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# Naturally occurring phytopathogens enhance biological control of water hyacinth (*Eichhornia crassipes*) by *Megamelus scutellaris* (Hemiptera: Delphacidae), even in eutrophic water

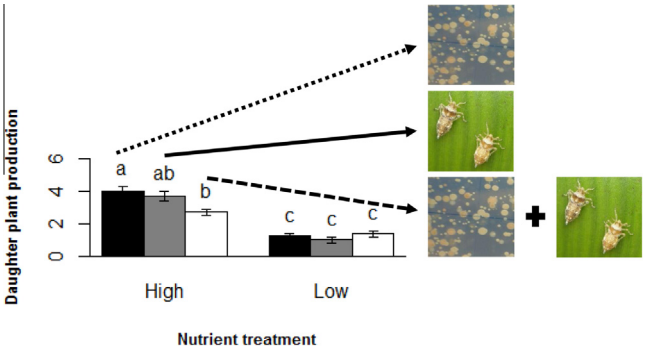
G.F. Sutton [a,*], S.G. Compton [a,b], J.A. Coetzee [c]

[a] Department of Zoology and Entomology, Rhodes University, P.O. Box 94, Grahamstown 6140, South Africa
[b] School of Biology, University of Leeds, LS2 9JT, United Kingdom
[c] Department of Botany, Rhodes University, P.O. Box 94, Grahamstown 6140, South Africa

## HIGHLIGHTS

- *Megamelus scutellaris* facilitated infection of water hyacinth by fungal pathogens.
- Synergy between phytopathogens and *M. scutellaris* reduced water hyacinth vigour.
- Synergy was observed in eutrophic waters, where the weed is most problematic.
- *Megamelus scutellaris* may complement mycoherbicides for improved weed management.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Insect biological control agents directly damage target weeds by removal of plant biomass, but herbivorous insects have both direct and indirect impacts on their host plants and can also facilitate pathogen infection. *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) was recently released into South Africa to help control invasive water hyacinth (*Eichhornia crassipes,* Pontederiaceae). We compared the impact of fungicide surface-sterilised and unsterilised *M. scutellaris* individuals and water hyacinth leaves on growth of the weed at two nutrient levels. The survival and reproduction of adult *M. scutellaris* was not reduced by sterilisation. Under high nutrient conditions, unsterilised *M. scutellaris* with unsterilised leaves reduced water hyacinth daughter plant production by 32%, length of the second petiole by 15%, chlorophyll content by 27% and wet weight biomass by 48%, while also increasing leaf chlorosis 17-fold, in relation to control plants under the same nutrient regime. Surface sterilisation of the insect and/or plant surfaces led to a general reduction in these impacts on water hyacinth growth and health. This contrast was less evident under low nutrient conditions. *Megamelus scutellaris* facilitated infection by fungal and other pathogens, thus its biology is compatible with pathogens that could be developed into mycoherbicides. This integrated approach may be ideal for management of infestations of water hyacinth in eutrophic water systems where control has been problematic, both in South Africa and elsewhere.



* Corresponding author.
*E-mail address:* guysutton41@gmail.com (G.F. Sutton).

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## 1. Introduction

Fungal pathogens are almost ubiquitous in both natural and agricultural environments (Peay et al., 2008). They can have devastating impacts on plant health (Dean et al., 2012), but more often have less obvious sub-lethal effects (Krokene et al., 2010). Some fungal infections are facilitated by insect feeding and the behaviour of phloem-feeding insects in particular aids transmission of plant diseases in general. Planthoppers (Auchenorrhyncha), such as the Delphacidae, are a prominent group of plant-feeders that are known to transmit a wide range of pathogens (viruses, mycoplasma-like organisms (MLOs), bacteria) as well as fungi (Harris and Maramorosch, 1980; Denno and Roderick, 1990). Not all plant-pathogen-vector relationships are economically harmful and the relationship between plant pathogens and their vectors can potentially be used to help control invasive plant species (Conway, 1976; Charudattan et al., 1978; Lambers et al., 2008).

Water hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laub.) (Pontederiaceae) is a free-floating aquatic macrophyte originating from the Amazon basin in South America (Bechara, 1996). It has colonised natural water courses worldwide (Gopal, 1987) and was introduced into South Africa in the early 20th century as an ornamental plant (Cilliers, 1991). Water hyacinth quickly gained the status of the country's most problematic aquatic weed (Hill and Olckers, 2001) with well documented negative socioeconomic impacts, health-related consequences and reductions in native biodiversity (Mailu, 2001; Midgley et al., 2006; Malik, 2007; Coetzee et al., 2014).

Until recently, six arthropods and one pathogen had been released as biological control agents against water hyacinth in South Africa (Coetzee et al., 2011), with notable successes attributed to two weevils, *Neochetina eichhorniae* Warner and *N. bruchi* Hustache (Coleoptera: Curculionidae) (Hill and Olckers, 2001). However, biological control programmes in South Africa and elsewhere have not achieved complete control, especially where the plant is growing in eutrophic, pollution-enriched water (Holm et al., 1977; Coetzee and Hill, 2012). Additional biological control agents have therefore been sought in an effort to achieve more widespread control over water hyacinth (Cordo, 1996; Hill and Olckers, 2001), one of which is *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) (Sosa et al., 2004). This phloem-feeding bug is native to those parts of South America where water hyacinth is present, including Argentina, Brazil, Peru and Uruguay (Sosa et al., 2007). It can reduce water hyacinth growth rates, induce significant tissue damage, and increase plant mortality rates (Tipping et al., 2011). *Megamelus scutellaris* was released first in the USA, in 2010 (Tipping and Center, 2010), and subsequently in South Africa in 2013 (J.A. Coetzee, pers. obs.).

The success of biological control agents against water hyacinth can largely be attributed to the reductions in vigour that are effected by tissue loss (Wilson et al., 2007). Herbivory by the control agents *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae) (Coetzee et al., 2005), *N. eichhorniae* and *N. bruchi* (Center et al., 2005) and *Cornops aquaticum* Bruner (Orthoptera: Acrididae) (Bownes et al., 2010) has been shown to reduce the competitive ability of water hyacinth plants. However, the effects of insect feeding cannot be attributed to herbivory alone (Ripley et al., 2008; Marlin et al., 2013). Venter et al. (2013) demonstrated that pathogens associated with *N. eichhorniae* contributed more than herbivory to a reduction of photosynthesis in water hyacinth. Pathogens are able to significantly decrease productivity and plant growth parameters, including overall fresh weight, photosynthetic rates and daughter progeny numbers (Conway, 1976; Lambers et al., 2008), and can lead to a gradual decline in water hyacinth populations (Charudattan, 1984).

The use of pathogens to control water hyacinth has received relatively little attention, both in South Africa and elsewhere (Charudattan, 2001; Ray and Hill, 2012a, 2012b), although the efficacy of fungal pathogens in controlling water hyacinth has been shown under both laboratory and field conditions (Shabana et al., 1995; Martínez Jiménez and Charudattan, 1998; Ray et al., 2008). Exposure to isolates of two species (*Alternaria eichhorniae* Nagraj and Ponappa and *Fusarium oxysporum* Schltdl) resulted in disease indices (pathogenicity) of 65% and 47% respectively, when applied as mycoherbicidal applications on water hyacinth under laboratory conditions (Ray and Hill, 2012a). Furthermore, the disease indices of these isolates were significantly increased when augmented with feeding by the weevil *N. eichhorniae*, whereby pathogenicity increased by 21.8% for *A. eichhorniae* and 45.2% for *F. oxysporum* treatments. Feeding by *Neochetina* weevils also achieves a significantly greater level of control over water hyacinth when augmented with *Cercospora piaropi* Tharp (Moran, 2005), as does the mite *Orthogalumna terebrantis* Wallwork (Acarina: Galumnidae) when present in combination with *Acremonium zonatum* (Sawada) Gams. (Sanders et al., 1982). These examples support the hypothesis that combined herbivore and fungal pathogen applications can provide greater control of water hyacinth than agents acting alone (Moran, 2005; Martínez Jiménez and Gomez Balandra, 2007).

The phloem-feeding behaviour of *M. scutellaris* suggests it may facilitate fungal disease initiation on water hyacinth (Harris and Maramorosch, 1980). The aims of this study were to determine whether *M. scutellaris* facilitates infection of water hyacinth by fungal pathogens, what the consequences of infection are for water hyacinth vigour, and whether the effects vary according to the water nutrient regime in which water hyacinth is growing.

## 2. Methods and materials

Cultures of water hyacinth and *M. scutellaris* were maintained at Rhodes University, Grahamstown, South Africa. A consignment of *E. crassipes* plants was collected from the Kubusi River (32.5926° S; 27.4218° E) near Sutterheim, South Africa and used to cultivate additional plants in 3000 L plastic pools housed in greenhouse tunnels made of clear plastic sheeting. Pools were supplied with a constant release nutrient supply (see Section 2.2) from two perforated plastic bottles suspended in the water column, which are replenished approximately every six months. *Megamelus scutellaris* (ex. Argentina via USDA, Fort Lauderdale) was obtained from a colony which was initiated in 2008 and maintained on *E. crassipes* plants.

### 2.1. Sterilisation of insects and plants

*Eichhornia crassipes* plants and *M. scutellaris* adults were surfaced sterilised to remove spores of fungal pathogens. Sterilisation of *M. scutellaris* adults was performed by applying a brief spray application of 1.5% Sporekill© (Hygrotech (Pty) Ltd, Pretoria, South Africa), a commercially available anti-fungal solution, to a 10 cm × 15 cm nylon mesh pouch containing 10 insects. *Eichhornia crassipes* leaves and stems were initially treated by rinsing the leaves and stems in tap water and then with sterile distilled water to remove unwanted particulate matter. They were then sequentially immersed for 30 s each in 70% ethyl alcohol (to improve the penetration of sodium hypochlorite), sodium hypochlorite (3.5% w/v), and finally three times in distilled water (Ray and Hill, 2012b). Control (unsterilised) plants and insects were obtained directly from the cultures.

To test the effectiveness of the sterilisation procedures, single *M. scutellaris* adults were vortexed for one minute in 1 ml of deionised water and single leaves of *E. crassipes* were vortexed in 2.5 ml of deionised water. 100 µl aliquots of each solution were then pla-

ted onto both Potato Dextrose Agar (PDA) and Rose Bengal Chloramphenicol Agar (RBCA) (Biolab©, Merck, Modderfontein, South Africa). The media were then incubated for 72 h at both 27 °C and 32 °C, and the colony forming units per ml (CFU/ml) counted. Each test of sterilisation effectiveness was replicated five times with each growth medium at each temperature, providing a total of 20 *M. scutellaris* and 20 *E. crassipes* leaf replicates. Negative controls were employed by plating an aliquot of 100 µl of deionised water.

Effectiveness of sterilisation was determined by performing a two-way analysis of variance (ANOVA) on the CFU/ml between sterilised and control insects and plants, with culture medium and incubation temperature as factors. All statistical analyses and graphing were performed in R Studio© ver. 2.15.3 (The R Foundation for Statistical Computing, 2013).

### 2.2. Herbivory and insect/plant sterilisation experiments

Herbivory and pathogen infection experiments were performed using *M. scutellaris* to determine the control agent's ability to facilitate fungal pathogen infection while feeding on water hyacinth. Additionally, bottom-up mediation was investigated by monitoring the effect of both the biological control agent and the presence/impact of any fungal pathogens on supressing growth parameters of water hyacinth plants maintained at two contrasting nutrient regimes. Healthy water hyacinth plants were obtained from the stock cultures and groups of five plants were placed into each of 18 plastic tubs (40 cm × 40 cm × 60 cm) filled with 50 L of tap water.

The tubs were divided into two nutrient treatments, eutrophic (high nutrient) and oligotrophic (low nutrient). Nutrient regimes were applied in accordance with Reddy et al. (1989), which were deemed suitable for growth of water hyacinth, and within the range of nutrients of South African waterbodies (Coetzee and Hill, 2012). The commercial controlled-release fertilizer Multicote™ 8 (15 N: 3 P: 12 K) (Haifa Chemicals Ltd., Cape Town, South Africa) was applied at 8.0 mg N $L^{-1}$ (high nutrient treatment) and 0.5 mg N $L^{-1}$ (low nutrient treatment). An initial treatment of $KNO_3$ was added to the high nutrient tubs at 40 mg N $L^{-1}$ (Saarchem, uniLAB©, Gauteng, South Africa) along with $KH_2PO_4$ at 1.55 mg P $L^{-1}$. Commercial iron chelate (13% Fe) was added to both nutrient regimes at 1.69 mg Fe $L^{-1}$ water to reduce chlorosis of the plants. The nutrient medium was replaced weekly. After three weeks any daughter plants, dead leaves and stems were removed. Wet weight biomass was measured, following a 5 min drip-drying period, using a digital bench-top kitchen scale (Clicks©, South Africa) and chlorophyll content was measured using an Apogee CCM-200 plus chlorophyll meter (ADC BioScientific Ltd., Hoddeson, United Kingdom).

The impact of *M. scutellaris* herbivory on water hyacinth and any correlation with fungal pathogen infection of its host plant was examined by placing groups of 10 brachypterous adults onto single expanded leaves with approximately 5 cm of petiole inside a fine mesh bag (mesh size: 0.5 mm × 1 mm). Leaf age was standardised by selecting leaf two (the second youngest leaf) (Center and Spencer, 1981). The combinations of sterile and unsterile treatments of both *E. crassipes* and *M. scutellaris* were employed to highlight the effect of each organism's pathogen load on *E. crassipes*. The herbivory and leaf sterilisation treatments applied were: (i) sterile insect/sterile plant (SI × SP); (ii) sterile insect/unsterile plant (SI × UP), (iii) unsterile insect/sterile plant (UI × SP) and (iv) unsterile insect/unsterile plant (UI × UP). Control leaves were enclosed in mesh bags which did not receive any *M. scutellaris* adults or sterilisation. Each plant in every tub received a single treatment, equating to nine replicates for the five treatments at both nutrient regimes.

The experiment ran for five weeks, with leaf production, daughter plant production, maximum petiole length and the length of petiole two recorded at weekly intervals. Leaf production by the plant meant that the longest and leaf two petioles measured each week were not necessarily the same as before. The chlorophyll content index was recorded at end of the experiment, rather than at weekly intervals, to minimise disruption and contamination of the leaf surfaces. At the end of the five weeks, wet weight biomass was measured as before, and the percentage of each abaxial and adaxial leaf surface displaying chlorosis was estimated through visual inspection (Coetzee et al., 2007). Insect performance indicators were recorded upon completion of the experiment by recording *M. scutellaris* adult abundance (survival) and presence of eggs and nymphs (reproductive capacity).

Two-way ANOVA followed by Tukey's HSD *post hoc* analysis examined differences in plant growth parameters across nutrient and sterility treatments at the start and end of the experiment, together with nutrient × treatment interactions. Nutrient regime, and herbivore and sterilisation treatment were fitted as factors.

### 2.3. Isolation and identification of pathogens

Upon completion of the experiment, water hyacinth leaves inoculated with *M. scutellaris* or control leaves displaying symptoms of fungal infection, such as necrotic flecks, necrotic lesions, leaf spots, zonate lesions and leaf blight, were removed and wrapped in paper towelling to absorb excess moisture (to reduce unwanted secondary microbial growth). Diseased leaf material (4 $mm^2$) was then excised from sites of fungal infection, rinsed first with tap water to remove unwanted particulate matter, and then with sterile distilled water before being immersed sequentially for 30 s in each of 70% ethyl alcohol, sodium hypochlorite (3.5% w/v) and three times in distilled, sterile water (Ray and Hill, 2012b). The sterilised leaf pieces were individually transferred onto PDA, RBCA and Malt Extract Agar (MEA) and cultured under sterile conditions at 27 ± 2 °C (mean ± S.D.).

The fungal samples isolated from diseased water hyacinth were aseptically purified by streak plating and sub-culturing protocols as outlined in Agrawal and Hasija (1986). The margins of growing colonies were isolated and serially transferred onto fresh growth media (PDA, MEA and RBCA) until a pure culture was obtained. Preparation of fungal specimens for identification was performed using a modified tape mount technique (Harris, 2000). A piece of transparent tape (4 cm × 1.5 cm) was pressed against the fungal isolate, radiating from the center to the edge of the culture. A drop of lactophenol blue was placed onto the tape, and mounted onto a microscope slide with a coverslip. The slide preparation was then examined under high power (400× magnification). A preliminary identification of fungal isolates was obtained using morphological, structural and growth characteristics and the ability of the fungi to produce pigmentation on the culture media (Gilman, 1959; Barnett, 1960; Mpofu, 1995; Shabana et al., 1995; Domsch et al., 2007).

## 3. Results

### 3.1. Sterilisation of insects and plants

The number of CFU/ml obtained from sterilised *M. scutellaris* was not significantly different when media were incubated at 27 °C and 32 °C (PDA medium: 216 ± 37.78 vs 256 ± 30.25; RBCA medium: 80 ± 9.42 vs 170 ± 40.73), on both PDA ($F_{1,28}$ = 1.18, $P$ = 0.188) and RBCA media ($F_{1,28}$ = 1.46, $P$ = 0.238). Similarly, the number of CFU/ml from sterilised *E. crassipes* leaves did not differ when media were incubated at 27 °C and 32 °C (PDA: 352 ± 28.51

vs 616 ± 36.49; RBCA: 152 ± 25.55 vs 308 ± 53.34), on both PDA ($F_{1,28}$ = 2.57, $P$ = 0.120) and RBCA media ($F_{1,28}$ = 2.05, $P$ = 0.162). Temperature treatments were therefore pooled in subsequent analyses.

Sterilisation of *M. scutellaris* adults was partially effective, resulting in a significant reduction in the number of CFU/ml cultured on both PDA (sterile: 236 ± 16.27 vs unsterile: 616 ± 30.47) ($F_{2,27}$ = 23.36, $P$ < 0.001) and RBCA media (sterile: 116 ± 12.29 vs unsterile: 224 ± 16.67) ($F_{2,27}$ = 7.73, $P$ = 0.002). Sterilisation of *E. crassipes* leaves was also partially effective, with a significant reduction in the number of CFU/ml on both PDA (sterile: 484 ± 20.78 vs unsterile: 2212 ± 89.68) ($F_{2,27}$ = 47.16, $P$ < 0.001) and RBCA media (sterile: 230 ± 31.26 vs unsterile: 1312 ± 65.21) ($F_{2,27}$ = 39.92, $P$ < 0.001).

### 3.2. Nutrient regime effects on plant growth

Water hyacinth growth responded more to water nutrient regime than to herbivory and leaf sterilisation treatments (Table 1) as indicated by consistently more growth observed in control treatments under high nutrient conditions than in low nutrient conditions (Fig. 1). Leaf production increased 1.8-fold (Fig. 1a), daughter plant production 3.2-fold (Fig. 1b), longest petiole length 1.2-fold (Fig. 1c), second petiole length by 1.3-fold (Fig. 1d), chlorophyll content index 1.8-fold (Fig. 1e), and wet weight biomass 1.5-fold (Fig. 1f).

### 3.3. Herbivory and fungal pathogen effects on plant growth

Herbivore and leaf sterilisation treatments had no appreciable impact on mean leaf production (Fig. 1a) and longest petiole length (Fig. 1c) after five weeks. However, these treatments resulted in significant differences in daughter plant production (Fig. 1b), second petiole lengths (Fig. 1d), relative chlorophyll content (Fig. 1e) and wet weight biomass (Fig. 1f). In combination with *M. scutellaris* herbivory, the unsterilised leaf treatment resulted in a greater reduction in water hyacinth vigour than the sterilised leaf treatments. Significant interactions between nutrient regime and herbivore and leaf sterilisation treatments were observed for mean daughter plant production and wet weight biomass (Table 1). These interactions indicate greater absolute reductions of both plant parameters when plants were cultivated in eutrophic, rather than oligotrophic water nutrient conditions, although the proportional reduction of plant parameters was comparable between nutrient regimes.

**Table 1**
Differences in water hyacinth growth parameters across *Megamelus scutellaris* herbivory and insect/leaf sterilisation treatments at high (n = 7) and low nutrient (n = 8) regimes upon completion of the five week experiment. *F*-statistics were obtained from univariate tests of significance. Significant effects on plant parameters due to nutrient, treatment and nutrient × treatment interactions are highlighted in bold.

| | $F$[a] | $P$ |
|---|---|---|
| Leaf production | | |
| Nutrient | 238.29 | **<0.001** |
| Treatment[b] | 2.15 | 0.147 |
| Nutrient × Treatment | 0.06 | 0.810 |
| Daughter plant production | | |
| Nutrient | 242.67 | **<0.001** |
| Treatment | 10.56 | **0.018** |
| Nutrient × Treatment | 3.30 | **0.016** |
| Longest petiole length | | |
| Nutrient | 219.32 | **<0.001** |
| Treatment | 2.18 | 0.145 |
| Nutrient × Treatment | 1.63 | 0.205 |
| Second petiole length | | |
| Nutrient | 171.29 | **<0.001** |
| Treatment | 28.24 | **<0.001** |
| Nutrient × Treatment | 3.18 | 0.079 |
| Chlorophyll content index | | |
| Nutrient | 26.52 | **<0.001** |
| Treatment | 24.39 | **<0.001** |
| Nutrient × Treatment | 2.682 | 0.107 |
| Wet weight difference | | |
| Nutrient | 164.28 | **<0.001** |
| Treatment | 36.92 | **<0.001** |
| Nutrient × Treatment | 11.69 | **0.001** |

[a] Degrees of freedom and sample sizes were (1,65) for nutrient regime, (4,65) for treatments and (4,65) for nutrient × treatment interactions.
[b] Herbivore and leaf sterilisation treatments (see text for details).

### 3.4. Herbivore and pathogen performance

The number of *M. scutellaris* adults recovered at the end of the experiment was greater in the high nutrient treatment (93%) than the low nutrient treatment (83%) ($F_{1,52}$ = 9.86, $P$ = 0.003) (Fig. 2a), but there were no herbivore and leaf sterilisation treatment effects on adult insect survival across treatments ($F_{3,52}$ = 0.41, $P$ = 0.744) (Fig. 2a). *Megamelus scutellaris* reproductive output was also greater on plants growing under high nutrient conditions, with 6 out of the 7 tubs (86%) containing nymphs, whereas no nymphs were present on any of the plants grown under low nutrient conditions. These findings suggest that the presence of fungal pathogens did not impact *M. scutellaris* reproduction and survival. The highest extent of leaf chlorosis was observed under high nutrient treatments (15%, compared with 8%), when both plants and insects were unsterilised (Fig. 2b), with a significant interaction between nutrient regime and treatment observed ($F_{4,65}$ = 6.50, $P$ = 0.013).

### 3.5. Fungal pathogens isolated from water hyacinth

*Eichhornia crassipes* leaves displayed various disease symptoms at the end of the five week experiment. These were cultured to obtain a baseline estimate of fungal pathogen community structure in the presence of *M. scutellaris*. A total of 35 isolates were cultured from the leaves, of which 17 could not be identified further because of contamination, failure to grow, sterility or a lack of useful morphological characteristics (Table 2).

The most frequently isolated genus was *Alternaria* Nees, with three species obtained from eight isolates. *Alternaria eichhorniae* Nag Raj & Ponappa was the most abundant species within this genus, with five isolates, followed by *A. tenuissima* (Nees ex Fr.) Wiltshire with two isolates and lastly *A. alternata* with a single isolate (Fr.) Keissler. The remaining isolates comprised *Fusarium moniliforme* Sheldon with three isolates, *Cladosporium* sp. with two isolates and single isolates from the genera *Acremonium* (Link ex. Fr) and *Ulocladium* Preuss.

## 4. Discussion

One reason that water hyacinth is so invasive is that it directs the majority of its resources into growth and maintenance of photosynthetic tissues rather than sexual reproduction (Coetzee and Hill, 2012), which allows the plant to respond rapidly to changes in nutrient regimes (Coetzee et al., 2007). Our study demonstrated that water hyacinth growth was significantly impacted by water nutrient status, which is in accordance with a large body of literature (Gossett and Norris, 1971; Reddy et al., 1989; Coetzee et al., 2007; Marlin et al., 2013). Under low nutrient conditions, our experimental plants were less healthy and productive than those grown under high nutrient conditions, which corroborates the findings of Coetzee et al. (2007), who showed that plants cultivated

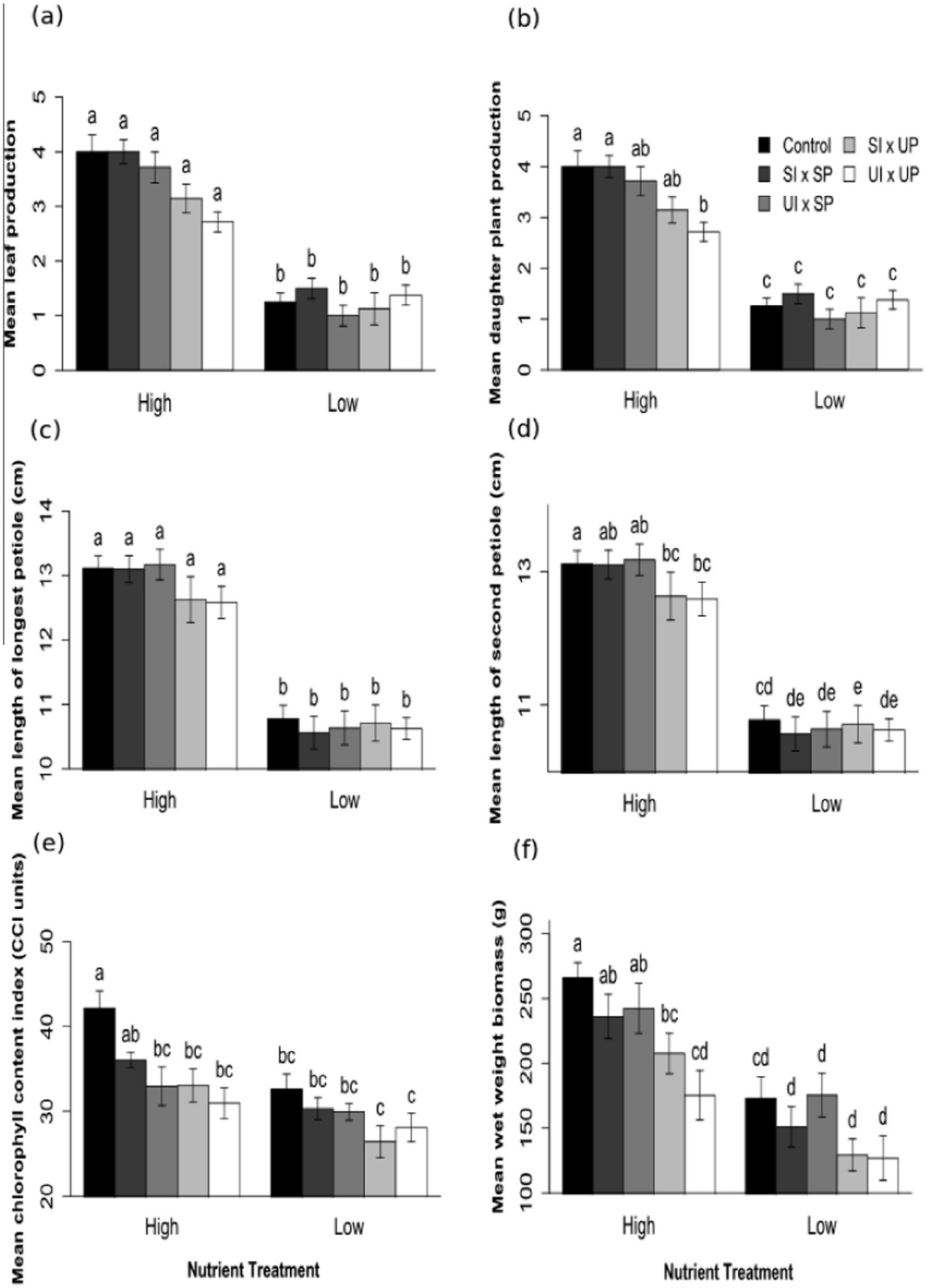


**Fig. 1.** Differences in water hyacinth growth parameters in relation to *Megamelus scutellaris* herbivory and insect/leaf sterilisation treatments upon completion of the five week experiment under high (n = 7) and low nutrient (n = 8) regimes for: (a) leaf production, (b) daughter plant production, (c) maximum petiole length, (d) second petiole length, (e) chlorophyll content index and (f) wet weight biomass. Treatments applied were: sterile insect/sterile plant (SI × SP); sterile insect/unsterile plant (SI × UP), unsterile insect/sterile plant (UI × SP), unsterile insect/unsterile plant (UI × UP) and control (which did not receive any *M. scutellaris* adults or sterilisation). Error bars indicate standard errors of the mean, those followed by the same letter are not significantly different from one another (Tukey's HSD, $P > 0.05$).

under low nutrient conditions displayed a short-petioled, bulbous growth form.

Herbivory by *M. scutellaris* did not have as appreciable an effect on water hyacinth growth as water nutrient status. Our results indicate that leaf chlorosis was the sole parameter that was significantly influenced by *M. scutellaris* herbivory alone (treatment: SI × SP), although reductions in several plant growth parameters were observed across the remaining treatments. This implies that although herbivory alone can impact water hyacinth productivity, our findings highlight the role of other less conspicuous factors required for a deleterious impact on water hyacinth vigour.

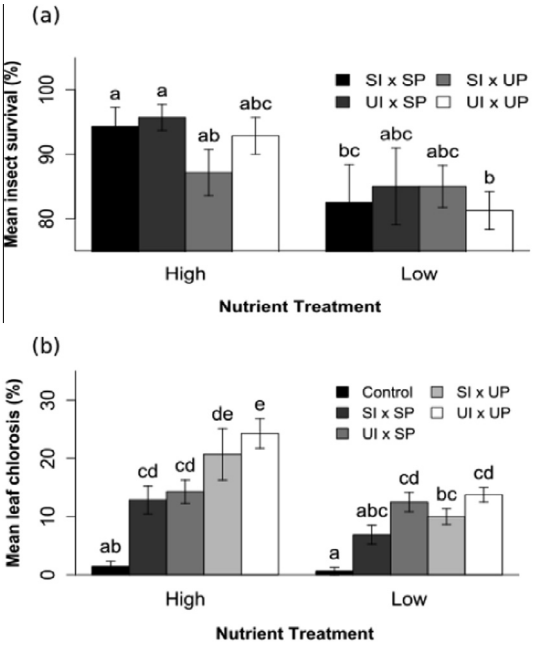


**Fig. 2.** Differences in herbivore and fungal pathogen performance across treatments upon completion of the five week experiment under high (n = 7) and low nutrient (n = 8) regimes for: (a) *Megamelus scutellaris* adult survival percentages and (b) combined herbivore/fungal pathogen inductions of leaf chlorosis. For the figure legend refer to Fig. 1. Error bars indicate standard errors of the mean, those followed by the same letter are not significantly different from one another (Tukey's HSD, $P > 0.05$).

**Table 2**
Fungal isolates identified morphologically from diseased water hyacinth plant tissues exposed to various sterilisation treatments of *Megamelus scutellaris* and water hyacinth.

| Fungal species | Frequency of isolation (%)[a] | Water hyacinth association (Country of report) |
|---|---|---|
| *Acremonium* sp. | 2.86 | Charudattan et al. (1978) (USA); Ray and Hill (2012b) (South Africa) |
| *Alternaria alternata* | 2.86 | Shabana et al. (1995) (Egypt); Ray and Hill (2012b) (South Africa) |
| *eichhorniae* | 14.28 | Shabana et al. (1995) (Egypt) |
| *tenuissima* | 5.71 | Barreto and Evans (1996) (Hong Kong); Ray and Hill (2012b) (South Africa) |
| *Cladosporium* sp. | 5.71 | Barreto and Evans (1996) (Hong Kong); Ray and Hill (2012b) (South Africa) |
| *Fusarium moniliforme* | 8.57 | Mpofu (1995) (Zimbabwe); Ray and Hill (2012b) (South Africa) |
| *Ulocladium* sp. | 2.85 | El-Morsy (2004) (Egypt); Ray and Hill (2012b) (South Africa) |
| Sterile/contaminated/ no ID | 57.14 | |

[a] Frequency of isolation = (number of isolates per species/total number of isolates (n = 35)) × 100.

Fungal pathogens have been implicated as a factor that can contribute to a reduction in water hyacinth growth and proliferation (Charudattan et al., 1978; Moran, 2005). The cumulative effect of herbivory, fungal pathogens associated with *M. scutellaris* and fungal pathogens harboured on water hyacinth (treatment: UI × UP) was required to reduce daughter plant production and length of the second petiole, while reductions in relative chlorophyll content, leaf chlorosis, length of the longest petiole and wet weight biomass required the combined impact of herbivory and fungal pathogens associated with either *M. scutellaris* or water hyacinth, respectively. This highlights the deleterious impact of fungal pathogens associated with water hyacinth leaves and pathogens associated with the herbivore *M. scutellaris*. Our results are in accordance with Venter et al. (2013) who demonstrated that pathogens associated with the weevil *N. eichhorniae* effected a significant reduction in water hyacinth leaf photosynthetic rate, and Ray and Hill (2015) who showed that the mirid *E. catarinensis* facilitated disease initiation of *A. zonatum* on water hyacinth, but we explicitly highlight the deleterious impact of fungal pathogens associated with water hyacinth, when in the presence of the herbivore *M. scutellaris*.

Avocanh et al. (2003) examined the efficacy of applications of the fungal pathogen *A. eichhorniae* on water hyacinth, and found that disease incidence and severity were significantly lower on plants growing under high nutrient conditions. This led Muniappan et al. (2009) to argue that mycoherbicidal applications are likely to be more effective against water hyacinth in low nutrient systems. Our results suggest that this is not necessarily the case when fungal pathogens are present in combination with insects that are feeding on the plants. When *M. scutellaris* was inoculated onto unsterilised water hyacinth leaf material there was a greater absolute reduction in mean daughter plant production and wet weight biomass at high nutrient conditions, although the size of the effect was similar between nutrient levels. Phloem-feeding insects such as *M. scutellaris* are likely to be particularly effective at facilitating fungal phytopathogen infection because their stylets pierce the epidermis, creating feeding scars that can act as entry sites for opportunistic pathogens (Galbraith, 1987). Pathogens associated with chewing insects such as the weevil *N. eichhorniae* can nonetheless significantly reduce rates of photosynthesis in water hyacinth leaves (Venter et al., 2013). Moran (2005) similarly showed that augmentation of the weevils *N. eichhorniae* and *N. bruchi* with the fungus *Cercospora piaropi* resulted in greater reductions in water hyacinth leaf production and plant densities in relation to control plots. Mode of feeding therefore does not appear to limit the insects that can facilitate the spread of pathogens.

A multi-faceted, integrated approach has been proposed as the most effective management strategy for controlling aquatic weeds (Pieterse, 1977; Charudattan, 2001) and the synergy between insect herbivores and plant pathogens has been highlighted as a potential management tool (Charudattan et al., 1978). Our results suggest that fungal pathogens may indeed contribute to reductions in water hyacinth growth and proliferation (Charudattan et al., 1978; Moran, 2005; Venter et al., 2013; Ray and Hill, 2015). Surface sterilisation of *M. scutellaris* and the leaves of water hyacinth neither increased nor decreased the insect's growth, survival and reproduction. This suggests that *M. scutellaris* has a casual, rather than a mutualistic, relationship with the fungi that it was transmitting, and that plant nutrient status, rather than plant disease is the major determinant of host plant suitability.

It can be concluded that *M. scutellaris* herbivory facilitates fungal pathogen infection. Unlike either herbivory or fungal phytopathogens alone, this has a deleterious impact on water hyacinth fitness. Mycoherbicidal applications on water hyacinth appear unlikely to impact negatively on *M. scutellaris*, although this needs to be tested explicitly, preferably with whole-plant and field trials. Mycoherbicide effects on other water hyacinth-feeding insects also need to be considered, but our results suggest that an integrated approach utilising *M. scutellaris* and mycoherbicide formulations may represent an effective control strategy against

water hyacinth when it is growing in eutrophic waters, where this weed is currently most problematic (Coetzee and Hill, 2012).

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