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Note

Impact of the fungal pathogen, SPFG, on the *Salvinia molesta* Mitchell biological control agent, *Cyrtobagous salviniae* (Coleoptera: Curculionidae)

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INTRODUCTION

Giant salvinia (*Salvinia molesta* Mitchell) is a free-floating, aquatic fern native to southeastern Brazil (McFarland et al. 2004). It was first reported outside its native range in Sri Lanka in 1939 (Doeleman 1989, Room et al. 1990), and it is now known to be a serious pest in more than 20 countries. It is recognized as one of the world's worst aquatic weeds, second only to waterhyacinth [*Eichhornia crassipes* (Mart.) Solms] (Thomas and Room 1986, McFarland et al. 2004). Giant salvinia was cultivated for the nursery trade in the United States for many years with no major issues (McFarland et al. 2004). However, it was first reported outside of cultivation in 1995 in North Carolina, and although that population was quickly eliminated using chemical treatments, new outbreaks of giant salvinia were reported in Texas and Louisiana in 1998. Economically important infestations can now be found from Hawaii to Florida to Puerto Rico, with some of the most problematic occurring in Louisiana, Texas, Mississippi, and South Carolina (Jacono et al. 2001, McFarland et al. 2004, Tipping et al. 2008, Mukherjee et al. 2014). Large infestations of giant salvinia are known to affect navigation, degrade water quality, impede recreational uses, negatively affect irrigation, and allow the formation of breeding areas for vectors of human disease (McFarland et al. 2004). Several traditional control options have been employed, including drawdowns, mechanical control (i.e., harvesting or shredding), and chemical treatments. Use of the biological control agent, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae) has proven to be effective in many locations (Tipping et al. 2008, Mukherjee et al. 2014, Martin et al. 2018). Although drawdowns and chemical- and mechanical-control options are effective in some cases, overall, only limited widespread control has been observed. The use of the biological control agent *C. salviniae* has proven to be effective in many locations (Tipping et al. 2008, Mukherjee et al. 2014, Martin et al. 2018). However, *C. salviniae* has demonstrated only limited effectiveness in controlling giant salvinia in the more-northern extremes of its distribution (above 31°18′N is optimum though this varies with local weather patterns and climatic changes; Cilliers 1991, Mukherjee et al. 2014).

New herbicidal compounds and management techniques for controlling giant salvinia are limited. Although new herbicide registrations have allowed the use of different chemistries and combinations of active ingredients for giant salvinia control (Mudge et al. 2016) new and innovative management techniques are needed. One example is the use of a pathogenic fungus native to the United States in the family Botryosphaeriaceae, a teleomorph of the fungus *Botryosphaeria rhodina* (Berkeley et Curtis) von Arx (SPFG), which has shown promise in the control of giant Salvinia (Boyette et al. 2021). Although still being researched, SPFG has shown great promise with rapid kill, restricted host-range, and apparently, no or very limited production of mycotoxins, a limitation of many fungal pathogens. Because of the promise shown by SPFG, we evaluated its effect on *C. salviniae* adults using direct (contact-based mortality) and indirect (plant-quality mediated) assays. Note, that formulation and dose have yet to be fully determined, so the maximum rates, as determined by efficacy trials, were used.

MATERIALS AND METHODS

Insects

Adult *C. salviniae* were collected from rearing ponds located in St. Gabriel, LA. Weevils were live extracted from giant salvinia using Berlese funnels for 48 h. After extraction, the weevils were packaged in ice chests on a small amount of plant material and shipped overnight to the National Biological Control Laboratory (NBCL)[1] for testing.

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Giant salvinia culturing

At the NBCL, giant salvinia was cultured outdoors between June and September 2018 at ambient temperature and light in cylindrical fiberglass tanks (approximately 120 cm tall, 70 cm diameter) with deionized, reverse-osmosis, carbon-filtered water, supplemented with 16 g per tank of Excel 15–5–15 Cal-Mag special fertilizer[2] and 4 g chelated iron per tank.

SPFG preparation

The SPFG starter inoculum (15.0 g soybean flour, 3.75 g cornmeal, 30.0 g sucrose, 3.0 g calcium carbonate L^{-1} of distilled water; 500 m) was grown in 2-L flasks and incubated in rotary shakers (185–200 rpm, 28 C) for 7 days. The same medium was also adopted for scaled-up production in laboratory fermenters.[3] Fermentations were conducted at 185 to 200 rpm and 28 C for 48 h. Harvested mycelia batches were filtered[4] and oven dried (80 C, 24 h), and dry weights were recorded to determine SPFG mycelia biomass (referred to as *dry mycelium equivalents*). The dry weight (mycelia and unspent medium) of a typical fermentation batch of SPFG mycelia was 0.05 to 0.06 g ml^{-1}. For all experiments, before spray application to plants, the fermentation product was homogenized in 500-ml aliquots with an electric blender[5] (at high speed for 20 s).

Direct toxicity (contact-based mortality)

Four treatments were assessed: 1) distilled water (control), 2) an adjuvant,[6] mixed with distilled water (1 ml L^{-1}), 3) SPFG (homogenized for 20 s before weevil application), and 4) SPFG with adjuvant[6] (1 ml L^{-1} homogenized for 20 s before weevil application). Weevils were selected randomly, and depending on weevil numbers collected on any one date, 7 to 10 weevils were placed in a 100 mm by 15 mm Petri dish[7] and served as a replicate within any given block. Weevils were not sexed because of the difficulty in that determination. The experiment was set up in blocks with the date as the blocking factor. Four blocks were run, ranging in date from 8 June 2018 to 29 June 2018. The following are the number of replications used for each block:

1) Block 1, three replications
2) Block 2, five replications
3) Block 3, five replications
4) Block 4, seven replications

Using a pipette, 20 µl of the designated treatment material was placed onto the dorsal surface of each weevil. A small amount (about two plants) of giant salvinia was placed on wetted cotton balls and placed into each Petri dish, and the top of the Petri dish secured, but not sealed, with parafilm, as needed. Weevils were held in an environmental chamber[8] at 28 C with 14 : 10 h light : dark light conditions. After 5 days, weevil mortality was determined, and the percentage of survival was calculated for each Petri dish.

Indirect (plant-quality mediated) assay

To determine the effect on weevils after plant material was inoculated with SPFG, the movement of weevils from treated to untreated plant material was quantified. To accomplish that, two 992-ml plastic storage containers[9] (each 15.2 by 10.2 by 6.4 cm) were glued together (Figures 1a and 1b) and served as the testing arena. Each side of the testing arena was filled with nutrient solution as described previously to approximately two-thirds of the total volume or from 600 ml to 700 ml. Giant salvinia was placed in each side of the attached containers so the entire surface area was covered. A small amount of giant salvinia was draped between the containers to allow easy movement between sides. Either 20 (block 1) or 30 (blocks 2 through 7) randomly selected adult weevils were placed on one side of the testing arena and allowed to acclimate and move freely for 1 h. Application of each treatment was applied to the weevil-stocked side only. Liquid treatments were applied using a small hand sprayer ensuring all the plant material was completely covered. Five treatments were tested and included: 1) water (60 ml; the control), 2) dry (giant salvinia placed onto a container containing just sand, no water; the negative control), 3) an adjuvant[6] plus distilled water (1 ml L^{-1}, 60 ml), 4) SPFG (homogenized for 20 s before weevil application), and 5) SPFG with adjuvant[6] (1ml L^{-1} homogenized for 20 s before weevil application). The test arenas were held in a greenhouse at about 30 C under ambient light levels until the giant salvinia in the negative control treatment was thoroughly dried. Nutrient solution was added, as necessary, to the other treatments. The number of days the containers was held ranged from 3 to 6 d. Weevils were extracted from each side for each treatment using Berlese funnels, collecting the weevils in 70% ethanol. The percentage of movement from the treated to untreated side was determined from the number of weevils obtained through Berlese funnel extraction. The experiment was conducted over several weeks with date as the blocking factor. The experiment was conducted starting 20 June 2018 through 21 September 2018. Ten replicates were used for each date (i.e., block).

Statistical analysis

ANOVA assumption tests, including the Levene test of homogeneity of variances and the Brown-Forsythe test of homogeneity of variances, and the post hoc Newman-Keuls test were performed using Statistica software[10] version 13 (TIBCO 2017). Those tests examined differences in mean survival 5 d after treatment (direct toxicity) and the percentage of movement from treated to untreated giant salvinia (indirect toxicity). Unless noted otherwise, significant differences were determined at $P \leq 0.05$.

RESULTS AND DISCUSSION

Survival of adult *C. salviniae* was not different among water treatment alone (86.7 ± 3.4%), adjuvant/water[6] (81.4 ± 5.0%), and SPFG (79.7 ± 5.6%) treatments ($P > 0.05$). However, only 57.9 ± 5.6% of the weevils survived direct application of the combination of SPFG and adjuvant[6] and

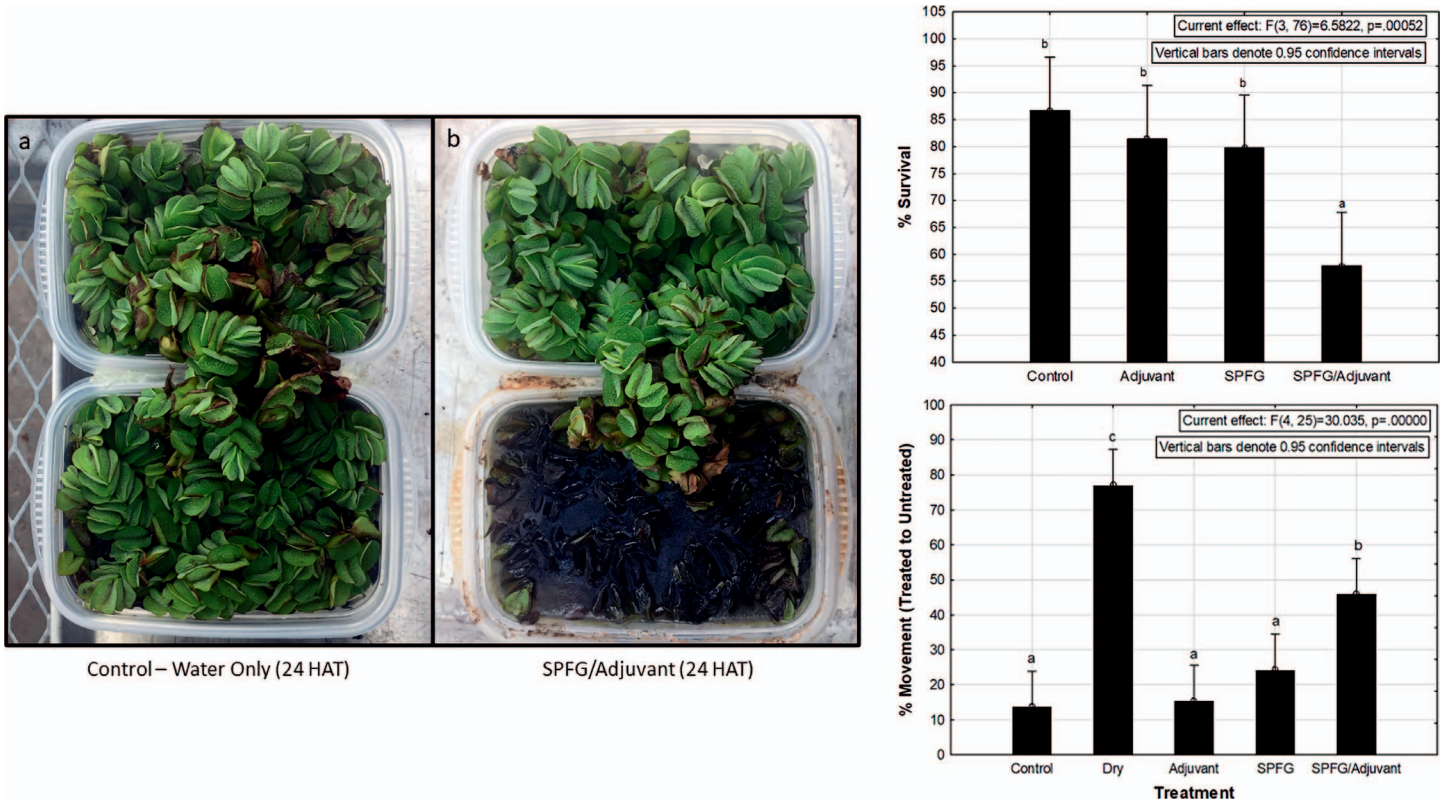

Figure 1. (a) Experimental setup for indirect (plant-quality mediated) assay (i.e., movement) 24 h after treatment (HAT) for the control or water-sprayed treatment. (b) Experimental setup for indirect (plant-quality mediated) assay (movement) 24 HAT for giant salvinia treated with a combination of SPFG and Silwet L77 adjutant. Note high degree of plant mortality only 24 HAT. (c) Percentage of survival of *C. salviniae* adults where four different treatment combinations were applied directly to the weevils. (d) Percentage of movement of *C. salviniae* from the treated giant salvinia to the untreated giant salvinia for five different treatment combinations.

was significantly different than all other treatments ($P = 0.00058$; Figure 1c) and represents an approximately 20% decrease in survival in comparison to the SPFG application. Such a large decrease, when combining both the fungus and adjuvant suggests there may be additive effects. However, although a significant decrease was detected overall, we speculate that such mortality is not overly concerning with a relatively large percentage of weevils still able to survive a direct application. Studies designed to evaluate the efficacy of different herbicide/adjuvant treatments on *C. salviniae* demonstrated limited effect when directly applied to the weevils. Mudge et al (2013) demonstrated that the greatest mortality occurred with the addition of adjuvants. In contrast, Wahl et al. (2018) only observed limited mortality with the application of 2,4-D and nonionic adjuvants. Reasons for such discrepancies are not clear, and more research is needed.

SPFG was a highly effective fungal agent against giant salvinia. Almost total kill was observed in only 24 h after application of a hyphal suspension (Figures 1a and 1b). The plants rapidly turned brown, then black, with eventual sinking of the damaged plant material. The hyphal suspension is relatively thick, and with the addition of the adjuvant,[6] it easily covers the fronds. The adjuvant[6] used is a wetting agent, reducing the surface tension of the suspension. The wetting agent is a necessity, especially for giant salvinia because the copious trichomes on the fronds limit contact of the hyphal suspension. It is essential that the suspension totally covers the plant because plant mortality does not occur unless contact is made. Although the exact action of SPFG on giant salvinia is unknown, it is believed that both a combination of hyphal penetration, phytotoxins, and enzymatic action are the cause of such rapid plant mortality. Based on unpublished data (C. Boyette), SPFG produces large amounts of laccase, an enzyme known to be involved in the delignification process (Mayer and Staples 2002) and may possibly be a main factor in the observed rapid mortality in giant salvinia. Other fungi have been shown to produce enzymes that cause rapid necrosis and, ultimately, plant death. One example is *Rhizoctonia* spp., which has been shown to produce several pectolytic enzymes that cause rapid cell degradation and necrosis (Bugbee 1990).

Because of the pathogen effects, it was necessary to determine whether *C. salviniae* adults could detect deterioration in plant quality. This was accomplished by quantifying adult weevil movement away from treated giant salvinia to fresh giant salvinia. Similar to the direct toxicity tests, only a few treatments were significantly different from each other, although differences did occur ($F_{4,25} = 30.04$, $P = 0.00001$). Only limited weevil movement was observed for plants treated with water alone (Figure 1c); i.e., there was

only 12% movement of the weevils from the water-sprayed side (i.e., the control). In contrast, the negative control (i.e., drying the plants) had almost 80% movement from plants desiccating over several days. It has been documented that aquatic or semiaquatic weevils can detect and respond quickly to desiccation of their host plant, including the waterhyacinth weevil (*Neochetina eichhorniae* Warner and *Neochetina bruchi* Hustache) (Coleoptera: Curculionidae) (Haag 1986, Grodowitz and Pellessier 1989). Based on field observations, *C. salviniae* responds similarly to desiccating plant material, rapidly moving onto nearby healthy plant material (unpub. data). This is not surprising because giant salvinia in the field will often wash up on the shore or be subjected to changes in water level causing desiccation, and the weevils need the ability to detect and be able to escape such changes. No significant differences were observed in the movement of weevils for the adjuvant-only treated plants (15% movement) as well as those plants treated with SPFG alone (24% movement). However, only limited movement was observed for those plants treated with water alone (12%). Interestingly, significant differences were observed in weevil movement for the SPFG and adjuvant[6] treatment, in comparison to the other treatments. More than 45% of the weevils in that treatment moved from the treated, to the untreated, side of the arena. Again, as with the direct-toxicity experiments, the combination of both SPFG and adjuvant[6] caused an increase in weevil movement, indicating some type of additive effect when combining the two. Interestingly, this seemingly corresponds relatively closely to the mortality of about 53% observed in direct-toxicity experiments. The desiccating plant treatment was significantly different in comparison to all other treatments. Overall, impact on the integrated use of the SPFG/adjuvant[6] and *C. salviniae* is significant, but the differences are minimal. Based on these studies, we can speculate that at least one-half of resident weevils will be conserved after application of the fungicide/adjuvant combination.

Although only adults were tested in these experiments, we expect that the larvae would respond similarly to the adults in direct-toxicity testing; i.e., limited mortality. However, we speculate that impacts to the immature forms, such as the larvae and pupae would be significantly different. Larvae and pupae of *C. salviniae* are less mobile than adults or are immobile, and as such, large-scale rapid movement away from the treated plant material is not expected, which may result in greater mortality with rapid plant death. Such was demonstrated for immature *Neochetina* spp., in the few studies conducted in Louisiana (M. Grodowitz, unpub. data) and is often mentioned in other publications (Jadhav et al. 2007). In addition, there appears to be a difference in cuticle thickness (i.e., immature forms have noticeably thinner cuticles), which may also influence direct toxicity, with increased absorbance through a thinner cuticle. More research is warranted.

Limited research has been conducted related to the effect of different control strategies on *C. salviniae*. Mudge et al. (2013) tested different herbicide and surfactants alone and in combination and showed that most herbicides had only minimal direct-toxic effect on the weevils. However, the addition of various surfactants increased mortality significantly up to 47%. This contrasts with Wahl et al (2018), where the application of 2,4-D and a nonionic adjuvant did not cause significant increases in mortality. Although only a single surfactant was assessed in these experiments, it did not cause increased mortality or movement when applied alone. When applied mixed with the pathogen SPFG, significant decreases in survival (57%) and increased movement away from the treated plant material were observed.

Similar results have been observed for other weevil species used as biological control agents. Hill et al. (2012) assessed direct mortality of *N. eichhorniae* when treated with various herbicides and surfactant combinations. Again, the addition of a surfactant in those studies increased mortality and was thought to be related to several factors, including the action of the surfactants removing the outer waxy layer of the insect's cuticle, causing water loss or a change in the surface tension of the herbicide, allowing for better and more-complete coverage. Other studies have also shown similar results with the application of herbicides and surfactants to *Neochetina* spp. (Haag 1986, Grodowitz and Pellessier 1989, Sushilkumar et al. 2008).

In summary, a series of experiments were conducted to assess the effect of the fungal pathogen SPFG on *C. salviniae*, a biological control agent for giant salvinia. SPFG was applied as a hyphal suspension and has great potential for the management of giant salvinia with almost complete control occurring in 24 to 48 h. It apparently causes only limited mortality when applied by itself to the salvinia weevil, although increased mortality occurred when used in conjunction with an adjuvant.[6] In addition, weevils reacted quickly to its application, with rapid movement away from the treated plant material. Additional research is needed that includes effects of the fungus on immature weevil stages, application of the fungus at more-realistic levels, and an understanding of the mechanisms behind the observed toxic effects of surfactants. In addition, although the present study provided some evidence for the compatibility between *C. salvinae* and SPFG, there are questions remaining regarding SPFG formulation and application rate, *C. salvinae* dispersal, and outcomes from entire waterbody-scale trials.

SOURCES OF MATERIALS

[1]Plant testing, National Biological Control Laboratory (NBCL), U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS), Stoneville, MS 38776.

[2]Excel 15–5–15 Cal-Mag special fertilizer, J.R. Peters, Inc., Allentown, PA 18106.

[3]Model MF-214 laboratory fermenter, New Brunswick Corp., Edison, NJ 08817.

[4]No. 40 Whatman filter paper, GE Healthcare Life Sciences, Chicago, IL 60661.

[5]Waring electric blender model BB155S, Conair Corporation, East Windsor, NJ 08520.

[6]Silwet® L77 adjuvant, PhytoTech Labs, Lenexa, KS 66215.

[7]Petri dish, Thermo Fisher Scientific, Waltham, MA 02451.

[8]Model I30VL environmental chamber, Percival Scientific, Perry, IA 50220.

[9]Glad plastic storage containers, Clorox, Oakland, CA 94612.

[10]Statistica software, version 13, TIBCO Software Inc., Palo Alto, CA 94304.

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