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Integrated management of hydrilla with two biological control agents and a herbicide

EMMA N. I. WEEKS, MARK V. HOYER, JASON P. BENNETT, MARK A. JACKSON, AND JAMES P. CUDA*

ABSTRACT

When left unmanaged, the invasive aquatic weed hydrilla [*Hydrilla verticillata* (L. f.) Royle; Hydrocharitaceae] causes serious environmental and economic impacts by choking out native plants and impeding flood control, navigation, and recreation. In Florida, millions of dollars are spent annually to control infestations of hydrilla, primarily with herbicides. However, during the past 15 yr, some hydrilla populations developed resistance to two of the most used aquatic herbicides. Since 2010, we have been testing a novel integrated weed management (IWM) system for hydrilla control that integrates selective insect herbivory by the hydrilla tip miner *Cricotopus lebetis* Sublette (Diptera: Chironomidae) with a disease-causing fungal pathogen [*Mycoleptodiscus terrestris* (Gerd.) Ostaz.] (Mt), and the reduced-risk aquatic herbicide imazamox. Over a period of 2 yr, field testing of the two biological control agents and imazamox was performed in four limnocorrals (1 m diam. by 1 m depth) installed in man-made ponds naturally infested with hydrilla to determine the most effective combination for use in hydrilla management. Establishment of the biological control agents and hydrilla damage were measured by collecting apical meristem samples and harvesting the hydrilla to count turions and calculate biomass. *Cricotopus lebetis* and Mt together and the two biological control agents in combination with imazamox caused the most damage to hydrilla, both in terms of short-term damage to apical meristems and long-term damage through reduced turion production and biomass. Overall, these findings indicate that a combination of different biological and chemical tactics can be used for integrated management of hydrilla.

Keywords: Chironomidae, *Cricotopus lebetis*, fungal pathogen, hydrilla tip miner, *Hydrilla verticillata*, imazamox, *Mycoleptodiscus terrestris*.

INTRODUCTION

Hydrilla verticillata (L. f.) Royle (Hydrocharitaceae), hereafter hydrilla, is an invasive aquatic weed causing environmental and economic damage outside of its native range (Schmitz et al. 1991, Langeland 1996). There are monoecious and dioecious plants, with both present in the United States; however, in Florida only the dioecious form is present. In the United States, hydrilla is one of the worst aquatic weeds; it is listed as a Federal Noxious Weed by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA–APHIS 2010) and as a Category I Invasive Exotic Species in North, Central, and South Florida by the Florida Exotic Plant Pest Council (FLEPPC 2019). Efforts to control hydrilla currently rely primarily on herbicides and nonselective biological control using the grass carp *Ctenopharyngodon idella* Val. (Dibble and Kovalenko 2009). Millions of dollars are spent every year to control infestations; for example, in Florida almost USD $9 million were spent in 2018 to 2019 controlling hydrilla on 24,000 acres (FWC 2019).

According to a U.S. Geological Survey (USGS) report, reliance on pesticides to control weeds and other organisms is adversely affecting the U.S. watersheds that support a diverse aquatic biota and are used for drinking water, recreation, crop irrigation, and aquaculture (Gilliom et al. 2006). The report concluded that several commonly used herbicides were detected in sediments, fish, and mollusks of both urban and agricultural streams. The findings of the USGS report only served to increase the public's negative perception of pesticides, especially when they are applied directly to the water to control invasive aquatic weeds such as hydrilla. At around the same time that the USGS report was issued, resistance to the systemic bleaching herbicide fluridone and the contact herbicide endothall was reported to be occurring in dioecious hydrilla in Florida (Michel et al. 2004, Netherland 2009, Berger and MacDonald 2011, Giannotti 2013). Resistance to herbicides develops when the same active ingredient or multiple active ingredients with the same mode of action are used for an extended duration, because individuals with resistance to the active ingredient survive and reproduce just as those that are susceptible are killed. The discovery of resistance to these two herbicides was unexpected but not surprising given their use patterns and target sites. Endothall has been used to treat dioecious hydrilla infestations since the 1960s and fluridone since the 1980s (Schardt and Netherland 2020). Moreover, these extensively used herbicides target a single enzyme (Schardt and Netherland 2020), which made resistance development in hydrilla more likely due to cross-resistance (Thum and Chorak 2020).

Harker et al. (2012) argued that more research on herbicide alternatives is needed to mitigate herbicide resistance. They contend that combinations of different

*First and fifth authors: Assistant Research Scientist and Professor (Retired), Entomology and Nematology Dept., University of Florida, Gainesville, FL 32611. Second and third authors: Director and Regional Coordinator, Florida LAKEWATCH, University of Florida, Gainesville, FL 32653. Fourth author: Research Microbiologist (Retired), USDA, Agricultural Research Service, Crop Bioprotection Research Unit, Peoria, IL 61604. Corresponding author's email: eniweeks@ufl.edu. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Received for publication January 19, 2021 and in revised form September 3, 2021.

tactics can lengthen the useful life of our valuable herbicide toolbox. Given the limited number of herbicides available for aquatic use, the best approach for long-term management of hydrilla in the United States is to reduce reliance on a single strategy (chemical control) by adopting the use of nonherbicide tactics in combination with herbicides, i.e., integrated weed management or IWM (Gettys and Enloe 2016). The incorporation of multiple efficacious tools and techniques into an IWM plan could slow the development of resistance to herbicides, thereby providing a more sustainable option for hydrilla management. However, limited knowledge and options have led to the continued frequent application of herbicides as a standalone tactic.

Classical (or importation) biological control of hydrilla has been investigated since the 1970s. Foreign exploration for insect natural enemies was conducted in Asia, Australia, and to a lesser extent Africa (Buckingham 1994, Purcell et al. 2019, Harms et al. 2021). Several insects were identified, and four insects were released, but only two ephydrid leaf-mining flies of the genus *Hydrellia* and the stem mining weevil *Bagous hydrillae* O'Brien (Coleoptera: Curculionidae) are established in the United States (Center et al. 1997, Grodowitz et al. 1997, Center et al. 2013). To date, the two *Hydrellia* leaf-miners have not effectively controlled hydrilla (Forno and Julien 2000, Grodowitz et al. 2010), and there is no evidence to suggest the weevil *B. hydrillae* has had any impact on hydrilla in southern Louisiana where it was discovered in 2009 (Center et al. 2013).

A naturalized midge, *Cricotopus lebetis* Sublette (Diptera: Chironomidae), was discovered in Crystal River, FL in the 1990s causing damage to apical meristems and stunting the growth of hydrilla (Epler et al. 2000, Cuda et al. 2002). *Cricotopus lebetis* was subsequently found to be distributed at low abundance in several waterbodies of central and south-central Florida (Stratman et al. 2013a). Despite low natural abundances, the presence of *C. lebetis* in hydrilla samples from multiple sites, along with a rapid development from egg to adult, high fecundity in adults, and established mass rearing procedures (Cuda et al. 2002), coupled with its ability to develop on both monoecious and dioecious hydrilla (Stratman et al. 2013b), make it a suitable option for augmentative biological control of hydrilla. Furthermore, its preference for the actively growing apical meristem over other plant tissue makes its feeding more likely to reduce the rapid expansion of hydrilla than previously evaluated biological control agents.

In addition to herbivory, pathogenic organisms, such as bacteria or fungi, also could be used to control hydrilla and other weeds (Joye and Cofrancesco 1991). An indigenous fungal pathogen *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (hereafter Mt), capable of causing disease in hydrilla was isolated in Texas (Joye 1990, Cuda et al. 2008) and has been under development as a mycoherbicide (Shearer 1998, Heilman 2012). Furthermore, in laboratory and field experiments, Mt increased the toxicity of sublethal concentrations of aquatic herbicides, including fluridone, endothall, and imazamox (Netherland and Shearer 1996, Shearer and Nelson 2002, 2009).

The systemic herbicide imazamox was registered for aquatic use in 2008 under the Reduced Risk Pesticide Program (Netherland 2009). Reduced risk herbicides are greener chemistries that can be applied at lower concentrations and, therefore, are compatible with IWM strategies (WSSA 2020). After being absorbed through the foliage and roots, imazamox travels through the xylem and phloem to areas of new growth where it targets the plant-specific enzyme, acetolactate synthase (ALS). ALS is involved in the synthesis of amino acids that are required for protein synthesis and cell growth. Therefore, treating hydrilla with imazamox suppresses growth and reduces biomass. These effects can continue for up to 7 mo and there are no restrictions on its use for drinking water and minimal restrictions for irrigation (Netherland 2009).

The potential for integrating a fungal pathogen with an insect natural enemy to control hydrilla was first investigated by Shabana et al. (1998, 2003). The hydrilla leaf mining fly, *Hydrellia pakistanae* Deonier (Diptera: Ephydridae), was found to be compatible with several plant pathogens (Shabana et al. 2003). Our previous greenhouse experiments have confirmed that *C. lebetis* is compatible with the plant pathogen Mt and the herbicide imazamox (Cuda et al. 2016). Integrating the tip miner with either the fungus Mt or the herbicide imazamox had a significant impact on hydrilla biomass and architecture (Cuda et al. 2016). However, it is not yet known how the treatments will work in a field setting or if a three-treatment combination of the insect, fungus, and herbicide would be compatible and result in hydrilla reduction. The aim of this study was to evaluate the effect of different combinations of the hydrilla tip miner *C. lebetis*, the herbicide imazamox, and the fungal pathogen Mt on hydrilla under field conditions.

MATERIALS AND METHODS

Study site

The experiments were conducted in four man-made ponds naturally infested with hydrilla and other plants at the University of Florida, Institute of Food and Agricultural Sciences (UF/IFAS) Center for Aquatic and Invasive Plants (CAIP) in Alachua Co., FL (29°43′37.62″N, 82°25′02.24″W). Three of the ponds (ponds 1, 2, and 3) were approximately the same size, 26 m long and 6 m wide, and depth (1 m). Therefore, each had a volume of approximately 151,416 L (40,000 gallons). The fourth pond was the same depth (1 m) but 37 by 29 m and approximately 893,357 L (236,000 gallons). Water samples of approximately 1 L were collected from each pond in Nalgene™[1] high density polyethylene bottles and analyzed for total phosphorus, total nitrogen, and color following Florida LAKEWATCH standard operating procedures (Canfield et al. 2002; Hoyer et al. 2012). The ponds had total phosphorus of 182 to 366 μg L^{-1}, total nitrogen of 1,040 to 1,120 μg L^{-1}, and color of 26 to 28 Pt Co Units. Baseline assessments of hydrilla tips showed low levels of apical meristem damage, indicating the presence of *C. lebetis* at low numbers. Apical meristem damage at baseline was 5.6%, 7.8%, and 1.5% in Experiments 1, 2, and 3, respectively. However, to assess the efficacy of the treatments in a true field setting with the midge as an augmentative biological control agent, no insecticides were applied to the limnocorrals or ponds.

Insect (*Cricotopus lebetis*)

A colony of the hydrilla tip miner *C. lebetis* was used for all experiments. The colony was established and maintained at the UF/IFAS Entomology and Nematology Department following the methods of Cuda et al. (2002). Hydrilla for maintaining the colony was collected from ponds located at the UF/IFAS Center for Aquatic and Invasive Plants (29°43′37.62″N, 82°25′02.24″W). Hydrilla tips, the top 12 cm of a stem including an apical meristem, were rinsed with water using a high-pressure hose to remove other herbivores such as snails and moth larvae. The hydrilla tips were then placed in aerated well water in 17-L plastic trays.

Trays of hydrilla were placed in a greenhouse maintained at 21 to 38 C. Ambient light was supplemented with 243.8 cm (8 ft) florescent tubes (60 watt, cool white) and incandescent bulbs (40 watt, 120 volt, soft white) to maintain a 14 : 10 h L : D photoperiod. *Cricotopus lebetis* eggs were placed into trays at a 1 : 1 tip to egg ratio. By day 10, trays were placed into white mesh cages (61 by 61 by 61 cm) for adult emergence, which occurred 14 to 21 d from the time the eggs were laid. During this period, adults were collected daily from the cages using a mouth aspirator comprising a hard plastic tube separated from a piece of flexible plastic tubing by a HEPA filter[2].

Adult *C. lebetis* were collected in plastic vials (1 cm diam. by 5 cm length), transported to a laboratory rearing room (23 C, 21% relative humidity [RH], and a 14 : 10 h L : D photoperiod) and placed into a 500 ml separatory funnel containing 300 ml well water using a mouth aspirator. Prior to being added to the funnel, the well water was conditioned by soaking with hydrilla stems, which was previously observed to induce oviposition (Cuda et al. 2002). An approximately 1 : 1 ratio of males to females was placed in the funnel for an approximate total of 100 adults. After 24 h, eggs that had been laid on the water surface were collected and fecundity (total eggs) and fertility (% fertile eggs) in each egg mass were recorded by viewing under a dissecting microscope. Egg masses then were used for rearing purposes or field release. Egg masses for release at the field site were enumerated and placed in 20 ml glass vials containing the appropriate pond water. At the study site, a low (40 midges 0.09 m^{-2}) or high (80 midges 0.09 m^{-2}) midge density was applied based on the results of previous research. Cuda et al. (2016) demonstrated that although a low midge density resulted in the greatest reduction in hydrilla growth in combination with Mt, a high midge density was needed in combination with the herbicide imazamox. Because the limnocorrals had a water surface area of 0.79 m^2, a total of 350 (low) or 700 (high) midges were added per limnocorral to achieve an equivalent inoculation rate, depending upon the experiment.

Herbicide (Imazamox)

The herbicide used in this study was Clearcast®[3]. The active ingredient of Clearcast is the ammonium salt of imazamox, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)-3-pyridinecarboxylic acid. The label rate for hydrilla ranges from 150–200 parts per billion (ppb) for control or 50–75 ppb for growth suppression. A low concentration of approximately 10 μg L^{-1} (10 ppb) was applied, because previous studies with this active ingredient found it to be compatible with Mt (Shearer and Nelson 2009) and the midge (Cuda et al. 2016) at this concentration. Depending upon the water depth at inoculation, the amount of herbicide added was approximately 65.5 μl per limnocorral (1 m depth).

Pathogen (*Mycoleptodiscus terrestris*)

Mt isolate TX-05 (NRRL 30559) used in these studies was from infected hydrilla collected at Sheldon Reservoir, Texas, USA (Dunlap et al. 2011). Stock cultures of Mt were grown on potato dextrose agar (PDA) plates for three weeks at room temperature, cut into 1 mm^2 agar plugs and stored in 10% glycerol at −80 C. Inocula for liquid culture microsclerotia production were obtained from PDA plates inoculated with a frozen stock culture of Mt and grown at room temperature (∼21 C) for 2 wk, as described previously (Shearer and Jackson 2006). Because Mt cultures do not sporulate on PDA, cultures were cut into 1 mm^2 agar pieces for use as inoculum. The composition and preparation of the media followed Jackson et al. (2011). The preparation of the Mt for experiments followed the methods of Dunlap et al. (2011).

Movement of Mt and Mt-infected plant material was completed under USDA–APHIS permits P526P-14-03276 and P526P-15-04391. Mt was applied as a liquid formulation to the limnocorrals. A high concentration of Mt was applied based on previous studies (Shearer and Nelson 2009, Cuda et al. 2016). Because prior research used a dry formulation at 0.06 μg L^{-1}, the equivalent concentration of Mt in liquid formulation was 200 CFU ml^{-1} (J. Shearer, pers. comm.). Based on the production concentration of Mt at 1×10^6 CFU ml^{-1}, a desired concentration of 200 CFU ml^{-1} and the volume of water in the limnocorral (approx. 790 L, 1 m depth by 1 m diam.), 0.314 ml of liquid Mt was added per limnocorral.

Experimental design

The general experimental design was the same for all experiments, which was a randomized complete block field trial. The field study was performed in four ponds naturally infested with hydrilla. Experiments 1 and 3 were blocked by pond with each pond (ponds 1, 2 and 3) containing one each of four treatments. Experiment 2 was conducted in pond 4 and blocked by groups of four limnocorrals. The experimental units were 12 1-m-diam by 1-m depth-benthic enclosures, or limnocorrals (Cruikshank et al. 1983). Treatments were applied and the hydrilla was left to grow for 45 d after application of the last agent.

Limnocorrals

Each limnocorral was constructed from a heavy-duty opaque polyethylene tube (2 m length 1 m diam.)[4] with a 25-cm-diam foam-filled corrugated PVC pipe ring to provide flotation (Figure 1A). The flotation ring was attached to the

Figure 1. Limnocorrals (1 m diam. by 2 m potential depth) constructed for the experiments (A). A polyethylene tube was suspended from a buoyant PVC pipe ring. A chain at the bottom provided anchorage into the sediment and two PVC pipe hoops provided shape. A net was placed over the top to prevent access of herbivores (B). Four limnocorrals were placed in each of three hydrilla-infested ponds (C). One treatment was randomly placed per limnocorral in each pond.

tube using hose clamps and O rings attached to grommets in the tube. A section of heavy chain was inserted into the hem at the bottom of the tube to provide anchorage. Two hoops constructed from PVC pipe (1.9 cm diam.) were used to maintain limnocorral shape in the water column at 1 and 2 m. Nets were constructed from fine mesh (240 µm) that fit over the flotation ring and could be drawn closed with polyester cord and secured with a plastic spring-loaded barrel cord lock (Figure 1B).

The limnocorrals were positioned in the center line of the ponds (Figure 1C), by placing over a section of the hydrilla stand. Then the tube that extended down to the bottom of the pond, was anchored by the chain, and sealed by pushing the bottom plastic ring into the sediment to prevent seepage. According to visual assessments the hydrilla isolated in each limnocorral was of uniform surface area and depth from the surface prior to applying the treatments. After harvesting the plant material inside the limnocorrals for biomass analyses, the limnocorrals were removed from the pond, washed, and scrubbed, then left to dry in the sun for several weeks between experiments.

Environmental monitoring

Dataloggers were used to record water temperature, ambient temperature, and relative humidity throughout the study. Water temperature was recorded in each pond (Experiments 1 to 3) and in one limnocorral per pond (Experiment 2 and 3 only) every 15 min using water temperature data loggers[5]. The data loggers in the ponds were placed in the water attached to a rope for retrieval and weighted to maintain the logger below the water surface. The data loggers in the limnocorrals were attached to the underside of the flotation rings, which rested below the water surface. Ambient temperature and relative humidity were recorded every 15 min using a temperature/relative humidity data logger[6] housed inside a solar radiation shield[7]. The shield containing the logger was on a PVC post at approximately 1 m above the ground equidistant between the limnocorrals.

Experiment 1: Assess the efficacy of combining *C. lebetis* with the fungal pathogen Mt.

There were four treatments: Control, Mt, midge (hydrilla tip miner, *C. lebetis*), and Mt+midge. Each treatment was repeated three times, once in each of three ponds. The treatments were randomly assigned to the limnocorrals within a pond. One rate of Mt (high) and one density of the midge (low) were applied to the limnocorrals containing established hydrilla plants. The midge was added 2 d before the Mt to cause feeding damage that would facilitate Mt infection. The control limnocorrals received no insects or fungi. Establishment of Mt and the midge was confirmed by microscopic examination of hydrilla shoot tips. This experiment was conducted once from 5 June to 21 July 2015. During the experiment, the average air temperature was 26.7 C (18.3–36.1 C) and the relative humidity was 85% (35–100%). The pond temperature was 27.2 C (23.9–28.9 C).

The limnocorrals were monitored every other day (at approximately the same time) to ensure that they were secure and pond water depth was recorded.

In each limnocorral prior to first treatment (baseline) and 45 d (two *C. lebetis* generations) after the last treatment was applied, the depth of the hydrilla plants (cm) was measured from the surface of the water, the surface area of the hydrilla (%) was visually approximated, and hydrilla tips were collected for microscopic examination. Photographs also were taken. The surface area of the hydrilla was approximated using a 30 by 30 cm PVC quadrat, that was placed in the center of the limnocorral. A trained technician then estimated the percentage of the quadrat that contained hydrilla stems to the nearest 5%.

In total, 50 hydrilla tips (apical 12 cm of a hydrilla stem) were randomly collected from the upper section of the water column per limnocorral. Collected hydrilla tips were placed into zip closure bags containing pond water and were transported back to the laboratory on ice for analysis. To assess hydrilla damage, each hydrilla tip was observed under a stereomicroscope with LED illumination at ×8 magnification. The hydrilla tips were examined for apical meristem damage (*C. lebetis*-specific damage) and the presence or absence of apical meristems. When hydrilla is treated with Mt, the first sign is chlorosis after a week; by the second week, leaves and stems become flaccid, lyse, and float to the surface (Shearer 1998). Hydrilla tips from Mt-treated limnocorrals were observed to have dark brown necrotic areas surrounded by chlorotic tissue. These areas of dead plant tissue were believed to be possibly due to the fungal pathogen and tips with such damage were counted.

Additionally, at 45 d the entire above sediment biomass of hydrilla from inside the limnocorral was harvested, placed in plastic bags, and brought back to the laboratory. The hydrilla was laid on a rack in a greenhouse and rinsed with well water to remove sediment, snails, and other debris. Although the vegetation in the ponds was predominantly hydrilla (approximately 95%) the ponds were naturally infested (not planted) and so other plant species were present in small amounts. Therefore, before drying, the plant material was examined to remove any other plant species as well as hydrilla roots and tubers. The plant material was then left to air dry on the racks at 21 to 38 C for approximately 4 d. The hydrilla was rotated daily to facilitate drying. When dry to touch, the hydrilla from each limnocorral was transferred into brown paper bags and then placed into a drying oven for 4 d at 70 C. Three empty paper bags as controls also were placed in the oven at the same time. After 4 d, the bags were removed from the oven and weighed immediately[8]. The average weight of the empty paper bags was subtracted from the biomass of each bag.

Experiment 2: Assess the efficacy of combining *C. lebetis* with the herbicide imazamox.

There were four treatments: Control, Ix (imazamox), midge, and Ix+midge. Each treatment was repeated three times in a single pond. The treatments were randomly assigned to the limnocorrals within a block. One rate of imazamox (low) and one density of the midge (high) were applied to limnocorrals containing established hydrilla plants. The midges were added 14 d after the imazamox treatment so that the imazamox had time to cause branching, creating new apical meristems and sites for midge invasion. The control limnocorrals received no insects or herbicide. Establishment of the midge was confirmed by microscopic examination of hydrilla shoot tips. This experiment was conducted once from 22 August to 14 October 2015. During the experiment, the average air temperature was 25.0 C (15.0–43.3 C) and the relative humidity was 91% (38–100%). The pond temperature was 26.7 C (22.2–30.0 C). and within the limnocorrals the water temperature was 27.8 C (22.2–35.6 C).

Monitoring and data collection were the same as for Experiment 1 except that the hydrilla tips were not examined for dead plant tissue because this experiment did not involve Mt.

Experiment 3: Assess the efficacy of combining *C. lebetis* with the fungus Mt and the herbicide imazamox.

There were four treatments: Control, Ix+midge, Mt+midge, and Mt+Ix+midge. Each treatment was repeated

three times, once in each of three ponds. The treatments were randomly assigned to the limnocorrals within a pond. The experiment was replicated three times, once in 2015 and twice in 2016. One rate of imazamox (low), one rate of Mt (high) and one density of the midge (high; applied twice, second time at 20 d) in combination were applied to the limnocorrals containing established hydrilla plants. All treatments were added at the same time. The control limnocorrals received no insects, fungi, or herbicide. Establishment of the midge was confirmed by microscopic examination of hydrilla shoot tips.

The experiment was completed for the first time in 29 October to 14 December 2015. Due to a clear advantage of combined over individual treatments in terms of increased hydrilla damage it was replicated two more times: 20 May to 13 July 2016, and 5 October to 18 November 2016. In the first replicate, air temperature and relative humidity data were not collected due to a technical issue with the data logger. The pond temperature was 20.6 C (12.8–26.7 C); within the limnocorrals, the water temperature was 21.1 C (13.9–28.9 C). For the second replicate, the average air temperature was 19.4 C (4.4–30.0 C) and the relative humidity was 83% (30–100%). The pond temperature was 21.1 C (12.8–27.8 C); within the limnocorrals the water temperature was 21.1 C (14.4–28.3 C). For the third replicate, the average air temperature was 25.6 C (7.2–35.0 C) and the relative humidity was 80% (29–100%). The pond temperature was 27.2 C (13.9–32.2 C), and within the limnocorrals the water temperature was 30.6 C (24.4–39.4 C).

Monitoring and data collection were the same as for Experiments 1 and 2 except for the following adaptations due to observations during the first two experiments. The hydrilla tips were not examined for dead plant tissue due to time constraints during the tip analysis process. To increase precision in the average estimates, 100 hydrilla tips (apical 12 cm of a hydrilla stem) were randomly collected per limnocorral. Due to feeding damage observed on the tips in the previous experiments, the presence of general damage also was recorded. General damage was defined as feeding on the leaves throughout the stem but not specifically on the apical meristem; due to the preference of *C. lebetis* for the apical meristem, this feeding was likely due to a different herbivore, e.g., moths or snails. It was possible for a tip to have both apical meristem damage and general damage if herbivory was present in both areas.

Additionally, for the second and third replicates of Experiment 3, the number of turions was counted. Turion count was added after the first replicate following observations that turion production might be affected.

Statistics

All statistical analyses were conducted in SAS version 9.4 (SAS Institute Inc. 2013). Damage variables (midge-specific, absence of apical meristems, general, and fungal) were percentages of the total number of tips examined. Fungal damage data were only collected in Experiment 1 and general damage data were only collected in Experiment 3. Biomass values were expressed in grams of dry weight per limnocorral. Turion production was a count of the number of turions per limnocorral per gram of dry weight biomass (turions g^{-1}) it was only measured in the second and third replicates of Experiment 3. Surface area (%) of hydrilla coverage and depth (cm) of hydrilla from the surface in each limnocorral at the end of the experiment were analyzed for Experiment 3.

In Experiment 1 the damage variables were analyzed by a general linear model procedure for normal and nonnormal data (PROC GLMMOD) based on a binomial distribution with a logit link considering the effects of pond (block) and treatment. Damage variables were not analyzed for Experiment 2 due to minimal damage. For biomass in Experiment 1 and 2 a general linear model procedure (PROC GLM) was used to fit a model with both pond/block and treatment as fixed effects.

In Experiment 3 all variables except turions g^{-1} were analyzed using a linear mixed model procedure (PROC MIXED) with the fixed effects of replicate (date), pond (block) within replicate, treatment, and its interaction with replicate; heterogeneous error variances were considered for each individual replicate. Turions g^{-1} was analyzed for each replicate individually using PROC GLM with the fixed effects of pond (block) and treatment. Least significant differences tests were used to compare means ($a = 0.05$). Means with standard errors of the mean were reported in results and figures.

RESULTS AND DISCUSSION

The goal of our study was to evaluate the effect of different combinations of the hydrilla tip miner *C. lebetis*, the herbicide imazamox, and the fungal pathogen Mt on hydrilla under field conditions. In short-term field experiments conducted in ponds naturally infested with hydrilla in Florida, we demonstrated that in general the combined treatments were superior in causing hydrilla damage compared with individual treatments. Furthermore, the three-treatment combination caused the most damage to hydrilla both in terms of short-term damage to apical meristems and long-term damage through reduced biomass and turion production.

Experiment 1: Assess the efficacy of combining *C. lebetis* with the fungal pathogen Mt.

When the efficacy of combining *C. lebetis* with the fungal pathogen Mt was evaluated, there was a significant five-fold increase in midge specific damage ($F = 35.59$, $df = 3,6$; $P = 0.003$, Figure 2A) and absence of apical meristems ($F = 32.78$, $df = 3,6$; $P = 0.004$, Figure 2B) following exposure to the combined treatment only. *Cricotopus lebetis* prefers feeding on the apical meristems of hydrilla (Cuda et al. 2002), so its presence in the combined treatments likely resulted in this consistently higher damage. There also was a significant increase in damage due to the fungus Mt when hydrilla was exposed to Mt alone (four-fold) and in combination with the midge (nine-fold; $F = 65.21$, $df = 3,6$; $P < 0.0001$, Figure 2C). In combination with the midge, the damage due to the fungus was significantly greater than when Mt was applied alone.

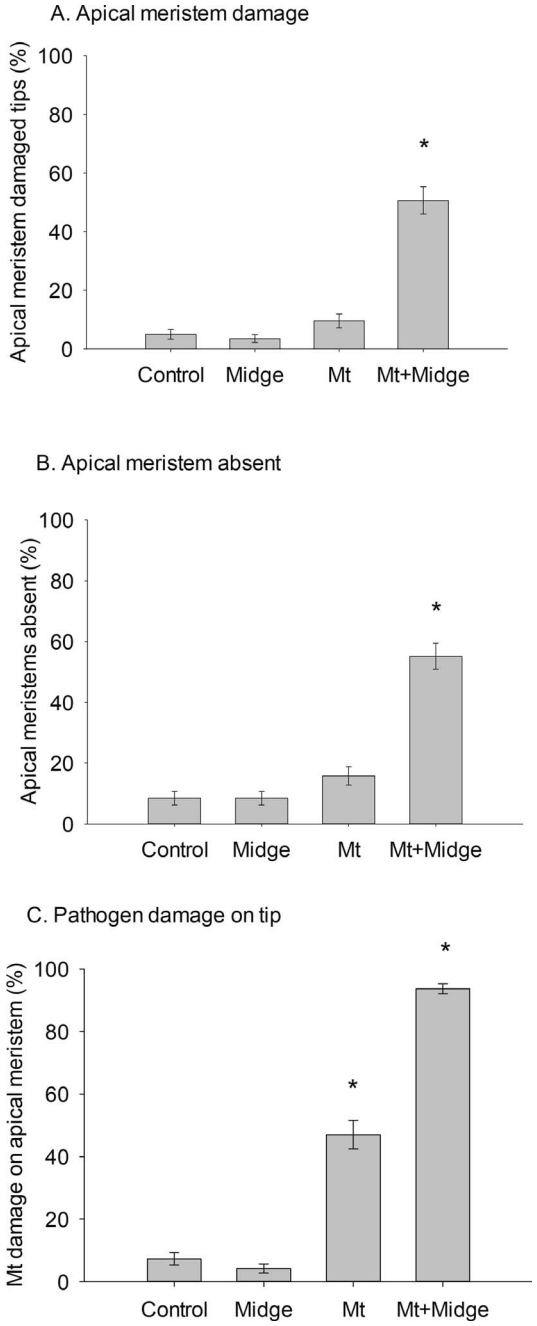

Figure 2. Effect of combining *Cricotopus lebetis* (midge) with the fungal pathogen *Mycoleptodiscus terrestris* (Mt) in Experiment 1 on the midge-specific damage to the apical meristem (A, %), on the absence of the apical meristem (B, %), on the presence of Mt damage on the tips (C, % tips with areas of dead plant tissue). Bars are means ± standard errors of the mean; asterisks denote statistically different means compared with the control.

There were no significant effects on biomass due to the biological control agents compared to the control ($F = 0.48$, $df = 3,6$, $P > 0.05$). The average biomass across all treatments at the end of the experiment was 131.0 ± 12.0 g (range 64.5–201.0 g).

The hydrilla depth from the surface was 0 cm in all but two limnocorrals at the end of the experiment. One Mt-treated limnocorral had a hydrilla depth of 2.5 cm and one Mt+midge-treated limnocorral had a depth of 5.0 cm. The surface area of the hydrilla was 100% in all but one control limnocorral in which it was 30%. These data were not subjected to statistical analysis due to the minimal differences recorded.

Experiment 2: Assess the efficacy of combining *C. lebetis* with the herbicide imazamox.

When the efficacy of combining *C. lebetis* with the herbicide imazamox was evaluated, there was minimal midge and tip damage with only three tips found with apical meristem damage and 12 tips found without an apical meristem. Therefore, these data were not subjected to statistical analysis. There were no significant effects on biomass due to the biological control agents compared to the control ($F = 0.29$, $df = 3,6$; $P > 0.05$). The average biomass across all treatments at the end of the experiment was 187.5 ± 13.0 g (range 111.0–264.5 g). This was not surprising, given the lack of midge-specific apical meristem damage recorded. Hydrilla depth from the surface was 0 cm in all limnocorrals at the end of the experiment. The surface area of the hydrilla was 100% in all but two limnocorrals in which it was 98% (midge treatment) and 99% (control treatment). These data (depth and surface area) were not subjected to statistical analysis due to the minimal differences recorded.

This minimal midge damage and consequential lack of biomass reduction was likely due to the high temperatures when the experiment was conducted. During this experiment, within the limnocorrals, the water temperature varied from 22.2–35.6 C. Stratman et al. (2014) showed that optimal temperatures for *C. lebetis* larval development were 20–30 C; at 32–35 C, there were severe impacts on survival. Therefore, it is likely that the effect of the midge in this experiment was reduced due to high temperatures and that release of midges for hydrilla management should be avoided when water temperatures are greater than 30 C. In north Florida, where this study was conducted, average temperature in a shallow lake (< 5.5 m) was lowest at 10.4 C in February and highest at 29.8 C in August (Beaver et al. 1981). In central and south Florida, average lake temperatures exceeded 30 C in the summer (Beaver et al. 1981).

Experiment 3: Assess the efficacy of combining *C. lebetis* with the fungus Mt and the herbicide imazamox.

When the efficacy of combining *C. lebetis* with the fungus Mt and the herbicide imazamox was evaluated, there was a significant five-fold increase in midge specific damage ($F = 3.66$, $df = 3, 11.3$; $P = 0.047$, Figure 3A) because of all three combined treatments compared with the control. However, general damage was only significantly increased by the three-treatment combination of midge, Mt, and imazamox (three-fold, $F = 7.7$, $df = 3, 10.8$; $P = 0.0049$, Figure 3B). There was no significant effect of treatment on the absence of apical meristems ($F = 0.58$, $df = 3, 10.3$; $P > 0.05$). The average percentage of absent apical meristems at the end of the experiment was 12.97% ± 1.46% (range 3–44%).

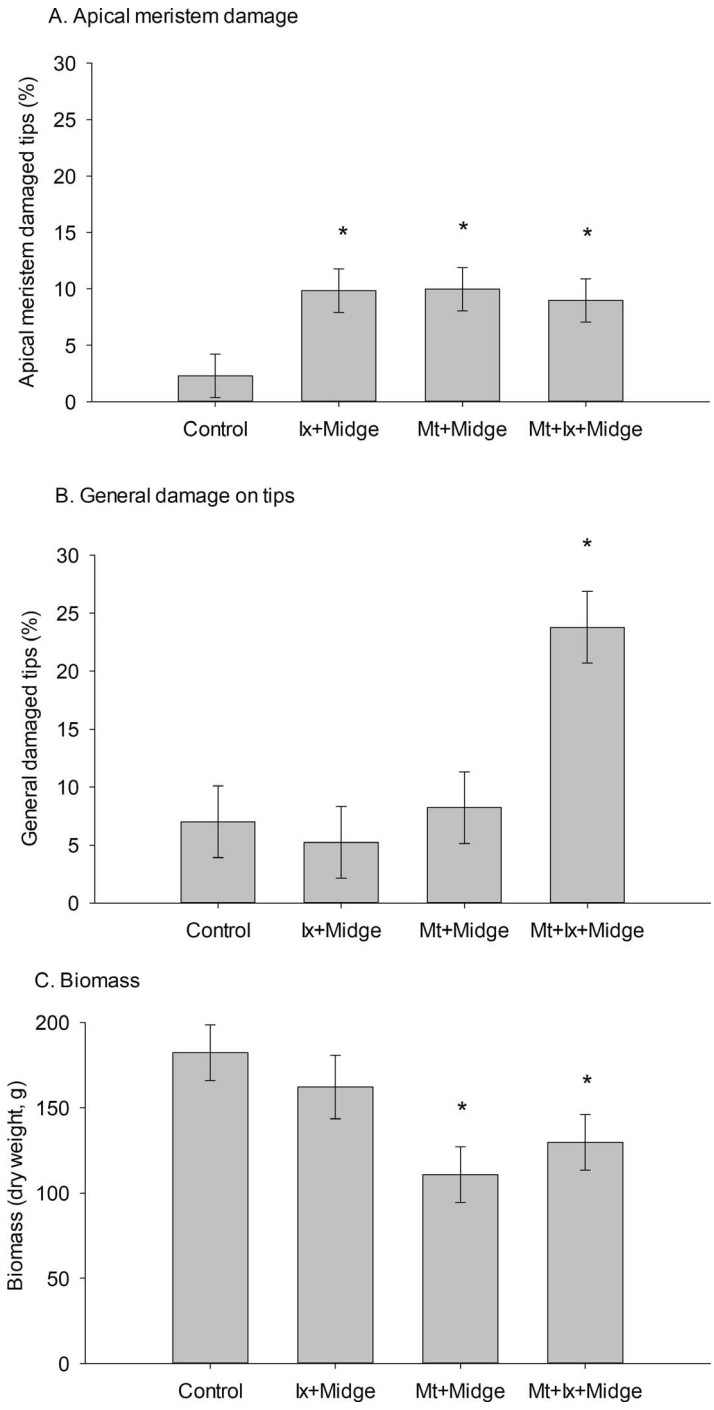

Figure 3. Effect of combining *Cricotopus lebetis* (midge) with the fungal pathogen *Mycoleptodiscus terrestris* (Mt) and the herbicide imazamox (Ix) in Experiment 3 on the presence of midge specific damage to the apical meristem (A, %), general tip damage (B, %), and biomass (C, g). Bars are means ± standard errors of the mean; asterisks denote statistically different means compared with the control.

In contrast to the earlier experiments, in Experiment 3 the three-treatment combination and the two-treatment combination of Mt+midge reduced the biomass by ~30% and ~40% compared to the control, respectively ($F = 3.8$, df = 3,13.9; $P = 0.0352$, Figure 3C). This reduction in biomass in Experiment 3 is promising and indicates that over time hydrilla management is possible with these combinations. Although the Mt+midge combination did not significantly reduce the biomass in Experiment 1, the difference between the two experiments is likely due to the second inoculation of midges that was implemented at day 20 in Experiment 3. These results are supported by those of Cuda et al. (2016), who reported a reduction in hydrilla biomass when 55 L aquaria were inoculated with corresponding numbers of midges and concentrations of Mt as used in the current study. Cuda et al. (2016) also reported a reduction in biomass due to the midge and imazamox that was not found in the current study. Unlike other herbicides, imazamox is known to stunt the upwards growth of hydrilla but stimulate branching. Although this branching is beneficial in combination with the midge, due to the provision of apical meristems for development, it might not result in as rapid a reduction in biomass as the combination of midge and Mt, or all three treatments combined. Shearer and Nelson (2009) found that although imazamox alone at 10 ppb did not decrease biomass, Mt alone and in combination with imazamox caused significant reductions in hydrilla biomass in 55-L aquaria (concentrations were the same as used in the current study). It should be noted that Shearer and Nelson (2009) reported that higher concentrations of imazamox of 25 and 50 ppb provided 58% and 86% hydrilla control, respectively, and that the label rate for hydrilla management is higher at 50 to 200 ppb depending upon the goal, i.e., growth suppression or control, respectively.

When the number of turions was counted per gram of biomass, there was a reduction in the autumn with all three combinations by more than 50%. However, the differences were only significant ($F = 16.20$, df = 3; $P = 0.0052$) with the three-treatment combination and the Ix+midge treatments compared to the control (Figure 4B). No treatments differed significantly in turion production in the spring ($F = 0.52$, df = 3; $P > 0.05$) (Figure 4A). To overwinter, hydrilla has two strategies: production of tubers under the sediment that can regrow if the plant dies, and turions that form above the sediment, which detach to germinate and grow into new plants. Turions develop from axillary buds at the axils of the leaves and branches (Yeo et al. 1984). They develop at the start of autumn, regulated by temperature and day length, and can survive the winter and enable the plant to get a head start the following spring (Adamec 2018). The Ix+midge, as well as the three-treatment combination, resulted in decreased turion production compared to the control in the replicate that occurred in October to November. No differences among the treatments were observed in the May to July replicate. Other studies with hydrilla biological control agents have reported a similar reduction in turion production associated with insect feeding (Grodowitz et al. 2007, Kariuki 2017), as well as with herbicide application (Miller et al. 1993). In a previous study, feeding by *C. lebetis* reduced turion production in hydrilla growing in pots in 200-L outdoor concrete tanks (Kariuki 2017). In north Florida, the months of March, October, and November have been shown to be those with the highest turion production (Miller et al. 1993). In our study, turion production in the control treatment was ~0.6 turions/g of dry weight biomass in the replicate conducted

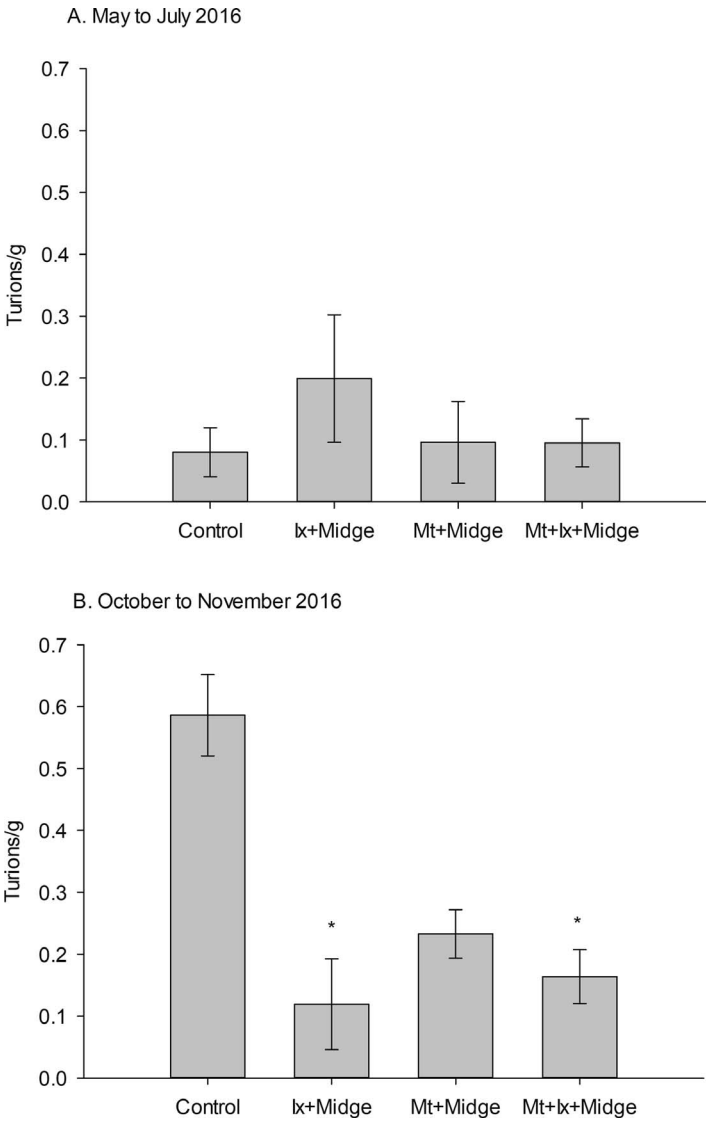

Figure 4. Effect of combining *Cricotopus lebetis* (midge) with the fungal pathogen *Mycoleptodiscus terrestris* (Mt) and the herbicide imazamox (Ix) in Experiment 3 on turion production in May to June (A) and October to November (B) of 2016. Bars are mean turion counts per gram of hydrilla biomass (turion g^{-1} dry weight) ± standard errors of the mean; asterisks denote statistically different means compared with the control.

in October to November but only ~0.1 turions/g in the replicate conducted in May to July. Therefore, reduction in turion production due to treatments applied in the appropriate season could result in effects that carry over into subsequent years, providing a longer-lasting impact on hydrilla abundance and density that has so far been difficult to achieve with other methods.

A trend towards a greater depth of the hydrilla from the surface and reduced surface area was visible in the data. For example, the average depth from the surface was greater than or equal to 2.5 cm in the Ix+midge treatment as well as in the three-treatment combination, compared to 0 cm in the control (data not shown). However, there was no significant effect of the treatment on the hydrilla depth from the surface ($F = 2.01$, $df = 2,8.15$; $P > 0.05$) or the surface area of the hydrilla ($F = 1.79$, $df = 2,12.3$; $P > 0.05$). The hydrilla depth from the surface was 1.7 cm ± 0.6 cm (range 0–12.5 cm). The surface area of the hydrilla was 91.5% ± 2.9% (range 45–100%). Possibly a longer experiment duration with additional midge generations would have enabled the differences observed to become more pronounced over time.

Study limitations

A limitation of the study was the successive changes that were made leading to differences in the protocol between experiments and replicates. For example, 50 hydrilla tips were analyzed in Experiment 1 and 2, and 100 tips were analyzed in Experiment 3. The goal of this change was to increase precision in the mean estimate. Furthermore, a second inoculation of midges was done in Experiment 3 but not in earlier experiments. This was done because high midge infestation but limited effects on biomass recorded in Experiment 1 indicated that a larger or second inoculation might cause a significant reduction in biomass. There were also changes in the variables recorded; areas of dead tissue potentially due to Mt infection were recorded only in Experiment 1, and general damage and turions per gram biomass were added in Experiment 3. These changes were made because of observations during the previous experiments to maximize the quantity and quality of the data collected. These differences likely explain the variation in apical meristem damage in the Mt+midge treatment between Experiment 1 (Figure 2A) and 3 (Figure 3A). Due to the increased sample size and precision greater emphasis should be placed on the results of Experiment 3.

A second limitation could be the use of the same ponds for the successive experiments. However, the minimum time between two experiments in the same ponds was 84 d, which is long enough for the ponds to reset to baseline conditions. Baseline assessments of the midge were completed prior to each experiment or replicate, and confirmed numbers returned to preinoculation levels. Additionally, according to its MSDS, the half-life of imazamox is 6.8 h in water and 35 to 50 d in soil. so prior treatment should not have influenced later replicates. Finally, Mt is typically found naturally in populations of hydrilla at low levels. The inundative use of Mt infects and kills the vegetative portion of the plant but does not infect or destroy the turions, thus requiring annual applications for control of the vegetation. Therefore, the experiments assume a low endemic level of Mt and the midge that would be returned to between each replicate and degradation of imazamox prior to the next treatment.

CONCLUSIONS

Our research demonstrated proof of concept that the integration of selective herbivory by the hydrilla tip miner *C. lebetis* with the native fungal pathogen Mt and the ALS-inhibiting herbicide imazamox could effectively reduce the growth of dioecious hydrilla populations in Florida. Treating hydrilla with a low concentration of imazamox stunts the growth of hydrilla and induces branching, which

leads to the formation of new shoot tips. The new shoot tips provide additional sites to increase development and reproduction of *C. lebetis*. The mining damage from *C. lebetis* also injures or kills the shoot tips, which increases susceptibility of hydrilla to infection by the fungus, Mt. Future efforts should focus on training herbicide industry representatives, aquatic plant managers, extension agents, and their clientele in how to use this system to overcome the bottleneck to adopting this hydrilla IWM strategy on a local, regional, and statewide basis (Peterson et al. 2018). A comprehensive Hydrilla IWM guide as well as additional educational and promotional materials and online and face-to-face training programs were developed specifically to address this need (Gillett-Kaufman et al. 2014, Weeks et al. 2020a,b).

SOURCES OF MATERIALS

[1]Nalgene™ high density polyethylene bottles, Thermo Scientific™, Fisher Scientific, Hampton, NH 03842.

[2]HEPA filter, Whatman™ HEPA-Vent Filter, GE Healthcare Life Sciences, Chicago, IL 60661.

[3]Clearcast®[3], SePRO Corporation, Carmel, IN 46032.

[4]Heavy-duty opaque polyethylene tube (2 m length by 1 m diam., Aquatic Research Instruments, Hope, ID 83836.

[5]HOBO® Water Temperature Pro ver. 2 Data Logger, Onset Computer Corporation, Bourne, MA 02532.

[6]HOBO® U3 Pro ver. 2 Temperature/Relative Humidity Data Logger, Onset Computer Corporation, Bourne, MA 02532.

[7]Solar Radiation Shield, Onset Computer Corporation, Bourne, MA 02532.

[8]OHAUS Ranger® 3000, Ohaus Corporation, Parsippany, NJ 07054.

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