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PRIMARY RESEARCH PAPER

More is not necessarily better: the interaction between insect population density and culture age of fungus on the control of invasive weed water hyacinth

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Abstract The possibilities of a positive or negative impact the biocontrol agents may have on each other as well as on the control of the weed itself, inspired us to study the interactions between the mirid, *Eccritotarsus catarinensis* and the phytopathogen, *Acremonium zonatum*, biocontrol agents of water hyacinth, *Eichhornia crassipes*. Observations were made on disease initiation time of *A. zonatum* grown for different time durations with different insect densities on water hyacinth. In absence of mirids, the lowest (3.1 days) and the highest (5.1 days) disease initiation time was observed using 21 and 42 days old culture respectively. In treatments involving mirids, the shortest (1.78 days) and the longest (13.22 days) disease initiation time by *A. zonatum* was observed on water hyacinth with 10 and 20 mirids/plant respectively. By the 30th day, maximum percentage damage (77.9%) was observed in the treatment of 21 day old fungal culture with 20 mirid density/plant despite of initial delay in disease initiation. This result suggests an initial development of a plant defense response due to mirid feeding delaying the pathogen from establishing. Extensive studies involving multitrophic interactions should be an essential part of prerelease assessments to enhance the success rates of biological control of weeds.

Keywords Biological control · Fungi · Insect · Phytopathogens · Water hyacinth

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Introduction

Water hyacinth, *Eichhornia crassipes* (Mart.) Solms-Laubach (Pontederiaceae) is considered one of the worst aquatic weeds throughout the tropical and subtropical regions of the world including South Africa (Coetzee et al., 2009). Biological control has been considered as a preferred method of control for large infestations of water hyacinth because it is economical and sustainable with no negative environmental impacts (DeBach, 1974; Center, 1994; Julien et al., 1996). For the management of this weed, in South Africa seven arthropod biocontrol agents including two weevils, *Neochetina eichhorniae* Warner and *N. bruchi* Hustache, the pyralid moth *Niphograpta albiguttalis* (Warren), a sap sucking mirid *Eccritotarsus catarinensis* (Carvalho), the water hyacinth grasshopper, *Cornops aquaticum* Brüner, a mite *Orthoga lumna terebrantis* Wallwork and the

planthopper, *Megamelus scutellaris* Berg, have been released. A plant pathogen *Cercospora piaropi* Tharp (=*Cercospora rodmanii* Conway, Tessmann et al. 2001), has been introduced (Morris, 1990; Hill & Cilliers, 1999; Morris et al., 1999; Coetzee et al., 2011) as well. Furthermore, fungi *Acremonium zonatum* (Sawada) Gams and *Alternaria eichhorniae* Nagraj and Ponappa have also been reported on water hyacinth in South Africa (Morris et al., 1999). Water hyacinth has been brought under complete control through the introduction of a suite of agents in some areas of the world, most notably Lake Victoria where the introduction of the two weevils, *N. eichhorniae* and *N. bruchi* reduced the weed infestation from 20,000 to 2,000 ha in a period of 5 years (Moorhouse et al., 2001). Spectacular biocontrol has been achieved with the weevils in India at Hebbal in Bangalore, with a 95% reduction in weed cover within a span of 2 years (Jayanth, 1988) and at the Loktak Lake in Manipur (Jayanth & Visalakshi, 1989). Similarly, introduced and native plant pathogenic fungi have been reported to cause severe damage to water hyacinth under field conditions in a number of countries (Conway et al., 1974; Conway, 1976; Martyn, 1985; Charudattan, 2001).

However, in several regions the biological control against water hyacinth has been less successful (PDBC, 1994; Coetzee et al., 2009). Such failures have been ascribed to eutrophicaltion, species interaction between control agents, management practices and climatic factors that slow the build-up of the biological control agent populations (Van et al., 1998; Navarro & Phiri, 2000; Julien, 2001; Madsen et al., 2001; Hill & Olckers, 2001; Sims-Chilton et al., 2010). Application of herbicides may also affect the agents or cause a reduction in the weed population thereby decimating the agent populations (Center et al., 1999). One of the overlooked aspects of failure in biological control programs has been the effect of the biological control agents on each other that could result in competitive interactions (Rosenheim et al., 1995). The possible negative interactions between these agents have not been investigated as possible cause for lack of success in some areas.

Previous studies showed positive interactions between the insect and fungal biological control agents of water hyacinth (Charudattan et al., 1978; Sanders et al., 1982; Charudattan, 1986; Galbraith, 1987; Moran, 2004). Often a successful biological control programme against enduring weeds requires the release and establishment of multiple agents exerting cumulative impacts (Syrett et al., 2000; Denoth et al., 2002; Moran, 2005). Interaction among different species of natural enemies and their host are common in nature as all may be exploiting the same plant part (Kluth et al., 2002; Kruess, 2002). Such interactions can result in synergistic (Shabana et al., 2003; Buccellato et al., 2012) or inhibitory effects (Hatcher & Paul, 2001) on plant performance. However it is difficult to predict the effect of such interactions on the host plants. For example, a phytopathogen may induce chemical alteration in the host plant leading to preferential consumption of infected tissue by the herbivore (Ramsell & Paul, 1990; Hatcher, 1995; Thaler et al., 2002; Hatcher et al., 2004). Conversely fungal infection may reduce the palatability of plant tissues (Karban et al., 1987), by inducing accumulation of high concentrations of defense compounds (Piening, 1972).

Since combinations of natural enemies are routinely used in the biological control of weeds, success will strongly depend on the degree to which herbivores and phytopathogenic fungi influence each other. Yet, there are few reports that quantify interactions between pathogen and insect biocontrol agents that simultaneously exploit weedy plants. However, the importance of herbivore-plant-pathogen interactions has been noted (e.g., Charudattan et al., 1978; Spencer & Sankaran, 1985; Hatcher, 1995; Caesar, 1996, 2000), with several authors emphasizing the need to integrate insects and pathogens (e.g., den Breeyen, 1998; Tinney et al., 1998; Caeser, 2011).

Some earlier studies integrations of biological control agents have been done involving the plant pathogenic fungi and water hyacinth weevils (Charudattan et al., 1978; Sanders et al., 1982; Charudattan, 1986; Galbraith, 1987; Moran, 2004, 2005; Martínez Jiménez & Gómez Balandra, 2007). However, no studies involving the water hyacinth mirid, *E. catarinensis*, have been conducted. Both nymphs and adults of *E. catarinensis* feed gregariously on water hyacinth leaf tissue. They pierce the leaf tissue to feed on the plant sap, causing chlorosis. This ultimately leads to premature death of the leaves and is therefore detrimental to plant growth and its competitive ability (Hill et al., 1999; Coetzee et al., 2005). Studies show that the mirid is unlikely to be an effective agent by itself, but it will be a useful complement to the existing

biological control agents of water hyacinth (Coetzee et al., 2005).

The phytopathogen *A. zonatum* is known to infect water hyacinth causing characteristic necrotic leaf spots formed of alternating concentric light and dark brownish grey bands on the leaves (Martyn & Freeman, 1978; Charudattan, 1996). This pathogen has been reported to cause disease on water hyacinth in many parts of the world including Australia, USA, and many countries of Asia, Central America, and South America (Charudattan, 2001). It is often, but not always associated in the field with infestations of the water hyacinth mite *O. terebrantis*. This pathogen is represented by several highly virulent strains in Mexico (Martínez Jiménez & Charudattan,1998) and has been recorded at a few sites in South Africa (Ray & Hill, 2012a).

Enhancement of pathogen infection by insect attack is a promising idea for biological weed control (Venter et al., 2012). Several studies show that herbivore attack may not only stress a plant, causing growth reductions, but also facilitate pathogen infection (Kluth et al., 2001, 2002). Both herbivores and pathogens may exert effective control, but little is known of their interactions with each other and the host plant. In this study, we quantified the effect of herbivory by the water hyacinth mirid, *E. catarinensis* on the virulence of different ages of *A. zonatum* to investigate whether a combination of the agents could result in a better level of water hyacinth control than pathogen infection alone.

Materials and methods

A culture of *A. zonatum* was isolated from diseased leaves of water hyacinth collected from naturally infested water hyacinth plants in one of our study sites, the Tongaat Sugar Estates, Tongaat, KwaZulu-Natal, South Africa (29.271 S/31.355 E). The stock culture of the pathogen was maintained on potato dextrose agar (PDA) slants and malt extract (ME) media and stored at 7°C in refrigerator for short-term storage and in −80°C in 15% glycerol for long-term storage (Kirsop & Doyle, 1991).

Acremonium zonatum was grown on PDA media and cultivated in walk-in-incubators for 7, 14, 21, 35 and 42 days at 26 ± 2°C and 12 h photoperiods. The spores and a mycelial suspension were prepared in sterile distilled water for all the treatments. *A. zonatum* grown on PDA plates were scraped out by flooding the petri-plates with sterile distilled water and scraped lightly with a sterile spatula to obtain a fungal suspension containing 10 g of scraped material per 50 ml of sterile distilled water. The conidial suspension of *A. zonatum* was observed to contain about 10^8–10^{10} spores ml^{-1} using haemocytometer. To this the surfactant Tween 20 (Oxysorbic polyxyethylene sorbitan monoleate) was added at the rate of 0.05/50 ml of fungal suspension (Ray, 2008).

Water hyacinth plants were obtained from local water bodies and grown in 1000 l plastic tubs in tap water enriched with a 15-3-12 N:P:K, slow release fertilizer (Multicote 8, Controlled Release Fertilizer, Haifa Chemicals Israel, RSA Ptv Ltd) at the rate of 10 g/1000 l water in plastic dispenser to enable its slow release. A commercial iron chelate (13% Fe) was also added to the water at a concentration of 2 g/23 l of water. Healthy individual plants were selected (old leaves and daughter plants were removed) and placed in 10 l plastic tubs. These plants were kept in controlled environmental chambers at 60–70% relative humidity, 27:25°C and photoperiod of 14:10 h (Light: Dark) for a week before starting the experiment to let them acclimatize.

Stock culture of *E. catarinensis* was collected from local water bodies and reared on healthy water hyacinth grown in 50 l tubs that were placed in fine meshed cages in a PVC tunnel at Rhodes University, Grahamstown, South Africa for 1–3 months. Adults were collected from this stock culture with an aspirator into plastic vials covered with a ventilated cap. Adults were released into each experimental tub containing plants at 5 different inoculation loads of insects (0, 5, 10, 15, 20 mirids/plant). Each tub containing the plants and insects was covered with fine nylon mesh cages of about 45 cm diameter and 85 cm height. Fifteen days after insect release, the plants were sprayed with a mycelial suspension (containing 10^8–10^{10} spores ml^{-1}) of *A. zonatum* of different culture age (7, 14, 21, 35 and 42 days). Three sets of control were taken. These included plants with no biocontrol agents, plants with release of insects only and fungal application alone. The treatment having plants with only insects (0 fungi), were applied with a suspension of distilled water and Tween 20. Observations were taken daily, noting the time from start of the experiment to disease initiation to water hyacinth. Thirty

days after fungal inoculation (i.e., 45 days after insect release) the damage caused to water hyacinth was visually assessed. The intensity of fungal infection and/or insect injury on water hyacinth was measured as plant damage (%). The total damage caused by necrosis (by *A. zonatum*) and/or chlorosis (by *E. catarinensis*) was scored by assessing each leaf individually using the 0-to-6 scale rating system where 0 = no damage; 1 = 1–10%; 2 = 11–25%; 3 = 26–50%; 4 = 51–75% and 5 ≤75% area damaged leaves; 6 = death of the plant (no regrowth), 30 days after fungal inoculation. Using this rating system (modified from Caesar, 2003), percentage plant damage was calculated taking into account individual leaf ratings using the following formula:

$$\text{Plant damage}\ (\%) = \frac{(0 \times N_0) + (1 \times N_1) + \cdots + (6 \times N_6) \times 100}{\text{Total } n_0 \text{ of leaves} \times \text{Maximum damage rating}}$$

N_0 = number of leaves with score 0, … N_6 - = number of leaves with score 6.

Statistical analysis

The experiment was repeated nine times, in a completely factorial randomized design with inoculation densities of insect (0, 5, 10, 15 and 20 insects) and culture age of fungi (0, 7, 14, 21, 35 and 42 days) as experimental groups and disease initiation time and percent damage as response variable. The data from the same treatment from the repeated studies were combined and pooled across experiments. The data for both the analyses were subjected to general linear model analysis of variance (GLM–ANOVA), followed by a Tukey's post hoc honest significant difference (HSD) test. The data for percent damage were arc-sine transformed before statistical analysis (Sokal & Rohlf, 1981; Gomez & Gomez, 1984). All analyses were made using the software STATISTICA Version 10 (StatSoft Inc).

Results

There was a significant impact of interaction ($F_{16,200} = 4.2$, $P < 0.0001$) between culture age of *A. zonatum* ($F_{4,200} = 38.7$, $P < 0.0001$) and inoculation density of *E. catarinensis* ($F_{4,200} = 187.84$, $P < 0.0001$) on time taken for disease initiation of *A. zonatum* on water hyacinth (Fig. 1). In the absence of mirids, the plants developed disease symptoms by the third or fourth day after inoculation. The 21-day-old culture of *A. zonatum* was found to be the most virulent causing disease initiation by 3.1 days which was significantly lower than the time taken for disease initiation (5.1 days) by 42 day old culture. There was no significant difference between the disease initiation caused by 7, 14, 21 and 31 days old culture of *A. zonatum* in treatments without mirid release. In the combination treatments with mirids and *A. zonatum*, water hyacinth plants with different inoculation loads of *E. catarinensis* were observed to develop disease symptoms differentially. Disease initiation time was low in plants with 5 and 10 *E. catarinensis* while in those with inoculation loads of 15 and 20 mirids disease initiation took considerably longer. The lowest disease initiation time of 1.78 days was measured in water hyacinth inoculated with 10 mirids and 21-day-old culture of *A. zonatum* while in plants inoculated with 20 mirids and 42-day-old culture of *A. zonatum* disease initiation time was the longest (13.22 days). For treatments with 5 and 10 mirids per plant, there was no significant difference in disease initiation times between culture ages of 7, 14, 21 and 35 days. But there was a significant difference between 21 and 42 day old cultures treatments with 10 mirids per plant. Similarly the disease initiation caused by 35 day old culture and 42 day old culture of *A. zonatum* for plants with 15 (12.9 days) and 20 mirids (13.2 days) was significantly higher as compared to all the other treatments.

By 30 days after fungal inoculation, there was a visual difference in damage caused to water hyacinth by *E. catarinensis* at different inoculation loads and *A. zonatum* at different culture ages. The interaction ($F_{20,240} = 3.8$, $P < 0.0001$) between culture age of *A. zonatum* and inoculation density of *E. catarinensis* significantly affected the percentage damage caused by *A. zonatum* (Fig. 2). Water hyacinth was more affected by different inoculation loads of *E. catarinensis* than the fungal applications at different culture age. Highest damage percentage (77.89%) was measured for the treatment with 20 *E. catarinensis* and 21 day old *A. zonatum* culture. This was at par to other treatments with 20 insects except the treatment with no *A. zonatum* application. Water hyacinth with 20 mirids and no fungal application, recorded low damage score

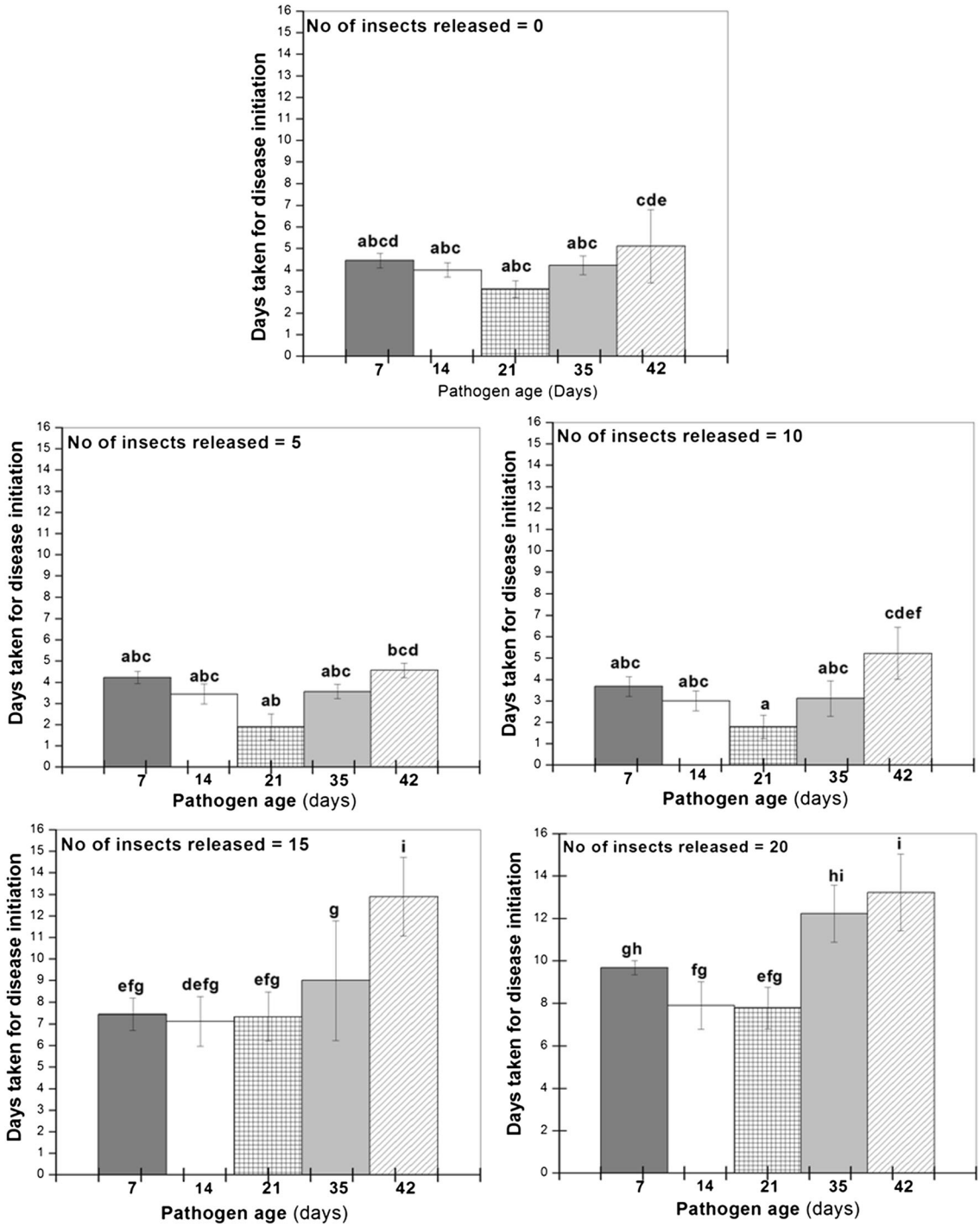

Fig. 1 Effect of culture age of *A. zonatum* and inoculation load of *E. catarinensis* on disease initiation of *A. zonatum*. Mean marked by same letter(s) are not significantly different from each other ($P = 0.05$) for all the graphs. Vertical bars denote 95% confidence intervals

(21.11%). Water hyacinth with an inoculation load of 15 *E. catarinensis* had no significant difference in damage caused when inoculated with *A. zonatum* of 7 (59.67% damage), 14 (61.44%), 21 (67.11%), 35 (64.00%) and 42 (62.44%) days culture age. There was no significant difference between water hyacinth

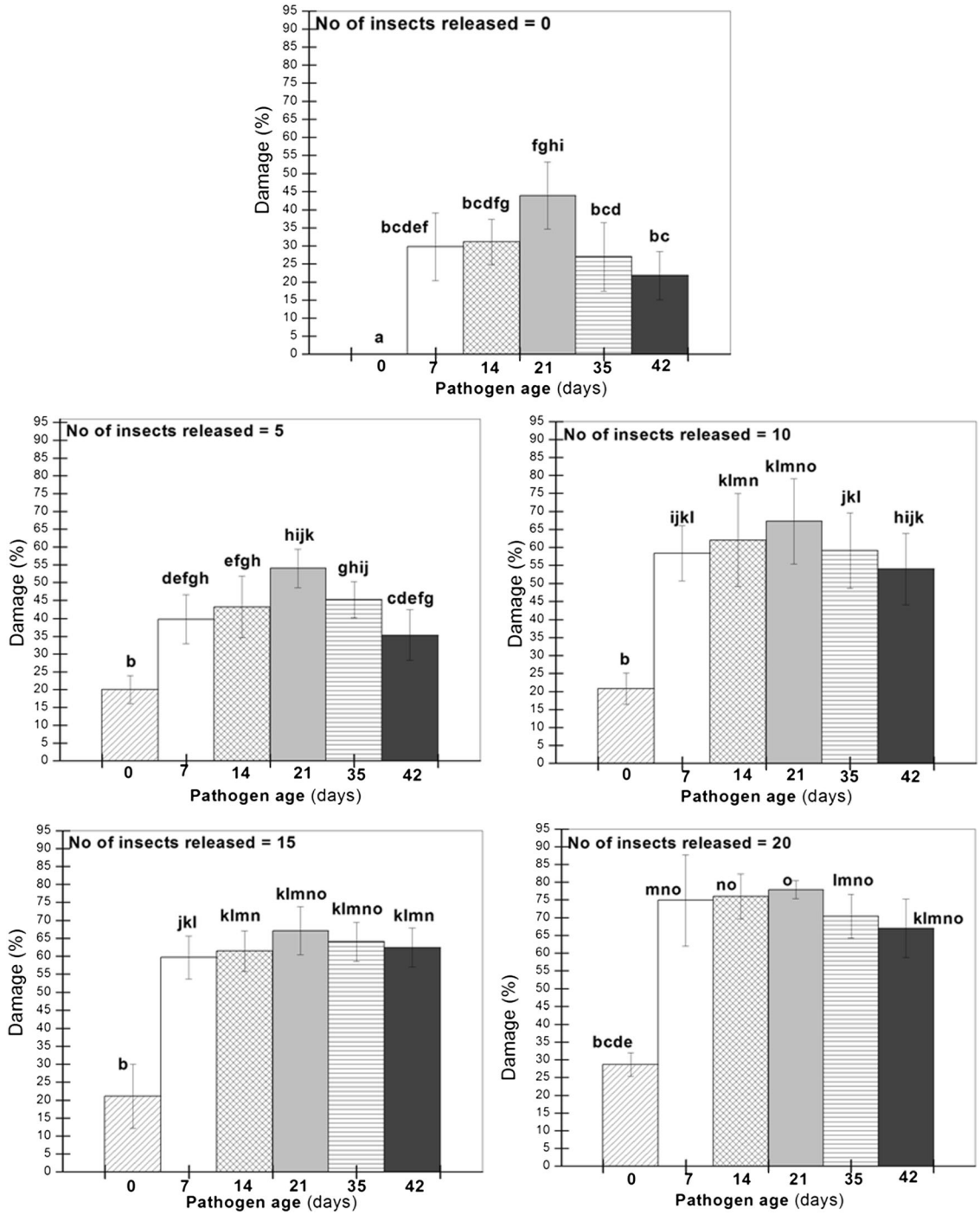

Fig. 2 Effect of culture age of *A. zonatum* and inoculation load of *E. catarinensis* on damage to *E. crassipes*. Mean marked by same letter(s) are not significantly different from each other ($P = 0.05$) for all the graphs. Vertical bars denote 95% confidence intervals

treated with 15 and 10 insects and *A. zonatum* at different culture age except the treatments with no *A. zonatum* application. Water hyacinth with an inoculation load of 10 insects per plant, damage was higher (67.22%) for treatment with 21 day old inoculum of *A. zonatum* as compared to those applied with

other culture ages of the fungi with five and ten mirids per plant. At an inoculation load of 5 *E. catarinensis* released per plant, the damage percentage was 54.0%, with application of 21 day old culture of *A. zonatum*. Damage to water hyacinth was significantly lower in plants treated with 45 day culture of *A. zonatum* (35.33%) and plants with no fungal application but having inoculation load of 5 insects per plant (20%). By the end of 30 days, the number of disease lesions were less visible on leaves damaged by *E. catarinensis* as compared to the plants without insects because of intensive feeding marks created by the mirids.

Discussion

Studies have shown that insect population density can have an effect on weed control success (Ray et al., 2009; Diop et al., 2010). Likewise, age of fungal culture also has an effect on its viability and virulence (Boyette et al., 1991; Hall et al., 1994; Texier et al., 2009). Kadir et al. (2011) reported that there is a significance regression trend between inoculums age and seedling mortality. Such studies prove that the ability of the conidia to incite disease first increases and then decreases with conidial age. Changes in surface property and biochemical composition of conidial wall with age have been observed by Ghamrawi et al. (2014). This in turn affects weed control where fungal pathogens are released as biological control agents. Further to be able to cause disease on the host, ability to adhere to the host surface by strong hydrophobic bond is necessary. So cell surface hydrophobicity are of major importance in the firm adhesion of microorganisms to their host substrate (Donlan & Costerton, 2002). Age of fungal culture may often be an important factor in hydrophobicity of fungi (Smits et al., 2003). A relationship of conidial age with their hydrophobicity has been reported by Peñas et al. (1998). There was possibly change in hydrophobicity with the increase in age of the culture causing increase in time taken for disease initiation and reduction in damage percentage in water hyacinth applied with *A. zonatum* of culture age more than 21 days.

The combination of *E. catarinensis* and *A. zonatum* had significant detrimental impact on water hyacinth. Several studies (Hatcher, 1995; Denoth et al., 2002) have shown that multiple-agent projects against weeds have been more successful than single agent release, with the biocontrol agents exerting cumulative impact on the target weed. For example, studies by Kremer & Spencer (1989) showed that not only the combined effect of fungi and seed feeding insects decreased the seed germination but also herbivory by plant bug, *Niesthrea louisianica* on weed, velvetleaf *Abutilon theophrasti* significantly enhanced the disease causing potential of seed- borne microorganisms like *Fusarium* and *Alternaria* in seed up to 98% compared to the average fungal infection (8%) in absence of insect damage. Morrison et al. (1998) also demonstrated that chrysomella beetles *Chrysolina hyperici* can augment biological control of St. John's wort *Hypericum perforatum* seedlings by transmitting the fungal pathogen *Colletotrichum gloeosporioides* f. sp. *hypericum* during foraging and feeding. Similar reports of enhanced fungal infection due to insect feeding have been reported by several other researchers (Gratwick, 1992; Alford et al., 2003; Ray and Hill, 2012b) as well. In the present study facilitative effect of insects on disease initiation by *A. zonatum* was only seen with low inoculation loads of *E. catarinensis* and 14–21 day old cultures of *A. zonatum*. Hence the fungal pathogen and insect are more compatible at low insect density. Excessive wounding at high mirid density (15–20 mirids) seemed to restrict pathogen disease induction once it penetrated the cuticle.

Although plants have been shown to evolve multiple defense mechanisms against pathogens, herbivores and various types of environmental stress (Lattanzio et al., 2006; Ashry & Mohamed, 2011; Mazid et al., 2011), low-level damage by herbivores or pathogens can enhance leaf and shoot production by the plant or induce production of chemical defenses that are antagonistic to the other agent (McNaughton, 1983; Trumble et al., 1993). Hatcher & Paul (2001) reported an interaction due to feeding by leaf beetle *Gastrophysa viridula* and pathogen infection on *Rumex obtusifolius* where feeding by the insect induced a systemic resistance that reduced the subsequent infection by the rust *Uromyces rumicis*. Similarly several other studies (Shivas & Scott, 1993; Crowe & Bourchier, 2006) have emphasized minimizing the number of biological control agents released that use similar resources on the target weed, to avoid negative interactions between control agents and potential reductions in biocontrol efficacy resulting from competition. Studies by Kruess (2002)

showed that in dual-choice tests, adults of leaf beetle *Cassida rubiginosa* preferred leaf discs from healthy thistles *Cirsium arvense*, over those thistles leaf discs infected by *Phoma destructiva*. Further, the mortality of early instars of *C. rubiginosa* increased when fed with *P. destructiva* -infected *C. arvense* leaves. Arthropod herbivory and phytopathogens are known to induce a variety of defense reactions in the host plant (Zidack, 1999; Alba et al., 2011). These reactions may be either physiological or physical responses to prevent further insect/pathogen damage. Fungal infection may induce a response in plants by accumulation of secondary compounds causing several phyto-chemical changes in the plants metabolism (Schaller & Ryan, 1996; Mazid et al., 2011). This may indirectly influence feeding, survival, and/or reproduction of the insect feeding on the plant which could directly or indirectly cause a cumulative, synergistic, additive or inhibitory effect on the control of the host plant. The lower disease lesions on plants with higher numbers of *E. catarinensis* released may be due to the plant resistance pathways (War et al., 2012) induced by the insect feeding damage which was not induced with lower number of insects. Such negative impact of arthropod feeding on disease development have been reported by several authors (Bultman and Mathews 1996, Hatcher & Paul 2001). Guevara et al., (2000) reported that two ciid beetle species, *Octotemnus glabriculus* and *Cis boleti* reduced the reproductive potential of a phytopathogenic fungus *Coriolus versicolor* by up to 50% alone and 64% in combination both under laboratory and field conditions. The mirids are known to puncture the epidermis while feeding on plant juices causing injury to the plant and often inducing systemic resistance in host plants. Studies by Frati et al. (2006) shows that mirids produce hydrolytic enzymes *Endo*-polygalacturonases (PGs), involved in the degradation of pectin, one of the major components of plant cell wall. Several plants are known to produce extracellular plant proteins like polygalacturonase-inhibiting proteins (PGIPs) which are known for their ability to inhibit fungal PGs and restrict fungal colonization. Similarly in another study by Halitschke et al. (2011) wild tobacco, *Nicotiana atenuata* attacked by mired bugs, *Tupiocoris notatus* becomes resistant against more damaging herbivores through mirid-induced direct and indirect defenses. Also mirid-induced resistance and tissue loss do not result in a reduction of plant fitness as it induced metabolic responses allowing the plant to compensate for the lost tissue and resources allocated to defenses by elevating photosynthetic activity in the plant.

In most biocontrol programs, several agents are introduced in the hope that the stress exerted on the invasive plant by their combined effects will be greater than that of one agent acting alone. But interactions between control agents and the plant may not always have a detrimental impact on the target plant (Denoth et al., 2002; Rayamajhi et al., 2006; April et al., 2011). In our study, low mirid densities (5 and 10 mirids/-plant) tended to enhanced the disease causing potential of *A. zonatum* initially, but high mirid densities (15-20 mirid/plant) reduced the initial disease development of the fungi on the test plants. Despite initial antagonistic effect caused by higher number of insect feeding in delaying disease development, an additive effect in damaging water hyacinth was observed in the present study since higher numbers of mirids were more effective in increasing the damage percentage of water hyacinth. Also the exhibition of some degree of antagonistic interactions should not deter the deployment of multiple agents, especially combinations of microbes and insects in invasive plant biological control programs because microbial agents can accelerate mortality of attacked plants when deployed in combination with insects (Rayamajhi et al., 2006). However since studies have shown the presence of wide range of potential interactions, studies on compatibility between agents should be given importance in the weed biocontrol projects involving multiple agents.

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