbicide through wounds or systemic movement through increased transpiration. However, adult lesser knapweed flower weevils (*Larinus minutus* Gyllenhal) reduce transpiration in spotted knapweed leaves (*Centaurea stoebe* L. ssp. *Micranthos*; Wooley et al. 2011). The ability of waterhyacinth weevils to generate damage was not adversely affected by any of the three herbicides. Negative impacts on weevil population dynamics could occur in the field because 45 to 60 days are required to complete a beetle generation in warm conditions (Center et al. 2002), a time frame exceeding time to death in treatments involving penoxsulam or triclopyr under summer conditions.

Irrespective of BC, the results demonstrate lethality of triclopyr against waterhyacinth at a foliar rate 4.2-fold below the low end of label recommendations, albeit in artificial tank mesocosms. Glyphosate applied at a rate 6.8-fold below label recommendations caused limited (~40%) plant mortality but did reduce shoot gain in the summer 2009 test. In the case of penoxsualm, a dose intermediate to those used here (0.0245 kg ai ha^{-1}) without BC killed all waterhyacinth plants within 5 WAT (Wersal and Madsen 2010), consistent with the present results. In past studies, additive effects of herbicides and biological control were apparent only when herbicide ap-

Table 1. Comparison of efficacy of three herbicides against waterhyacinth with biological control damage in a winter 2008 test.

Herbicide (dose)	Days to death (median)[1]	Change in shoot density[2]	Final DW (g)[2]
Penoxsulam + BC (0.035 kg ai ha^{-1})	43	-47.0 ± 5.0 c	100.7 ± 31.3 a
Triclopyr + BC (0.42 kg ae ha^{-1})	62	6.0 ± 24.8 bc	111.3 ± 27.2 a
Glyphosate + BC (0.56 kg ae ha^{-1})	67	50.8 ± 13.2 ab	161.9 ± 33.3 a
BC alone	75	110.5 ± 13.7 a	222.9 ± 39.1 a

[1]Test terminated 75 days after treatment.
[2]Means ± SE. Means with the same letter(s) are not significantly different in Tukey mean comparisons ($\alpha = 0.05$).

Table 2. Comparison of efficacy of three herbicides against waterhyacinth with and without biological control in a summer 2009 test.

Herbicide (dose)	Days to death (median)[1]	Change in shoot density[2]	Final DW (g)[2]
Penoxsulam (0.035 kg ai ha^{-1})	54	8.3 ± 16.9 b	31.7 ± 9.0 c
Penoxsulam + BC	40	1.3 ± 8.3 b	23.3 ± 23.3 c
Triclopyr (0.42 kg ae ha^{-1})	21	12.0 ± 14.0 b	60.3 ± 12.9 bc
Triclopyr + BC	21	-0.7 ± 5.0 b	84.0 ± 23.2 bc
Glyphosate (0.56 kg ae ha^{-1})	47	18.0 ± 6.8 b	9.3 ± 7.6 c
Glyphosate + BC	54	10.0 ± 11.8 b	86.0 ± 40.0 bc
BC alone	57	110.3 ± 9.8 a	219.1 ± 54.5 ab
Untreated	70	102.0 ± 10.6 a	246.6 ± 50.5 a

[1]Test terminated 70 days after treatment.
[2]Means ± SE. Means with the same letter(s) are not significantly different in Tukey mean comparisons ($\alpha = 0.05$).

plication rates were adjusted to sublethal levels. In the present study, both penoxsulam and triclopyr without BC caused waterhyacinth shoot death prior to test termination, possibly obscuring some efficacy-enhancing effects of biological control. The enhanced negative effects of penosxulam + BC compared to penoxsulam alone on new shoot gain could be important in the field to prevent dispersal of detached daughter plants not directly exposed to foliar spray herbicide.

Additive or synergistic effects of herbicides and biological control may manifest as a reduction in herbicide dose needed to achieve control in a desired timeframe (Williams et al. 2004, Metzger et al. 2008, Nelson and Shearer 2008, 2009), or, as in the present study, as a decrease in the timeframe itself (Lym and Nelson 2002). Additional studies are needed to develop integrated weed management recommendations for waterhyacinth by evaluating several below-label doses of herbicides and multiple levels of weevil infestation against waterhyacinth under field conditions.

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SOURCES OF MATERIALS

[2]Aquashade®, Applied Biochemists, W175 N11163 Stonewood Dr., Suite 234, Germantown, WI 53022

[3]CP Kelco U.S., 100 Parkwood Circle, Suite 1000, Atlanta, GA 30339.

[4]Brewer International, PO Box 690037, Vero Beach, FL 32969.

[5]Sigma-Aldrich Inc., PO Box 2060, Milwaukee, WI 53201.

[6]Galleon® SC , SePRO Inc., 11550 North Meridian St., Suite 600, Carmel, IN 46032

[7]Renovate® 3, SePRO Inc.

[8]Aqua Neat®, Nufarm Americas Inc, 150 Harvester Dr., Suite 200, Burr Ridge, IL 60527.

[9]Phase® MSO, Loveland Products, PO Box 1289, Greeley, CO 80632.

[10]RL Flo-Master® Model 1998TL, Root-Lowell Manufacturing, 1000 Foreman Rd., PO Box 289, Lowell, MI 49331.

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