Control of Water Hyacinth with *Neochetina* spp. and *Alternaria alternata* in the Rift Valley of Ethiopia

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Abstract

Water hyacinth [*Eichhornia crassipes* (Mart.) Solms] remains one of the worst aquatic weeds worldwide. Its presence in Ethiopia was officially reported in Koka Lake and Awash River about 60 years ago. A study was conducted for two years to evaluate the integrated use of host-specific herbivorous weevil species *Neochetina eichhorniae* and *N. bruchi*, and an indigenous fungal plant pathogen (*Alternaria alternata*) for controlling water hyacinth in the lathhouse in the Rift Valley of Ethiopia. Water hyacinth plants that grown in caged tanks were exposed to one of eight treatments: control; only either of the weevil species (*N. eichhorniae* or *N. bruchi* alone); the two weevil species (*N. eichhorniae* + *N. bruchi*); the fungal spray (foliar application of *A. Alternate*); combination of the weevils and fungal spray (*N. bruchi*, *N. eichhorniae* or *N. bruchi* and *N. eichhorniae* + foliar fungal application). Water hyacinth plants that received the two weevils combined with *A. alternata* showed a disease index (DI) of 90% compared with DI values of 70% and 60% recorded in *N. bruchi* combined with *A. alternata* and *N. eichhorniae* with *A. alternata*, respectively. Application of both weevils combined with *A. alternate* showed about 97% and 85% reduction in number of new ramets and fresh weight, respectively. Thus, it is concluded that application of the three agents together had an overall synergistic effect in controlling water hyacinth.

Keywords: *Alternaria alternata*, aquatic weeds, biological control, *Eichhornia crassipes*, *Neochetina bruchi*, *Neochetina eichhorniae*, water hyacinth

Introduction

Water hyacinth, *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae), is renowned as the world's most noxious aquatic weed that causes environmental, economic, and social difficulty in the tropics and the sub-tropics (Holm et al., 1977). In the Rift Valley of Ethiopia, this floating plant forms impenetrable mats

across waterways like irrigation canals and drainage structures, and on stagnant water bodies such as lakes, dams and reservoirs,. These mats result in blockage of irrigation canals, and meddling with hydro-electric power generation, sugarcane and vegetable production (Firehun et al., 2013). However, management of water hyacinths by manual, mechanical, and chemical methods is costly and unending (Center et al., 1999a).

The host-specific weevils *Neochetina eichhorniae* Warner and *N. bruchi* Hustache have been used already for a long time as biological agents for control of water hyacinth in different parts of the world (Harley, 1990). In 2011, efforts to control water hyacinth using weevils were made in Ethiopia. The *N. eichhorniae* and *N. bruchi* were introduced to the country from the Biological Control Unit, Namuloge Agricultural and Animal Production Research Institute, based in the Republic of Uganda. Adaptability, host-specificity to ecological as well as economic plant species and pre-release impact assessment studies confirmed their suitability for release in the Rift Valley of Ethiopia (Firehun et al., 2015, 2016).

Insects alone have generally not caused the necessary damage level (Perkins, 1978; Center et al., 1982; Martinez et al., 2001). However, it is known that their effects are heightened when they are applied in combination with plant pathogens (Martinez and Gomez, 2007). Several fungal pathogens have been reported to attack water hyacinth in various parts of the world. Various strains in the genera, *Acremonium*, *Alternaria*, *Cercospora*, and *Myrothecium* have been studied intensively as biocontrol agents and shown to be effective under experimental conditions (Shabana et al., 1995a, b, 1997, 2000; Charudattan, 2001b; Martinez and Gutierrez, 2001; Mohan et al., 2003; Praveena and Naseema, 2004).

A survey of fungal pathogens was made in the Rift Valley of Ethiopia (Firehun et al., 2017) with the aim of identifying at least one indigenous fungus with prospect for development as a mycoherbicide to boost the effects of the insect biological control agents. *Alternaria alternata* (Fr.) Keissler has been selected as one of the fungi with the largest potential. Moreover, the *Alternaria alternata* has been described as a pathogen of water hyacinth in Australia (Galbraith, 1987), Egypt (El-Morsy, 2004; Elwakil et al., 1990; Shabana et al., 1995b), Bangladesh (Bardur-ud-Din, 1978) and India (Aneja and Singh, 1989). Mohan et al. (2003) highlighted the potential to use the *A. alternata* as biological control agent of water hyacinth without negative effects on plants of economic and ecological importance.

Many biological control projects involve the release of multiple agents exerting cumulative impacts (Syrett et al., 2000; Denoth et al., 2002). Associations among weed biological control agents may arise directly or indirectly. The direct association arises, if influx by one agent directly alters the ability of others to pervade the target (Caesar, 2003). The indirect association arises, if attack alters target plant quality, indirectly influencing the feeding, survival and/or reproduction of other agent(s) (Milbrath and Nechols, 2004). Positive interactions between insect herbivores and plant pathogenic

fungi are potentially useful in biological water hyacinth control.

The few attempts made so far to utilize this potential for the management of water hyacinth demonstrated the feasibility and commercial potential of augmenting weevils with pathogens (Moran and Graham, 2005; Martinez and Gomez, 2007). The weevils' feeding wounds facilitate entry of fungal pathogens, and weevils can also deliver fungal inoculums directly onto cuticular surfaces. Therefore, the current study was initiated to evaluate the integrated use of *N. eichhorniae* and *N. bruchi*, the host-specific herbivorous weevil species, and an indigenous plant pathogen (*A. alternata*) for controlling water hyacinth in the Rift Valley of Ethiopia.

Materials and Methods

Plants, insects and pathogen

Water hyacinth plants were grown in untreated irrigation water supplemented with 2.5 ppm nitrogen and potassium, 9.5 ppm phosphorous, and 2 ppm iron. Blue dye (0.01% v/v) was added to the growth medium to inhibit algal growth. The plants were acclimatized to the growing condition for a month.

Water hyacinth weevils were collected (from August 2012 to 2014) from a mass rearing site at Wonji Research Station, located in the Rift Valley of Ethiopia (8° 31' N; 39° 20' E; 1540 m a.s.l.). Water hyacinth weevils were collected from infested water hyacinth plants. An insect colony was established with 200 weevils (1:1 ratio of male to female), which were placed into a 60 l tented tank containing water hyacinth shoots. Weevils needed for the experimental studies were obtained from this colony.

Indigenous strain of the fungal pathogen *A. alternata* (Wonji-WH-4) was isolated(in May 2011) from surface-sterilized leaf disks (0.5 cm) cut from experimentally infected leaves collected at Wonji Research Station sites. Disks and colony transfers were cultured on solid potato dextrose agar (39 g l^{-1}) containing 5 g l^{-1} yeast extract (Difco, Detroit, Michigan). Two-week-old sporulating cultures were used for inoculations. Spores were harvested by flooding the plates with distilled water and lightly scraping the surface. The resulting spores were suspended in a formulation, and the concentration was adjusted to 1×10^6 spores ml^{-1}. The formulations consisted of 3 ml corn oil, 15 ml of an emulsifier (Tween 80) and 500 ml water.

Treatments and experimental design

The experiment was performed five times in a randomized complete block design with three replications. The water hyacinth plants were maintained in 30 L tanks in the lathhouse (n = 8 plants per treatment) for about two months during each experimental period (i.e., August 2012 to 2014). The weevils were sorted by both sex and species following the procedure developed by CSIRO scientists in Australia (Julien et al., 1999), and subsequently released onto plants at a density of one weevil per plant.

The experiment consisted of the following eight treatments, each containing eight plants: control (no weevils, no foliar fungal application); only one weevil species (*N. eichhorniae* or *N. bruchi*, no *A. alternata*); only the

two weevil species (*N. eichhorniae* + *N. bruchi*, no *A. alternata*); only fungal spray (foliar application of *A. alternate*, no weevils); and combination of weevils and fungal spray (*N. bruchi* + foliar fungal application, *N. eichhorniae* + foliar fungal application or *N. bruchi* + *N. eichhorniae* + foliar fungal application).

Application of agents

Forty-eight weevils (1 male: 1 female) per replication were added onto plants selected for exposure to the weevils alone and to both the weevils and the *A. alternata* fungus. In order to prevent the weevils ovipositing on the non-treated plants, all treatments were placed in separate cages. The cages were covered with fine white netting.

Two weeks after the release of the weevil, a suspension of spores having a formulation of about 1×10^6 spores ml^{-1} was sprayed until run-off by using a hand-held airbrush sprayer onto the foliage of plants selected for exposure to either the *A. alternata* fungus alone or to both the *A. alternata* fungus and the weevils. The control plants were sprayed with sterile distilled water containing Tween 80. Each plant was then covered overnight with a moistened clear plastic bag to provide optimal conditions for fungal infection in the treatments with application of *A. alternata*.

To restrict the spread of the pathogen among the treatments, plants in the non-pathogen treatments were sprayed with the broad-spectrum fungicide TILT® at a rate of 5 ml each time. Plants that were artificially inoculated with the pathogens were sprayed with the same volume of water.

Data collection and analysis

Disease intensity and severity were rated by visual observations during a total period of 30 days. Disease intensity was evaluated visually on the basis of initiation of disease and increase in disease area every day after application of the inocula, using a score chart framed by Freeman and Charudattan (1984) that rated disease intensity as excellent (+++), good (++), poor (+), and no infection (−) after 5, 10, 15, 20, 25 and 30 days. Disease was scored using a 0 to 5 scale rating system where 0 = no symptoms; 1 = 1-10%; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%, and 5 = ≥75% area covered by spots on leaves, until 30 days after fungal inoculation.

All the ratings from each experiment were then averaged and a disease index (DI) was calculated according to Chaube and Singh (1991):

$$\text{Disease Index (DI)} = \frac{Sum\ of\ all\ ratings * 100}{Total\ number\ of\ leaves\ measured * Maximum\ disease\ index}$$

where, the sum of all numerical ratings is (0 × N0) + (1 × N1) + (2 × N2) + (3 × N3) + (4 × N4) + (5 × N5); with N0, number of leaves with score 0; N1, number of leaves with score 1; and . . . . N5, number of leaves with score 5.

Leaf scarring by the weevils was monitored weekly. Two month after the start of the treatments (i.e., 6 weeks after inoculation), total numbers of shoots

(rosettes) and flowers were counted in each tank, and leaf counts, petiole length, root and shoot fresh weight as well as asexual plant production via axillary buds were assessed on a subset of five plants per tank.

The effects of treatments were analysed using Analysis of Variance (ANOVA) (SAS. 2008). The percentage data recorded for evaluating disease index of different fungi were transformed first with arcsine transformation prior to being compared using one-way analysis of variance. The treatment means were compared with Fisher's honest least significant difference (LSD) at 5% level of significance.

Results and discussion

Effect on disease development and feeding scars

Water hyacinth leaves in four treatments inoculated with either only *A. alternata* or *A. alternata* in combination with the individual or both weevils developed disease symptoms within 4-7 days after inoculation. The disease development results indicated that there was a significant difference ($P < 0.05$) among the four treatments with fungus application when introduced alone or in combination with weevils.

Among the treatments, 10 days after inoculation, the maximum disease rating(DI=29%, $P < 0.05$) was recorded in water hyacinth plants that received the *A. alternata* combined with two weevil species (NB+NE) followed by water hyacinth plants that received the *A. alternata* with *N. bruchi* (DI=22%) and *A. alternata* with *N. eichhorniae* (DI=17%). As the number of days after inoculation increased, the disease score also increased (Figure 1).

Twenty days after inoculation, the combined application of the *A. alternata* augmented with two weevil species showed a DI of 70% ($P < 0.001$), which was much higher than that of the treatment with only *A. alternata* (DI=19%). Thirty days after inoculation, water hyacinth plants that received *A. alternata* combined with the two weevils showed a DI of 90% whereas DI levels of 70% and 60% were recorded in *A. alternata* combined with *N. bruchi* and *N. eichhorniae*, respectively.

These findings confirmed that *A. alternata* can heavily infect water hyacinth (El-Morsy, 2004; Ray, 2006). However, the spread of *A. alternata* on more mature plants was slower and limited to the lower leaves and the stem. Similarly, Charudattan (2005) reported that on plants that have the ability to regenerate quickly, spread of pathogens was limited to lower leaves.

Analysis of results on weevil feeding scars indicated that there was a significant difference ($P < 0.05$) among the weevil treatments when introduced alone or combined. Adult feeding by both species removed large areas of the laminal cuticle. Among the weevil treatments, the maximum number of feeding scars per plant was recorded in water hyacinth plants that received the combination of the two weevils (220 ± 14, $P < 0.05$) followed by water hyacinth plant that received *N. bruchi* (190 ± 10) and then *N. eichhorniae* (140 ± 15) alone. Average plant disease damage levels

were significantly higher in plants that received application of *A. alternata* augmented with the two weevil species (mean ± SE; 0.89 ± 0.009). Plants where *A. alternata* was augmented with *N. bruchi* (0.75 ± 0.01) and *N. eichhorniae* (0.71 ± 0.01) exhibited higher disease damage than plants that received only *A. alternata* (0.65 ± 0.01).

Twenty days after weevil infestation, necrosis development was 2.8 and 1.6 fold greater in plants that received *A. alternata* augmented with both weevils and *A. alternata* combined with a weevil alone, respectively, than in plants received only *A. alternata*. Thirty days after weevil infestation, the percentage of leaf area covered by lesions increased by 2.2 fold in plants augmented with weevils.

A correlation analysis showed a strong and significant positive correlation between number of feeding scars and DI ($r = 0.93$; $P < 0.0001$; Table 1). This indicates that the higher number of feeding scars due to the *Neochetina* weevils enabled a better disease development. Galbraith (1987) reported that feeding by *N. eichhorniae* increased infection by *Acremonium zonatum*.

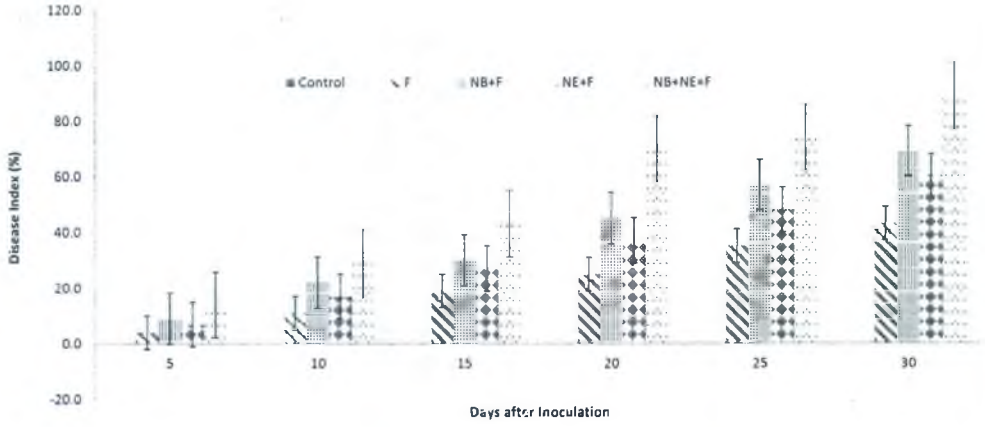

Figure 1. Impact of herbivory by *Neochetina* weevils augmented with the fungal pathogen *A. alternata* on disease index. Treatments: C (control treatment), F (applying *Alternaria alternata*), NB+F (*N. bruchi* augmented with *A. alternata*), NE+F (*N. eichhorniae* augmented with *A. alternata* and NB+NE+F (both weevil species augmented with *A. alternata*). Error bars represent the standard error of the mean.

In the present study, the disease symptoms on water hyacinth caused by the fungus were more severe on weevil-damaged leaves. In various earlier studies (Charudattan et al., 1978; Galbraith, 1987; Moran, 2005; Martínez and Gómez, 2007), the disease causing efficiency of *A. zonatum* and *Cercospora piaropi* was considerably enhanced when the pathogens were applied to water hyacinth in the presence of *Neochetina* weevils. The feeding by the weevils gave access for the fungal pathogens and facilitated infection of water hyacinth (Charudattan et al., 1978). Ajuonu et al. (2003) reported an increase in disease caused by *M. rodidum*, with an increase in the number of feeding scars of adult weevils. Moran (2005) reported that leaf scarring by the weevils *N. eichhorniae*

and *N. bruchi* enhanced efficiency of the pathogen *C. piaropi* to cause disease on water hyacinth leaves.

Table 1. Effects of augumented application of *Neochetina* weevils with *A. alternata* (August 2012 to 2014)

Treatments	Fscar	Disease index (%)	Plant damage (%)	Increase in necrosis (@20DAI)	Increase in necrosis (@30DAI)
C	0	0	0	0	0
F	0	40	65	1	1
NB+F	190	69	75	1.8	1.7
NE+F	140	60	71	1.5	1.4
NB+NE+F	220	89	89	2.8	2.2
Correlation (Fscar and DI)		0.93			

Fscar= Feeding scar; DAI = days after infestationTreatments: C (control treatment), F (only *A. alternata*), NB+F (*N. bruchi* augmented with *A. alternata*), NE+F (*N. eichhorniae* augmented with *A. alternate)* and NB+NE+F (both weevil species augmented with *A. alternata*).

Effect on vegetative growth and inflorescence

The effects of release of *Neochetina* weevils augmented with the fungal pathogen *A. alternata* on the numbers of ramets, leaves and inflorescences were significant ($P < 0.05$) (Figure 2A,B,C). Water hyacinth plant treated with *Neochetina* weevils alone as well as augmented with *A. alternata* showed a significant negative reduction on the reproductive potential.

Petiole length is one of the best proxies of the impact of stress applied to water hyacinth. The percentage of petiole length reduction by application of *Neochetina* weevils augmented with *A. alternata* followed a similar pattern as that with the number of leaves (Figure 2B). However, since individual weevil species only destroyed a fraction of each petiole, the percentage of petiole length destroyed by the respective weevils was rather low compared to the proportion of petiole length affected by the individual weevil species augmented ,with *A. alternata*. Moran (2005) reported that inoculation of *C. piaropi* augmented with *Neochetina* weevils had 20% lower live leaf counts per plant and 38% lower plant densities than control plots.

The average numbers of ramets, leaves and inflorescences per plant recorded during week 8 were 0.45, 0.63 and 0.1 (Figure 2A, B, C), respectively, in water hyacinth plants treated with both *Neochetina* weevils augmented with *A. alternata*. These values were significantly lower ($P < 0.05$) than plants treated with *N. bruchi* or *N. eichhorniae* augmented with *A. alternata* as well as in those treated with the combined application of *Neochetina* weevils. However, both weevil species restricted flowering in a similar way when combined and when individual weevil species were augmented with A. *alternata* (Figure 2C). Eight weeks after establishment of insects and pathogens, the number of green leaves per plant diminished by 95% and the number of new ramets was reduced by 97% due to

combined application of the two weevils with *A. alternata*.

The present findings indicate that reduced vegetative growth by application of both weevils augmented with *A. alternata* led to reduced vigour. Similarly, Martinez and Gomez (2007) indicated that combined application of *Neochetina* weevils with *A. zonatum* showed 65% reduction in number of green leaves and 85% reduction in new ramets.

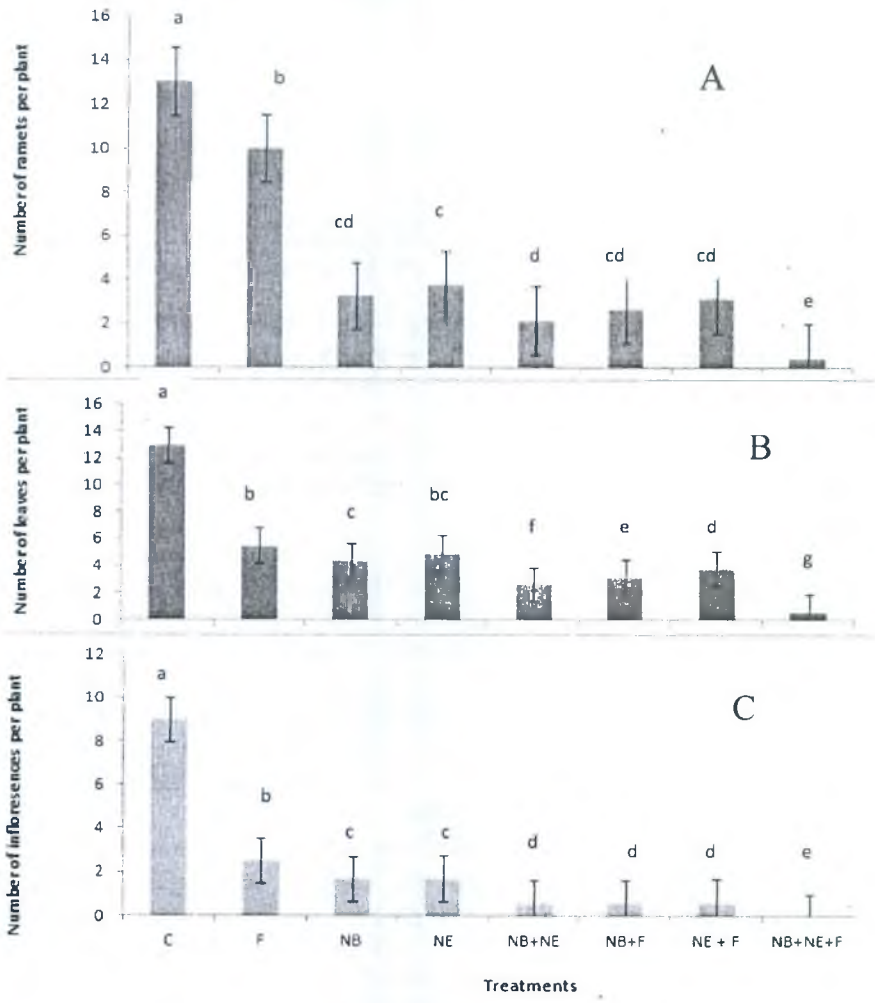

Figure 2. Impact of herbivory by *Neochetina* weevils augmented with the fungal pathogen *A. alternata* after eight weeks on the mean numbers of ramets (A), leaves (B), and inflorescences (C). Treatments: C (control), F (applying *Alternaria alternata*), NB (*N. bruchi*), NE (*N. eichhorniae*), NB+NE (both weevil species), NB+F (*N. bruchi* augmented with *A. alternata*), NE+F (*N. eichhorniae* augmented with *A. alternata* and NB+NE+F (both weevil species augmented with *A. alternata*). Means compared by two-way ANOVA; those with the same letter were not significantly different (Fisher's honest, $P < 0.05$). Error bars represent the standard error of the mean.

Leaf number and ramet production are among the critical growth factors that affect water hyacinth survival (Center and Van, 1989; Heard and Winterton, 2000; Coetzee et al., 2007). Vegetative multiplication is a key for the density and spread of water hyacinth populations. Therefore, a reduction in this reproductive mechanism would reduce expansion of water hyacinth mats and its invasiveness (Byrne et al., 2010).

Hatcher (1995) and Turner et al. (2010) indicated that the interaction between the agents may be synergistic, additive, equivalent or inhibitory. The present study revealed that whilst the weevils were predominantly responsible for the greatest control of the vegetative growth of water hyacinth, the pathogen *A. alternata* played a predominant role in reducing vegetative reproduction and inflorescence development. Combining the impacts of the three agents acting together on different sexual and asexual growth variables led to an overall synergistic effect on the damage caused to water hyacinth.

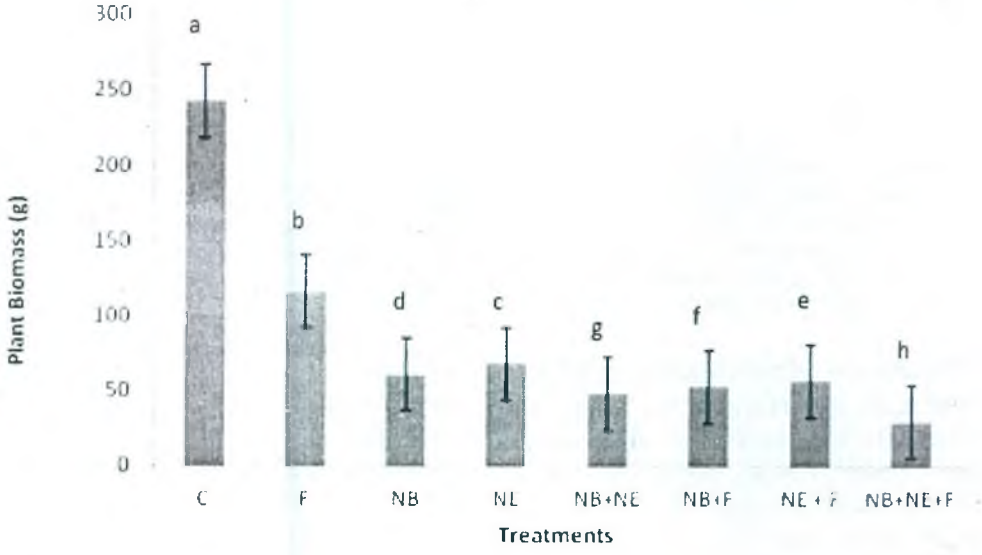

Figure 3. Impact of *Neochetina* weevils augmented with the *A. alternata* on plant biomass. Treatments: C (control), F (applying *Alternaria alternata*), NB (*N. bruchi*), NE (*N. eichhorniae*), NB+NE (both weevil species), NB+F (*N. bruchi* augmented with *A. alternata*), NE+F (*N. eichhorniae* augmented with *A. alternata* and NB+NE+F (both weevil species augmented with *A. alternata*). Means compared by two-way ANOVA; those with the same letter were not significantly different (Fisher's honest, $P < 0.05$). Error bars represent the standard error of the mean.

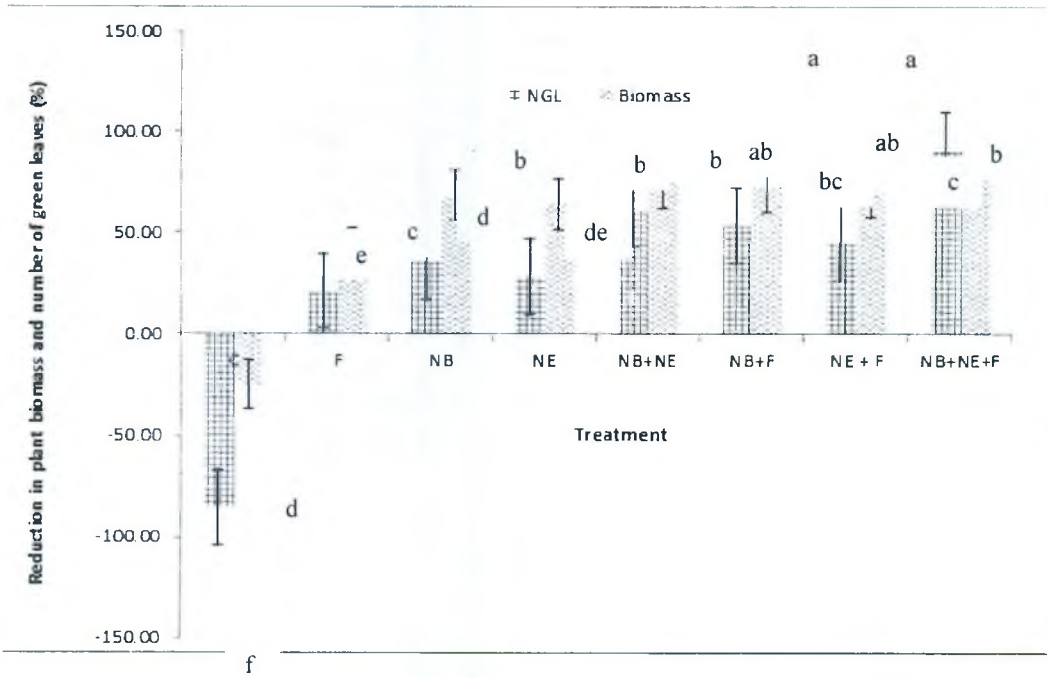

Figure 4. Impact of the Neocnetina weevils augmented with the A. alternata on plant biomass and number of green leaves (NGL). Treatments: C (control), F (applying only Alternaria alternata), NB (N. bruchi), NE (N. eichhorniae), NB+NE (both weevil species), NB+F (N. bruchi augmented with A. alternata), NE+F (N. eichhorniae augmented with A. alternata) and NB+NE+F (both weevil species augmented with A. alternata). Means compared by two-way ANOVA; those with the same letter were not significantly different (Fisher's honest, $P < 0.05$). Error bars represent the standard error of the mean.

Effects on plant fresh weight

Plant fresh weight difference among the treatments was significant at 8 weeks after release of the herbivory treatments. Among the treatments, the fresh weight of the plants that received *A. alternata* and augmented release of the two weevils was very significantly reduced ($P < 0.01$) as compared to the untreated plants (Figure 3). The plant weight difference among the herbivory treatments was more remarkable in plants that received all agents (weevils and fungal pathogen). In plants that received the two agents separately, the differences were low. Plant fresh weight was higher in plants that received only *A. alternata* than in plants that received weevils and application of weevils augmented with *A. alternata*, possibly because of no herbivory effect. Direct effects of herbivory on water hyacinth through biomass consumption and fungal pathogens through leaf and stem consumption have also been reported earlier to influence plant biomass (De Mazancourt and Loreau, 2000).

Plant fresh weight differed among treatments and was highest in the control treatment (no herbivory and no fungal application). Plants augmented with agents had 85% lower plant fresh weight than the control (Figure 4). Reduction in plant fresh weight was significantly

higher ($P < 0.001$) in plants with the two weevils augmented with *A. alternata* (mean ± SE; 84.6 ± 1.94) and plants that received only the weevils (75.3 ± 1.49) compared to plants that received only *A. alternata*. This indicates that the integrated effects of the weevils and the fungal pathogen infection created satisfactory stress on the plants to cause a very significant reduction in plant size and density. Similarly, Center and Van (1989) indicated that weevil herbivory resulted in a decrease in leaf and petiole length, an increase in leaf mortality and an overall reduction in plant biomass.

Conclusion

The two *Neochetina* weevils and the fungus *A. alternata* were together able to reduce the vegetative growth and fresh weight of water hyacinth plants considerably. The fungal pathogen inhibited plant growth, and this was exaggerated by leaf scarring of the weevils. In conclusion, the three agents together had an overall synergistic effect on water hyacinth control in the lathhouse.

Besides, it is important to note that further work is still needed for a better understanding and optimization of water hyacinth management using bioagents. Thus, the following major future research areas are recommended as a follow-up work, namely:

- To simplify large scale application of the fungal pathogens there is a need to develop a formulation/mycoherbicide for the fungal pathogens that shows better efficacy and safety;
- As demonstrated in the current study, there exists a clear synergy between fungal pathogens and the two weevil species, but there is still a need to investigate in greater detail the effects of combined application of two or more fungal pathogens together with the two weevil species; and
- Although the joint use of the two weevil species and fungal pathogens showed better efficacy and safety, there is a need to solve the practical challenges related to mass production of inocula and the two weevils species.

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