An insect-plant pathogen interaction between two biocontrol agents, the stem-gall fly and leaf-spot pathogen released against crofton weed, *Ageratina adenophora.*

Lisa Buccellato

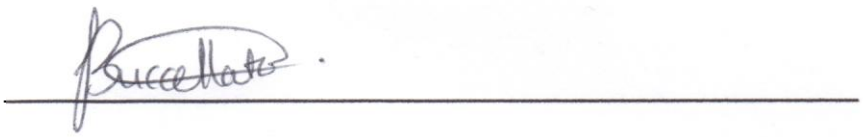
A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the academic requirements for the degree of Doctor of Philosophy.

November 2012

Johannesburg

Declaration

I declare that this thesis is my own, unaided work, unless otherwise noted within the text. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



1st day of November 2012 in Johannesburg

Acknowledgements

Firstly, I would like to thank my two supervisors, Prof. Marcus Byrne and Prof. Ed Witkowski, for their never-ending support, patience, guidance and invaluable knowledge. Thank you for believing in me and making this PhD a reality, it has been an amazing journey for me as an individual and student.

I am grateful for funding from the Working for Water Capacity Building Programme of DWAF, the National Research Foundation (NRF2069152) and the University of the Witwatersrand.

Dr. Stefan Neser is thanked for his invaluable knowledge of biocontrol, crofton weed and the two biocontrol agents in South Africa, information regarding crofton weed infestations, guidance at the proposal stage and introducing me to relevant people along the way. The Plant Protection Research Institute in Stellenbosch is thanked for identifying the pathogen collected from the Barberton field sites. Thank you to John Hoffmann for sending me the first culture of flies at the beginning of my research. I am grateful to the Laburn family and Kloofwaters staff for their unlimited access and help to the Magaliesberg field site, SAPPI Escarpment Plantation for access to the Barberton field sites and Dr M. Morris for his information about crofton weed infestations in the KwaZulu-Natal area. The South African Weather Service is thanked for rainfall data at the field sites. Thank you to Prof. Dave Mycock for help at the proposal stage and assistance relating to the pathogen throughout my research. Thank you to the many people I have met along the way, particularly at the Weeds Workshops and biocontrol conferences, who have provided guidance and advice on my research.

This research required many hours of work in the greenhouse and field, none of which would have been possible without the amazing research assistance I received from Kate Lorentz, Anthony King, Ryan Brudvig,

Samantha Bush, Lone Poulsen, Pedro Buccellato and Andrew Petrie. Thank you for all the time and energy you contributed, as well as the companionship to keep me sane through it all.

This has been an incredible experience for me, as well as the joys there has been blood, sweat and many tears along the way. I am incredibly grateful to my close friends who have kept me sane along the way with many coffee breaks and chats. To my wonderful mom, dad and brother thank you for always loving, supporting and motivating me unconditionally throughout my life, and especially through this research. Your love, shoulders to cry on, hugs and belief in me are how I have persevered through this journey. To my amazing husband, who has been with me through every step of this study, your love, support and patience is more than I could ever have asked for, without you this PhD would not have materialised. Lastly, to my incredible son, thank you for enduring the stress of my write up while you were still unborn and for giving me the motivation to complete the corrections.

Abstract

Classical biological control (biocontrol) of invasive plants involves the deliberate introduction of biocontrol agents, termed natural enemies, such as insects, mites and pathogens, from the country of origin into the invaded country to control an invasive alien plant (weed) infestation. This thesis evaluates the biocontrol of crofton weed, *Ageratina adenophora*, in South Africa, with a stem gall fly, *Procecidochares utilis*, and a leaf-spot pathogen, *Passalora ageratinae*. The issues of multiple biocontrol agents, pathogen-insect interactions, the assessment of agent efficacy and post-release evaluations in biocontrol are addressed using crofton weed biocontrol as a case study.

Laboratory trials showed an additive interaction between the fly and pathogen on crofton weed control. The fly inhibited vertical stem growth, with the gall acting as a nutrient sink, but crofton weed compensated with increased sideshoot growth. The pathogen inhibited sideshoot (vegetative reproduction) growth. Field trials showed an equivalent interaction between the two biocontrol agents. The pathogen inhibited sideshoot growth, however the fly did not inhibit stem height but the galled stems had less biomass allocated to bare stems, than sideshoots or live leaves, indicating weakened stems. Growth of crofton weed stems was slower in the field than the laboratory, therefore the effect of the biocontrol agents on the vegetative growth of crofton weed may be different in the laboratory, or field trials may need to run for a longer period to see an effect of the biocontrol agents. The fly reduced the reproductive output of crofton weed by 53.8% in both the laboratory and field, and the pathogen reduced the reproductive output by 26.7%. There was an equivalent effect with the agents in combination.

The fly and pathogen together have an equivalent effect on crofton weed ecophysiology, with the pathogen being the predominant agent. The pathogen reduced the transpiration, stomatal conductance and photosynthetic rate, as well as the functioning of Photosystem II of crofton

weed leaves. These ecophysiological results show that crofton weed compensated for infection by investing resources into vertical growth with healthy new leaves, thereby leaving fewer resources for sideshoot growth.

Three crofton weed infestations were surveyed at Barberton (pathogen present), Magaliesberg (pathogen present) and Pietermaritzburg (pathogen and fly present). The Barberton site was located under a pine forest canopy, with 30-50 stems/m^2 ranging in height from 100-1200mm. The Magaliesberg site, along a stream bank, had 20-50 stems/m^2 with stems of 100-2200mm in height, even after manual clearing. Stems at Pietermaritzburg, along a roadside, were 100-2000mm high and stem density was 80 stems/m^2. The pathogen infected up to 95% of stems, but only infected 1-30% of leaves per stem. Fly prevalence was low, 20% of stems were galled, and repeated galling of stems was rare. Parasitism was expected to explain the low fly population, however only 30% of galls were parasitised. The fly did however reduce the reproductive output of crofton weed stems. These post-release evaluations highlight the necessity to define success and collect pre-release data in biocontrol programmes prior to agent releases.

Surveys for new biocontrol agents for crofton weed have been undertaken. The selection of the new agent will need to consider the interaction with the fly and pathogen. In addition, based on this study, the new agent will need to inflict damage which will minimise compensatory growth in crofton weed.

.

Keywords: Crofton weed, *Procecidochares utilis*, *Passalora ageratinae*, multiple biocontrol agents, insect-pathogen interactions, post-release evaluations

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 5

Chapter 6

Chapter 1: Introduction

1.1 Background

Infestations of invasive alien plants ('weeds') worldwide have resulted in major ecological and economic impacts, instigating widespread concern and the initiation of control programmes. Invasive weeds pose major problems for natural environments. They compete with other plants for nutrients, water and light, and can be allelopathic (produce growth-inhibiting toxins) (Anderson, 1996; Mack *et al*., 2000). This often leads to major alterations of ecosystem functioning and native biodiversity (Witkowski, 1991; Day *et al*., 2003; Barton *et al*., 2007). Economically, weeds may entail a high cost in their control and eradication (Mack *et al*., 2000).

Once invasive species have established and spread it is generally not possible, or feasible, to eradicate them, therefore programmes are instigated to control and reduce the invasions. Three principal methods of controlling weeds are currently in use; namely chemical, mechanical and biological control (Wittenberg & Cock, 2001). However, recently there has been a move towards integrating all three of these methods into control programmes of invasive weeds (Zimmermann & Neser, 1999; Ainsworth, 2003; Hatcher & Melander, 2003).

Classical biological control (biocontrol) involves the deliberate introduction of biocontrol agents, termed natural enemies, such as insects, mites and pathogens, from the country of origin into the invaded country (McFadyen, 1998; Müller-Schärer & Schaffner, 2008). The aim of biocontrol is not to eradicate the weed population but to reduce the invasion to 'acceptable levels', where the plants may survive and reproduce but do not invade or impact negatively on the surrounding environment. The natural enemies reduce the target weed population by affecting plant performance such as reducing biomass and decreasing reproductive output (Müller-Schärer & Schaffner, 2008)

Biocontrol has many positive aspects that make it a preferred method of invasive weed control in comparison to chemical and mechanical control

(McFadyen, 1998; Wittenberg & Cock, 2001, Zimmermann *et al.*, 2004; Müller-Schärer & Schaffner, 2008). Firstly, it is safe and often referred to as 'environmentally friendly'. There are no records of weed biocontrol agents becoming economically important pests and weed biocontrol agents are almost exclusively host-specific (McFadyen, 1998). There are very few recorded instances of non-target effects of weed biocontrol agents (Louda *et al.*, 1997; Pemberton, 2000). One of the most advantageous aspects of biocontrol is that it is self-sustaining. Once agents are established in the field, their populations ideally increase and spread throughout the weed invasion, so that no further introductions are usually necessary. Also, as the host plants are not eradicated, small populations of agents persist in the field, and can control the weed if it reinvades. The self-sustainability of biocontrol leads to substantial economic savings in weed management programmes and as the weed populations are no longer invasive there is an increase in land productivity resulting in financial returns (van Wilgen *et al*., 2004, van Wilgen & De Lange, 2011).

Worldwide more than 400 species of biocontrol agents have been released against approximately 280 weed species in more than 75 countries (Julien & Griffiths, 1998). In South Africa, 284 biocontrol agents have been considered, of these 284 organisms 106 have been released, and 75 of these have established on 48 plant species (Klein, 2011). Of the 48 target weeds, 21% are completely controlled by the biocontrol agents, so that no other control measures are necessary, and 38% of the target weeds are under substantial control through biocontrol. Additional management practices are needed to keep these weeds at acceptable levels, but the intensity of these practices is reduced after the introduction of biocontrol methods (Klein, 2011). Twenty-nine percent of the target weeds are under negligible control, where agents damage the target weed but are not adequate for total control, necessitating the use of additional control methods to reduce the weed infestations. The impact of biocontrol agents is unknown for 10% of the target weeds (Klein, 2011).

Ageratina adenophora, (Sprengel) King and Robinson (syn. *Eupatorium adenophorum* Sprengel) (Asteraceae), also known as crofton weed or the Mexican Devil weed, originates from Mexico, and was probably first introduced into South Africa as an ornamental before 1948 (Hilliard, 1977). It is naturalised and an invasive weed in several countries worldwide, including Australia, New Zealand, Hawaii, India, Thailand, Taiwan and China (Peng *et al*., 1998, Trounce, 2003). Within South Africa crofton weed is found in KwaZulu-Natal, Western Cape, North West, Mpumalanga, Limpopo and Gauteng provinces (Henderson, 2001; Heystek *et al*., 2011) (Fig. 1.1). The initial major infestation was along the Natal mistbelt, where it spread rapidly causing some concern (Plant Protection News, 1988). Since 1997 crofton weed has appeared and spread rapidly in the Magaliesberg, a natural heritage site, particularly in the Easter Kloof (Heystek *et al*., 2011).

In South Africa crofton weed is a Category 1 invasive plant under the Conservation of Agricultural Resources Act (CARA) and is referred to as the inland equivalent of its close relative chromolaena, *Chromolaena odorata* King and Robinson (Asteraceae) (Plant Protection News, 1987a). It is listed as a widespread-abundant, special effect weed, indicating that its range and abundance significantly impacts on natural and semi-natural environments, degrading their value or purpose (Henderson, 2001; Nel *et al*., 2004). Crofton weed has many adverse affects on the areas in which it invades, necessitating its control (Erasmus *et al*., 1992; Land Protection, 2001; Page & Lacey, 2006). Firstly, it is a serious weed on agricultural land, reducing the carrying capacity and crop yields. Forestry is negatively impacted where expansive dense stands prohibit the growth of seedlings. When eaten by horses it causes a fatal lung disease, and it is toxic to other livestock (Plant Protection News, 1988). Conservation areas are adversely affected by this weed, as it is allelopathic, alters soil microbial communities and displaces natural vegetation (Erasmus *et al*. 1992; Henderson, 2001; Niu *et al*., 2007).

In South Africa a relatively small biocontrol programme for the control of crofton weed was initiated in 1984 (Plant Protection News, 1987b). Two biocontrol agents were released against crofton weed, a stem gall fly, *Procecidochares utilis* Stone (Tephritidae), and a leaf-spot pathogen, *Passalora ageratinae* Crous and A.R. Wood (Mycosphaerellaceae). The stem gall fly was released near Pietermaritzburg between 1984 and 1987 (Kluge, 1991), and later in the Magaliesberg. The leaf-spot pathogen, the second pathogen to be imported for biocontrol in South Africa, was released in Natal (now KwaZulu-Natal) and south-west Cape in 1987 (Plant Protection News, 1988), and in the Magaliesberg. Both agents are established in South Africa.

The gall fly has been very successful in Hawaii, clearing large infestations of crofton weed (Bess & Haramoto, 1959, 1972). Up to 100% of crofton weed stems were galled, with 1-7 galls per stem, which resulted in stunted plants, weakened stems and death of some plants (Bess & Haramoto, 1972). In Australia, the gall fly initially galled most stems, but now galls less than 50% of stems in crofton weed infestations (Page & Lacey, 2006). In China, galling levels are low with 10-37% of stems galled (Zhang *et al*., 2008). The gall fly and fungal pathogen together have slowed the encroachment of crofton weed and thinned infestations along the east coast of Australia (Dodd, 1961; Page & Lacey, 2006). The fungal pathogen has led to death of seedlings and major or complete defoliation of stems in Australia in the drier months (Page & Lacey, 2006), and high levels of galling reduced plant vigour and killed plants (Dodd, 1961). Bennett (1986) initially evaluated the effectiveness of the fly in South African laboratory trials, however since then no formal evaluation of the efficacy of the fly and pathogen on crofton weed control has been undertaken in South Africa. Surveys for new biocontrol agents for crofton weed in South Africa are currently underway (Heystek *et al*., 2011).

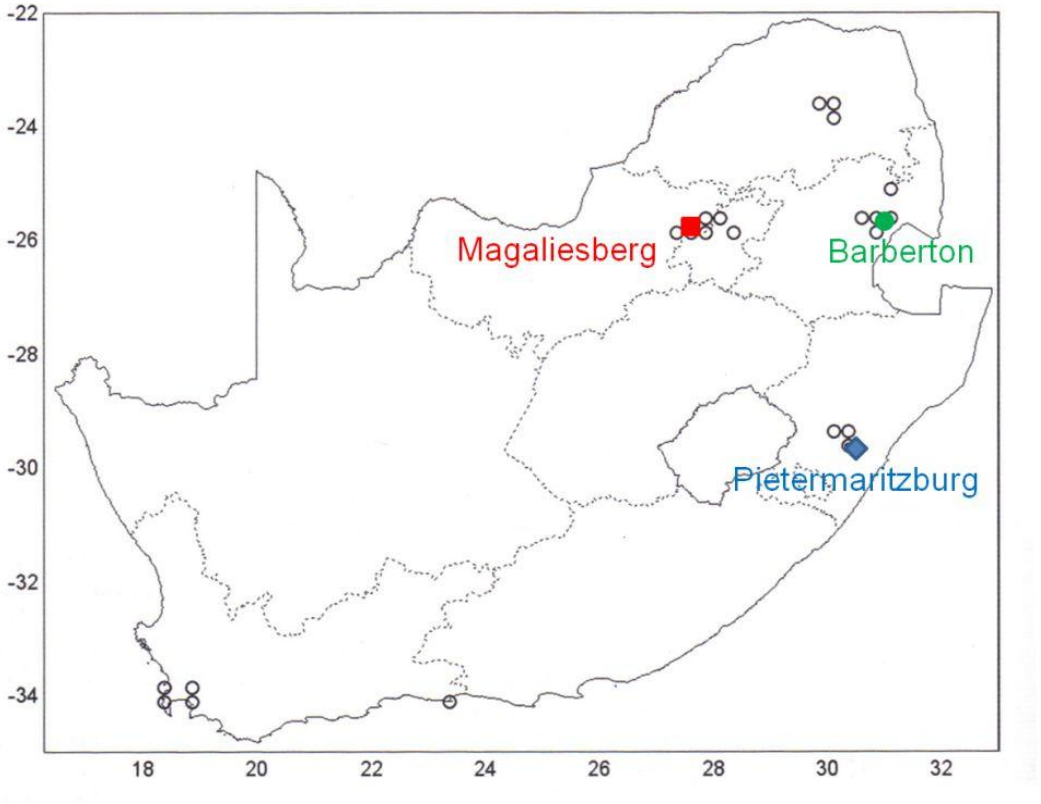


Figure 1.1: Distribution of *Ageratina adenophora* (O) in South Africa. (Drawn by L. Henderson; data source: SAPIA data base, ARC-Plant Protection Research Institute, Pretoria), and location of three field sites, Barberton (●), Magaliesberg (■) and Pietermaritzburg (◆), where *A. adenophora* infestations were surveyed for this study.

1.2 Aims and objectives

The overall aim of this study was to evaluate the effectiveness of *Procecidochares utilis* and *Passalora ageratinae* as biocontrol agents, individually and in combination, for *Ageratina adenophora* in South Africa. The study consisted of two components. Firstly, laboratory trials of the two agents in various combinations on crofton weed at the University of the Witwatersrand and field trials of the two agents in various combinations on crofton weed in the Magaliesberg (Fig. 1.2), and secondly, field surveys of the two biocontrol agents and crofton weed populations in the Pietermaritzburg, Magaliesberg, and Barberton areas.

1.2.1 Objectives of the laboratory and field trials

1.2.1.1 Assessment of the impact of both biocontrol agents on the vegetative growth of *A. adenophora*.

- Measure the vegetative growth of *A. adenophora* without biocontrol agents.
- Reassess the impact of *P. utilis* on the vegetative growth of *A. adenophora* (done by Bennett, 1986).
- Assess the impact of *P. ageratinae* on the vegetative growth of *A. adenophora*.
- Assess the combined impact of both *P. utilis* and *P. ageratinae* on the vegetative growth of *A. adenophora*.

1.2.1.2 Assessment of the impact of both biocontrol agents on the reproductive output of *A. adenophora*.

- Measure the reproductive output of *A. adenophora* without biocontrol agents.
- Reassess the impact of *P. utilis* on the reproductive output of *A. adenophora* (done by Bennett, 1986).
- Assess the impact of *P. ageratinae* on the reproductive output of *A. adenophora*.
- Assess the combined impact of both *P. utilis* and *P. ageratinae* on the reproductive output of *A. adenophora*.

1.2.1.3 Compare results obtained from laboratory trials and field trials.

1.2.2 Objectives of the field surveys

- Survey the *A. adenophora* population, in terms of plant density, size distribution and biomass, over four seasons.
- Survey the *P. utilis* population, in terms of galling prevalence and severity of gall attack, over four seasons.

- Survey the *P. ageratinae* populations, in terms of disease prevalence and severity of infection, over four seasons.
- Survey the reproductive output of *A. adenophora* in the field.
- Survey the level of parasitism of *P. utilis* in the field, over four seasons.

(a)

(b)

Figure 1.2: Photographs of *Ageratina adenophora* plants in cages during laboratory (a) and field (b) trials.

1.3 Study species

1.3.1 Ageratina adenophora

Ageratina adenophora, (Sprengel) King and Robinson (syn. *Eupatorium adenophorum* Sprengel) (Asteraceae), crofton weed, is a perennial herb, with a woody rootstock, and many stems reaching up to 2m in height (Bess & Haramoto, 1959; Henderson, 2001; Page & Lacey 2006). Stems consist of purplish to chocolate-brown branches, and are covered in stalked glandular hairs (Muniappan *et al*., 2009). The leaves are dark green, rhombic, opposite, purple underneath and are approximately10cm long (Muniappan *et al*., 2009) (Fig. 1.3). As the plant grows the bottom leaves senesce, eventually falling off. The tall main stems bend over and trail along the ground, rooting where they touch the soil, forming dense, tangled impenetrable stands (Dodd, 1961). Sideshoots develop in the nodes of tall stems, therefore where the horizontal stems root, these sideshoots root and grow, so that many stems may arise from one main stem (Wang *et al*., 2011). This original stem eventually forms a rudimentary rhizome. Crofton weed has a shallow tap-root system with short, lateral rootstocks in the upper soil, which may reach depths of 40cm (Bennett, 1986).

The inflorescences are arranged in terminal capitula (flower-like heads made up of many small white florets) surrounded by protective bracts (Peng *et al*., 1998) (Fig. 1.4). In South Africa flowering occurs between August and December (Henderson, 2001). The fruits are glabrous achenes (smooth, hard dry fruits) approximately 2mm long, with 8-10 apical bristles (Dodd, 1961; Peng *et al*., 1998). Crofton weed seeds prolifically, each plant can produce up to 100 000 achenes per season, and dense stands can contribute up to 60 000 viable seeds per m^2 (Muniappan *et al*., 2009). The seeds are dispersed by wind over long distances, as well as by water and humans, allowing invasion of new areas. Crofton weed is apomictic, producing seeds without fertilisation

(Rambuda & Johnson, 2004). Therefore, crofton weed can seed and spread in the absence of other individuals or pollinators.

The weed favours moist areas near water and along stream banks, but may also invade margins of forests, agricultural plantations and roadsides (Henderson, 2001). Crofton weed often grows in inaccessible areas, such as high up on waterfalls and in the crevices of mountain ridges, where these infestations are very difficult to clear manually.

(a)

(b)

Figure 1.3: (a) *Ageratina adenophora* shoot and inflorescence (A). (Drawn by G. Condy, South African National Biodiversity Institute, Pretoria. First published in Kluge (1991)). (b) *Ageratina adenophora* foliage.

Figure 1.4: *Ageratina adenophora* synflorescences and achenes.

1.3.2 Procecidochares utilis

Procecidochares utilis Stone (Tephritidae) originates from Mexico and was first introduced into Hawaii in 1945; and later into Australia in 1952 (Bess & Haramoto, 1959; Dodd, 1961). Populations of the fly, imported from Australia, were released in South Africa in 1984 (near Stellenbosch) and in 1987 (near Pietermaritzburg) (Kluge, 1991), and more recently in 2009 in the Magaliesberg area (Heystek *et al*., 2011). The gall fly has persisted and is now considered to be widely established in South Africa, although this has not been systematically tested.

One female gall fly can produce up to 160 eggs, laying eggs in batches, of 2-23 eggs, on or near the apical bud (Bennett & Van Staden, 1986). Larvae hatch 3-7 days later, after which they feed on young leaf tissue as they move to the base of the leaves and tunnel into the stem, where they feed on plant tissues, resulting in the formation of galls (Bennett, 1986). The gall fly has 3 larval instars. Before pupating the third larval instar excavates a tunnel from the centre of the gall to the outside of the gall, leaving only the epidermis intact, creating an emergence window (Fig. 1.5). The larval stage usually lasts for 20 days. Pupation occurs in the galls with adult flies emerging 2-3 weeks later. The life-cycle of the gall fly ranges from 48 days in summer to 92 days in winter, with an average of five generations per year (Dodd, 1961).

Laboratory trials show that the fly is an effective biocontrol agent that should significantly reduce weed populations (Bennett, 1986). The fly is capable of halting stem growth, and if the stem is subjected to repeated generations of flies (i.e. more than one gall per stem) the stem may die, and even whole plants may die (Erasmus *et al*., 1992). The above laboratory trials also showed that the fly reduces the reproductive potential of crofton weed by approximately 60% (Erasmus *et al*., 1992). The galls did not reduce the viability of the seeds, but the number of seeds produced was significantly reduced. This reduction was achieved as galled stems produced underdeveloped capitula and a reduced number of

capitula, in comparison with ungalled stems. However, in contrast to these laboratory results, Kluge (1991) reported no visible impact of the fly on the size or density of the crofton weed infestations in Pietermaritzburg.

Parasitism of the gall fly has been recorded in South Africa. Bennett (1986) identified three wasp parasitoids; a member of the Pteromalidae family, *Dimeromicrus* spp. and an *Eupelmus* spp..

Figure 1.5: *Procecidochares utilis* gall with emergence holes on a flowering *Ageratina adenophora* stem, and a *P. utilis* gall developing on the leaf petiole.

1.3.3 Passalora ageratinae

Passalora ageratinae Crous and A.R. Wood (Mycosphaerellaceae) (previously named *Cercospora eupatorii* Peck or *Phaeoramularia* sp.) (Crous *et al*., 2009) is a fungal leaf-spot pathogen native to Central America. The pathogen was first recorded in Queensland, Australia in 1954, where it was accidentally introduced with a shipment of the gall fly (Dodd, 1961). It was found that when adult flies were artificially laden with spores they were able to carry viable spores on their legs and among their body hairs, and therefore transmit the pathogen between host plants (Dodd, 1961). The pathogen spores are also spread by wind and water (Dodd, 1961).

Cultures of the pathogen were imported from Australia and released in South Africa in 1987 and 1988 near Stellenbosch and into areas surrounding Pietermaritzburg (Morris, 1991). A further three releases were made in Natal (now KwaZulu-Natal) in 1989. The pathogen established and some defoliation was recorded but there has been little monitoring on the impact of the pathogen since then (Morris, 1991). The introduction of the pathogen into the Magaliesberg kloofs (riverine gorges) was not a large-scale, formally organised biocontrol programme. Dr. Stefan Neser, of the Plant Protection Research Institute (PPRI) Pretoria, inoculated crofton weed infestations in the Easter Kloof, Magaliesberg, with the pathogen opportunistically in the late 1990's. A survey in 2004 showed that the pathogen has established and spread to other kloofs within the Magaliesberg (Buccellato, 2004).

The pathogen only infects the leaves of crofton weed. A dew period of 24 hours, 24/19°C (day/night), is required (Wang *et al*., 1997). Conidia germinate at 20-25°C and the optimum temperature for fungal growth is 25°C. Conidia germinate within 24 hours of inoculation, germ tubes from the conidia branch across the leaf surface and penetrate the stomata within five days (Morris, 1989; Wang *et al*., 1997). The penetration hyphae then branch in the substomatal chambers and form networks in the mesophyll tissue. Conidiophores are produced from the hyphal networks or on the surface hyphae. Leaf-spots are visible on the leaves three weeks after inoculation and five to seven weeks later the infected leaves die (Morris, 1989) (Fig. 1.6).

A survey in the Magaliesberg in 2004 showed that the pathogen may increase the proportion of dead leaves on plants, but does not seem to significantly influence any other part of the plant (Buccellato, 2004). The lower leaves on the stems of mature plants naturally senesce and fall off, therefore it is predicted that the predominant impact of the pathogen on crofton weed is to reduce the life-span of leaves on a plant (i.e. increase the number of leaves senescing on a plant) and thereby possibly reduce the amount of nutrients and energy available for other parts of the plant.

Any possible effect of the pathogen on the reproductive potential of crofton weed is not known. However, if a shorter leaf life-span reduces the availability of nutrients and energy, it is possible that the reproductive potential of the plant will be reduced.

Currently crofton weed is considered to be under negligible control by either of the biocontrol agents in South Africa. Even though there is obvious damage by the agents the management of crofton weed generally relies on other means of control, such as chemical and mechanical (Zimmermann *et al*., 2004).

Figure 1.6: *Passalora ageratinae* leaf-spots on the lower leaves of *Ageratina adenophora* stems.

1.4 Multiple biocontrol agent releases

Often weed biocontrol programmes involve the release of more than one agent. However, the number of biocontrol agents needed for the successful control of a weed has not been determined. A review of 59 weed biocontrol projects showed that multiple species released against weeds did not adversely affect the establishment rate of agents, and most importantly the success of biocontrol projects increased with multiple agents (Denoth *et al.,* 2002). Of the 59 projects, 28 projects were successful using multiple agents, 75% of which involved between two and

five agents, and 25% of which involved from six to 25 species (Denoth *et al.,* 2002). However, of all the projects 54% attributed success to only one of the agents and two to three species were responsible for the success of 39% of the projects.

Two models have been proposed to explain the higher success rate of biocontrol projects with multiple agents in comparison with single agents. The cumulative stress model proposes that several agents are involved in the control of the weed (Harris, 1985). In this model the agents interact synergistically, increasing the stress on the plant and thereby increasing the efficacy of biocontrol on the target weed (Hoffmann & Moran, 1998; Stiling & Cornelissen, 2005; Campanella *et al*., 2009; Rayamajhi *et al*., 2010; Turner *et al*., 2010). The lottery model predicts that only one agent is responsible for the control of the weed, and that the release of multiple agents increases the chance that this 'right' agent will be released (Myers, 1985, 2008; McEvoy & Coombs, 2000). The lottery model also proposes that different agents may be more or less suitable in different locations.

The biocontrol programme in Hawaii and New Zealand, for mistflower, *Ageratina riparia* (Regel) R. King and H. Robinson (Asteraceae), closely related to crofton weed, has been successful using three biocontrol agents. Together the leaf pathogen, *Entyloma ageratinae* Barreto and Evans (Ustilaginomycetes), leaf-attacking plume moth, *Oidaematophorus benefices* Yano and Heppner (Pterophoridae), and stem galler, *Procecidochares alani* Steyskal (Tephritidae), have resulted in substantial to complete control of mistflower throughout Hawaii (Gardner & Davis, 1982; Julien & Griffiths, 1998). The stem galler is capable of controlling mistflower by halting stem growth (Morin *et al*., 1997). Further research in both Hawaii and New Zealand shows that the fungus is the primary biocontrol agent of mist flower, significantly reducing mist flower infestations (Morin *et al*., 1997; Trujillo, 2005; Barton *et al*., 2007). The gall fly is established at some of these infestations and provides control along with the fungus. The plume moth is not as significant in controlling

mistflower, but is the preferred biocontrol agent in drier areas, which are not suitable for the leaf pathogen (Morin *et al*., 1997).

Synergistic relationships between insects and pathogens are responsible for the first successes in biocontrol for *Opuntia stricta* and *Hypericum perforatum* (Caesar, 2000). However, biocontrol agent selection has focused largely on insect species, with little attention given to plant pathogens. In South Africa, of the 284 biocontrol agents that have been considered, 83% were phytophagous insects and only 15% plant pathogens (Klein, 2011). Biocontrol programmes may improve their success by including plant pathogens that work synergistically with the insects released (Caesar, 2000, 2003). The relationship between insects and pathogens may be synergistic in terms of pathogen transmission by the insect, and insects in addition to pathogens may increase the impact on the target weed (Hatcher, 1995).

One way to understand the interaction between two biocontrol agents of a target weed is to measure the impact they have on the plant (Hatcher & Paul, 2001). Hatcher (1995) proposed four response categories; synergistic, additive, equivalent and inhibitory; to explain the effects of the interactions between an herbivorous insect and a fungal pathogen on plant productivity. These categories are defined as the interactions that cause a reduction in a plant variable:

- significantly greater than that obtained from adding damage from the insect and pathogen acting alone (synergistic)
- equivalent to that obtained from adding damage from the insect and pathogen acting alone (additive)
- equivalent to the damage obtained from either the insect or the pathogen alone (equivalent)
- significantly less than that caused by the weaker of the two agents alone (inhibitory) (Hatcher, 1995).

Turner *et al*. (2010) further modified the additive category to that the combined impact of the two agents is greater than the highest impacting

agent acting alone, but less than or equal to the sum of the impacts caused by each agent acting alone.

1.5 Post-release evaluations

There is a skewed distribution of priorities in biocontrol programmes, with preference given to selecting, screening and releasing agents and little attention given to post-release evaluations (Thomas & Willis, 1998; Kluge, 2000; McEvoy & Coombs, 2000; Morin *et al*., 2009). However, post-release evaluations are very important in biocontrol programmes, providing valuable information on (i) the effectiveness of the agent, (ii) ecological interactions occurring in the field and, (iii) evaluations of plant ecology and response to the agents. All of which provide improved understanding, prediction and management guidelines for controlling weed invasions (McEvoy & Coombs, 2000; Raghu *et al*., 2006).

(i) The only way to evaluate the effectiveness of a biocontrol agent in the field is through post-release evaluations (Kluge, 2000). If the evaluation shows the biocontrol agent to be effective, the programme can be reported as successful and provide lessons and guidance for future programmes. For instance, the example of the *Azolla filiculoides* Lamarck (Azollaceae) biocontrol programme in South Africa (McConnachie *et al*., 2004) shows how adequate release and establishment techniques, as well as good climatic matching enhanced the potential for success. In addition, if the agent is successful, other control mechanisms, such as mechanical and chemical control, which are expensive and time-consuming can be reduced or stopped. If the agent is ineffective, the post-release evaluation can show why and steps can be taken to improve the biocontrol programme. Specifically, post-release evaluations allow a biocontrol programme on a weed to be an iterative process.

(ii) Post-release evaluation provides information on the ecological interactions of the agent, target weed and components of the ecosystem in which the weed is invasive. It reveals the factors influencing the

effectiveness of the biocontrol programme. Firstly, these evaluations often show any interactions between the introduced agent and native enemies (Thomas & Willis, 1998). This knowledge informs scientists that a new agent may be needed, and that taxa which are not susceptible to parasitism by native enemies should be given priority. Secondly, any interaction of the agent with native vegetation can be assessed (Thomas & Willis, 1998). Thereby, alerting scientists to any early non-target affects, increasing the potential to rectify the situation, and also to prohibit further releases of the agent.

(iii) Another valuable contribution of post-release evaluation is an improved understanding of the target weed's ecology and response to the biocontrol agent in the 'invaded' country. Firstly, knowing the weed's life cycle, such as at what time of the year flowering occurs, and relating this to the life-cycle of the agent can highlight phenological asynchrony. For example, the damage caused by an agent may be too late in the weeds life-cycle to reduce seed output, such as with *Solanum sisymbriifolium* Lamarck (Solanaceae) (King *et al*., 2011). Secondly, understanding the response of weeds to the biocontrol agents is beneficial in understanding the ecology of the weed (Pearson & Callaway, 2003).

Failure of lantana biocontrol programmes is attributed to many factors, one of which is indecision over the best part of the plant to attack (Broughton, 2000), as can be seen from the range of lantana feeders introduced into South Africa (Urban *et al*., 2011). Many of the currently released agents were collected opportunistically, but future agent selection programmes should move towards more rational and disciplined approaches (Kluge, 2000; Morin *et al*., 2009). That is, instead of collecting natural enemies of the weed in the country of origin, screening them for host-specificity and then releasing them into the field; agent selection should involve investigation into the weed and its weaknesses, giving guidelines for the type of agent which should be sought in the country of origin (Briese, 2006; Raghu *et al*., 2006). One such suggestion is to simulate herbivory

on different parts of the target weed, allowing insight into which part of the plant is best attacked for optimal control (Raghu & Dhileepan, 2005).

In South Africa, no comprehensive post-release evaluations have been conducted to assess whether the fly and/or pathogen are controlling crofton weed infestations, or if another agent is needed. A recent survey of the pathogen and crofton weed populations in the Magaliesberg, suggests that the pathogen is not having an effective impact on the vegetative growth of crofton weed (Buccellato, 2004). A post-release evaluation and assessment of crofton weed and the two biocontrol agents released against it allows for the opportunity to examine some key issues in biocontrol. Namely multiple versus single agent releases, pathogen and insect interactions, parasitism of biocontrol agents, and new agent selection.

1.6 Field sites

The field surveys in this study were undertaken at three field sites across South Africa (Fig. 1.1, 1.7). Initially it was decided to survey crofton weed infestations in three provinces of South Africa based on the presence or absence of the fly and pathogen. Pietermaritzburg (KwaZulu-Natal) was chosen as both the fly and pathogen are present, and Magaliesberg (North West Province) was chosen as only the pathogen was present when the surveys were conducted. Barberton (Mpumalanga) was chosen as it was believed not to have any agents present, because the infestations were previously unknown and no release of biocontrol agents had been undertaken in this area. However, the pathogen was subsequently found on plants in Barberton, and it is believed that the pathogen dispersed over 300km on its own from the Magaliesberg area (Heystek *et al*., 2011). The Magaliesberg site is along a river on a privately owned farm, which is mainly used for school camps. There were two sub-sites chosen at the beginning of the study at Pietermaritzburg (along a roadside and in a forest area) and at Barberton (along a roadside and under a pine forest

canopy), however the forest site in Pietermaritzburg and roadside site in Barberton were completely cleared after the first survey, and therefore were subsequently excluded from this study.

Figure 1.7: Photographs of the three *Ageratina adenophora* infestations surveyed in the post-release evaluation conducted in this study.

1.7 Structure of the thesis

This thesis is divided into seven chapters, each chapter is written as a stand-alone paper, and therefore unfortunately some repetition occurs between the chapters. In order to reduce the repetition, detailed descriptions of the study species have not been included in all the chapters, and where methodology is repeated between chapters the initial chapter is referred to. The thesis layout is as follows:

- Chapter 1: A general introduction to the thesis, study species, aims and objectives, and thesis layout.
- Chapter 2: Assesses the impact of the biocontrol agents on crofton weed vegetative growth under laboratory conditions.
- Chapter 3: Assesses the impact of the biocontrol agents on crofton weed vegetative growth under field conditions.
- Chapter 4: Assesses the impact of the biocontrol agents on crofton weed reproductive output under laboratory and field conditions.
- Chapter 5: Assesses the impact of the biocontrol agents on the ecophysiology of crofton weed under laboratory conditions.
- Chapter 6: Field surveys of crofton weed, and the fly and pathogen populations at three field sites in South Africa.
- Chapter 7: A general discussion of the thesis overall findings, as well considerations for the future biocontrol of crofton weed in South Africa.

Chapter 2: Interactions between a stem gall fly and a leaf-spot pathogen in the biological control of *Ageratina adenophora*.

Parts of this chapter have been published in the journal Biological Control.

Buccellato, L., Byrne, M.J. and Witkowski, E.T.F. (2012). Interactions between a stem gall fly and a leaf-spot pathogen in the biological control of *Ageratina adenophora*. *Biological Control, 61,* 222-229.

2.1 Abstract

Many biological control projects involve the release of multiple agents. *Ageratina adenophora* (crofton weed) has had two biocontrol agents, *Procecidochares utilis*, a stem gall fly, and *Passalora ageratinae*, a leaf-spot fungal pathogen, released against it in South Africa. This study investigated whether the two biocontrol agents, individually or together, increased or decreased the impact on crofton weed under greenhouse conditions. Six month old plants were exposed to one of six treatments (n=15 plants/treatment): control (no agents), pathogen-only, single-galled only, double-galled only, pathogen-single galled, and pathogen-double galled, all for a period of six months. Individually, both agents, reduced stem height and percentage of live leaves; however there was no synergistic effect of the two agents together. Pathogen-double galled plants had significantly fewer pathogen infected leaves relative to the other pathogen infected treatment plants, suggesting a negative interaction between the two agents on pathogen establishment. Pathogen infection did not affect the size of the fly's galls. Double galling by the fly inhibited stem growth above the gall position on the stem. Crofton weed compensated for galling through vegetative reproduction, by increasing the number of sideshoots. The pathogen inhibited sideshoot growth, thereby adding to the stress on the weed. Overall, repeated galling by the fly caused the greatest impact on the growth of crofton weed and the pathogen inhibited sideshoot growth, resulting in an additive interaction between the two agents on the biocontrol of crofton weed.

2.2 Introduction

Are two biocontrol agents better than one? Weed biocontrol programs often involve the release of more than one agent (Julien & Griffiths, 1998, McEvoy & Coombs, 2000). However, the ideal number of biocontrol agents needed for the successful control of a weed has not been established (Myers, 1985; Denoth *et al.,* 2002; Stiling & Cornelissen, 2005). If several agents are released the question arises as to whether their interactions will be synergistic, complimentary, or antagonistic and if biological control will be improved or deterred (Myers, 1985).

A review of 59 weed biocontrol projects showed that multiple species released against weeds did not affect the establishment rate of the agents, and most importantly, the success of biocontrol projects increased with multiple agent releases (Denoth *et al.,* 2002). However, of all the projects, 54% attributed success to only one of the agents and two to three species were responsible for the success of 39% of the projects (Denoth *et al.,* 2002).

Ageratina adenophora, (Sprengel) King and Robinson (syn. *Eupatorium adenophorum* Sprengel) (Asteraceae), also known as crofton weed or Mexican Devil weed, originates from Mexico, and is an invasive weed in several countries worldwide, including South Africa, Australia, New Zealand, Hawaii, India and China (Julien & Griffiths, 1998). Crofton weed is a perennial herb, with a woody rootstock, and many stems reaching up to 2m in height (Bess & Haramoto, 1959; Henderson, 2001; Page & Lacey 2006). Trailing crofton weed stems root where they touch the soil, resulting in dense infestations (Bess & Haramoto, 1959; Morris, 1991). Crofton weed was probably first introduced into South Africa as an ornamental before 1948 (Hilliard, 1977). Within South Africa crofton weed invades steep slopes and wet areas along streams, roadsides, forests and plantations in the KwaZulu-Natal, Western Cape, North West, Limpopo, Mpumalanga and Gauteng provinces (Henderson, 2001; Heystek, 2008).

In South Africa, two biocontrol agents have been released against crofton weed, a stem gall fly, *Procecidochares utilis* Stone (Tephritidae), and a leaf-spot fungal pathogen, *Passalora ageratinae* Crous and A.R. Wood (Mycosphaerellaceae) (previously named *Cercospora eupatorii* Peck or *Phaeoramularia* sp.) (Plant Protection News, 1987a; Kluge, 1991; Crous *et al*., 2009). The stem gall fly was released near Pietermaritzburg, KwaZulu-Natal, between 1984 and 1987, and more recently in the Magaliesberg, North West Province (Kluge, 1991; Heystek *et al*., 2011). The leaf-spot pathogen was released in KwaZulu-Natal and the south-western Cape in 1987 (Plant Protection News, 1988), and later in the Magaliesberg, North West Province (Heystek *et al*., 2011). Both agents are well established in South Africa (Kluge, 1991).

Female gall flies lay eggs on or near the apical bud; larvae move to the base of the leaves and tunnel into the stem, where they feed on plant tissues, resulting in the formation of galls (Haseler, 1965; Bennett, 1986; Bennett & Van Staden, 1986). Previous laboratory trials concluded that the fly is an effective biocontrol agent that should significantly reduce weed populations in the field (Bennett, 1986). Erasmus *et al*. (1992) found that the fly is capable of halting stem growth in the laboratory, and if the stem is subjected to repeated generations of flies (i.e. more than one gall per stem) the stem may die, and even whole plants may die.

In South Africa, the pathogen has to date only had a negligible impact on crofton weed (Olckers, 2004; Heystek, 2008). A preliminary survey in the Magaliesberg, North West, in 2004 showed that the pathogen may increase the proportion of dead leaves on plants, but does not significantly influence any other plant variable (L.Buccellato, unpublished data).

The gall fly has however successfully controlled crofton weed infestations in Hawaii (Bess & Haramoto, 1959, 1972). Along the east coast of Australia both the gall fly and pathogen have reduced plant vigour and slowed encroachment of crofton weed populations (Page & Lacey, 2006).

The control of crofton weed infestations in New Zealand has been partially successful as a result of the release of the gall fly (Fowler *et al*., 2000).

Insect-plant pathogen interactions may be synergistic when the action of one agent increases the ability of the other agent to attack the target weed (Caesar, 2000, 2003; Moran, 2005). However, it is also possible for insect-plant pathogen interactions to be antagonistic. In Hawaii, two biocontrol agents, a gall fly, *Procecidochares alani* Steyskal (Tephritidae), and a white smut fungus, *Entyloma ageratinae* Barreto and Evans (Ustilaginomycetes), were released against mist flower, *Ageratina riparia* (Regel) R. King and H. Robinson (Asteraceae). It was initially suggested that antagonism might arise if the fungal infection defoliated the whole plant, thereby removing the gall fly oviposition sites, which are the new leaves at the shoot tip (Morin *et al*., 1997). However, the biocontrol programme of mist flower in both Hawaii and New Zealand has been successful with the combination of both agents. Further research in both Hawaii and New Zealand shows that the fungus is the primary biocontrol agent of mist flower, significantly reducing mist flower infestations (Morin *et al*., 1997; Trujillo, 2005; Barton *et al*., 2007). The gall fly is established at some of these infestations and provides control along with the fungus.

One way to understand the interaction between two biocontrol agents of a target weed is to measure the impact they have on the plant (Hatcher & Paul, 2001). Hatcher (1995) proposed four categories; synergistic, additive, equivalent and inhibitory; to explain the effects of the interactions between an herbivorous insect and a fungal pathogen on plant productivity. These categories are defined as the interactions that causes a reduction in a plant variable: (a) significantly greater than that obtained from adding damage from the insect and pathogen acting alone (synergistic), (b) equivalent to that obtained from adding damage from the insect and pathogen acting alone (additive), (c) equivalent to the damage obtained from either the insect or the pathogen alone (equivalent) and (d) significantly less than that caused by the weaker of the two agents alone (inhibitory) (Hatcher, 1995). Turner *et al*. (2010) further modified the

additive category to that the combined impact of the two agents is greater than the highest impacting agent acting alone, but less than or equal to the sum of the impacts caused by each agent acting alone.

While previous laboratory trials have investigated the fly, *P. utilis*, as a biocontrol agent for crofton weed in South Africa (Bennett, 1986, Erasmus *et al*., 1992), no evaluation of the leaf-spot pathogen, *P. ageratinae,* as a biocontrol agent has been done (Morris, 1991). The aim of this study was to evaluate the effectiveness of *P. utilis* and *P. ageratinae* as biocontrol agents, individually and jointly, on the vegetative growth of *A. adenophora*, and to assess what type of interaction occurs between these two biocontrol agents, under controlled laboratory conditions.

2.3 Material and methods

In September 2005, achenes (fruits) were randomly collected from several plants within *A. adenophora* infestations in the Magaliesberg area, South Africa (25˚49'45.1"S, 27˚26'26.0"E). Achenes were dried and stored in brown paper bags, at room temperature, in a laboratory at the University of the Witwatersrand, Johannesburg, Gauteng (26˚11'20.97"S, 28˚01'55.37"E) for 11 months, and then used to grow plants for laboratory trials. A stock culture of the pathogen was garnered by collecting leaves infected with *P. ageratinae*, from several plants at the same site in Magaliesberg, in October 2006. These infected leaves were tied to crofton weed plants in the greenhouse, on the University of the Witwatersrand campus. Fungal leaf spots were visible on the plants eight weeks later. These infected plants were then kept outside the greenhouse and used as a source of fresh, pathogen-infected leaves for the laboratory trials. In order to initiate a fly breeding programme for the laboratory trial, galls were collected in December 2006, from *A. adenophora* infestations in Pietermaritzburg (29˚33'12.4"S, 30˚20'04.0"E). Pupae were dissected out of the galls and placed on moist cotton wool in glass vials, and kept at room temperature until adults emerged. *Procecidochares utilis* adults were

allowed to mate and then placed onto plants, in cages, in the above greenhouse. These plants were successfully galled, and emerging flies were allowed to mate and gall the plants further, providing a stock culture of flies for the laboratory trial.

2.3.1 Laboratory trials

The laboratory trial was run in a separate section of the above greenhouse, which had 30% shading, and summer temperatures of 26.7±1.5ºC (mean ± SE) and winter temperatures of 15.6±1.5ºC. The relative humidity ranged from 41% to 100% (mean=78.5%) in the summer months (December 2006 to February 2007), and 30% to 100% (mean=77.6%) in the winter months (June 2007 to August 2007).

Approximately 100 crofton weed seedlings were grown from stored achenes, sown in August 2006, into 5-litre plant bags filled with potting soil. Seeds were sown just below soil surface and were not given any germination pre-treatment. Seedlings started to emerge after seven days and continued for a further 21 days. In January 2007 the approximately 50cm tall plants were cut back to soil level, to promote new stem and leaf growth. Plants were well watered once a day for half an hour, in the morning, and fertilized once a month with *Nitrosol* (N 8%: P 2%: K 5.8%).

At the end of March 2007, 15 plants were randomly allocated to each of the following six treatments:

(i) no biocontrol agents (control);
(ii) plants infected with the fungal pathogen *P. ageratinae* only (hereafter referred to as pathogen-only);
(iii) plants exposed to one release of *P. utilis* (hereafter referred to as single-galled);
(iv) plants exposed to two releases of *P. utilis* (hereafter referred to as double-galled);
(v) plants infected with *P. ageratinae* and exposed to one release of *P. utilis* (hereafter referred to as pathogen-single galled);

(vi) plants infected with *P. ageratinae* and exposed to two releases of *P. utilis* (hereafter referred to as pathogen-double galled).

In order to restrict the spread of the pathogen between the treatments, plants in the non-pathogen treatments were sprayed with a broad-spectrum fungicide, AMISTAR ®. A mechanical pressurized sprayer was calibrated to a spray volume of 60-litre/ha; AMISTAR was sprayed at 500ml/ha every four weeks. Plants that were infected with the pathogen were sprayed with the same volume of water.

A control trial was run, from March 2007 to November 2007, to measure any stimulatory or inhibitory influence AMISTAR may have had on *A. adenophora* growth. Thirty plants were grown concurrently in the greenhouse, under the same conditions as the laboratory trial plants. Fifteen of these plants were sprayed with the fungicide AMISTAR, as above, and the other fifteen plants were sprayed with water as above.

In order to prevent *P. utilis* ovipositing on non-fly treatment plants, the plants of all treatments were placed in separate cages. The cages were 1m tall x 1.2m x 0.9m frames of conduit piping, covered in fine white netting.

The trial *A. adenophora* plants in the pathogen treatments were infected with the pathogen by tying fresh infected leaves, from the stock culture, onto their stems in mid-April 2007. Plants from all treatments were then covered with plastic bags, to create a dew period of 24 hours (Wang *et al*., 1997). The first fungal leaf spots appeared in late May 2007, and continued to naturally infect new leaf growth under greenhouse conditions. Pathogen infection occurred before stems were galled in these trials. However, up to 95% of crofton weed stems are infected with the pathogen from an early stage in the field, therefore in the field stems are infected with the pathogen before galling occurs (L. Buccellato, unpublished data).

The first flies were released onto plants at the beginning of June 2007. One pair of flies was released per plant into the cages of the double galled treatments (treatments iv and vi). Single galled treatment plants were not

exposed to flies in the initial release as there were insufficient flies to infest these plants. Flies emerged from galls 10 weeks later and were collected, for 3 weeks, in order to stop the emerging flies from further ovipositing on the treatment plants. The second release of flies took place in September 2007. One pair of flies was released per plant onto all four of the single and double galled treatments (treatments iii–vi).

Due to the large number of stems per plant (approximately 15 to 20 stems), four stems were randomly selected per plant for measurements of vegetative growth. Pre-treatment and then monthly post-treatment measurements per trial plant included stem height, number of sideshoots, number of living leaves, number of dead leaves attached to the stem and number of abscised leaves, from January 2007 to November 2007. The dry biomass of the roots of the whole plants were weighed, however there was no significant interaction or effect and the results have not been included. Prior to the first fly release, oviposition sites (apical leaf buds) were marked, with a piece of sewing thread, on all the stems in order to measure the growth of the stem above the point of oviposition, and the internode length below and above the oviposition site. Oviposition site therefore refers to the position of these apical leaf buds, on all of the stems of the six treatments, at the time of the first fly release. Oviposition sites were not marked for the second fly release as the stems were flowering and no apical leaf buds were present. Monthly assessments also included the number of leaves per stem infected with the pathogen. Gall measurements included gall length and diameter (at the longest/broadest point respectively); which were used to calculate a gall size index (gall diameter x gall length, as calculated by Bennett (1986)). The number of emergence holes was counted 10 weeks after flies were released, and used as an index of the number of adult flies successfully emerging from galls.

2.3.2 Data analysis

Student t-tests were used to assess differences in gall indices between the first generation of flies on the double-galled and pathogen-double galled stems during the first *P.* utilis release in June 2007. One-way ANOVA's were used to assess differences in gall indices between the galled treatment stems after the second *P. utilis* release in September 2007. The influence of the biocontrol agents on the vegetative growth of crofton weed over the trial period was assessed using repeated measures ANOVA with nesting (stems nested within plants) (GLM procedure, StatSoft, 2007). One-way ANOVA with nesting and LSD tests were used to assess differences in stem measurements between treatments for each month. Two-way ANOVA's were also used to determine significant interactions between single galled and pathogen infection, and double galled and pathogen infection.

2.4 Results

2.4.1 Impact of AMISTAR ® on Ageratina adenophora *growth*

The fungicide, AMISTAR, caused no significant impact on stem height ($F_{1,89}$=0.51, P=0.482), percentage of live leaves ($F_{1,89}$=2.64, P=0.108) and the number of sideshoots ($F_{1,89}$=0.04, P=0.836) relative to untreated plants during the laboratory trials.

2.4.2 Agent establishment and infection severity

The pathogen did not infect more than 40% of living leaves per stem at any time during the six months of the experiment (Fig. 2.1). A repeated measures ANOVA showed a significant difference between the treatments ($F_{5,103}$=405.04, P<0.001). At the end of the trial there was no significant interaction between single galled and pathogen infection ($F_{1,132}$=1.66, P=0.199). However, in July ($F_{1,132}$=7.25, P=0.008), August ($F_{1,132}$=5.03, P=0.027) and September ($F_{1,132}$=3.76, P=0.044) there was a significant

interaction, in the percentage of leaves infected with the pathogen, between double galled and pathogen infection. Stems that were galled twice had a lower percentage of leaves infected with the pathogen, indicating an antagonistic relationship between the two biocontrol agents.

Neither the gall size index (t_{69}=1.62, P=0.110), nor the number of adult flies emerging (t_{69}=1.29, P=0.201) from the first generation galls differed significantly on pathogen infected and uninfected stems in June 2007 (Fig. 2.2). Similarly the gall size index ($F_{3,147}$=1.29, P=0.280) and the number of adult flies emerging from the galls ($F_{3,147}$=1.89, P=0.133) did not differ significantly between treatments after the second fly release in September 2007 (Fig. 2.3).

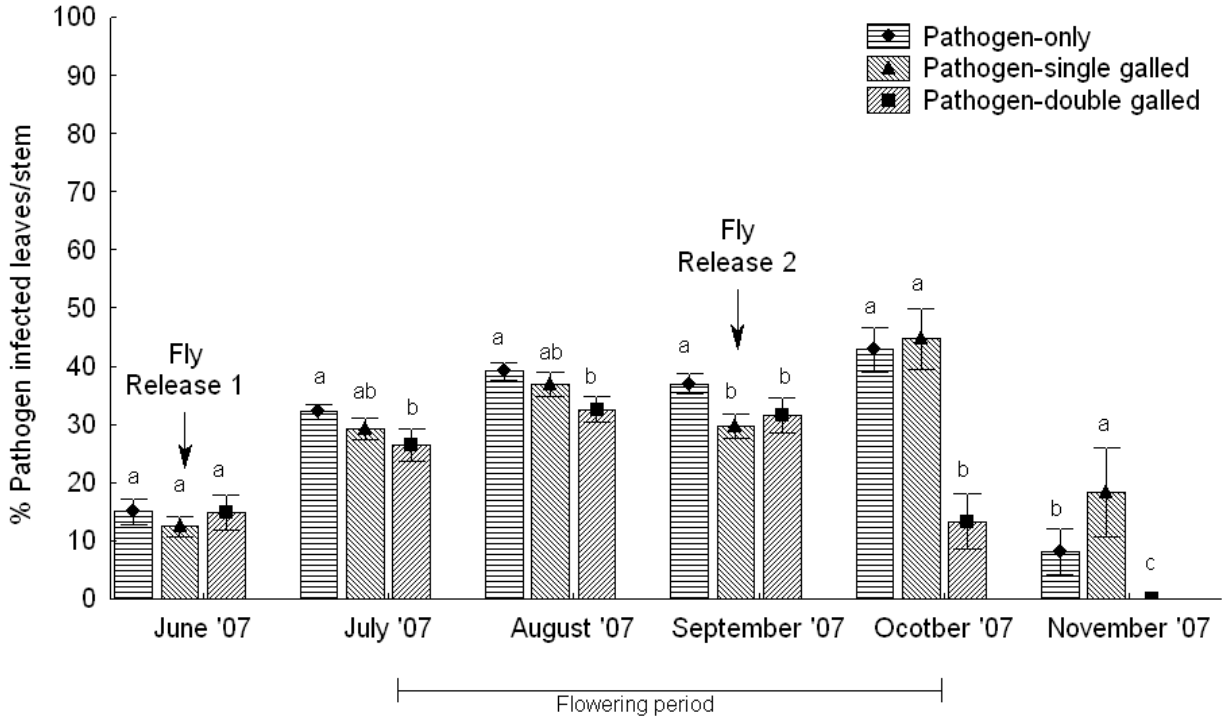


Figure 2.1: The percentage of living leaves infected with the fungal pathogen *Passalora ageratinae* per *Ageratina adenophora* stem, when either galled or ungalled by the fly *Procecidochares utilis*, from June 2007 to November 2007. For each month, means followed by the same letter are not significantly different, at P<0.05 (LSD). (Means ± SE).

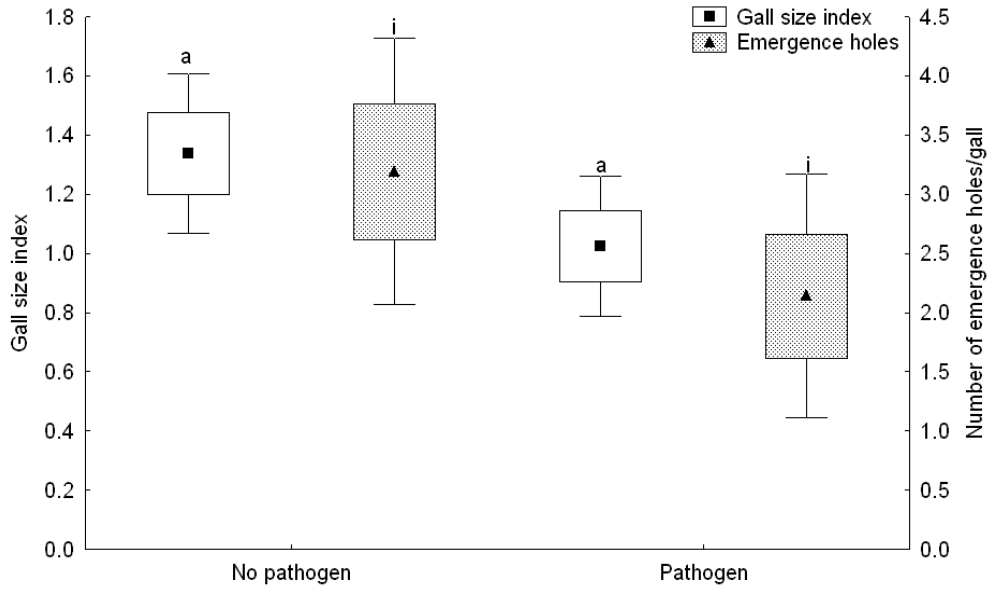


Figure 2.2: Gall size index (gall diameter x length) of the first generation of *Procecidochares utilis* on *Ageratina adenophora* double galled treatment stems infected and uninfected with the fungal pathogen *Passalora ageratinae*. The number of first generation *P. utilis* adults (represented by the number of emergence holes) emerging from galls on double galled stems infected and uninfected with the pathogen; from the first release of *P. utilis* in June 2007. Means followed by the same letter are not significantly different, at *P*<0.05 (t-test). (Mean; Box: Mean ± SE; Whisker: Mean ± 1.96*SE).

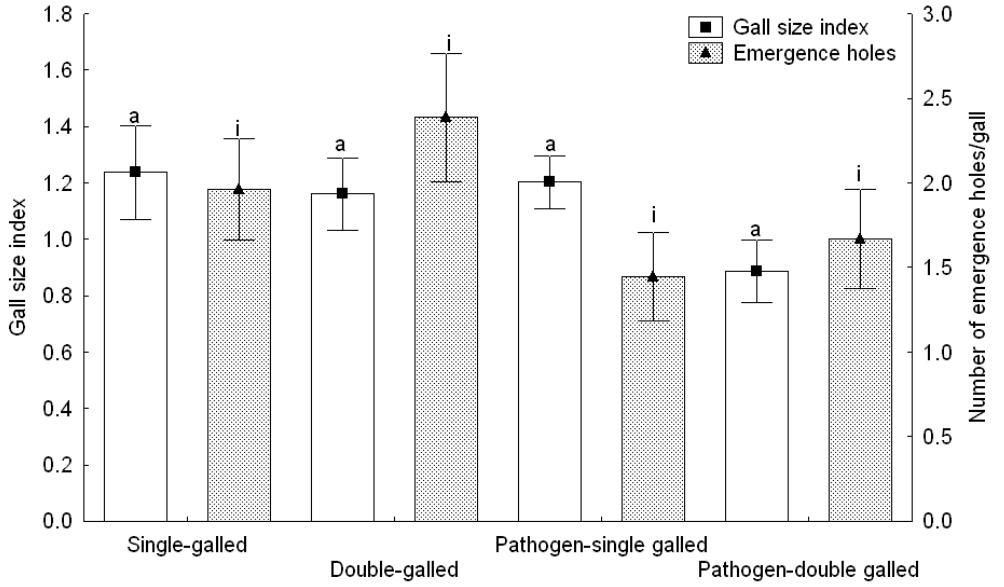


Figure 2.3: Gall size index (gall diameter x length) of the first generation (single galled stems) and the second generation (double galled stems) of *Procecidochares utilis* on *Ageratina adenophora* stems infected and uninfected with the fungal pathogen *Passalora ageratinae*. The number of *P. utilis* adults (represented by the number of emergence holes) emerging from the first generation galls (single galled stems) and the second generation galls (double galled stems) on pathogen infected and uninfected stems; from the second release of *P. utilis* in September 2007. Means followed by the same letter are not significantly different, at *P*<0.05 (LSD). (Means ± SE).

2.4.3 Stem height

Over the duration of the trial there was a general trend for stem height to increase into the flowering period, thereafter it decreased in all treatments when the flower stalks were shed, there was also a significant difference between the treatments ($F_{5,103}$=2.69, P=0.025) (Fig. 2.4). Within two months of the initial fly release, growth in stem height slowed on double-galled and pathogen-double galled plants ($F_{5,103}$=4.16, P=0.002). The pathogen alone suppressed stem growth from September onwards. At the end of the trial, both of the biocontrol agents, individually and in combination, had significantly inhibited stem height ($F_{5,103}$=4.92, P<0.001). The control stems were the tallest in November 2007, having resumed vertical growth after the flowering period. Stems that had been galled twice, with or without the pathogen, were significantly the shortest. There was no significant interaction between one fly release and pathogen infection ($F_{1,132}$=0.05, P=0.818) or two fly releases and pathogen infection ($F_{1,132}$=1.35, P=0.247) on stem height. In terms of stem height, there was an equivalent interaction between the agents, with the fly causing the greatest damage.

2.4.4 Stem growth above the oviposition site

Monthly stem growth above the oviposition site differed significantly over the trial period ($F_{5,103}$=23.85, P<0.001) (Fig. 2.5). Double galling clearly suppressed growth above the oviposition site. By November the control and pathogen-only treatment stems had a significantly greater growth above the oviposition site compared to the double galled treatments ($F_{5,103}$=4.29, P<0.001). Double galling halted growth above the oviposition site after flowering. There was no significant interaction between the pathogen and single galled treatments ($F_{1,132}$=0.09, P=0.755). However, the pathogen and double galled treatments showed a significant interaction in July ($F_{1,132}$=4.76, P=0.031). Stems that had been galled and infected with the pathogen had a slower growth above the oviposition site.

The interaction between the fly and pathogen is equivalent, and as with stem height the fly caused the greatest damage.

2.4.5 Internode length above oviposition site

Reduced stem growth was also seen in shorter internode lengths above the oviposition site of the double-galled (mean±SE = 22.9 ± 3.34mm) and pathogen-double galled (25.8 ± 3.26 mm) treatments. In comparison, the control (57.98 ± 2.91 mm), pathogen-only (55.05 ± 2.63 mm), single-galled (44.65 ± 4.94 mm) and pathogen-single galled (45.25 ± 4.77 mm) treatments had much longer internodes ($F_{5,103}$=7.15, P<0.001).

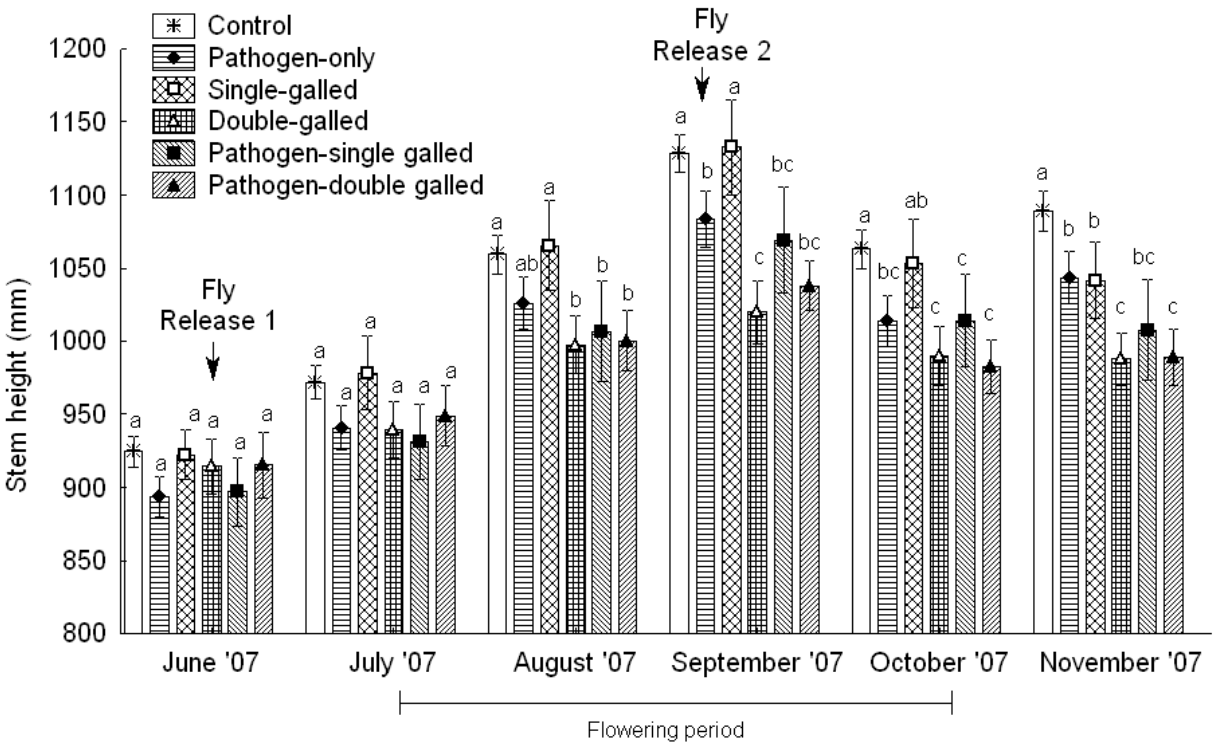


Figure 2.4: Height of *Ageratina adenophora* stems in response to six different treatment conditions, of pathogen infection and/or fly galling (single or double), from June 2007 to November 2007. For each month, means followed by the same letter are not significantly different, at P<0.05 (LSD). (Means ± SE).

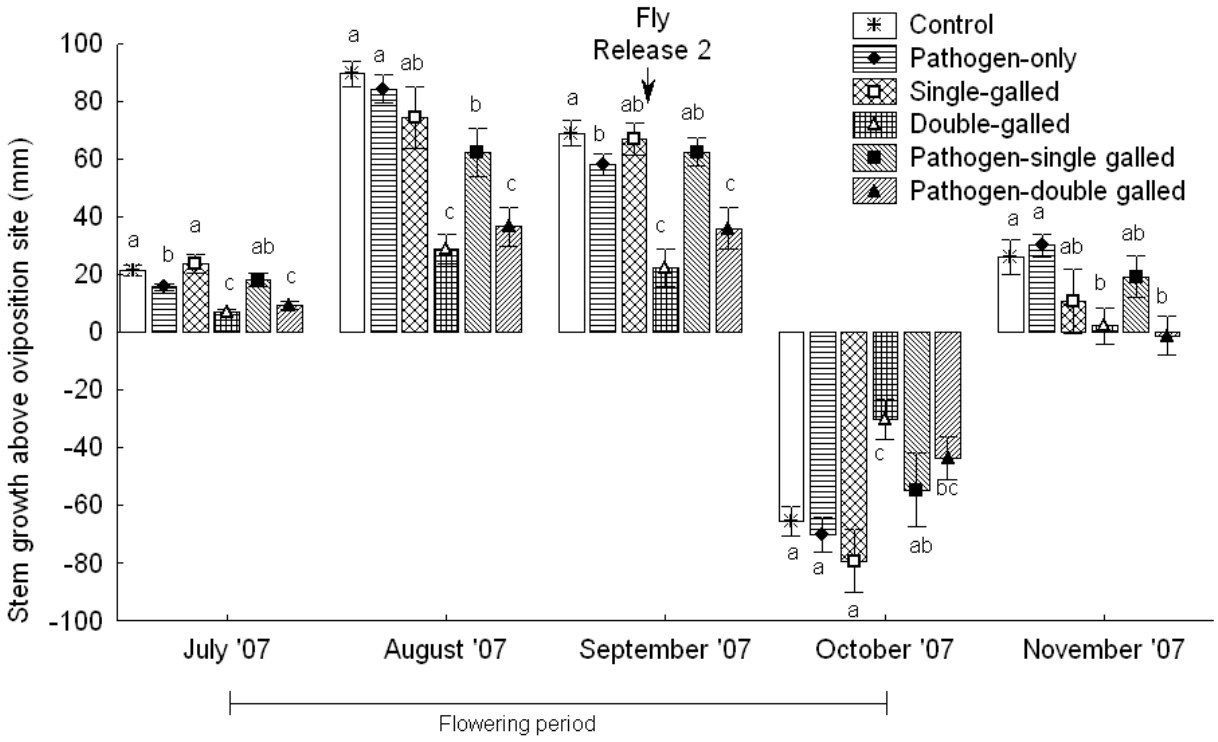


Figure 2.5: Monthly growth of *Ageratina adenophora* stems above the oviposition site, in response to six different treatment conditions, of pathogen infection and/or fly galling (single or double), after the first fly release in June 2007 until November 2007. For each month, means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

2.4.6 Percentage of live leaves per stem

Overall there was a general decrease in live leaves per stem in all treatments; however this decrease became significantly different from the control stems over time ($F_{5,103}=16.91$, $P<0.001$) (Fig. 2.6). The agent-infected stems had significantly fewer leaves than the control in August ($F_{5,103}=12.99$, $P<0.001$), September ($F_{5,103}=9.72$, $P<0.001$), October ($F_{5,103}=25.99$, $P<0.001$) and November ($F_{5,103}=5.02$, $P<0.001$). In November there was no significant interaction between the pathogen and single galled ($F_{1,132}=0.42$, $P=0.519$), but there was a significant interaction between the pathogen and double galled ($F_{1,132}=5.81$, $P=0.017$). Stems that were infected with the pathogen and double galled had fewer live

leaves, than stems that were double galled or infected with the pathogen only, resulting in an additive interaction between the two biocontrol agents.

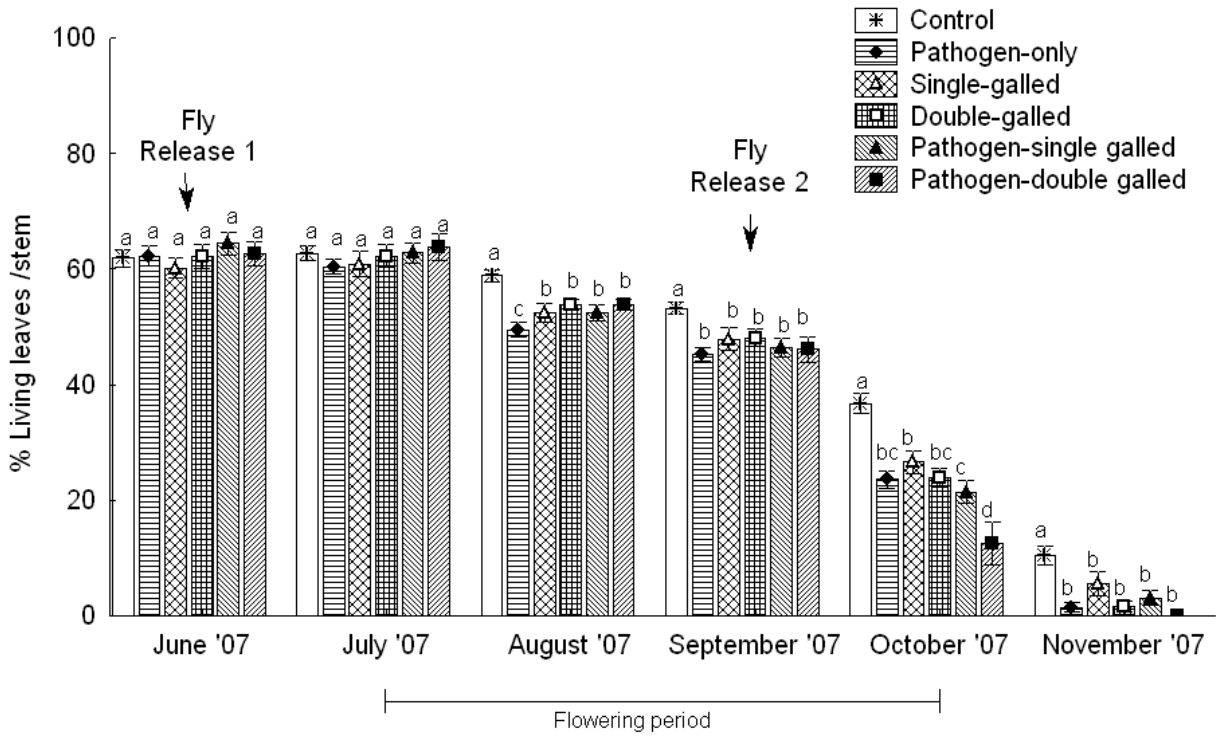


Figure 2.6: Percentage of living leaves on *Ageratina adenophora* stems in response to six different treatment conditions, of pathogen infection and/or fly galling (single or double), from June 2007 to November 2007. For each month, means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

2.4.7 Number of sideshoots per stem

The number of sideshoots per stem differed significantly between treatments by the end of the trial ($F_{5,61}=5.1$, $P<0.001$) (Fig. 2.7). Sideshoot growth was promoted on galled stems without the pathogen present. There was no significant interaction between the pathogen and single galled ($F_{1,132}=0.22$, $P=0.102$), but there was between the pathogen and double galled ($F_{1,132}=4.06$, $P=0.045$). Stems that were galled twice and infected with the pathogen had fewer sideshoots than stems that were galled twice without pathogen infection. Once again there is an equivalent

interaction between the two agents; however in contrast to stem growth the pathogen caused the greatest damage.

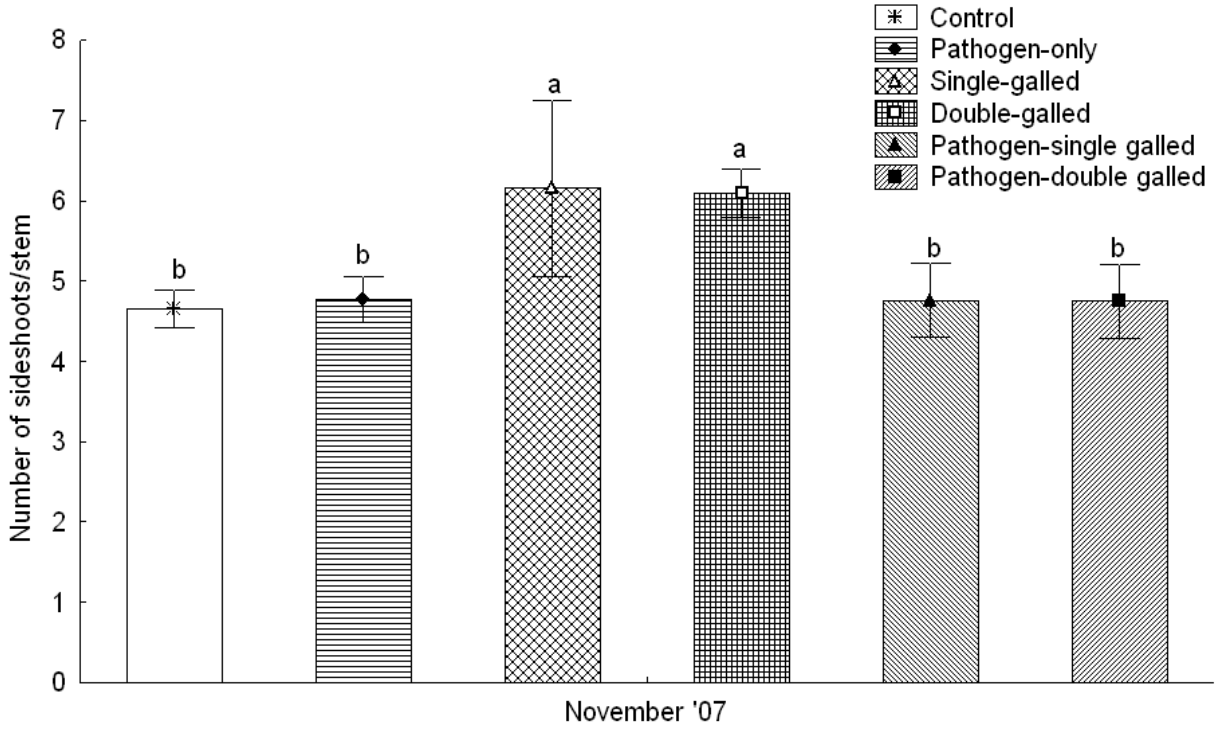


Figure 2.7: The number of sideshoots on *Ageratina adenophora* stems in response to six different treatment conditions, of pathogen infection and/or fly galling (single or double), at the end of November 2007. Means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

2.5 Discussion

Two models have been proposed to explain the higher success rate of weed biocontrol projects with multiple agents in comparison to those with single agents. The lottery model predicts that only one agent is responsible for the control of the weed, and that the release of multiple agents increases the chance that this 'right' agent will be released (Myers, 1985, 2008; McEvoy & Coombs, 2000). The cumulative stress model proposes that all the agents are needed to achieve successful control (Harris, 1985). In this model the agents interact synergistically, increasing the stress on the plant and thereby increasing the efficacy of biocontrol on the target weed (Hoffmann & Moran, 1998; Stiling & Cornelissen, 2005;

Campanella *et al*., 2009; Rayamajhi *et al*., 2010; Turner *et al*., 2010). The interaction between the agents may be synergistic, additive, equivalent or inhibitory (Hatcher, 1995; Turner *et al*., 2010). The present study revealed that whilst the gall fly, *P. utilis*, is predominantly responsible for the greatest control of the vegetative growth of crofton weed, *A. adenophora*, the pathogen, *P. ageratinae* plays the predominant role in reducing vegetative reproduction (sideshoot growth). Combining the impacts of the two agents acting together on different plant variables leads to an overall additive effect on the damage caused to crofton weed. This study also shows that the impact of biocontrol agents can differ, depending on what is measured, such as stem height, at different points in the phenology of the weed.

The use of multiple agents in the cumulative model is based on the hypothesis that if the agents utilize different parts of the plant, there should be little negative interaction between them in terms of establishment and survival on the target weed (Denoth *et al*., 2002). However, although the gall flies attack the apical meristem while the pathogen infects the lower leaves of crofton weed in this system, they may still influence the establishment of each other in terms of the severity of each agent's impact on the plant.

Double galled plants had the lowest percentage of leaves infected with the pathogen, suggesting an antagonistic relationship between the fly and pathogen infection of leaves. It is possible if the gall is acting as a nutrient sink, that it reduces the health of the lower leaves (Florentine *et al*., 2005; Dorchin *et al*., 2006; Moseley *et al*., 2009), making them less likely to become infected. However, the severity of pathogen infection was generally low even on ungalled plants, and field observations indicate that the pathogen only infects up to 30% of live leaves per stem at sites where only the pathogen is present (L. Buccellato, unpublished data). Even when leaf loss was high after the flowering period, the pathogen infected a small percentage of the remaining live leaves. Field observations in New Zealand and greenhouse trials in Australia have shown that the pathogen

can heavily infect and even kill crofton weed seedlings (Wang *et al*., 1997; Page and Lacey, 2006). In contrast, the spread of the pathogen on more mature plants is slower and limited to the lower leaves on the stem, as seen in this trial. This is not unusual in weed-pathogen systems, if the weed has the ability to regenerate quickly (Charudattan, 2005).

The fly larvae tunnel into tissues at the tip of crofton weed stems (Bennett & van Staden, 1986), higher on the stems than the leaves infected by the pathogen. Stress on the plant from the pathogen-infected lower leaves could influence the stem tissue higher up for the gall fly, as seen on *Lantana camara* infested with a sap-sucking lace bug and a root-feeding flea beetle (Simelane, 2006). However, there were no significant differences between gall indices from pathogen infected and uninfected plants, indicating that the pathogen does not antagonistically influence the interaction between the fly population and the weed.

Both of the biocontrol agents on crofton weed, individually, reduced stem height in comparison to the control. Stems exposed to single or double galling, regardless of pathogen infection, were constrained to similar levels. These results indicate that there was an equivalent effect of both agents together on stem height of crofton weed, with the greatest impact on stem height seen with repeated galling by the fly. Previous trials claimed death of stems galled more than once (Dodd, 1961; Kluge, 1991; Erasmus *et al*., 1992), but stem death was not observed in these trials, nor has it been observed at crofton weed infestations in South Africa where the fly is present (L. Buccellato, unpublished data). Galls may exhibit the greatest effect on young, vigorously growing stems, as plants in the study conducted by Erasmus *et al.* (1992) were only approximately 200mm tall when the initial fly releases were made, compared with 900mm in this study.

By the end of these trials the pathogen alone did not inhibit further vertical stem growth, highlighting the limited impact of the pathogen on more mature plants. The effect of the pathogen is possibly at its greatest in the

early stages of infection and on younger plants, thereby killing seedlings, but not influencing adult plant growth over a longer period. It is possible for weeds to overcome the pressure of disease infection from pathogens by rapidly increasing vegetative (compensatory) growth (Charudattan, 2005, 2010). Crofton weed may initially be affected by the pathogen infection, but is able to outgrow the disease if it survives through the seedling stage. Galls are known to act as nutrient sinks by redirecting plant resources to the galls, causing an imbalance in source-sink tissues of the weed (Florentine *et al*., 2005; Dorchin *et al*., 2006; Moseley *et al*., 2009). The reduction in stem growth and the shorter internode lengths above the oviposition site may indicate that *P. utilis* galls act as nutrient sinks, with the weed supplying nutrients to tissues within the galls for larval feeding (Bennett & van Staden, 1986; Erasmus *et al*., 1992). The larvae of the fly tunnel into the apical and differentiating tissues of the stem, thereby slowing vertical stem growth (Bennett & van Staden, 1986). The control stems increased their vertical growth after flowering, whereas the double galled stems halted any vertical growth after flowering. These results indicate once again an equivalent interaction, where repeated galling has the largest impact on growth of the stem, regardless of pathogen infection.

The percentage of live leaves decreased more quickly on stems attacked by the biocontrol agents, both individually and together, relative to the control stems, which also lost their leaves naturally. There was an additive interaction at the end of the flowering period (October 2007), where pathogen-double galled stems had fewer live leaves than the double-galled and pathogen-only stems. Plants may compensate for pathogen infection by shedding diseased leaves more quickly, so that new leaf growth is uninfected (Charudattan, 2005, 2010). An example is the ability of water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae), to accelerate senescence of leaves infected with the pathogen, *Cercospora rodmanii* Conway (Hyphomycetes), so that new leaves may grow unaffected (Charudattan *et al*., 1985). Therefore, this

impact on crofton weed may not have a long term control effect, as leaf loss is not sufficient to completely defoliate stems in a short span of time.

Tall *A. adenophora* stems eventually bend over and trail along the soil, where the sideshoots can then root and subsequently grow into new plants (Muniappan *et al*., 2009). Stems that were only galled, without pathogen infections, produced a higher number of sideshoots than the control or pathogen-only treatments. The loss of apical dominance as a result of galling promotes the growth of sideshoots (Erasmus *et al*., 1992). However, the presence of the pathogen inhibits the number of sideshoots produced by the stems on galled plants, resulting in an equivalent interaction between the agents, with the pathogen causing the greatest damage.

This study highlights that measuring the effects of multiple agents on the biocontrol of a target weed may not always be straightforward. In order to accurately assess the impact of the interaction between biocontrol agents, consideration needs to be given to what plant parameters are measured, and when these measurements are taken in terms of the plant's life-cycle. For example, if sideshoot growth was not measured, or if monthly measurements had not been taken, the conclusion about the efficacy of the agents in combination would be different. The study was conducted under controlled greenhouse conditions, which could under- or overestimate the impacts of the agents, as these optimum conditions are not always representative of conditions in the field (Morin *et al*., 2006, 2009). The efficacy of the agents may differ during different times of the year and at different crofton weed infestations across South Africa. In Hawaii the fly is less successful in high rainfall areas (Bess & Haramoto, 1959, 1972), and in Australia the pathogen is more successful during the drier months of the year (Dodd, 1961).

2.6 Conclusion

There is an overall additive effect of the two agents together increasing the stress on *A. adenophora.* Repeated galling by the fly is responsible for the greatest impact on the stem growth of crofton weed. There was an additive effect of the two agents together on the percentage of live leaves per stem after the flowering period; however this is only a short-term effect as crofton weed eventually loses its leaves naturally. The pathogen inhibits sideshoot growth, which is promoted by galling. Therefore, the sum of the impacts of both the fly and pathogen, on reduced stem growth and limited sideshoot growth, suggests the two agents together have an overall additive effect on crofton weed biocontrol. Based on this laboratory trial, we should expect to observe some control of crofton weed in the field. Hence a study of the two agents in the field has been undertaken. The results presented here indicate that in terms of assessing biocontrol agent efficacy, the interactions between agents may not always be clear and may depend on what plant growth parameters are measured and when these measurements are taken.

Chapter 3: Can laboratory trials testing agent efficacy be extrapolated to field conditions? A test case using multiple agents in the biocontrol of crofton weed.

3.1 Abstract

Two biocontrol agents, a leaf-spot pathogen, *Passalora ageratinae*, and a stem gall fly, *Procecidochares utilis*, have been released against crofton weed, *Ageratina adenophora*, in South Africa. An earlier controlled greenhouse trial using both the fungal pathogen and the gall fly, individually and in combination, predicted an additive interaction between the two biocontrol agents on the control of crofton weed. With galling reducing stem height and growth, and the pathogen reducing sideshoot growth. This study investigated whether the pathogen and fly work in combination to control the vegetative growth of crofton weed, under field conditions, in South Africa, as predicted by greenhouse studies. The field trial was conducted at a site in the Magaliesberg. Four month old stems were exposed to one of the following three treatments (n=20plants/treatment): pathogen-only, pathogen-single galled and pathogen-double galled, for a period of 11 months. The percentage of leaves on stems infected with the pathogen ranged from 10% to 80% through the trial period, with no significant difference in infection between galled and ungalled stems. Small galls developed during the flowering period, as a result of more galls developing in leaf nodes compared to apical leaf buds. Growth above the oviposition site was slower on galled stems; however there was no significant effect of any treatment on overall stem height. Crofton weed compensated for repeated galling with increased sideshoot (vegetative reproduction) growth. Overall, the total biomass of the treatment stems did not differ, but more biomass was allocated to sideshoots, in comparison to leaves and bare stems, on galled stems. The interaction between the agents did not lead to cumulative control of crofton weed growth. However, the fly may reduce stem vigour over a longer time period, and the pathogen may reduce the compensatory effect of increased sideshoot growth. This study showed that greenhouse trials are not always predictive of field conditions, as the interaction between the biocontrol agents in the field was not an additive interaction as predicted by greenhouse trials.

3.2 Introduction

Classical biocontrol of invasive alien plants involves the deliberate introduction of biocontrol agents, such as insects and pathogens, to negatively influence the invasive plant's growth parameters and population dynamics (McFadyen, 1998; Zimmermann *et al*., 2004, Morin *et al*., 2006). Over the years, more than one biocontrol agent has often been released onto a specific target weed, based on the theory that the combined effects of multiple biocontrol agents will increase control of the weed (Myers, 1985, 2008; Julien & Griffiths, 1998; Denoth *et al*., 2002; Stiling & Cornelissen, 2005). Of these, synergistic relationships between insects and pathogens are considered responsible for the first successes in biocontrol of *Opuntia stricta* (Caesar, 2000, 2003).

There is an increasing call to not release ineffective agents (Raghu *et al*., 2006), therefore the effectiveness of biocontrol agents is often first assessed under controlled conditions in laboratory or greenhouse studies (Morin *et al*., 2009). However, under such controlled conditions the impact of the agents can be under- or overestimated, as these are not always representative of conditions in the field (Rosskopf *et al.*, 1999; Morin *et al*., 2006, 2009). For example, the Eucalyptus weevil, *Gonipterus "scutellatus"* Gyllenhal (Curculionidae), was found to be more selective in feeding and oviposition behaviour on *Eucalyptus* species in the field in comparison to laboratory trials. This has implications for the realised host range of the weevil in comparison to the predicted host range. There is also the need for biocontrol research to move beyond the initial host-specificity and greenhouse trials research stage into field and post-release evaluation studies (Raghu *et al*., 2006; Morin *et al*., 2009). This work examined the impact of two biocontrol agents in the field, released against *Ageratina adenophora*, following greenhouse trials of the efficacy of these two biocontrol agents.

Originating from Mexico, *Ageratina adenophora*, (Sprengel) King and Robinson (syn. *Eupatorium adenophorum* Spreng.) (Asteraceae), also

known as crofton weed or the Mexican Devil is an invasive weed in several countries worldwide, including South Africa, Australia, New Zealand, Hawaii, India and China (Julien & Griffiths, 1998). Crofton weed is a perennial herb, with a woody rootstock, and many stems reaching up to 2m in height (Bess & Haramoto, 1959; Henderson, 2001; Page & Lacey 2006). Trailing crofton weed stems root when in contact with the soil, resulting in dense infestations (Bess & Haramoto, 1959; Morris, 1991). Crofton weed invades steep slopes and wet areas along streams, roadsides, forests and plantations (Dodd, 1961; Henderson, 2001; Trounce, 2003; Page & Lacey, 2006). Conservation areas are adversely affected by this weed, as it is allelopathic, alters soil microbial communities and displaces natural vegetation (Erasmus *et al*., 1992; Henderson, 2001; Niu *et al*., 2007). Crofton weed also reduces crop yield, the carrying capacity of grazing land, is unpalatable to cattle and causes a fatal lung disease in horses (Plant Protection News, 1988; Land Protection, 2001; Page & Lacey, 2006).

In 1984 a small, opportunistic biocontrol programme for the control of crofton weed was initiated in South Africa (Plant Protection News, 1987a; Kluge, 1991). Two biocontrol agents, a stem gall fly, *Procecidochares utilis* Stone (Tephritidae), and a leaf-spot pathogen, *Passalora ageratinae* Crous and A.R. Wood (Mycosphaerellaceae) (previously named *Cercospora eupatorii* Peck or *Phaeoramularia* sp.) (Crous *et al*., 2009), have been released in South Africa (Kluge, 1991). The biology and impact of the fly have been described by Haseler (1965) and Bennett (1986) and the pathogen has been described by Morris (1989), Wang *et al*. (1997) and Crous *et al*. (2009). Heystek *et al*. (2011) reviews the crofton weed biocontrol programme in South Africa to date.

A controlled greenhouse trial using both the fungal pathogen and the gall fly, individually and in combination, showed that the fly reduced plant height and growth of crofton weed, in particular, repeated galling by the fly was the most effective (Chapter 2). The pathogen reduced the number of sideshoots, representing vegetative reproduction of crofton weed, which

was promoted by galling (Chapter 2). The combined effects of the interaction between the two agents led to additive control of crofton weed.

In South Africa, recent anecdotal field observations suggested that neither the fly nor fungal pathogen are successfully controlling crofton weed (Heystek *et al*., 2011), however the impact of the two agents has not been formally assessed in the field in South Africa. The aim of this study, therefore, was to evaluate whether the pathogen and fly in combination control the vegetative growth of crofton weed, under field conditions, in South Africa, as predicted by the greenhouse studies.

3.3 Material and methods

3.3.1 Field site

The field trial was run on the 150ha Kloofwaters farm (1380m ASL), in the Magaliesberg, North West Province, South Africa (25˚49'45.1"S, 27˚26'26.0"E). The farm is privately owned, and predominantly used for school camps; however a low stocking level of cattle is present. The field site was located within *A. adenophora* infestations along a flat stream embankment, facing eastwards. Plants were in full sunshine, with late afternoon shading and only the fungal pathogen, *P. ageratinae*, was present. The field site is on Moot Plains Bushveld (central bushveld, Savanna Biome), with thorny savanna, dominated by *Acacia* species and woodlands (Mucina *et al*., 2006a; Rutherford *et al*., 2006). The Magaliesberg experience summer rainfall and very dry winters with frost (Mucina *et al*., 2006a). In winter, temperatures at the field site ranged from 2.6ºC to 18.3ºC (mean = 9.9ºC), and from 14.0ºC to 29.5ºC (mean = 20.5ºC) during summer. The mean relative humidity was 55% (min = 10.2%, max = 100%) during the winter months (June 2007 to August 2007) and 87.5% (min = 45.0%, max = 100%) during the summer months (December 2007 to February 2008).

3.3.2 Field trials

In February 2007 all crofton weed plants within a 50m^2 area within the field site were cut back to soil level and labelled. Plants were cut back to promote growth of new stems from the woody rootstock.

The fungicide, AMISTAR ®, used in greenhouse trials to restrict *P. ageratinae* infection, resulted in phytotoxic symptoms (chlorotic spots) on crofton weed seedling leaves. Therefore, it was not possible to chemically exclude the pathogen from the new stems, as they had already been infected when they were at the seedling stage from surrounding plants. Fungal leaf spots were visible on new stems by April 2007.

In June 2007, 20 plants were randomly allocated to each of the following three treatments:

(i) plants infected with the fungal pathogen, *P. ageratinae* (pathogen-only);
(ii) plants infected with the fungal pathogen, *P. ageratinae*, and exposed to one release of the fly, *P. utilis* (hereafter referred to as pathogen-single galled);
(iii) plants infected with the fungal pathogen, *P. ageratinae*, and exposed to two releases of the fly, *P. utilis* (hereafter referred to as pathogen-double galled).

Cages were used to restrict flies from ovipositing on non-galled treatment plants. The cages were constructed from fine white netting, attached to a 1m central pole with a steel ring at the top, and pegged into the soil. All plants in all treatments were covered with cages. Cages were removed two weeks after flies were released, as the average life span of adult flies is 10 days. No galling on pathogen-only stems was observed during the trial period.

Adult *P. utilis* were collected from a colony on galled plants at the University of the Witwatersrand, Johannesburg, Gauteng (26˚11’20.97”S, 28˚01’55.37”E). The first release of flies was made in August 2007. One pair of flies per plant was released into the cages with plants of the

pathogen-double galled treatment (treatment iii). Flies emerged from galls 12 weeks later (November 2007), and were collected weekly from the cages with a pooter, over a five week period, to prevent further galling of the treatment plants. In January 2008 one pair of flies per plant was released onto the pathogen-single galled and pathogen-double galled plants (treatments ii and iii).

Four stems per plant were randomly selected for measurements of vegetative growth. Monthly measurements of stem height, number of living, dead and abscised leaves per stem, and the number of sideshoots per stem, were recorded from June 2007 to April 2008. The number of leaves per stem infected with the pathogen was also recorded monthly. On the day of the fly release the oviposition site (apical leaf bud) was marked on all the treatment stems with a piece of sewing thread. Monthly measurements of stem growth above this oviposition site were then taken for all treatment stems. These measurements were only taken after the second fly release, as stems were flowering during the first fly release and no apical leaf buds were present. Gall length and diameter (at the longest and broadest point respectively) were measured and used to calculate a gall size index (Bennett, 1986). The number of emergence holes per gall was used as an index of the number of adult flies successfully emerging per gall. The position of galls was classified into two categories, stem or side (galls formed on the leaf petiole or leaf node). At the end of the trial the treatment stems were cut, collected and separated into bare stems, live leaves, dead leaves and sideshoots. The bare stems, leaves and sideshoots were then dried in a forced-draught oven for seven days at 60ºC and weighed. The total dry mass of the treatment stems was calculated. In addition the allocation of biomass to live leaves, bare stems and sideshoots per stem was calculated as a percentage of stem total biomass.

3.3.3 Data analysis

Repeated measures ANOVA with nesting (stems nested within plants) were used to assess the influence of the biocontrol agents on the vegetative growth of crofton weed over the trial period (GLM procedure, StatSoft, 2007). One-way ANOVA with nesting and LSD tests were used to assess differences in stem measurements between treatments for each month. One-way ANOVA's were used to assess differences in the gall size index and number of emergence holes per gall between the single and double galling treatments. A Chi-squared 2x3 contingency table was used to compare the association between stem death and treatments. Chi-squared 2x2 contingency tables were used to compare the association between the position of galls and the incidence of flowering. The difference in biomass between the three treatments was assessed with a one-way ANOVA with nesting.

3.4 Results

3.4.1 Stem death

Over the trial 15% of the pathogen-only stems died, while 6% of pathogen-single galled stems died and 10% of pathogen-double galled stems died. There was no significant association between stem death and treatment (χ^2=3.31, df=3, *P*=0.653).

3.4.2 Agent establishment and infection severity

The pathogen infected 10-40% of living leaves from June to October 2007 (Fig.3.1). The number of infected living leaves increased to 70-80% by November 2007, and thereafter 50% of living leaves were infected with the pathogen. There was no significant difference in the percentage of leaves infected with the pathogen on galled and ungalled stems over the trial period ($F_{2,45}$=1.14, *P*=0.331).

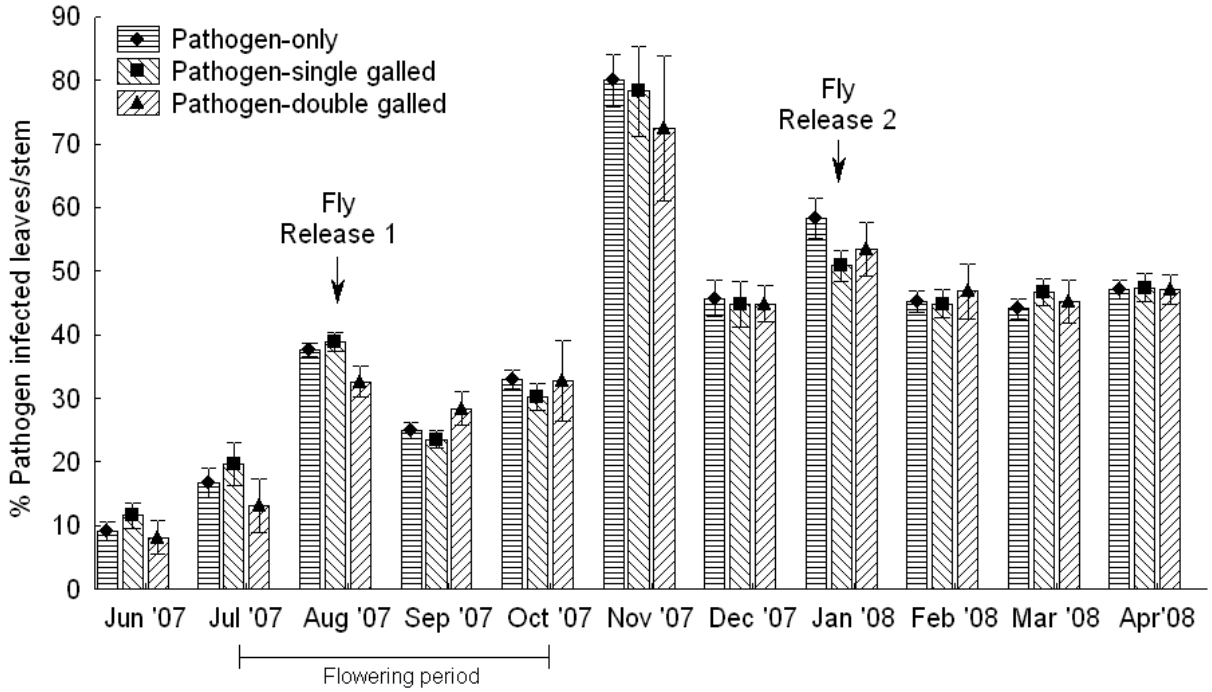


Figure 3.1: Percentage of live leaves infected with the fungal pathogen *Passalora ageratinae* per *Ageratina adenophora* stem, when either galled (single or double) or ungalled by the fly *Procecidochares utilis*, from June 2007 to April 2008. For each month, means followed by different letters are significantly different, at $P<0.05$ (LSD). (Means ± SE).

The gall size index differed significantly between release 1 (August 2007) and release 2 (January 2008) ($F_{2,93}=3.05$, $P=0.049$) (Fig. 3.2a). Galls that formed in August 2007, during the flowering period, were smaller than galls formed in January 2008. There was an association between the position of galls and flowering ($X^2=4.09$, df=1, $P=0.043$), with more galls formed on stems, in comparison to the side of stems, when crofton weed is not flowering. There was no significant difference between the sizes of galls formed on stems for either the first or second time, in January 2008. The number of adult flies emerging did not differ significantly between release 1 and 2, or between single and double galled stems ($F_{2,93}=2.29$, $P=0.106$) (Fig. 3.2b).

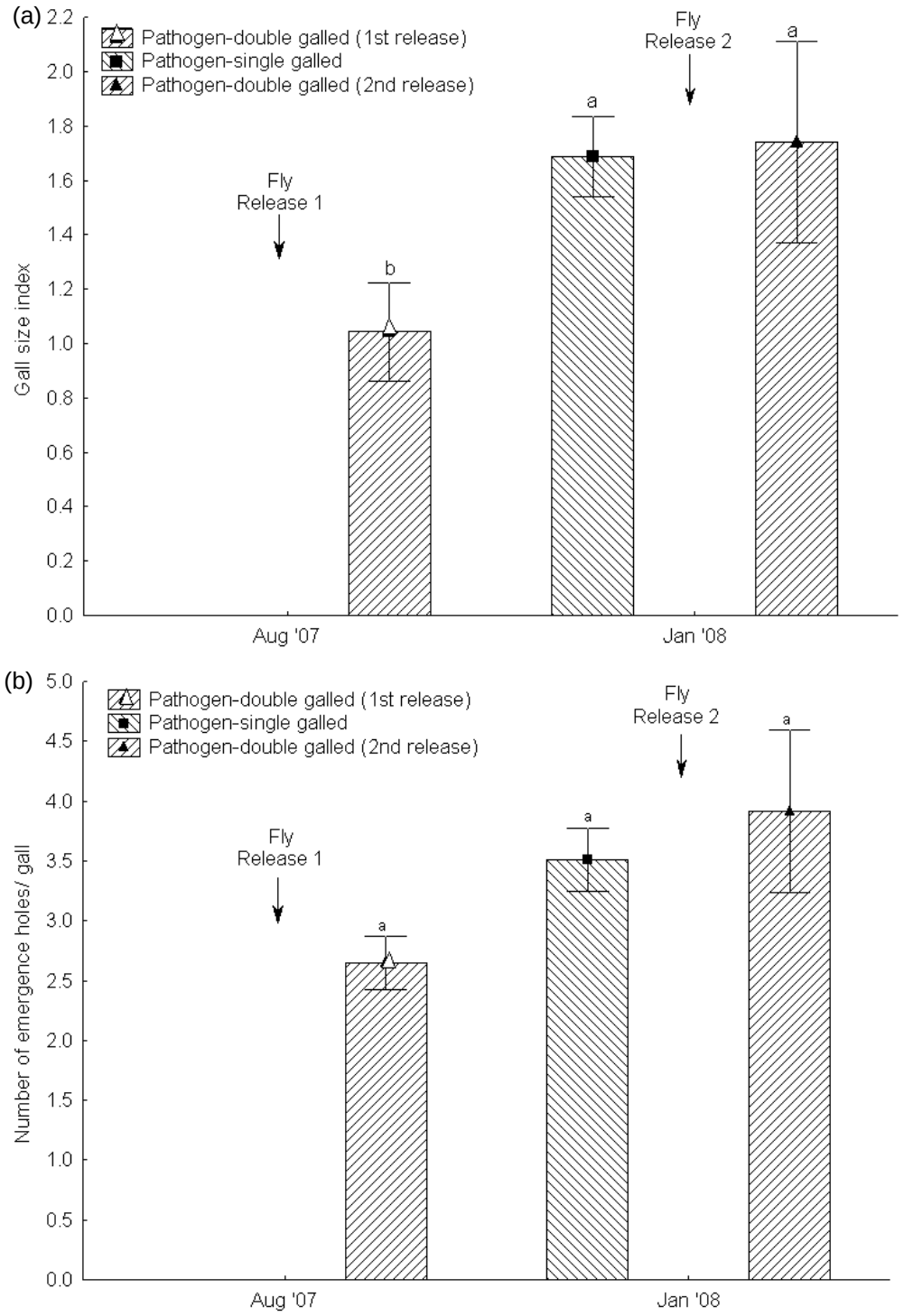


Figure 3.2: (a) Gall size index (gall diameter x length) of *Procecidochares utilis* on *Ageratina adenophora* stems infected with the fungal pathogen *Passalora ageratinae*, and (b) the number of *P. utilis* adults (represented by the number of emergence holes) emerging from galls on pathogen infected stems; from the release of *P. utilis* in August 2007 and January 2008. Means followed by different letters are significantly different, at $p<0.05$ (LSD). (Means ± SE).

3.4.3 Stem height

The stem height under all the treatments increased until the flowering period, but decreased after flowering, and thereafter increased again for the remainder of the trial period (Fig. 3.3). A repeated measures ANOVA showed no overall significant difference between the treatments ($F_{2,41}$=0.92, P=0.405). However, in September ($F_{2,41}$=5.63, P=0.007) and October ($F_{2,41}$=6.41, P=0.004), the pathogen-double galled treatment stems were significantly shorter than the pathogen-only and the pathogen-single galled stems.

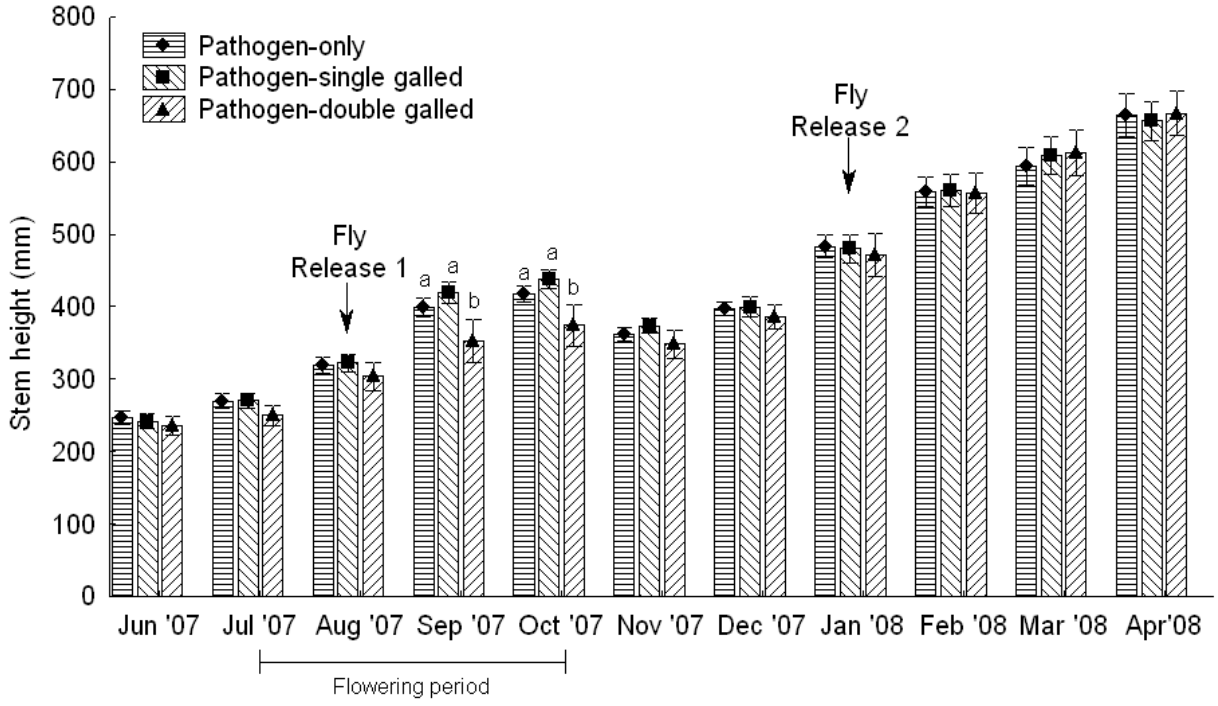


Figure 3.3: Height of *Ageratina adenophora* stems in response to three different treatments, of pathogen infection with or without galling (single or double), from June 2007 to April 2008. For each month, means followed by different letters are significantly different, at P<0.05 (LSD). (Means ± SE).

3.4.4 Stem growth above the oviposition site

Growth above the oviposition site differed significantly between the three treatments after the second fly release in January 2008 ($F_{2,41}$=4.69,

P=0.015) (Fig. 3.4). Pathogen-galled stems showed significantly less growth above the oviposition site compared to pathogen-only stems.

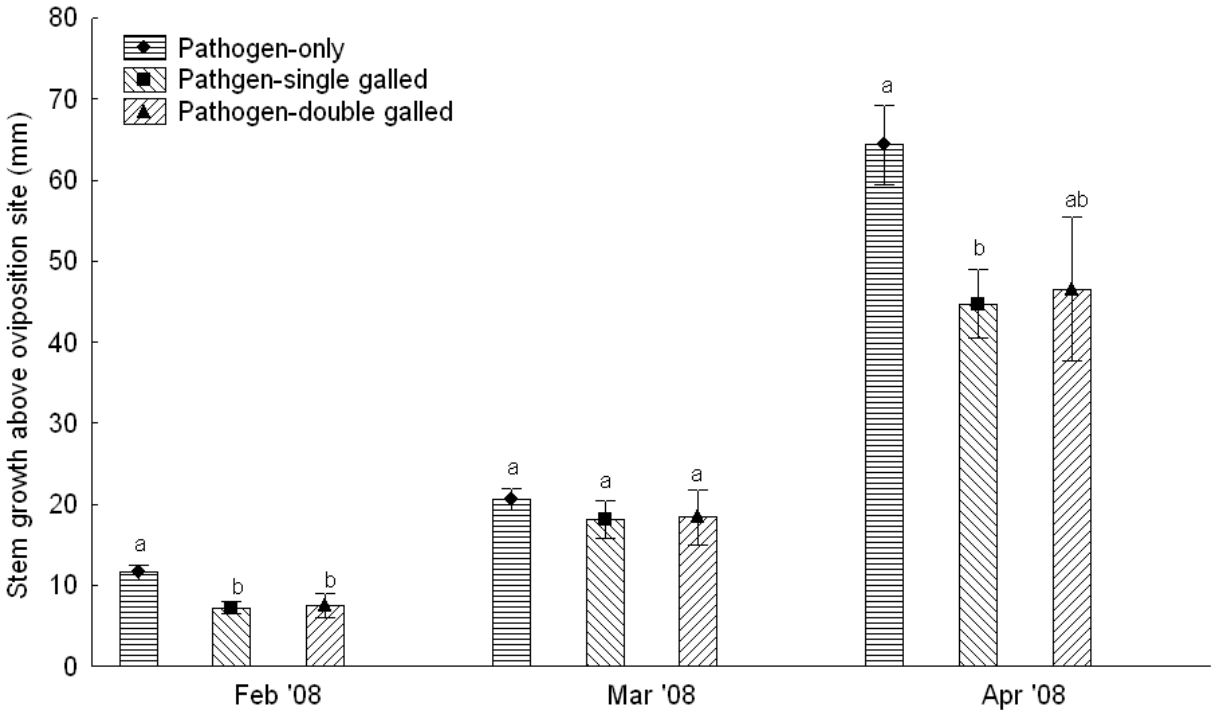


Figure 3.4: Growth of *Ageratina adenophora* stems above the oviposition site, in response to three different treatments, of pathogen infection with or without galling (single or double), after the second fly release in January 2008 until April 2008. For each month, means followed by different letters are significantly different, at *P*<0.05 (LSD). (Means ± SE).

3.4.5 Percentage of live leaves per stem

The percentage of live leaves decreased until the end of the flowering period, after which stems maintained 30-40% live leaves (Fig. 3.5). There was no significant difference in the percentage of live leaves between the treatments ($F_{2,39}$=0.11, *P*=0.893).

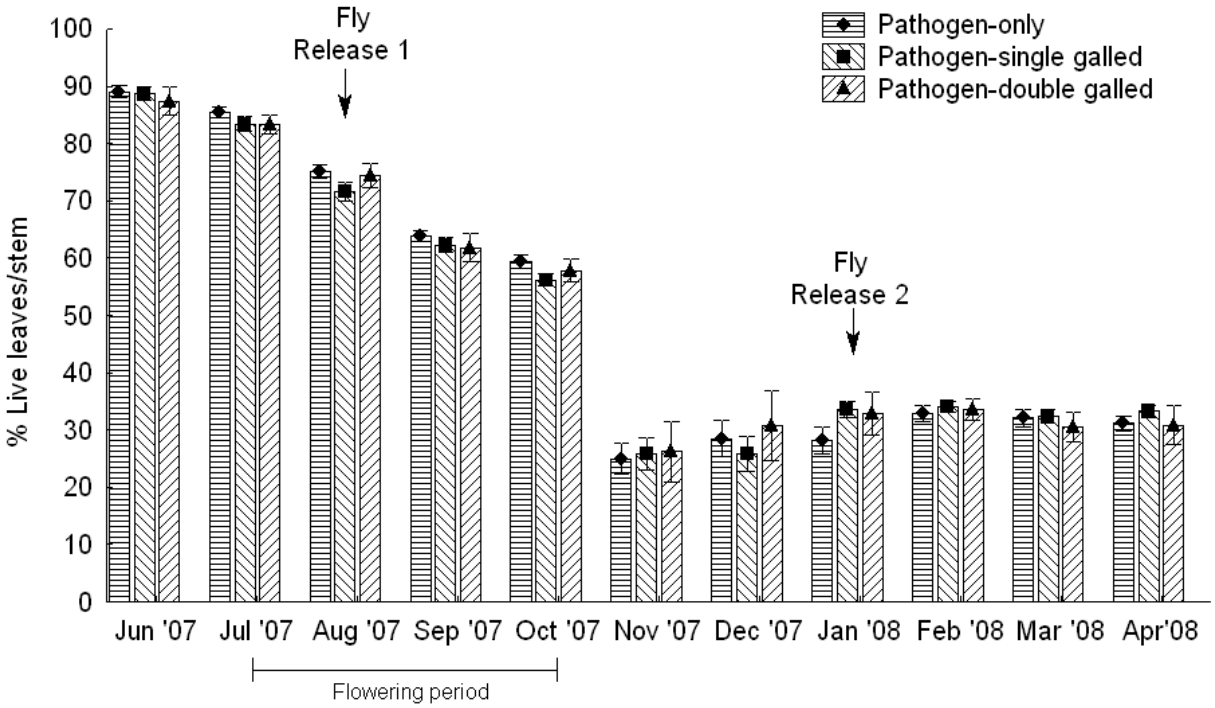


Figure 3.5: Percentage of live leaves on *Ageratina adenophora* stems in response to three different treatments, of pathogen infection with or without galling (single or double), from June 2007 to April 2008. For each month, means followed by different letters are significantly different, at $P<0.05$ (LSD). (Means ± SE).

3.4.6 Number of sideshoots per stem

At the end of the trial the number of sideshoots differed significantly between treatments ($F_{2,39}=3.26$, $P=0.049$) (Fig. 3.6). Repeated galling of stems promoted sideshoot growth in comparison to pathogen-only stems.

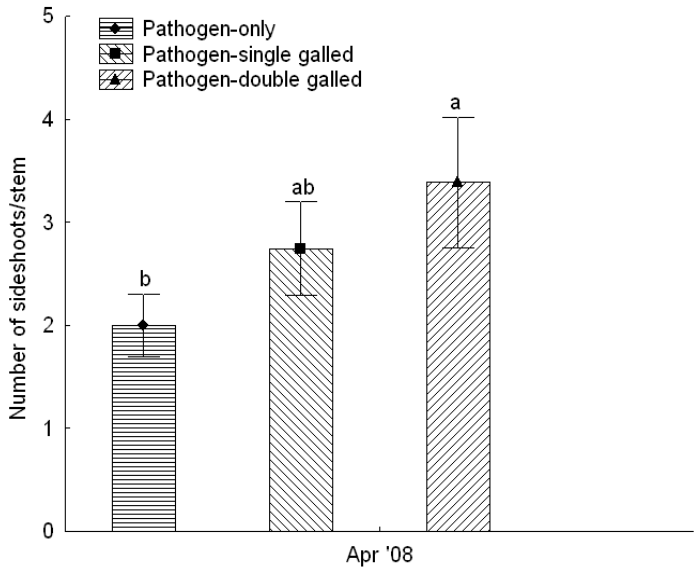


Figure 3.6: Number of sideshoots on *Ageratina adenophora* stems in response to three different treatments, of pathogen infection with or without galling (single or double), at the end of April 2008. Means followed by different letters are significantly different, at $P<0.05$ (LSD). (Means ± SE).

3.4.7 Biomass

The galled stems allocated more biomass to sideshoots than pathogen-only stems ($F_{2,41}$=4.74, P=0.014) (Fig. 3.7). The pathogen-only stems had a higher percentage of their biomass allocated to live leaves ($F_{2,41}$=4.12, P=0.024), and bare stems ($F_{2,41}$=3.33, P=0.046) (Fig. 3.7). Although the three treatments allocated their biomass to different areas of the stem there was no significant difference in the total biomass of the stems ($F_{2,41}$=0.14, P=0.873) (Fig. 3.7).

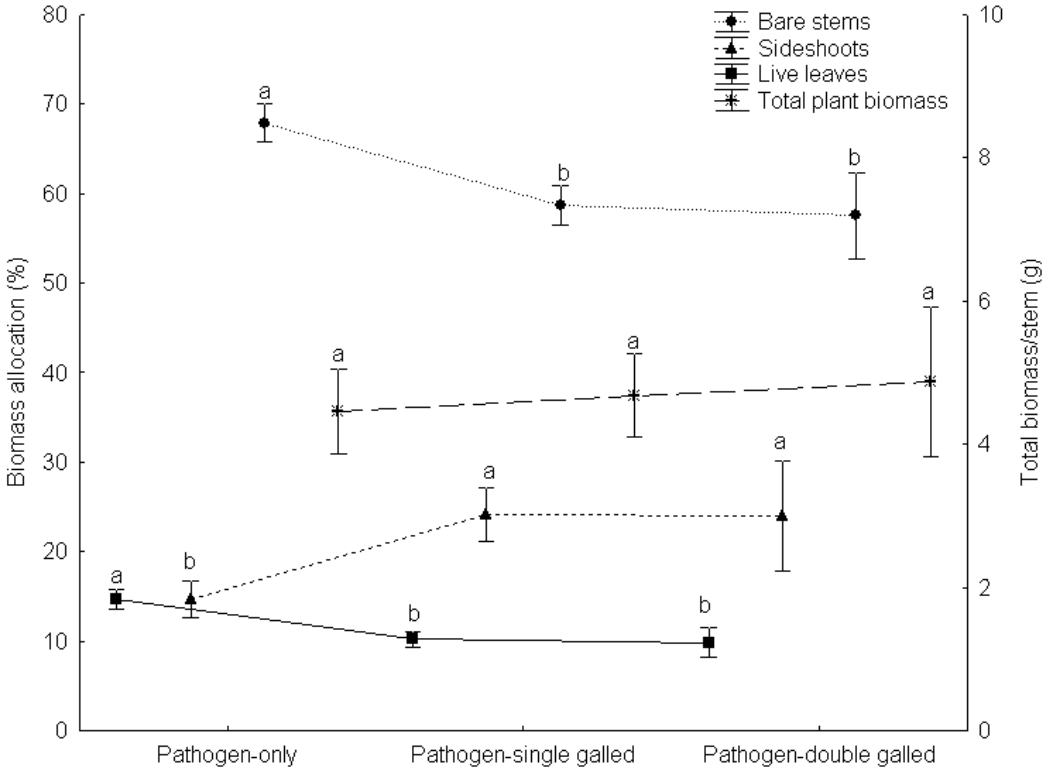


Figure 3.7: The percentage biomass allocation of *Ageratina adenophora* stems to live leaves, sideshoots and bare stems, and the total biomass (g) of stems in response to three different treatment conditions, of pathogen infection with or without galling, at the end of April 2008. Means followed by different letters are significantly different, at P<0.05 (LSD). (Means ± SE).

3.5 Discussion

Two models have been proposed to explain the higher success rate of biocontrol projects with multiple agents in comparison with single agents. The lottery model predicts that only one agent is responsible for the control of the weed (Myers, 1985, 2008; McEvoy & Coombs, 2000), and the cumulative stress model proposes that more than one of the released agents contribute to successful control of the target weed (Harris, 1985; Hoffmann & Moran, 1998; Stiling & Cornelissen, 2005; Campanella *et al*., 2009; Rayamajhi *et al*., 2010; Turner *et al*., 2010). This study revealed that there is no overall cumulative effect of both the pathogen and gall fly in combination on the control of crofton weed vegetative growth parameters, in contrast to predictions from greenhouse trials.

In this insect-pathogen system the agents partition the resources of crofton weed plants in space by the fly galling the shoot tips, and the pathogen infecting the lower leaves. These results show that in the field, galling does not influence the severity of pathogen infection of crofton weed. The percentage of pathogen infected leaves peaked at 80%, when leaf loss was high after flowering, and thereafter only half the leaves were infected at a time. Death of seedlings and severe or complete defoliation of pathogen infected stems was seen in Australia (Wang *et al*., 1997; Page & Lacey, 2006). While death of seedlings was observed in the field, most stems were able to limit pathogen infection to leaves on the lower half of the stem.

Flowering stems have fewer apical leaf buds or oviposition sites available (Bennett, 1986), therefore smaller galls develop in the leaf nodes. In the greenhouse trials there was no significant difference between the gall size index or the number of adult flies emerging from galls on pathogen infected or uninfected stems (Chapter 2). As the pathogen could not be excluded from stems in the field, it was not possible to account for antagonism or synergism between the pathogen and fly establishment. However, galls in the field were significantly larger in size (1.54±0.12,

mean ± SE) compared to those observed in the greenhouse (1.16±0.06) (t-test, t_{364}=2.47, *P*<0.001).

In greenhouse trials stem height was shortest when crofton weed was galled more than once (Erasmus *et al*., 1992; Chapter 2). However, in these field trials galls did not significantly affect stem height. The greenhouse trials suggest that galls should have a large impact on younger, shorter crofton weed plants (Erasmus *et al*., 1992). This was not seen in the field, even though the field plants were shorter than the greenhouse plants. Regrowth of stems after having been cut back was not as vigorous in the field as under more favourable growing conditions in a greenhouse. Stems grew slower in the field (250mm stem height increment in 5 months) than in the greenhouse (900mm stem height in 6 months), after being cut down to the root stock.

The pathogen with single or double galling slowed stem growth above the oviposition site, in comparison to pathogen-only stems. Weeds are often able to compensate for the pressure of diseased leaves by increasing vegetative growth (Charudattan, 2005, 2010). The slowed growth above the oviposition site highlights the ability of the fly galls to potentially act as nutrient sinks, as seen in other systems (Florentine *et al*., 2005; Dorchin *et al*., 2006; Moseley *et al*., 2009).

All treatment stems maintained approximately 30% of their living leaves. The pathogen alone, and the combination of pathogen and galling, was not sufficient to completely defoliate crofton weed as has been seen in Australia (Dodd, 1961). As crofton weed naturally sheds its lower leaves, this impact may not be sufficient to control crofton weed.

Tall *A. adenophora* stems eventually bend over and trail along the soil, where the sideshoots can then root and eventually grow into new plants (Muniappan *et al*., 2009). As galling influences apical dominance, as seen with growth above the oviposition site, crofton weed compensates by producing more sideshoots (Erasmus *et al*., 1992; Chapter 2). Pathogen-only stems had significantly less sideshoots. In the greenhouse the

pathogen was able to reduce the number of sideshoots produced, in comparison to stems that were only galled. Considering only the sideshoot growth there is an antagonistic effect between these biocontrol agents on the control of crofton weed, however we do not know what sideshoot growth would be like on galled stems, without pathogen-infection, in the field.

There was no cumulative effect of both biocontrol agents on the total biomass of crofton weed stems. Stems infected only with the fungal pathogen had less biomass allocated to sideshoots, however galling allocated less biomass to the bare stems and live leaves of stems. The lower bare stem biomass in galled stems indicates that although galled plants have similar heights to pathogen-only stems, the galled stems may be weaker and thinner than the pathogen-only stems. Therefore, the fly may be effective over a longer time period if growth above the oviposition site is weak. Stems galled in Hawaii were weaker and thinner than ungalled stems (Bess & Haramoto, 1959, 1972). In addition a smaller biomass being allocated to live leaves on galled stems supports the hypothesis that *P. utilis* galls may act as nutrient sinks, removing assimilates and nutrients from the leaves to provide nutrition for the fly larvae developing within the galls (Harris & Shorthouse, 1996).

Morin *et al*. (2009) reviews how field trials in the introduced range are necessary to understand the response of the agents to the new environmental conditions, as well as the response of the weed to the agents. Research and funding is focused on the initial host-specificity stages of a biocontrol programme, however it is necessary to continue research into the field to fully understand the dynamics of a biocontrol programme (Briese, 2006; Raghu *et al*., 2006; Morin *et al*., 2009). Factors which may affect the success or failure of biocontrol agents include parasitism (King *et al*., 2011), climate (Zachariades *et al*., 2011) and acquired natural enemies (Muniappan & McFadyen, 2005); which are not always present in the controlled conditions of greenhouse trials.

Based on this comparison of results the greenhouse trials were not predictive of the field results. However, based on the growth of crofton weed in the field and environmental conditions, more time may be needed for field trials to show any effect of the agents. In addition, these field trials have assessed the impact of the fly and pathogen on crofton weed vegetative growth within one area of South Africa, where the fly had not been previously released and therefore no parasitoids were present. Field surveys of crofton weed infestations across South Africa may yield different results, as based on the effect of the fly and pathogen in other countries, the impact of the agents may differ depending on the severity and prevalence of agent attack, parasitism of the fly, and environmental factors. In Hawaii crofton weed has been successfully controlled by the gall fly alone, where crofton weed has been eliminated over large tracks of land, in low rainfall areas (Bess & Haramoto, 1959, 1972; Muniappan *et al*., 2009). Heavy galling in Hawaii, up to seven galls per stem, resulted in stunted plants, weakened stems and death of some plants (Bess & Haramoto, 1972), but the fly was not as successful in high rainfall areas (Bess & Haramoto, 1959, 1972). The gall fly and fungal pathogen together have slowed the encroachment of crofton weed and thinned infestations along the east coast of Australia (Dodd, 1961; Page & Lacey, 2006). The pathogen has led to death of seedlings and major or complete defoliation of stems in Australia in the drier months (Page & Lacey, 2006), and high levels of galling reduced plant vigour and killed plants (Dodd, 1961). In addition, this study has only assessed vegetative growth of crofton weed, the efficacy of agents to control reproductive output of the target weed is also important in biocontrol (Pyšek & Richardson, 2007; Morin *et al*., 2009).

3.6 Conclusion

From the greenhouse trial, control of crofton weed by the fly in the field was expected, with repeated galling controlling stem growth, and the

fungal pathogen reducing sideshoot growth. However, in the field the fungal pathogen alone is the predominant biocontrol agent, and no additional cumulative control is achieved with the addition of the gall fly. The pathogen decreases vegetative reproduction, by reducing the number of sideshoots. However, the allocation of biomass is different when both biocontrol agents are used. The reduction in stem biomass by galling may be effective in controlling crofton weed over a longer time period. Is it better to reduce the quality of stems and live leaves, or to reduce the biomass of sideshoots which may become new plants? This study has highlighted that caution should be used when using only greenhouse trials, with controlled conditions, to predict the effectiveness of multiple biocontrol agents.

Chapter 4: Do multiple agents reduce the reproductive output of the invasive alien plant, *Ageratina adenophora*, in laboratory and field trials?

4.1 Abstract

Biocontrol aims to reduce the population density and spread of target weeds, and one way this can be achieved is by reducing the reproductive output of the target weed. Two biocontrol agents, a stem gall fly, *Procecidochares utilis*, and a leaf-spot pathogen, *Passalora ageratinae*, have been released against crofton weed, *Ageratina adenophora,* in South Africa. This study investigated whether the two biocontrol agents, individually or together, affected crofton weed reproductive output, under both greenhouse and field conditions. Six month old plants were exposed to one of six treatments (n=15plants/treatment) in the greenhouse: control (no agents), pathogen-only, single-galled, double-galled, pathogen-single galled, and pathogen-double galled before the flowering period. The field trial was conducted at a site in the Magaliesberg. Four month old stems were exposed to one of the following two treatments (n=20plants/treatment): pathogen-only and pathogen-single galled before the flowering period. Similar results were found in both the greenhouse and field trials. Galling by the fly resulted in fewer synflorescences per stem, capitula per synflorescences and filled achenes per capitula, in comparison to the ungalled treatments. Overall, galled stems had 53.8% less filled and germinable achenes than control stems. The pathogen did not reduce the number of synflorescences per stem or the number of capitula per stem. However pathogen infected stems did have 26.7% less filled and germinable achenes per stem in comparison to the control. The combination of the two agents together provided equivalent control, with the fly being the most damaging. The percentage germination of filled achenes was lower in the field (71%) in comparison to the laboratory (74%). Whilst the gall fly is the most effective agent in terms of reducing crofton weed sexual reproductive output, crofton weed compensates for galling with increased sideshoot (vegetative reproduction) growth. The pathogen inhibits the growth of sideshoots. Therefore, the pathogen may not reduce the sexual reproductive output of a stem to the extent of the fly, but it may reduce the number of potentially asexual reproductive

sideshoots in the next flowering season. The fly does not gall all the stems in the field, and therefore both agents in combination are better for the control of crofton weed in South Africa.

4.2 Introduction

Classical biological control (biocontrol) of weeds involves the deliberate introduction of biocontrol agents, termed natural enemies, such as insects, mites and pathogens, from the country of origin into the invaded country (McFadyen, 1998; Müller-Schärer & Schaffner, 2008; Morin *et al*., 2009). The aim of biocontrol is not necessarily to eradicate the weed population but to reduce the invasion to 'acceptable levels', where the plants may survive and reproduce but do not invade or impact negatively on the surrounding environment. When successful, biocontrol reduces the weed population's density, distribution and/or rate of spread (Zimmermann *et al*., 2004). Biocontrol agents that reduce the reproductive output of weeds are often used to reduce the invasiveness of a weed population (van Klinken *et al*., 2004; Morin *et al*., 2009). Some desired outcomes may include reducing the number of seeds, seed quality and dispersal characteristics (van Klinken *et al*., 2004). There is some debate over whether seed and flower feeders improve the success of biocontrol programmes (Myers & Risley, 2000). However, some plant traits, such as few large seeds and low seed viability, have been highlighted that may allow for better success with seed and flower feeders (van Klinken *et al*., 2004).

Ageratina adenophora, (Sprengel) King and Robinson (syn. *Eupatorium adenophorum* Spreng.) (Asteraceae), also known as crofton weed or the Mexican Devil, originates from Mexico and is an invasive weed in several countries worldwide, including South Africa, Australia, New Zealand, Hawaii, India and China (Julien & Griffiths, 1998). The weed favours moist areas near water and along stream banks, but may also invade forest margins, agricultural plantations and roadsides (Dodd, 1961; Henderson, 2001; Trounce, 2003; Page & Lacey, 2006). Crofton weed often grows in

inaccessible areas, such as high up on waterfalls and in the crevices of mountain ridges, where these infestations are very difficult to clear manually.

Crofton weed inflorescences are arranged in terminal corymbs; flower-like heads made up of many small white capitula surrounded by protective bracts, hereafter referred to as synflorescences (Peng *et al*., 1998; Henderson, 2001; Muniappan *et al*., 2009). In South Africa flowering occurs between August and December. The fruits are glabrous achenes (smooth, hard dry fruits) approximately 2mm long, with 8-10 apical bristles (Peng *et al*., 1998; Henderson, 2001; Muniappan *et al*., 2009). Crofton weed seeds prolifically, adult plants may produce 10 000 - 100 000 seeds per season, contributing up to 60 000 viable seeds/m^2 to the seed bank annually (Trounce, 2003; Muniappan *et al*., 2009). The very light seeds are dispersed by wind and water as well as by humans on clothing and vehicles, over long distances, allowing invasion of new areas (Muniappan *et al*., 2009) and rapid rates of spread. Crofton weed is apomictic, producing seeds without fertilisation (Rambuda & Johnson, 2004). Therefore, crofton weed can seed and spread in the absence of other individuals or pollinators.

In South Africa, two biocontrol agents, a stem gall fly, *Procecidochares utilis* Stone (Tephritidae), and a leaf-spot fungal pathogen, *Passalora ageratinae* Crous and A.R. Wood (Mycosphaerellaceae) (previously named *Cercospora eupatorii* Peck or *Phaeoramularia* sp.) (Crous *et al*., 2009), have been released against crofton weed (Plant Protection News, 1988; Kluge 1991). Laboratory trials predict that repeated galling reduces stem growth and the pathogen reduces sideshoot growth, resulting in an additive effect on crofton weed biocontrol (Chapter 2). Field trials show that there is no increased control, on vegetative growth of crofton weed, when the two biocontrol agents are used in combination, however the fly may reduce stem vigour over a longer time period, and the pathogen may reduce the compensatory effect of increased sideshoot growth (Chapter 3).

The aim of this study was to evaluate whether the pathogen and fly, individually and in combination, reduce the seed production of crofton weed, under both laboratory and field conditions, in South Africa.

4.3 Material and methods

4.3.1 Laboratory trials

The laboratory trial was run in a greenhouse at the University of the Witwatersrand, Johannesburg, Gauteng (26˚11'20.97"S, 28˚01'55.37"E). A detailed description of greenhouse conditions, biocontrol agent rearing and trial set up is provided in Chapter 2.

Approximately 100 crofton weed seedlings were grown from stored achenes, sown in August 2006, into 5-litre plant bags filled with potting soil. In January 2007 the approximately 50cm tall plants were cut back to soil level, to promote new stem growth.

At the end of March 2007, 15 plants were randomly allocated to each of the following six treatments:

(i) no biocontrol agents (control);
(ii) plants infected with the fungal pathogen *P. ageratinae* only (hereafter referred to as pathogen-only);
(iii) plants exposed to one release of *P. utilis* (hereafter referred to as single-galled);
(iv) plants exposed to two releases of *P. utilis* (hereafter referred to as double-galled);
(v) plants infected with *P. ageratinae* and exposed to one release of *P. utilis* (hereafter referred to as pathogen-single galled);
(vi) plants infected with *P. ageratinae* and exposed to two releases of *P. utilis* (hereafter referred to as pathogen-double galled).

In order to restrict the spread of the pathogen between the treatments, plants in the non-pathogen treatments were sprayed with a broad-spectrum fungicide, AMISTAR ®. A control trial was run concurrently to

measure any stimulatory or inhibitory influence that AMISTAR may have had on *A. adenophora* sexual reproduction. In order to prevent *P. utilis* ovipositing on non-fly treatment plants, the plants of all treatments were placed in separate cages.

The trial *A. adenophora* plants in the pathogen treatments were infected with the pathogen by tying fresh infected leaves, from a stock culture, onto their stems in mid-April 2007. Plants from all treatments were then covered with plastic bags, to create a dew period of 24 hours (Wang *et al*., 1997). The first fungal leaf spots appeared in late May 2007.

One pair of flies was released per plant into only the cages of the double galled treatments (treatments iv and vi), in June 2007 before the flowering period. Flies emerged from galls 10 weeks later and were collected for 3 weeks, in order to stop the emerging flies from further ovipositing on the treatment plants. The second release of flies took place in September 2007 during the flowering period. One pair of flies was released per plant onto all four of the single and double galled treatments (treatments iii–vi).

4.3.2 Field trials

The field trial was run on the 150ha Kloofwaters farm, in the Magaliesberg, North West Province, South Africa (25˚49’45.1”S, 27˚26’26.0”E). A detailed description of the field site, biocontrol agent rearing and trial set up is provided in Chapter 3.

In February 2007 all crofton weed plants within a 50m^2 area within the field site were cut back to soil level and labelled. Plants were cut back to promote growth of new stems from the woody rootstock.

It was not possible to chemically exclude the pathogen from the new stems in the field, as they were infected as seedlings from surrounding plants, and AMISTAR is phytotoxic in small plants. Fungal leaf spots were visible on new stems by April 2007.

In June 2007, 20 plants were randomly allocated to each of the following two treatments:

(i) plants infected with the fungal pathogen, *P. ageratinae* (pathogen-only);

(ii) plants infected with the fungal pathogen, *P. ageratinae*, and exposed to one release of the fly, *P. utilis* (hereafter referred to as pathogen-single galled);

Cages were used to restrict flies from ovipositing on non-galled treatment plants. All plants in the two treatments were covered with cages. Flies were released in August 2007, just before flowers appeared. One pair of flies per plant was released into the cages with plants of the pathogen-single galled treatment (treatment ii). Flies emerged from galls 12 weeks later (November 2007), and were collected from the cages with a pooter, over a five week period, to prevent further galling of the treatment plants. No unaccounted galling on non-treatment plants was observed during the trial period.

4.3.3 Reproductive output measurements

Due to the large number of stems per plant (approximately 15 to 20), four stems were randomly selected per plant for measurements of reproductive potential. All measurements were taken at the end of the flowering period in October 2007.

Seed production

For the laboratory and field trials, the total number of synflorescences per stem was counted. A subsample of five synflorescences was randomly selected and the number of capitula per synflorescence was recorded and a mean calculated.

Erasmus *et al*. (1992) recorded three categories of achenes: filled, empty and aborted (shrunken and undeveloped). A subsample of 15 capitula per

stem was randomly chosen and the number of achenes in each category per capitula counted. Very few aborted achenes were recorded; therefore they were grouped in the empty achenes category. Empty and aborted achenes are not viable (Erasmus *et al*., 1992). The total number of filled achenes per stem was calculated as follows:

Number filled achenes/ stem = (synflorescences/stem) x (mean capitula/synflorescence) x (mean filled achenes/capitula).

Number of germinable achenes/stem = (synflorescences/stem) x (mean capitula/synflorescence) x (mean filled achenes/capitula) x %germination/100.

Seed germinability and viability

Ten filled achenes per stem were randomly selected for germination trials to test percentage viability and germinability of achenes. Achenes for germination trials were placed on moist Whatman No.1 filter paper in 12cm petri-dishes and placed in a Phytotron controlled growth chamber, at the University of the Witwatersrand, Johannesburg, set at 20^{o}C with continuous white light or 12 hours of every 24 hours (as per Erasmus *et al*., 1992). The filter paper was moistened daily as required, and sprayed with the fungicide Benlate to control any fungal growth. Germination of achenes was recognised by protrusion of the radical by 2mm, and observed daily for 28 days. Germinated achenes were removed once they had been recorded.

4.3.4 Data analysis

One-way ANOVA with nesting and LSD tests were used to assess differences in reproductive output measurements between the six treatments for the laboratory trials (GLM procedure, StatSoft, 2007). Two-way ANOVA's were also used to determine significant interactions between single galled and pathogen infection, and double galled and pathogen infection. Student t-tests were used to assess differences in

reproductive output measurements between the two treatments for the field trials.

The laboratory and field trials cannot formally be statistically compared as they are two different experiments; however there is advantage and value to make these comparisons. Therefore, student t-tests were used to compare pathogen-only stems in the field and laboratory trials, and pathogen-single galled stems in the field and laboratory.

4.4. Results

4.4.1 Impact of AMISTAR ® on Ageratina adenophora *reproductive output*

In the laboratory, the fungicide, AMISTAR, caused no significant impact on the number of synflorescences per stem (t_{118}=0.05, P=0.957), the number of capitula per stem (t_{118}=0.64, P=0.523), the number of filled (t_{118}=0.48, P=0.633), empty and aborted (t_{118}=1.21, P=0.228) and total (t_{118}=1.09, P=0.274) achenes per capitulum, and the number of filled achenes per stem (t_{118}=0.48, P=0.636) in the laboratory trials. There was also no significant difference in percentage germination of achenes between plants sprayed with AMISTAR and unsprayed plants (t_{118}=0.02, P=0.983).

4.4.2 Number of synflorescences per stem

A One-way ANOVA showed a significant difference in the number of synflorescences per stem between the six treatments in the laboratory trials ($F_{5,164}$=7.75, P<0.001) (Fig. 4.1). The control and pathogen-only stems had the most synflorescences per stem, and stems galled twice had the lowest number of synflorescences per stem. There was no significant interaction between one fly release and pathogen infection ($F_{1,193}$=0.19, P=0.662) or two fly releases and pathogen infection ($F_{1,189}$=0.11, P=0.742) on the number of synflorescences per stem. The effect of the interaction between the fly and pathogen is equivalent, with the primary agent being the fly.

A student t-test showed no significant difference in the number of synflorescences per stem between pathogen-only and pathogen-single galled stems in the field (t_{121}=0.24, P=0.808) (Fig. 4.1).

The pathogen-only stems in the field had significantly more synflorescences per stem in comparison to pathogen-only stems in the laboratory (t_{129}=2.99, P=0.003), similarly the pathogen-single galled stems had more synflorescences per stem in the field, in comparison to the laboratory (t_{93}=4.85, P<0.001) (Fig. 4.1).

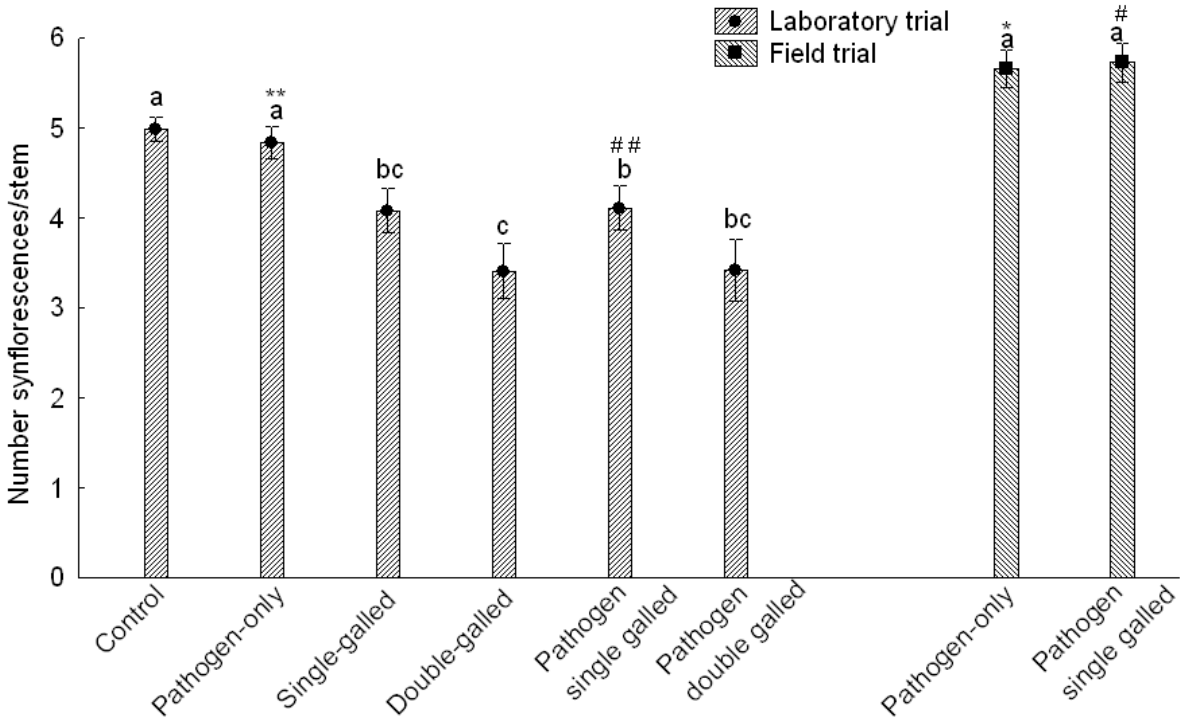


Figure 4.1: Number of synflorescences per *Ageratina adenophora* stem in response to different treatments, of pathogen infection and/or fly galling (single or double) in a laboratory and field trial. For field and laboratory trials separately, means followed by the same letter are not significantly different, at P<0.05 (LSD: laboratory trial, t-test: field trial). (Means ± SE). For comparing specific treatments between the field and laboratory trials, means followed by the same number of * or # are not significantly different, at P<0.05 (t-test).

4.4.3 Number of capitula per synflorescence

The number of capitula per synflorescence differed significantly between treatments in the laboratory trials ($F_{5,164}$=3.81, P=0.003). Control and

pathogen-only stems had a higher number of capitula per stem, in comparison to the other four treatments (Fig. 4.2). There was no significant interaction between one fly release and pathogen infection ($F_{1,193}$=2.57, P=0.111) or two fly releases and pathogen infection ($F_{1,189}$=0.19, P=0.662) on the number of capitula per synflorescence. The interaction between the agents led to an equivalent effect, the fly reduced the number of capitula per synflorescence the most.

There were significantly more capitula per synflorescence on pathogen-only stems compared to pathogen-single galled stems in the field (t_{121}=3.62, P<0.001) (Fig. 4.2).

The number of capitula per synflorescence was lowest in pathogen-only stems (t_{129}=3.16, P=0.002) and pathogen-single galled stems (t_{93}=4.42, P<0.001) in the field, relative to the same treatments in the laboratory (Fig. 4.2).

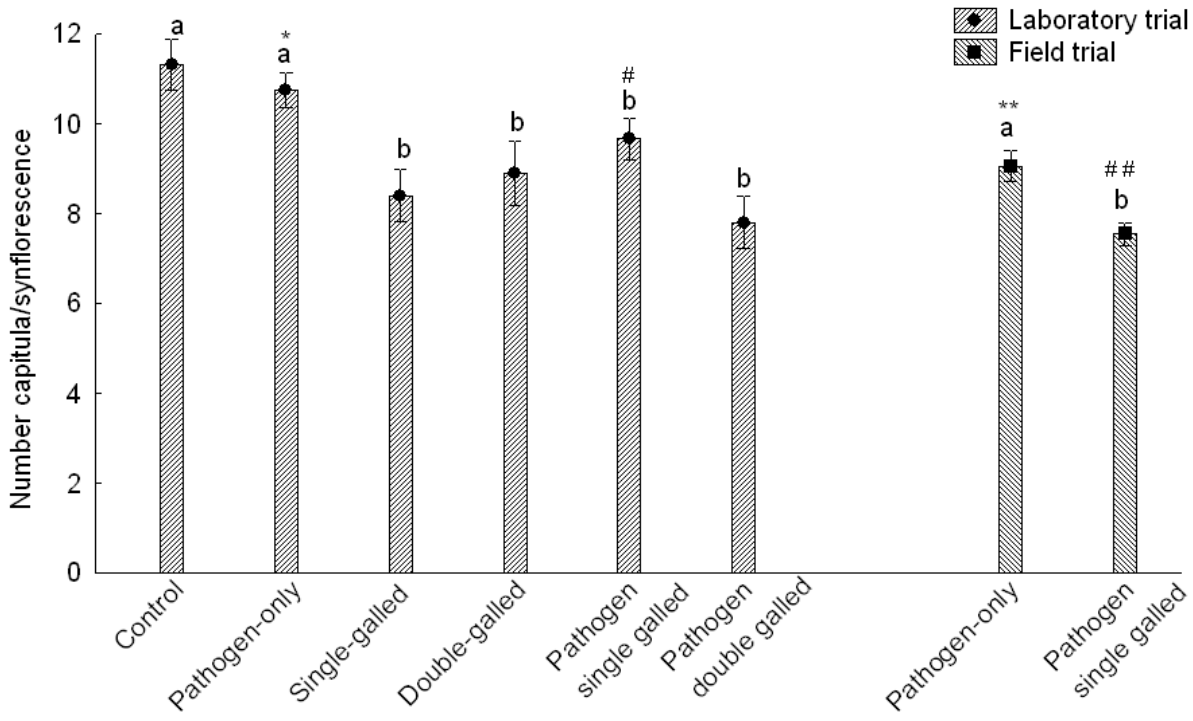


Figure 4.2: Number of capitula per *Ageratina adenophora* synflorescence in response to different treatments, of pathogen infection and/or fly galling (single or double) in a laboratory trial and field trial. For field and laboratory trials separately, means followed by the same letter are not significantly different, at P<0.05 (LSD: laboratory trial, t-test: field trial). (Means ± SE). For comparing specific treatments between the field and laboratory trials, means followed by the same number of * or # are not significantly different, at P<0.05 (t-test).

4.4.4 Number of filled, empty and aborted achenes per capitulum

The treatments in the laboratory trial had a significantly different number of filled ($F_{5,164}$=84.93, P<0.001), empty and aborted ($F_{5,164}$=12.63, P<0.001) and total ($F_{5,164}$=23.40, P<0.001) achenes per capitulum (Fig. 4.3). Control stems had the highest number of filled and total achenes per capitulum, and the lowest number of empty and aborted achenes per capitulum. Double-galled stems, regardless of pathogen infection, had the lowest number of filled achenes per capitulum. There was a significant interaction between one fly release and pathogen infection on the number of filled ($F_{1,193}$=16.43, P<0.001) and total achenes per capitulum ($F_{1,193}$=14.61, P<0.001), but not with the number of empty and aborted achenes per capitulum ($F_{1,193}$=0.02, P=0.877). The interaction of two fly releases and pathogen infection was significant for the number of filled ($F_{1,189}$=28.15, P<0.001), empty and aborted ($F_{1,189}$=4.41, P=0.037) and total ($F_{1,189}$=4.98, P=0.027) achenes per capitulum. The interaction between the agents has an equivalent effect on the number of filled, empty and aborted, and total achenes, where the fly caused the greatest damage.

There were significantly more empty and aborted achenes on pathogen-single galled stems compared to pathogen-only stems in the field (t_{121}=3.01, P=0.003) (Fig. 4.3). However, there was no significant difference between the two treatments in terms of filled achenes (t_{121}=1.66, P=0.099) and the total number of achenes (t_{121}=0.63, P=0.527) per capitulum.

In comparison to the laboratory, the pathogen-only stems (t_{129}=3.45, P<0.001) and pathogen-single galled stems (t_{93}=3.61, P<0.001) in the field had fewer filled achenes per capitulum (Fig. 4.3). The number of empty and aborted achenes per capitulum was not different between pathogen-only stems in the field and laboratory (t_{129}=1.71, P=0.090), but was higher in the pathogen-single galled field stems (t_{93}=2.28, P=0.025). The total number of achenes per capitulum was lowest on field pathogen-only stems

(t_{129}=2.29, P=0.023), but was similar between pathogen-single galled stems in the field and laboratory (t_{93}=1.15, P=0.254).

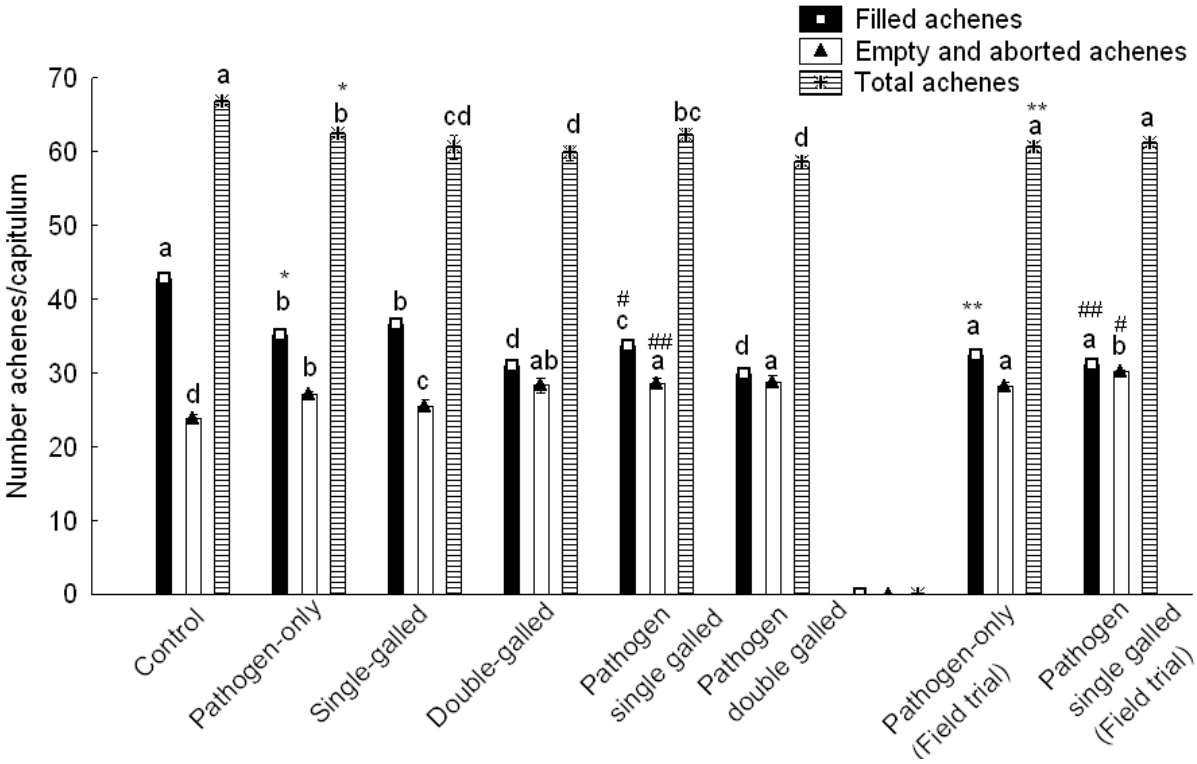


Figure 4.3: Number of filled, empty/aborted and total achenes per *Ageratina adenophora* capitula in response to different treatments, of pathogen infection and/or fly galling (single or double) in a laboratory trial and field trial. For field and laboratory trials separately, means followed by the same letter are not significantly different, at P<0.05 (LSD: laboratory trial, t-test: field trial). (Means ± SE). For comparing specific treatments between the field and laboratory trials, means followed by the same number of * or # are not significantly different, at P<0.05 (t-test).

4.4.5 Number of filled and germinable achenes per stem

There was a significant difference between the treatments in the laboratory trials in terms of the number of filled achenes per stem ($F_{5,164}$=16.46, P<0.001) (Fig. 4.4) and germinable achenes per stem ($F_{5,164}$=16.09, P<0.001) (Fig. 4.5). The control stems had the highest number of filled and germinable achenes per stem. There was a significant interaction between single fly releases and pathogen infection on the number of filled ($F_{1,193}$=5.99, P=0.015) and germinable ($F_{1,193}$=6.22, P=0.013) achenes per

stem. However, there was no significant interaction between double fly releases and pathogen infection on the number of filled ($F_{1,193}$=1.81, P=0.180) and germinable ($F_{1,193}$=1.55, P=0.215) achenes per stem. The effect of the interaction between the agents is equivalent, with the fly being the most damaging agent as regardless of pathogen infection; galled stems had the lowest number of filled and germinable achenes per stem.

Pathogen-only stems had significantly more filled achenes (t_{121}=2.77, P<0.001) (Fig. 4.4) and germinable achenes (t_{121}=2.89, P=0.004) (Fig. 4.5) per stem than pathogen-single galled stems in the field.

The number of filled (t_{129}=0.04, P=0.967) and germinable (t_{129}=0.59, P=0.559) achenes per stem did not differ between pathogen-only stems in the field or laboratory, similarly the number of filled (t_{93}=0.18, P=0.856) and germinable (t_{93}=0.45, P=0.653) achenes per stem did not differ between pathogen-single galled stems in the field or laboratory (Fig. 4.4, Fig. 4.5).

4.4.6 Percentage germination of achenes

The percentage germination of achenes did not differ significantly between treatments in the laboratory trials ($F_{5,164}$=0.29, P=0.918) (Fig. 4.6), or in the field trials (t_{121}=1.13, P=0.262) (Fig. 4.6).

The percentage germination of filled achenes was lower in the field than the laboratory on pathogen-only stems (t_{129}=3.08, P=0.003) and pathogen-single galled stems (t_{93}=3.25, P=0.002) (Fig. 4.6).

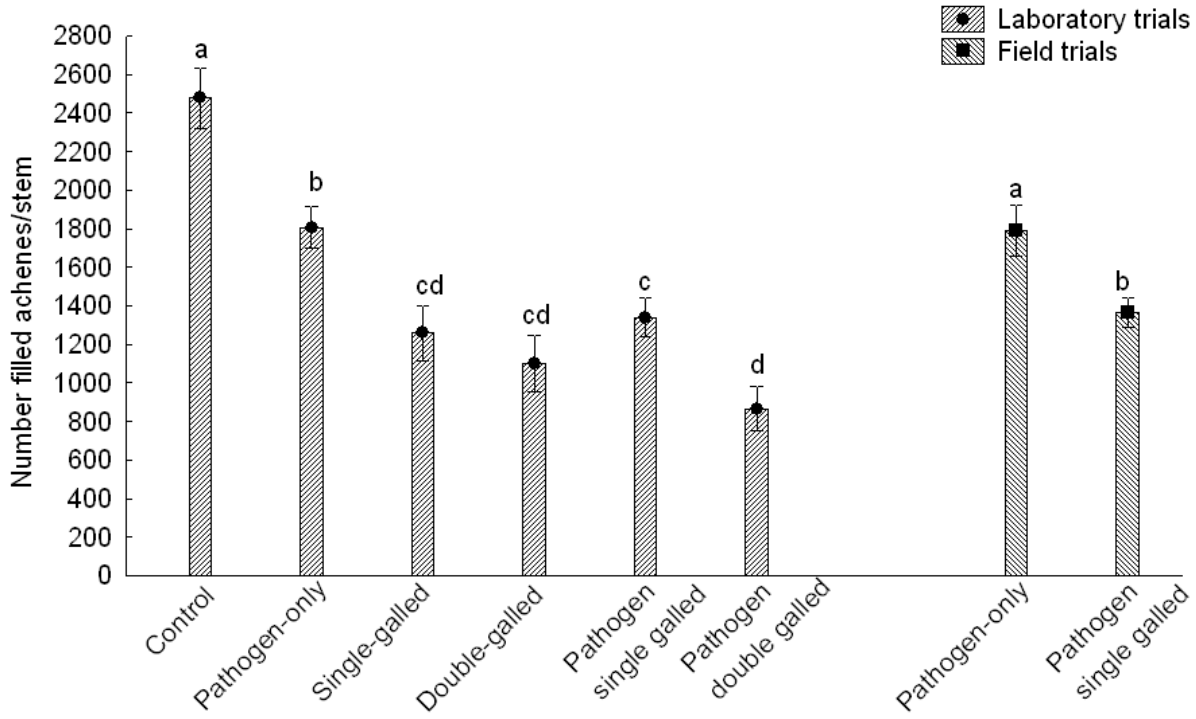


Figure 4.4: Number of filled achenes per *Ageratina adenophora* stem in response to different treatments, of pathogen infection and/or fly galling (single or double) in a laboratory trial and field trial. For field and laboratory trials separately, means followed by the same letter are not significantly different, at $P<0.05$ (LSD: laboratory trial, t-test: field trial). (Means ± SE). For comparing specific treatments between the field and laboratory trials, means followed by the same number of * or # are not significantly different, at $P<0.05$ (t-test).

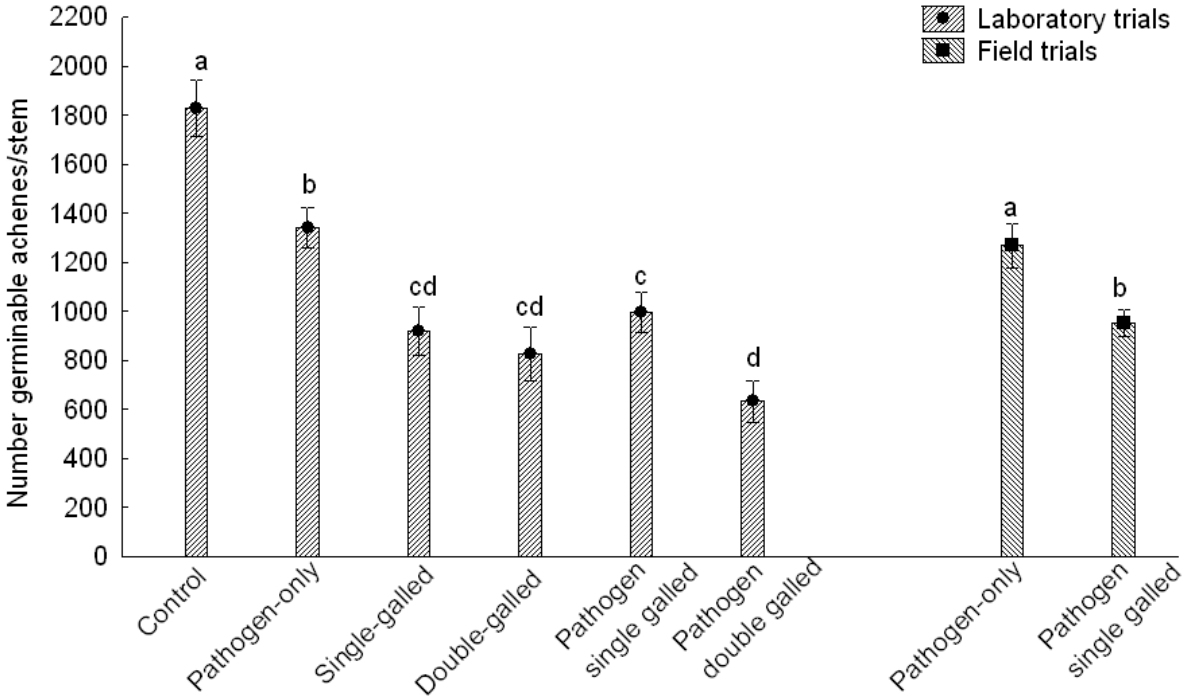


Figure 4.5: Number of germinable achenes per *Ageratina adenophora* stem in response to different treatments, of pathogen infection and/or fly galling (single or double) in a laboratory trial and field trial. For field and laboratory trials separately, means followed by the same letter are not significantly different, at $P<0.05$ (LSD: laboratory trial, t-test: field trial). (Means ± SE). For comparing specific treatments between the field and laboratory trials, means followed by the same number of * or # are not significantly different, at $P<0.05$ (t-test).

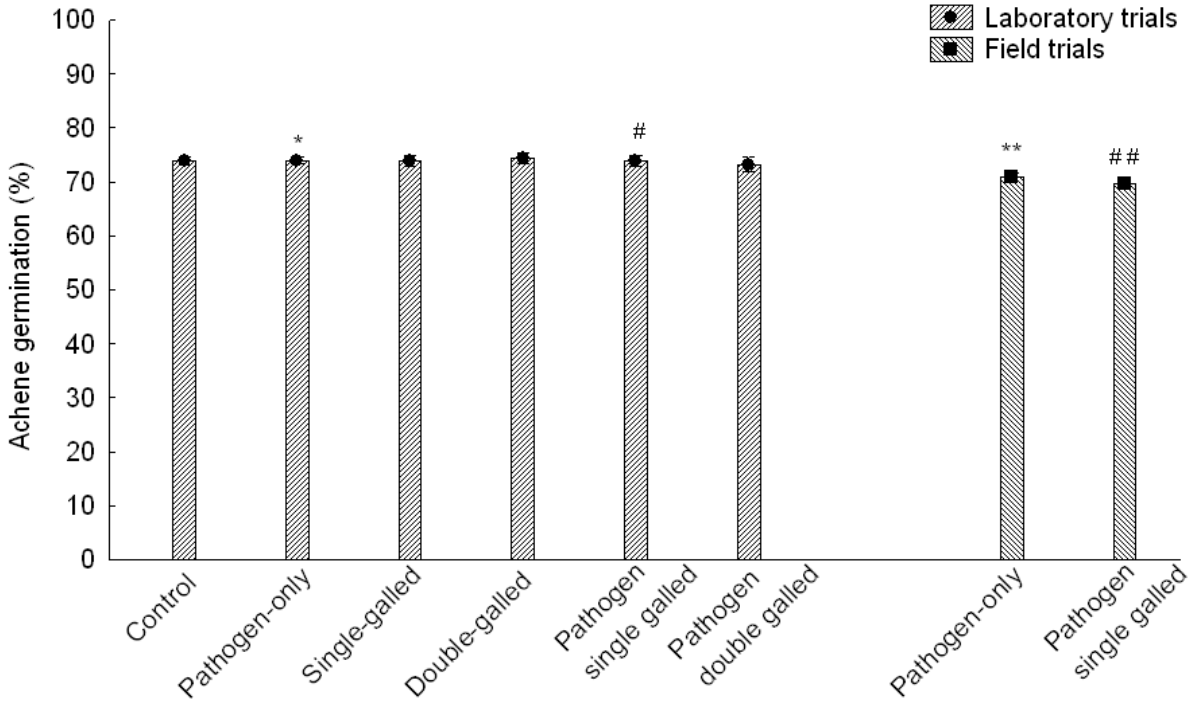


Figure 4.6: Percentage germination of *Ageratina adenophora* achenes in response to different treatments, of pathogen infection and/or fly galling (single or double) in a laboratory trial and field trial. For field and laboratory trials separately, means followed by the same letter are not significantly different, at $P<0.05$ (LSD: laboratory trial, t-test: field trial). (Means ± SE). For comparing specific treatments between the field and laboratory trials, means followed by the same number of * or # are not significantly different, at $P<0.05$ (t-test).

4.5 Discussion

It is not easy to identify a set of traits that predict the invasiveness of plants (Pyšek & Richardson, 2007). However, there are some reproductive traits that are important in invasive plants, such as early and extended flowering periods, efficient seed dispersal, seed size and long-term seed banks which allow them to spread and persevere in new areas rapidly (Rejmánek & Richardson, 1996; Pyšek & Richardson, 2007). *Ageratina adenophora* has the ability to produce a high number of light, wind-dispersed seeds. From the laboratory trials an individual stem can produce 1827±114 (mean±SE) germinable achenes, this calculates to potentially 36 540 germinable seeds per 20-stemmed plant. In addition crofton weed is apomictic, and is not reliant on the availability of pollinators to produce

these high numbers of viable seeds (Rambuda & Johnson, 2004). It is for this reason that manual clearing, within the Magaliesberg, is implemented before the flowering period. However crofton weed grows in inaccessible areas within the mountain range, therefore leaving a few plants behind still leads to a high number of achenes produced. The stem gall fly, *P. utilis*, reduces the reproductive output of each stem by an average of 53.8%, particularly with repeated galling (65.3%); the leaf-spot pathogen, *P. ageratinae*, has a lesser (reduced by 26.7%) but still significant effect on the reproductive output of the stems.

Galls are known to act as nutrient sinks, because resources destined for growth sites (sinks) from production or storage areas (sources) are redirected towards the gall, resulting in an imbalance of the plant's source-sink relationship (Larson & Whitman, 1997; Florentine *et al*., 2005; Dorchin *et al*., 2006; Moseley *et al*., 2009). This imbalance may lead to less resources being available for seed production (Woods *et al*., 2008). *Procecidochares utilis* galls are nutrient sinks (Bennett & van Staden, 1986; Erasmus *et al*., 1992), vegetative trials have shown decreased stem growth above the position of the gall (Chapter 2 and 3). Fewer resources reach the apex of the stem, in both single and double galled stems, leading to a reduced number of synflorescences and fewer capitula per synflorescence, in comparison to the control stems. Within the capitula, fewer filled achenes, and more empty and aborted achenes develop, particularly with stems galled twice. This results in 49.67% (single-galled), 45.5% (pathogen-single galled), 54.8% (double-galled) and 65.3% (pathogen-double galled) less filled and germinable achenes per stem, in comparison to the control stems.

In both the laboratory and field trials only 20-40% of the leaves on crofton weed stems were infected with the pathogen before flowering (Chapter 2 and 3). Weeds can shed pathogen infected leaves and increase the vegetative growth of new uninfected leaves, in order to outgrow the disease pressure (Charudattan, 2005, 2010); this was observed on crofton weed stems in both the laboratory and field trials (Chapter 2 and 3). The

level of the leaf-spot pathogen infection on crofton weed stems is not severe enough to decrease the number of synflorescences, or the number of capitula per synflorescence on pathogen-only stems in comparison to control stems. However, the disease pressure does result in fewer resources for the number of filled (viable) achenes per capitulum, resulting in a 26.7% reduction of filled achenes and hence seed production on pathogen-only stems. However, this impact of the pathogen is less than on galled-only stems. The extent to which a pathogen influences a weed depends on the severity of the pathogen infection (Charudattan, 2005), therefore defoliation by the pathogen may need to be more severe for a longer period of time to decrease the reproductive output of crofton weed to a higher level.

Two theories explain successful biocontrol programmes using multiple biocontrol agents (Denoth *et al*., 2002), the cumulative stress model states that the stress of all the agents in combination increases the impact on the target weed (Harris, 1985; Hoffmann & Moran, 1998; Turner *et al*., 2010), whilst the lottery model states that only one agent is needed to sufficiently control the target weed (Myers, 1985, 2008; McEvoy & Coombs, 2000). In these laboratory trials, the stem gall fly and pathogen in combination provided better control than the pathogen-only treatment, with reduced numbers of synflorescences, capitula per synflorescence, filled achenes per capitula and filled achenes produced on each crofton weed stem. However, the combination of the agents did not increase the cumulative stress on the reproductive potential of the crofton weed stems in comparison to the galled-only stems. The percentage germination of filled achenes did not differ between any of the treatments, including the absence of agents (control). In the field trials pathogen-single galled stems did not differ significantly from pathogen-only stems in terms of the number of synflorescences, but the addition of galling did reduce the number of capitula within the synflorescence and the number of filled achenes per stem. Therefore, the overall interaction between the biocontrol agents in the laboratory and field leads to an equivalent effect on crofton weed

reproductive output (Hatcher, 1995; Hatcher & Paul, 2001). That is the effect of the two agents together is equivalent to the highest impacting agent, which in this system is the fly. However, crofton weed compensates for galling with increased sideshoot (vegetative reproduction) growth (Chapter 2 and 3). These sideshoots will reproduce sexually in the next flowering season; therefore if these sideshoots are not galled in the following season, the reproductive output of the stem could increase over a longer term period. The pathogen inhibits sideshoot growth (Chapter 2 and 3); therefore whilst the pathogen may not directly increase the cumulative control of crofton weed reproductive output immediately, over the longer term period it may have a role to play in reducing sideshoot growth, thereby reducing future sexual reproduction induced by galling. In addition, field observations indicate that up to 95% of stems are infected with the pathogen and only 20% are galled, therefore the pathogen may play a role in reducing the reproductive output of ungalled stems. The agents may also differ in their efficacy under different environmental conditions.

The success of biocontrol agents is often predicted by using laboratory trials; however these controlled conditions are not always representative of field conditions (Rosskopf *et al*., 1999; Morin *et al*., 2006, 2009). Laboratory trials predicted an additive effect on crofton weed vegetative growth with both the fly and pathogen used in combination; however the field trials showed the agents in combination did not have a cumulative effect, with the pathogen being the highest impacting agent (Chapter 2 and 3). Conversely, these trials on reproductive output show similar results between the laboratory and field, the number of germinable achenes per stem did not differ between pathogen-only or pathogen-single galled stems in the laboratory and field.

This study has shown that galling and pathogen infection can reduce the reproductive output of individual crofton weed stems. Does this impact translate to a population level impact on infestations of crofton weed? Plant populations may be seed-limited, where not enough seeds are

produced to increase the plant's population, or micro-site limited where there are not enough suitable micro-sites available to increase the plant population (Turnbull *et al*., 2000; Clark *et al*., 2007; Herrera & Laterra, 2009). Factors affecting seed-limitation include disturbance, seed size and seed bank longevity (Clark *et al*., 2007). However, if plant populations are not seed-limited herbivory of reproductive structures has a small effect at the population level (Turnbull *et al*., 2000). Therefore, if the level of agent attack is not severe and prevalent throughout the whole infestation, then there may not be an overall net reduction in seed output (Rieder *et al*., 2001; Woods *et al*., 2008). For example, the flower-bud destroying *Trichapion lativentre* reduces the seed set of *Sesbania punicea* by 98%, which reduces the density of immature plants but there is no effect on the density of mature plants (Hoffmann & Moran, 1998). If crofton weed is not seed-limited, then the reduction of reproductive output by these agents may not be enough to successfully control the spread of crofton weed. Weeds are able to maintain high density levels through soil seed banks (Witkowski & Wilson, 2001; Davis, 2006). If the soil seed bank of crofton weed is only composed of 50% of seeds produced, then the reduction of seed output through galling may not have a population effect on crofton weed infestations. However, crofton weed may not be micro-site limited, as infestations occur along roadsides and water courses, and are easily carried to areas outside of the adult population. Therefore, a reduction in the number of viable seeds being carried to new suitable areas will be beneficial for the control of crofton weed in South Africa.

4.6 Conclusion

The gall fly and pathogen reduce the reproductive output of crofton weed by 53.8% and 26.7% respectively. However, the most effective single agent is the gall fly, particularly if there is repeated galling on a stem. The combination of the gall fly and pathogen is equivalent as the agents together do not cumulatively decrease crofton weed reproductive output in

comparison to the gall fly alone. However, as crofton weed compensates for galling with increased sideshoot growth, the pathogen is still important in the biocontrol programme.

Chapter 5: Ecophysiological response of the invasive alien plant *Ageratina adenophora* to multiple biological control agents.

5.1 Abstract

As photosynthesis is vital to a plant's survival, any negative influence on it could result in reduced growth and reproduction. *Ageratina adenophora* (crofton weed) has had two biocontrol agents, *Procecidochares utilis*, a stem gall fly, and *Passalora ageratinae*, a leaf-spot fungal pathogen, released against it in South Africa. This study investigated the effect of the two biocontrol agents, individually and together, on a number of leaf ecophysiological responses in crofton weed. Crofton weed plants were exposed to one of six treatments (n=10plants/treatment): control (no agents), pathogen-only, single-galled only, double-galled only, pathogen-single galled, and pathogen-double galled, all for a period of six months. Pathogen-only and pathogen-galled plants had significantly lower transpiration, stomatal conductance and photosynthetic rates in comparison to the control and galled-only plants. The instantaneous water-use efficiency was highest for the control plants in comparison to agent infected plants. The chlorophyll fluorescence parameters show that galling stresses the efficiency of photosystem II in the lower (older) leaves of crofton weed plants, but the most stress is inflicted by the pathogen. Galled plants compensate for this stress by increasing sideshoot growth, whilst pathogen infected plants compensate for the stress by increasing vertical growth with new healthy leaves. Chlorophyll content was lowest in the lower (older) leaves with visible leaf-spots. These results correspond well with what was predicted from laboratory and field trials. Overall the interaction between the agents results in an equivalent effect on crofton weed, with the pathogen causing the most direct damage to the lower, older leaves of the plants.

5.2 Introduction

Classical biocontrol of weeds is the deliberate introduction of a target weed's natural enemies from the native range, into the invaded area, to reduce the weeds abundance (Morin *et al*., 2009). Biocontrol agents affect the growth, reproductive output and survival of the target weed (van Klinken & Raghu, 2006; Morin *et al*., 2009). Insect feeding affects the photosynthetic capacity and functioning, transpiration, fluid and nutrient transport, and chlorophyll content of plants (Buntin *et al*., 1993; Macedo *et al*., 2007; Nabity *et al*., 2009). However, with a few exceptions such as water hyacinth, *Eichhornia crassipes*, (Ripley *et al*., 2006), few studies have tested the ecophysiological response of weeds to their biocontrol agents.

Ageratina adenophora, (Sprengel) King and Robinson (syn. *Eupatorium adenophorum* Spreng.) (Asteraceae), crofton weed, is an invasive weed in South Africa, Australia, New Zealand, Hawaii, India and China (Julien & Griffiths, 1998). Two biocontrol agents, a stem gall fly, *Procecidochares utilis* Stone (Tephritidae), and a leaf-spot fungal pathogen *Passalora ageratinae* Crous and A.R. Wood (Mycosphaerellaceae) (previously named *Cercospora eupatorii* Peck or *Phaeoramularia* sp.) (Crous *et al*., 2009) have been released against crofton weed and are established in South Africa (Kluge, 1991; Heystek *et al*., 2011).

Previously biocontrol success has predominantly been defined as a reduction in density of weed populations (Hoffmann & Moran, 2008). In Hawaii the gall fly alone has been very successful at reducing the density of crofton weed infestations (Bess & Haramoto, 1959, 1972; Muniappan *et al*., 2009). However, success in biocontrol may be more subtle than reducing the density of weed infestations, such as reducing the rate of plant growth (Hoffmann & Moran, 2008). Laboratory trials predict the interaction between the two agents leads to an additive effect on crofton weed, that is repeated galling by the fly reduces stem growth, and the pathogen inhibits sideshoot growth promoted by galling (Chapter 2). Field

trials showed an equivalent effect (Hatcher, 1995; Hatcher & Paul, 2001) of the two agents together, the gall fly may reduce stem vigour over a longer time period and the pathogen may reduce the compensatory effect of increased sideshoot growth (Chapter 3). Laboratory and field trials showed that the fly reduced the number of germinable achenes per crofton weed stem by 53.8% and the pathogen reduced germinable achenes by 26.7% (Chapter 4).

The galls may act as a nutrient sink redirecting plant growth resources for their own growth, and the pathogen, through direct damage to the leaf, may interfere with the photosynthetic capacity of leaves. Photosynthesis, the process of absorbing light energy in the chloroplasts to produce organic compounds, is vital to plant growth and survival (McDowell, 2002; Lambers *et al*., 2008). Therefore, another way of evaluating plant stress is by measuring leaf physiological variables such as photosynthesis, stomatal conductance, transpiration, water use efficiency, chlorophyll fluorescence kinetics and chlorophyll pigment content of the plant (Lambers *et al*., 2008; Resco *et al*., 2008; Govender *et al.*, 2009). These measurements have been used to compare the photosynthetic capacity of invasive and non-invasive plant species (McDowell, 2002; Feng *et al*., 2007a, 2007b), under different environmental conditions (Field *et al*., 1983; Meir *et al*., 2007; Santiago & Wright, 2007; Resco *et al*., 2008), plants with different leaf life spans (Witkowski *et al*., 1992), the effect of biocontrol agents under different nutrient conditions (Ripley *et al*., 2006) and to ground truth remote sensing data (Govender *et al*., 2009).

Previous studies have evaluated the effect of low temperature and low irradiance levels on the photosynthetic capacity of crofton weed (Feng *et al*., 2007a, 2007b; Li *et al*., 2008). Crofton weed is able to tolerate low temperatures and low irradiance levels. However, no studies have evaluated the effect of the stem gall fly and leaf-spot pathogen on the photosynthetic system of crofton weed leaves. The aim of this study was to evaluate the effects of the pathogen and fly, individually and in combination, on the ecophysiology of crofton weed, and whether these

results correspond with the effects of the agents seen on crofton weed's vegetative growth and reproductive output.

5.3 Material and methods

5.3.1 Laboratory trials

The laboratory trial was run in a greenhouse on the University of the Witwatersrand campus, Johannesburg (26˚11'20.97"S 28˚01'55.37"E). The greenhouse had 30% shading, with mean summer temperatures of 25.9ºC (min=18 ºC, max=35 ºC) and mean winter temperatures of 16.3ºC (min=5 ºC, max=27 ºC). The relative humidity ranged from 43% to 100% (mean=81.5%) in the summer months (December 2007 to February 2008), and 28% to 100% (mean=67.8%) in the winter months (June 2008 to August 2008).

In December 2007, 100 crofton weed seeds (collected from plants grown in the greenhouse in September 2007) were sown in 5-litre plant bags filled with potting soil. Three weeks later seedlings emerged. Plants were well watered once a day for half an hour, in the morning, and fertilised once a month with *Nitrosol* (N 8%: P 2%: K 5.8%).

In mid- March 2008, 10 plants, approximately 30cm tall, were randomly allocated to each of the following six treatments:

(i) no biocontrol agents (control);
(ii) plants infected with the fungal pathogen, *P. ageratinae* (pathogen-only);
(iii) plants exposed to one release of *P. utilis* (hereafter referred to as single-galled);
(iv) plants exposed to two releases of *P. utilis* (hereafter referred to as double-galled);
(v) plants infected with *P. ageratinae* and exposed to one release of *P. utilis* (hereafter referred to as pathogen-single galled);

(vi) plants infected with *P. ageratinae* and exposed to two releases of *P. utilis* (hereafter referred to as pathogen-double galled).

In order to prevent the pathogen spreading to non-pathogen treatment plants, plants in treatment i, iii and iv were sprayed with a broad-spectrum fungicide AMISTAR ®. A mechanical pressurised sprayer was calibrated to a spray volume of 60-litre/ha; AMISTAR ® was sprayed at 500ml/ha every four weeks. Plants that were infected with the pathogen were sprayed with the same volume of water.

Plants were infected with the pathogen by tying fresh infected leaves, from a stock culture at the University of the Witwatersrand, onto the stems at the end of March 2008. Plants from all treatments were then covered with plastic bags, to create a dew period of 24 hours (Wang *et al*., 1997). At the end of April 2008 the first fungal leaf-spots became visible, but throughout the trial no more than 30-40% of the leaves on plants were infected.

To prevent galling on non-fly treatment plants, the plants of all treatments were placed in separate cages. The cages were 1m tall x 1.2m x 0.9m frames of conduit piping, covered in fine white netting.

One pair of flies was released per plant for the galled treatments (treatment iii-vi) at the end of March 2008. Flies emerged from galls 10 weeks later and were collected, for 3 weeks, in order to stop the emerging flies from ovipositing on the treatment plants. The second release of flies took place in July 2008. One pair of flies was released per plant onto the double-galled treatments (treatments v–vi).

5.3.2 Leaf ecophysiological measurements

All measurements were taken using the top and/or seventh fully-expanded leaves of the treatment plants at the end of August 2008. The top leaf was above the gall/s (on galled plants) and did not have visible leaf-spots (on pathogen infected plants), the seventh leaf was below the

gall/s (on galled plants) and had visible leaf-spots (on pathogen infected plants).

Photosynthesis, Stomatal Conductance, Transpiration, Water-Use Efficiency

Transpiration, stomatal conductance and photosynthesis of only the seventh fully-expanded leaf were measured using a LI-6200 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA). The leaves were measured in the morning, between 09:00 and 11:00, on the 20th and 21st August 2008. The weather during measurements was clear and cloudless. Leaf measurements were alternated between the treatments to minimise any variation in environmental conditions during the time of readings. Instantaneous water-use efficiency was calculated as photosynthesis/transpiration.

Chlorophyll fluorescence kinetics

Chlorophyll fluorescence parameters give an indication of how well photosystem II (PSII) is functioning in terms of light energy use during photosynthesis (Lambers *et al*., 2008; Li *et al*., 2008). A decrease in the F_v/F_m value shows that PSII is not functioning efficiently, and therefore the plant is under physiological stress. Chlorophyll fluorescence parameters were measured with a portable OS5-FL chlorophyll fluorometer (Opti-Sciences, Tyngsboro, MA, USA). The top and seventh fully-expanded leaves of the treatment plants were dark adapted for 10 minutes before been measured, on clear, cloudless mornings between 09:00 and 11:00, on the 29th and 31st August 2008. Each leaf was sampled twice over the two days.

Chlorophyll content

The chlorophyll content of leaves was measured using a SPAD-502 Chlorophyll Meter (Spectrum Technologies Inc., Illinois, USA). The top and seventh fully-expanded leaves per plant in each of the six treatments were used for chlorophyll content measurements. Three measurements were taken along the length of the leaf and the mean chlorophyll content calculated, on the 28th August 2008, between 14:00 and 16:00.

5.3.3 Data analysis

One-way ANOVA and LSD tests were used to assess differences in leaf physiological measurements between the six treatments for the laboratory trials (GLM procedure, StatSoft, 2007). Two-way ANOVA's were also used to determine significant interactions between single galled and pathogen infection, and double galled and pathogen infection.

5.4 Results

5.4.1 Transpiration, Stomatal Conductance, Photosynthesis and Instantaneous Water-Use Efficiency (WUE).

There was no significant difference (P>0.05) in the air temperature, leaf temperature or relative humidity whilst measurements were been taken between treatments (Table 5.1). Transpiration rate ($F_{5,52}$=5.88, P<0.001) (Fig. 5.1) and stomatal conductance ($F_{5,52}$=9.81, P<0.001) (Fig. 5.2) were significantly higher for the control and galled-only treatments, resulting in more water loss and movement through the stomata, in comparison to pathogen-only and pathogen-galled treatments. Pathogen-only and pathogen-galled treatment leaves had the lowest photosynthetic rate ($F_{5,52}$=11.82, P<0.001) (Fig. 5.3). There was no significant interaction between single fly releases and pathogen infection for transpiration ($F_{1,33}$=2.46, P=0.126), stomatal conductance ($F_{1, 33}$=2.60, P=0.117) or photosynthetic rate ($F_{1,33}$=0.12, P=0.733). There was also no significant

interaction between double fly releases and pathogen infection for transpiration ($F_{1,45}$=1.57, P=0.216) and stomatal conductance ($F_{1, 45}$=0.02, P=0.897), however the interaction was significant for the photosynthetic rate ($F_{1,45}$=4.93, P=0.019). The effect of the interaction between the two agents on crofton weed transpiration, stomatal conductance and photosynthesis was equivalent, with the pathogen causing the most ecophysiological stress. All the treatment plants had a significantly lower instantaneous water-use efficiency in comparison to the control ($F_{5,52}$=4.42, P=0.002) (Fig. 5.4).

Table 5.1: Air and leaf temperatures and relative humidity (mean±SE) during the measurements of transpiration, stomatal conductance and photosynthesis for leaves of *Ageratina adenophora* in response to six different treatments, of pathogen infection and/or fly galling (single or double), in the greenhouse trial over two days. (NS, P>0.05 (LSD)).

Factor	Day	Control	Pathogen-only	Single-galled	Double-galled	Pathogen-single galled	Pathogen-double galled	*P*
Air temperature (^{o}C)	20/08/08	28.8±0.7	28.4±0.7	28.7±0.7	29.4±1.1	28.5±0.6	27.8±1.3	NS
	21/08/08	26.8±0.9	27.0±0.8	26.9±1.4	26.8±0.6	25.2±1.9	27.7±1.2	NS
Leaf temperature (^{o}C)	20/08/08	28.3±0.6	28.4±0.6	28.2±0.7	28.9±0.8	27.5±0.7	28.4±1.3	NS
	21/08/08	26.5±0.9	27.1±0.7	26.8±1.3	26.5±0.4	28.8±0.6	28.0±1.3	NS
Relative humidity (%)	20/08/08	62.3±1.2	55.1±4.3	61.4±3.4	58.2±1.3	59.0±3.0	57.5±5.3	NS
	21/08/08	69.1±2.1	67.4±2.4	66.9±2.4	64.5±3.7	64.9±3.7	57.8±4.8	NS

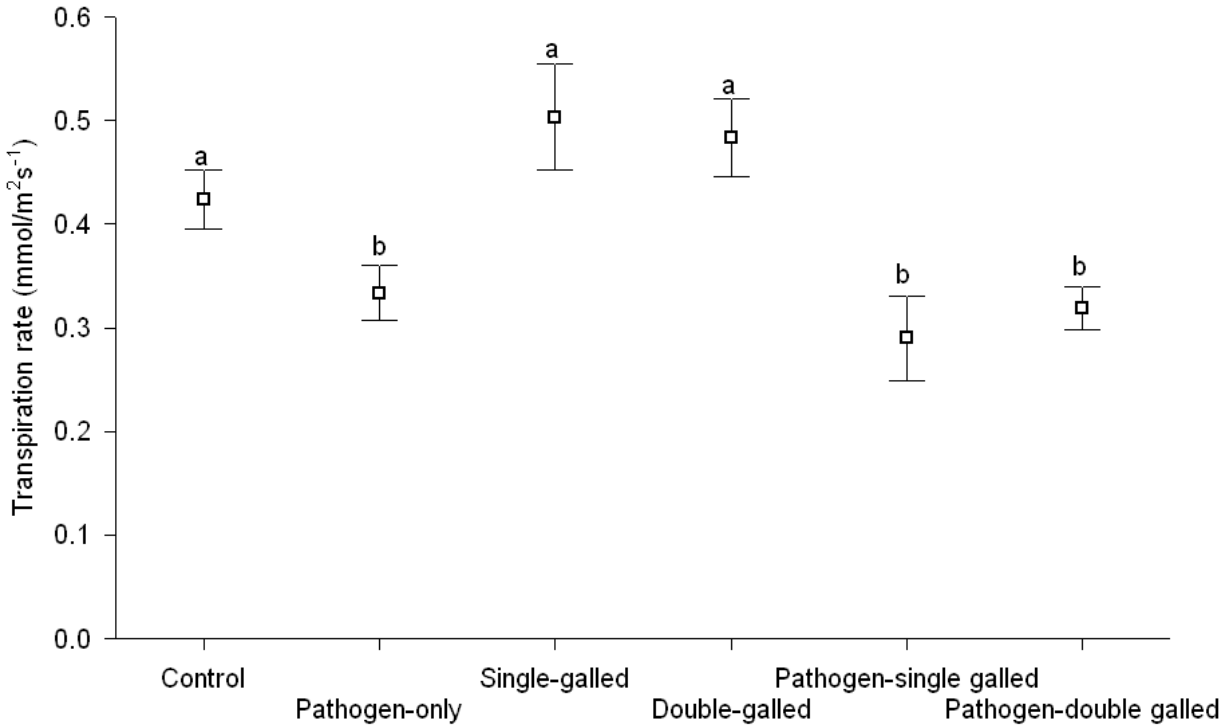


Figure 5.1: Transpiration rate of the seventh fully-expanded *Ageratina adenophora* leaf in response to six different treatments, of pathogen infection and/or fly galling (single or double). Means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

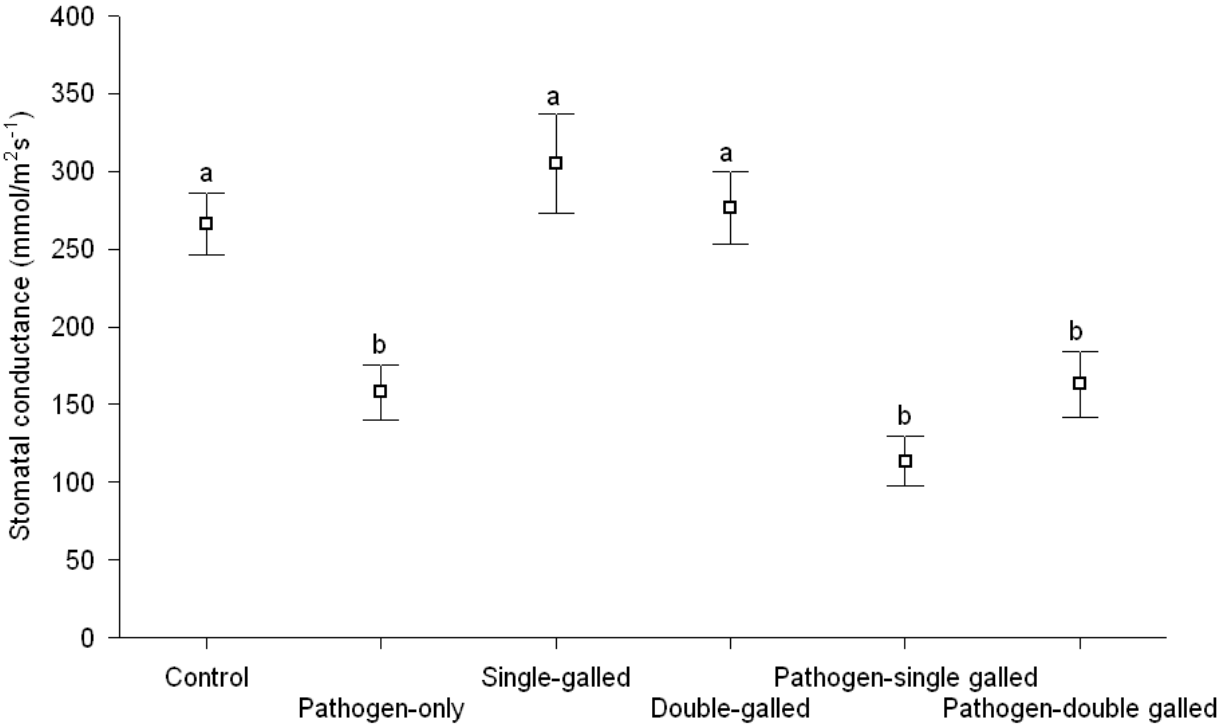


Figure 5.2: Stomatal conductance of the seventh fully-expanded *Ageratina adenophora* leaf in response to six different treatments, of pathogen infection and/or fly galling (single or double). Means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

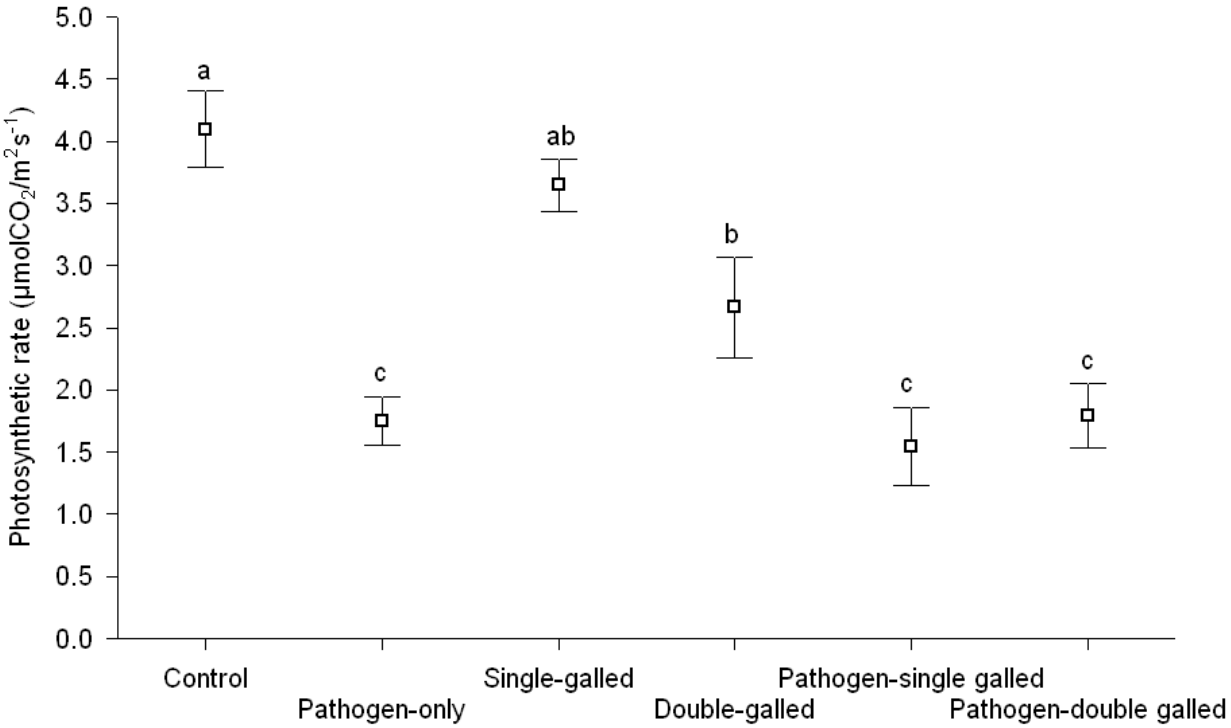


Figure 5.3: Photosynthetic rate of the seventh fully-expanded *Ageratina adenophora* leaf in response to six different treatments, of pathogen infection and/or fly galling (single or double). Means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

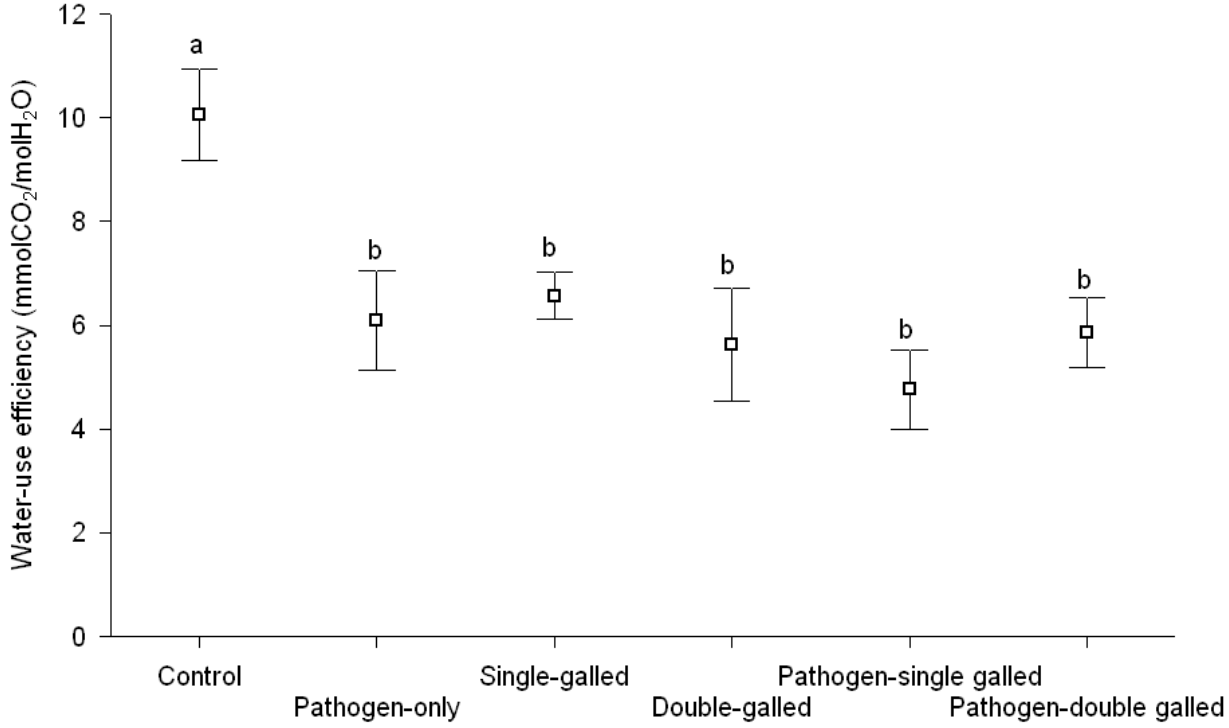


Figure 5.4: Instantaneous water-use efficiency (WUE) of the seventh fully-expanded *Ageratina adenophora* leaf in response to six different treatments, of pathogen infection and/or fly galling (single or double). Means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

5.4.2 Chlorophyll fluorescence kinetics

The fluorescence (F_v) and F_v/F_m values for the top leaf of the plants were not significantly different across treatments ($F_{5,102}$=0.47, P=0.801; $F_{5,102}$=0.49, P=0.781 respectively) (Fig. 5.5); therefore the top leaves were not considered to be under physiological stress from the biocontrol agents. There was a significant difference between treatments in terms of F_v ($F_{5,102}$=36.53, P<0.001) and F_v/F_m ($F_{5,102}$=40.82, P<0.001) for the seventh leaf of the plants (Fig. 5.5). There was a significant interaction between single galled and pathogen infection on the F_v ($F_{1, 69}$=12.25, P<0.001) and F_v/F_m ($F_{1, 69}$=5.23, P=0.025) values for the seventh leaf of the plants. Similarly the F_v ($F_{1, 94}$=9.65, P=0.003) and F_v/F_m ($F_{1, 94}$=8.44, P=0.005) values for the seventh leaf of the plants showed a significant interaction between double galled and pathogen. The pathogen-only and pathogen-galled plants were under physiological stress (in terms of photosystem II) from the pathogen infection. Therefore, combining the two agents has an equivalent effect with the pathogen influencing photosystem II the most.

5.4.3 Chlorophyll content

The chlorophyll content did not differ significantly between treatments on the top leaf ($F_{5,52}$=0.79, P=0.562), however there was a significant difference between treatments for chlorophyll content in the seventh leaf ($F_{5,52}$=11.35, P<0.001) (Fig. 5.6). The pathogen-only and pathogen-galled treatment plants had the lowest chlorophyll content in the seventh leaf, in comparison to the other treatments. There was no significant interaction between single galled and pathogen ($F_{1, 45}$=3.38, P=0.072) and double galled and pathogen ($F_{1, 33}$=1.43, P=0.240) on the chlorophyll content of the seventh leaf. As with the other measurements the interaction between the two agents is equivalent, with the pathogen decreasing chlorophyll content the most.

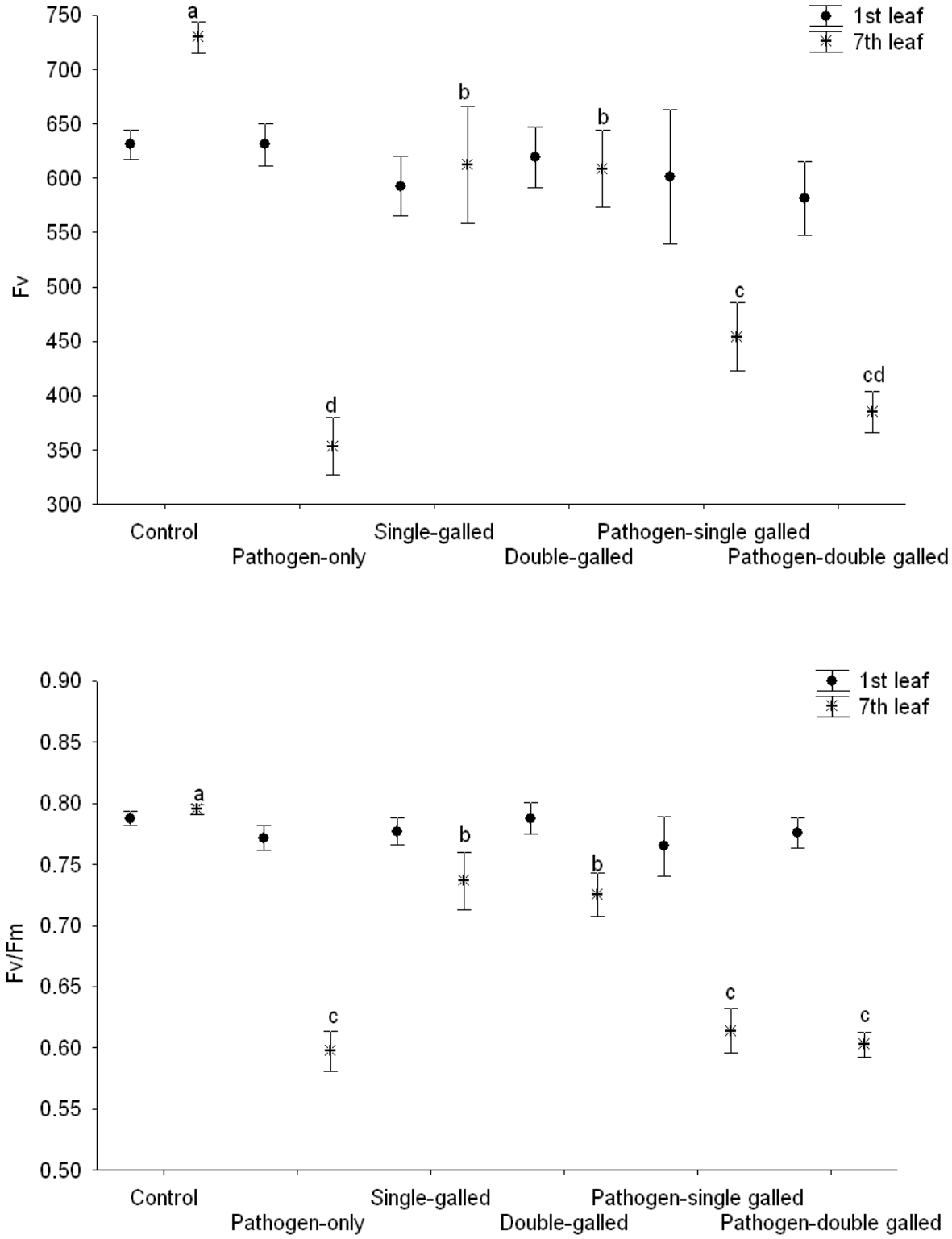


Figure 5.5: Chlorophyll fluorescence parameters of the top (youngest) and seventh (older) fully-expanded leaves of *Ageratina adenophora* in response to six different treatments, of pathogen infection and/or fly galling (single or double). Means followed by a different letter are significantly different, at *P*<0.05 (LSD). (Means ± SE).

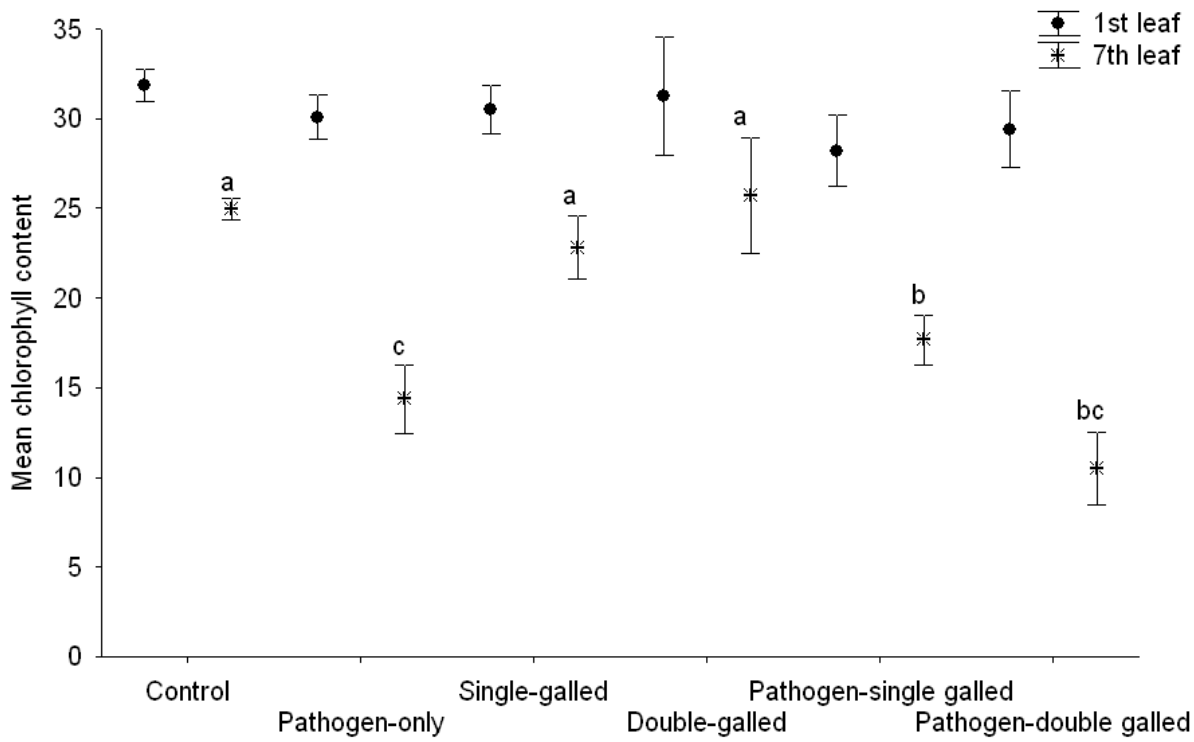


Figure 5.6: Chlorophyll content of the top (youngest) and seventh (older) fully-expanded leaves of *Ageratina adenophora* in response to six different treatments, of pathogen infection and/or fly galling (single or double). Means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

5.5 Discussion

Photosynthesis is vital to a plant's survival, providing the products used for growth and reproduction (Feng *et al*., 2007b; Lambers *et al*., 2008). A reduction in photosynthetic capacity of plants leads to less resources being available for these life processes (McDowell, 2002). The results from this study, on the influence of biocontrol agents on the photosynthetic capacity of crofton weed, correspond well with the results from previous laboratory and field trials on the effect of the fly and pathogen on crofton weed vegetative growth and seed production. This study provides information on how crofton weed responds physiologically to infection by the agents.

Galls act as nutrient sinks, taking available plant resources from other parts of the plant for their own growth (Florentine *et al*., 2005; Dorchin *et al*., 2006; Moseley *et al*., 2009). The *P. utilis* gall acts as a nutrient sink, as repeated galling alone by the fly on crofton weed leads to reduced vertical stem growth and decreased germinable seed production by 53.8% (Chapter 2 and 4). However crofton weed compensates for galling with increased sideshoot growth. Galls may increase (Dorchin *et al*., 2006) or decrease photosynthetic rates (Florentine *et al*., 2005) of weeds. In this study photosynthesis in double-galled plants was significantly lower than the control plants, leading to fewer resources for growth and reproduction. Under such conditions crofton weed reallocates the limited resources to compensatory growth of sideshoots, which may develop into new plants (Muniappan *et al*., 2009; Chapter 2 and 3) and these will therefore not be stressed by the galls; rather than investing further resources into the growth and reproduction of the original stressed stem.

Fungal leaf-spots are usually only visible on the lower (older) leaves of crofton weed, and no significant effect of the pathogen on the vertical vegetative growth of crofton weed is seen. However there is less sideshoot growth on pathogen infected stems (Chapter 2 and 3). But if pathogen infection is not aggressive, weeds are able to overcome the pressure of the pathogens by shedding the infected leaves and increasing vegetative (compensatory) growth (Charudattan, 2005, 2010). The reduced stomatal conductance and transpiration rate in pathogen-only and pathogen-galled treatment plants resulted in less water-use and CO_2 available for photosynthesis (Lambers *et al*., 2008). This is seen with pathogen-only and pathogen-galled plants having a significantly lower photosynthetic rate, compared to the control and galled-only treatment plants. The reduced resources available to crofton weed, as a result of pathogen infection on the lower leaves, are used to produce new healthy leaves on the upward vertical growth of stems, therefore leaving fewer resources available for sideshoot growth. The new compensatory leaf growth on the stem produces resources for reproduction, however the initial effect of

infection of the lower, older leaves is not completely eliminated and seed production is still reduced by 26.7% in comparison to the control stems (Chapter 4).

Weeds may be good invaders as they are able to have a high rate of photosynthesis relative to water loss, and hence a high water-use efficiency (WUE) (McDowell, 2002). Control plants had significantly higher WUE than agent infected plants. However, there was no significant difference in the WUE between the different types of agent infection; this may be a result of the experiment being run in a greenhouse where water was readily available. The results may be more variable under field conditions, where water availability is highly variable over time (Rosskopf *et al.*, 1999; Morin *et al*., 2006, 2009).

Chlorophyll fluorescence values provide an indication of the photochemical efficiency of photosystem II (PSII), quantifying stress on a plant (Witkowski *et al*., 1992; Lambers *et al*., 2008; Li *et al*., 2008; Resco *et al*., 2008; Govender *et al*., 2009). A healthy plant will have high chlorophyll content and an F_v/F_m value of about 0.8 (Lambers *et al*., 2008; Resco *et al*., 2008; Govender *et al*., 2009). The chlorophyll fluorescence and chlorophyll content results further correspond with the photosynthetic results; indicating that crofton weed is under physiological stress from both the pathogen and fly. The top (youngest) leaves of crofton weed were not stressed by the fly or pathogen, whilst the lower (older) leaves had significantly lower F_v/F_m values and chlorophyll content on pathogen-only and pathogen-galled plants. This further highlights that whilst the pathogen negatively affects the lower leaves, crofton weed is able to compensate by producing new leaf growth higher up on the stem that functions normally.

Ecophysiological measurements may be a good way to assess the influence of agents on weed growth during host-specificity testing at the beginning of a biocontrol programme, and to highlight what type of agents will be successful. These results show that crofton weed is negatively affected by leaf attack, and a leaf-chewer or leaf-galler may be good future

biocontrol agents for crofton weed. The overall interaction between the pathogen and fly is equivalent, with both agents affecting the state of crofton weed plants. This is important, as the fly and pathogen populations may differ at crofton weed infestations across South Africa, depending on the environmental conditions. Field observations indicate that neither of the agents are controlling crofton weed in South Africa (Heystek *et al*., 2011). However, they do influence the health of crofton weed, and the infestations in South Africa may have spread quicker, or be denser, if they had not been introduced.

5.6 Conclusion

This study highlights how we see success in biological control. Whilst the agents may not seem to have drastic visible impacts on the vegetative growth of crofton weed, they are inflicting stress on crofton weed at a physiological level. However, crofton weed is able to compensate for this stress by producing sideshoots in terms of the fly, and producing healthy leaf growth in terms of the pathogen. Therefore, the combination of the two agents complement each other, as the fly restricts upward growth and the pathogen inhibits sideshoot growth. These ecophysiological results correspond well with the laboratory and field results.

Chapter 6: Post-release evaluation of two biocontrol agents released against *Ageratina adenophora* (Asteraceae) at three sites in South Africa.

6.1 Abstract

Ageratina adenophora is a multi-stemmed invasive shrub in South Africa. Two biocontrol agents a stem gall fly, *Procecidochares utilis,* and a leaf-spot pathogen, *Passalora ageratinae*; have been released against *A. adenophora* in South Africa. This study surveyed three *A. adenophora* infestations across South Africa in Barberton (Mpumalanga), Magaliesberg (North West) and Pietermaritzburg (KwaZulu-Natal) from December 2005 to December 2006. The pathogen was present at all three field sites; and the fly was only present at Pietermaritzburg. The infestation in Barberton was located under dense shade from a pine forest canopy, predominantly composed of short (100 to 1200mm high) younger plants, with little seedling recruitment after flowering and with 30-50 stems per m^2. The infestation in Magaliesberg was along an unshaded stream bank, and was manually cleared in June 2006. The *A. adenophora* population recovered quickly after clearing, with 20-50 stems per m^2, adults of 100 to 2200mm in height and seedling recruitment after flowering. The Pietermaritzburg site, located along a road on abandoned land, was in full sunlight and had an established *A. adenophora* population, with 80 stems per m^2 and stems of 100 to 2000mm in height. *Ageratina adenophora* above-ground biomass per m^2 was lowest at Barberton, whilst Magaliesberg and Pietermaritzburg were similar (except after clearing), indicating that the agents may affect the health, for example the thickness, of the stems. The pathogen was prevalent at all three sites, infecting up to 95% of stems. However the severity of infection was low, no more than 50% of living leaves were infected at a time, and in general only 1-30% of leaves were infected. The gall fly population was low in Pietermaritzburg, only 20% of stems were galled, and repeated galling of stems was rare. Parasitism levels of the stem gall fly were 30%. The gall fly reduced reproductive output of *A. adenophora* stems, in comparison to stems only infected with the pathogen at Pietermaritzburg. This study highlights the importance of defining success at the start of a biocontrol programme and collecting baseline data prior to biocontrol agent introductions, in order to evaluate

the efficacy of biocontrol agents. In addition this study will provide baseline data for future studies, as investigations into new biocontrol agents for *A. adenophora* in South Africa are currently underway.

6.2 Introduction

Biocontrol involves the introduction of biocontrol agents, termed natural enemies, such as insects, mites and pathogens, from the country of origin into the invaded country to reduce the abundance of a weed population (McFadyen, 1998; Müller-Schärer & Schaffner, 2008). Many biocontrol programmes have focused on the initial stages of biocontrol, such as finding biocontrol agents, testing for host-specificity and biocontrol agent releases, with less focus on post-release evaluations (Morin *et al*., 2009). However, post-release evaluations are essential to understanding the effectiveness of the biocontrol agents in the invaded country and determining the success of the biocontrol programme. Invasions of the weed may differ between different localities depending on climate, land-use, disturbance and native species composition (Lonsdale, 1999). Agent efficacy and establishment may also differ between localities depending on climate and environmental conditions (Müller-Schärer & Schaffner, 2008).

Ageratina adenophora (Sprengel) King and Robinson (syn. *Eupatorium adenophorum* Spreng.) (Asteraceae), also known as Crofton weed or Mexican devil weed is a multi-stemmed perennial herb, native to Mexico, with a woody rootstock, and stems reaching up to 2m in height (Bess & Haramoto, 1959; Henderson, 2001; Page & Lacey 2006). Crofton weed is invasive in many countries, including China, Thailand, Australia, New Zealand, India and South Africa (Julien & Griffiths, 1998, Muniappan *et al*., 2009). In South Africa, six of the nine provinces, KwaZulu-Natal, Western Cape, North West, Limpopo, Mpumalanga and Gauteng, are invaded by crofton weed (Henderson, 2001; Heystek, 2008). Crofton weed is found in moist habitats, with high rainfall, along stream banks, roadsides, under

forest canopies and on steep slopes (Dodd, 1961; Henderson, 2001; Trounce, 2003; Page & Lacey, 2006; Heystek *et al*., 2011).

The biological control programme of *A. adenophora* in South Africa has involved the release of a stem gall fly *Procecidochares utilis* Stone (Tephritidae), and a leaf-spot pathogen *Passalora ageratinae* Crous and A.R. Wood (Mycosphaerellaceae) (previously named *Cercospora eupatorii* Peck or *Phaeoramularia* sp.) (Crous *et al*., 2009). The stem gall fly was released near Pietermaritzburg, KwaZulu-Natal, between 1984 and 1987 (Kluge, 1991), and more recently has been released in the Magaliesberg, North West Province (Heystek *et al*., 2011). The leaf-spot fungal pathogen was released in KwaZulu-Natal and the south-western Cape in 1987 (Morris, 1991), and later on in the Magaliesberg, North West Province (Heystek *et al*., 2011). The pathogen has also been found on crofton weed infestations in the Limpopo and Mpumalanga Provinces, even though it was never officially released in these areas, it is assumed to have dispersed the approximately 300km from the nearest infestation in the Magaliesberg (Heystek *et al*., 2011).

No formal post-release evaluation has been done in South Africa since the release of these two biocontrol agents; however field observations indicate that crofton weed is spreading and has the potential to invade a larger area than it currently does (Heystek, 2008; Heystek *et al*., 2011). Surveys for additional agents have been undertaken in 2007, 2008 and 2009, and new potential biocontrol agents are being evaluated (Heystek *et al*., 2011).

Little is known about the crofton weed infestations and its biocontrol agent populations within the invaded areas of South Africa. Therefore, the aim of this study was to undertake a post-release evaluation survey of crofton weed, as well as its two biocontrol agents, the gall fly and leaf-spot pathogen, within three provinces of South Africa. The objectives included evaluating crofton weed populations in terms of stem and seedling density, size structure, above-ground biomass and reproductive output over a year. The biocontrol agent populations were assessed in terms of prevalence

and severity of infection over a year (Morin *et al*., 2009). In addition parasitism of the gall fly was measured. This survey will allow for a better understanding of the current populations and whether agents have established, as well as provide baseline data for comparison of future crofton weed and agent populations if new agents are to be released.

6.3 Material and methods

6.3.1 Field sites

Three *A. adenophora* infestations at Barberton, Magaliesberg and Pietermaritzburg were surveyed in South Africa in December 2005, April 2006, July 2006, October 2006 and December 2006 (Fig. 6.1). The three sites chosen are in areas of known infestations where biocontrol measures are currently been implemented and researched.

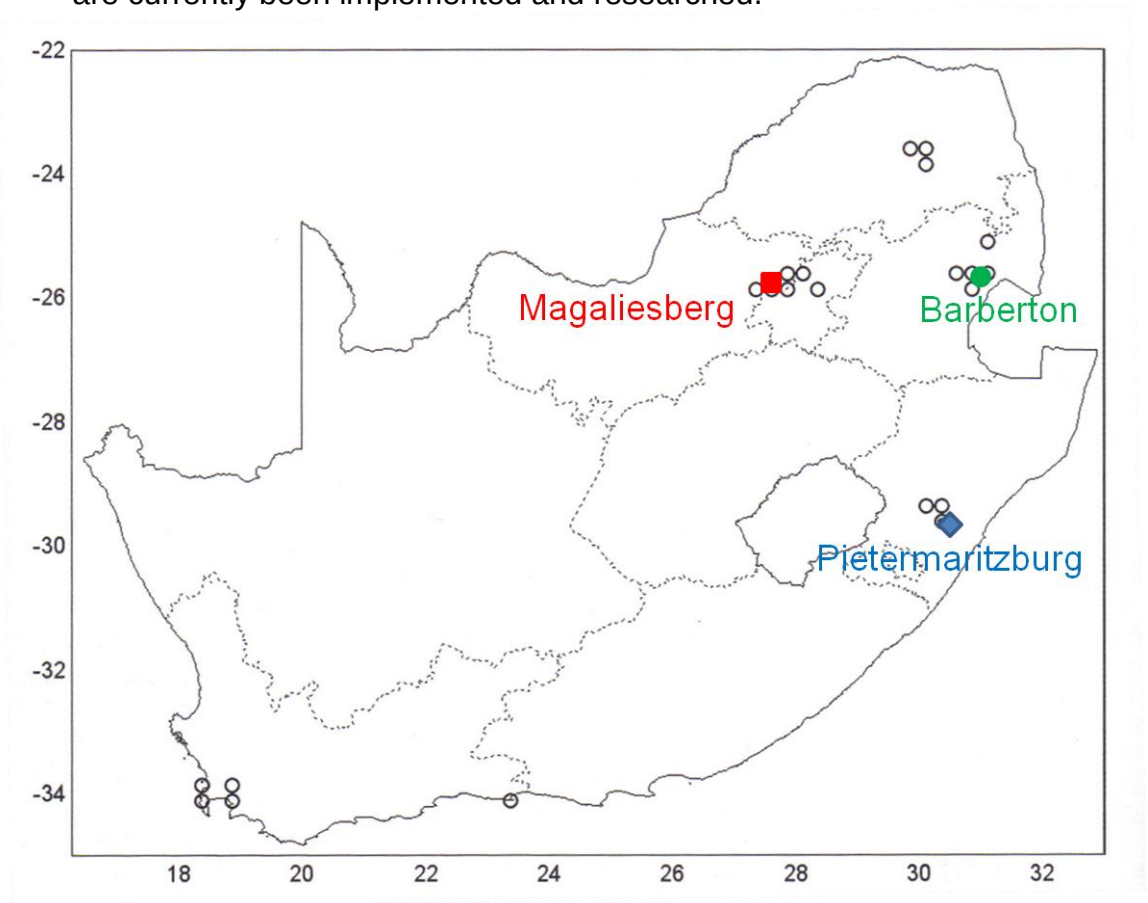


Figure 6.1: Distribution of *Ageratina adenophora* (O) in South Africa. (Drawn by L. Henderson; data source: SAPIA data base, ARC-Plant Protection Research Institute, Pretoria), and location of three field sites, Barberton (●), Magaliesberg (■) and Pietermaritzburg (◆), where *A. adenophora* infestations were surveyed for this study.

Barberton

The Barberton field site was located in the pine forests of the SAPPI Escarpment Plantation, outside the city of Barberton, Mpumalanga Province, South Africa (25˚37'33.8"S, 30˚46'41.9"E) (Table 6.1). The *A. Adenophora* infestation was along a dirt forest road, which is typical of all the crofton weed infestations in this area. Only the fungal pathogen, *P. ageratinae* is present in the Barberton area. The field site is located on Northern Mistbelt Forest (Zonal and Intrazonal units, Forests); within tall, evergreen afrotemperate mistbelt forests (Mucina *et al*., 2006a, Mucina & Geldenhuys, 2006). The area has high summer rainfall and dry winters (Mucina & Geldenhuys, 2006).

Magaliesberg

Ageratina adenophora infestations in the Magaliesberg area are located along stream embankments and in crevices within the mountain rock formations. The Magaliesberg field site was located on the 150ha Kloofwaters farm, in the Magaliesberg, North West Province, South Africa (25˚50'0.25"S, 27˚26'20.9"E) (Table 6.1). Kloofwaters farm is privately owned, and predominantly used for school camps; however a low stocking level of cattle is present. The *A. adenophora* infestation was along a flat stream embankment, facing eastwards, with only the fungal pathogen, *P. ageratinae* present. The field site is on Moot Plains Bushveld (central bushveld, Savanna Biome), with thorny savanna, dominated by *Acacia* species and woodlands (Mucina *et al*., 2006a; Rutherford *et al*., 2006). The Magaliesberg experiences summer rainfall and very dry winters with frost (Rutherford *et al*., 2006). The Magaliesberg field site was manually cleared in October 2005 and June 2006. Manual clearing involved groups of people walking through the Magaliesberg, hand-pulling crofton weed plants out at the root and then leaving them along the stream bank to senesce. Manual clearing of crofton weed occurs before the flowering period.

Pietermaritzburg

Many of the crofton weed infestations in the KwaZulu-Natal region are along roadsides and abandoned land; therefore the infestations are often manually cleared and cut by the municipality. The Pietermaritzburg field site was located on abandoned land, on the corner of Quarry Road, Hilton, outside the town of Pietermaritzburg, KwaZulu-Natal Province, South Africa (29˚33'31.8"S, 30˚18'11.2"E) (Table 6.1). The *A. Adenophora* infestation was along an urban road, with both the fungal pathogen, *P. ageratinae*, and the gall fly, *P. utilis* present. The field site is on Midlands Mistbelt Grassland (Sub-Escarpment Grassland, Grassland Biome), a hilly landscape dominated by forb-rich, sour *Themeda triandra* grasslands (Mucina *et al*., 2006a, 2006b). This area experiences summer rainfall and frequent occurrences of mist (Mucina *et al*., 2006b).

Table 6.1: Details of three *Ageratina adenophora* field sites sampled from December 2005 to December 2006.

	Barberton	**Magaliesberg**	**Pietermaritzburg**
Altitude (m ASL)	1500	1380	1195
Area of infestation (m^2)	120	165	410
Aerial cover (%)	90	30	0
Mean annual rainfall (mm) (Zucchini & Nenadić, 2006)	1300	690	1233
Vegetation type (Mucina *et al*., 2006a)	Northern Mistbelt Forest	Moot Plains Bushveld	Midlands Mistbelt Grassland

6.3.2 Field sites soil texture and fertility

At each site five samples of soil (0cm-10cm depth) were collected and sent for analysis of soil texture and fertility to the Soil Fertility and Analytical Services, KwaZulu-Natal Department of Agriculture and Environmental Affairs (Table 6.2).

Table 6.2: Comparison of soil texture and fertility of three *Ageratina adenophora* field sites. Comparisons between the three sites were made using one-way ANOVA's, different superscripts within rows are significantly different, at $P<0.05$ (LSD). (Means ± SE). Significant *P*-values are in bold.

Soil property	**Barberton**	**Magaliesberg**	**Pietermaritzburg**	$F_{2,12}$	***P*-value**
Texture	Sandy loam	Loamy sand	Sandy clay loam		
Clay (<0.002mm) (%)	8.4±0.68^{b}	10.4±0.24^{b}	25.0±0.89^{a}	186.64	**<0.001**
Fine silt (0.02-0.002mm) (%)	14.2±1.16^{a}	4.4±0.24^{b}	13.4±0.75^{a}	45.33	**<0.001**
Coarse silt & sand (0.02-2mm) (%)	77.0±1.67^{b}	85.2±0.47^{a}	61.8±1.39^{c}	84.92	**<0.001**
Available P (mg/L)	2.0±0.00^{b}	10.2±1.07^{a}	2.2±0.26^{b}	55.63	**<0.001**
Exchangeable K (mg/L)	110.8±1.02^{b}	122.8±14.99^{b}	247.8±11.69^{a}	47.62	**<0.001**
Exchangeable Ca (mg/L)	962.2±48.32	1267.0±198.51	819.2±56.05	3.50	0.064
Exchangeable Mg (mg/L)	416.8±3.65^{a}	378.2±26.56^{a}	201.6±15.58^{b}	41.07	**<0.001**
Total cations (cmol/L)	8.6±0.26^{ab}	9.8±1.14^{a}	6.6±0.39^{b}	5.05	**0.026**
pH (KCl)	4.6±0.01^{b}	4.8±0.11^{a}	4.3±0.05^{c}	12.26	**0.001**
Total Zn (mg/L)	1.8±0.07^{b}	7.9±1.54^{a}	8.8±0.43^{a}	16.81	**<0.001**
Total Mn (mg/L)	7.2±0.49^{b}	4.2±0.66^{b}	38.0±2.86^{a}	118.25	**<0.001**
Total Cu (mg/L)	2.1±0.12^{c}	4.4±0.15^{b}	5.3±0.27^{a}	77.56	**<0.001**
Total N (%)	0.1±0.01^{b}	0.2±0.03^{a}	0.2±0.03^{a}	14.49	**<0.001**
Organic C (%)	1.8±0.06^{b}	4.9±1.26^{a}	2.5±0.10^{b}	5.17	**0.024**

6.3.3 Rainfall, temperature and humidity

A mean annual rainfall value for the sites was obtained from the South African Rainfall Atlas (Zucchini & Nenadić, 2006). Mean monthly rainfall data for the three sites from December 2005 to December 2006 was provided by the South African Weather Service (SAWS). Temperature and humidity were measured every 90 minutes from December 2005 to December 2006 at each site using Hygrochron iButtons (DS1923, Maxim Dallis, Semiconductor Corporation). The iButtons were placed in a nearby tree at a height of 2m and out of direct sunlight.

6.3.4 Field measurements

At each site, a transect of ten quadrats (1m x 1m) was taken through the middle of the infestation, parallel to the road or river, running from one margin of the infestation to the other side. The quadrats were placed at equal distances from each other, with a maximum distance of 10m between quadrats, to spread across the infestation. These were used to measure all the traits listed below.

Density and size distribution of *A. adenophora* populations

As crofton weed infestations are often tangled stands of trailing and upright stems it is difficult to distinguish individual plants, therefore measurements were taken for individual stems that emerge upright from the ground. Within each $1m^2$ quadrat the total number of stems was counted, and the stem height of each stem was measured. A 15cm x 15cm quadrat was placed in the middle of each $1m^2$ quadrat and the number of seedlings counted. Seedlings were defined as stems less than 10cm high.

Biomass of *A. adenophora* populations

At each site, in December 2005, April 2006, July 2006 and October 2006, 50 randomly selected stems were cut at ground level, collected and separated into stem, live leaves and dead leaves, placed in brown bags and brought back to the laboratory at the University of the Witwatersrand. These were dried in a forced-draught oven at 60^oC for seven days, and weighed. The allometric relationship between stem height and total above-ground biomass was determined by regression analyses. These regression equations were then used to estimate the biomass of each stem, along the transect, to calculate a measure of the above-ground biomass at each site.

Reproductive output of *A. adenophora* populations

In July 2006 flowering had started and the number of stems with flowers was counted at each site. In October 2006 the total number of synflorescences per stem was counted. A subsample of five synflorescences was randomly selected from 50 stems and the number of capitula per synflorescence was recorded and a mean calculated.

Erasmus *et al*. (1992) recorded three categories of achenes: filled, empty and aborted (shrunken and undeveloped). A subsample of 15 capitula per stem was randomly chosen and the number of achenes in each category per capitula counted. Very few aborted achenes were recorded; therefore they were grouped with the empty achenes category. Empty and aborted achenes are not viable (Erasmus *et al*., 1992).

The total number of filled achenes per stem was calculated as follows:

Number filled achenes/stem = (synflorescences/stem) x (mean capitula/synflorescence) x (mean filled achenes/capitula).

The total number of germinable seeds per stem was calculated as follows:

Number germinable seeds/stem = (synflorescences/stem) x (mean capitula/synflorescence) x (mean filled achenes/capitula) x %germination/100.

Ten filled achenes per stem were randomly selected for germination trials to test percentage viability and germinability of achenes. Achenes for germination trials were placed on moist Whatman No.1 filter paper in 12cm petri-dishes and placed in a Phytotron controlled growth chamber, at the University of the Witwatersrand, Johannesburg, set at 20^{o}C with continuous white light for 12 hours per 24 hours (Erasmus *et al*., 1992). The filter paper was moistened daily as required, and fungi controlled by spraying the filter paper with the fungicide Benlate. Germination of achenes was recognised by protrusion of the radical by 2 mm, and observed daily for 28 days. Germinated achenes were removed once they had been recorded.

Prevalence and severity of *P. ageratinae* populations

The number of stems with and without pathogen-infected leaves was counted within each $1m^2$ quadrat. Each pathogen-infected stem was recorded as having 1-10%, 10-30%, 30-50%, 50-70% or 70-100% leaves infected, also referred to as the level of biocontrol agent attack.

Prevalence and severity of *P. utilis* populations

The number of ungalled and galled stems was counted in each $1m^2$ quadrat. The number of galls (also referred to as the level of biocontrol agent attack), and whether the galls were new or old (dried and withered), was recorded for each galled stem.

Fifty galls, with intact emergence windows, were collected in April, July and December 2006 and brought back to the laboratory at the University of the Witwatersrand. The galls were dissected and the pupae placed on moist cotton wool in glass vials. The number of gall flies and parasitoids emerging from the pupae was counted.

6.3.5 Data analysis

Stem and seedling density and above-ground biomass were compared between the three field sites across the sampling period using repeated measures ANOVA (GLM procedure, StatSoft, 2007). Differences in the size structure between the field sites were assessed using Kolmogorov-Smirnov tests. One-way ANOVA and LSD tests were used to assess differences in density and biomass measurements between sites for each month. Regressions were used to test for relationships between stem height and above-ground biomass of the three sites during each sample period. Chi-squared tests were used to assess associations between the level of parasitism and time of year, and the percentage flowering plants at the three field sites at different times of the year. Two-way ANOVA's were

used to assess the influence of site and level of biocontrol agent attack on reproductive output variables.

6.4 Results

6.4.1 Rainfall, temperature and humidity

All three sites experience summer rainfall and dry winter months (Fig 6.2). The temperature and relative humidity of all three sites decreases during the winter months (Fig. 6.3). Magaliesberg tends to have higher temperatures in comparison to the other two sites. Barberton and Pietermaritzburg have a higher relative humidity in comparison to the Magaliesberg field site, and these two sites experience mist frequently.

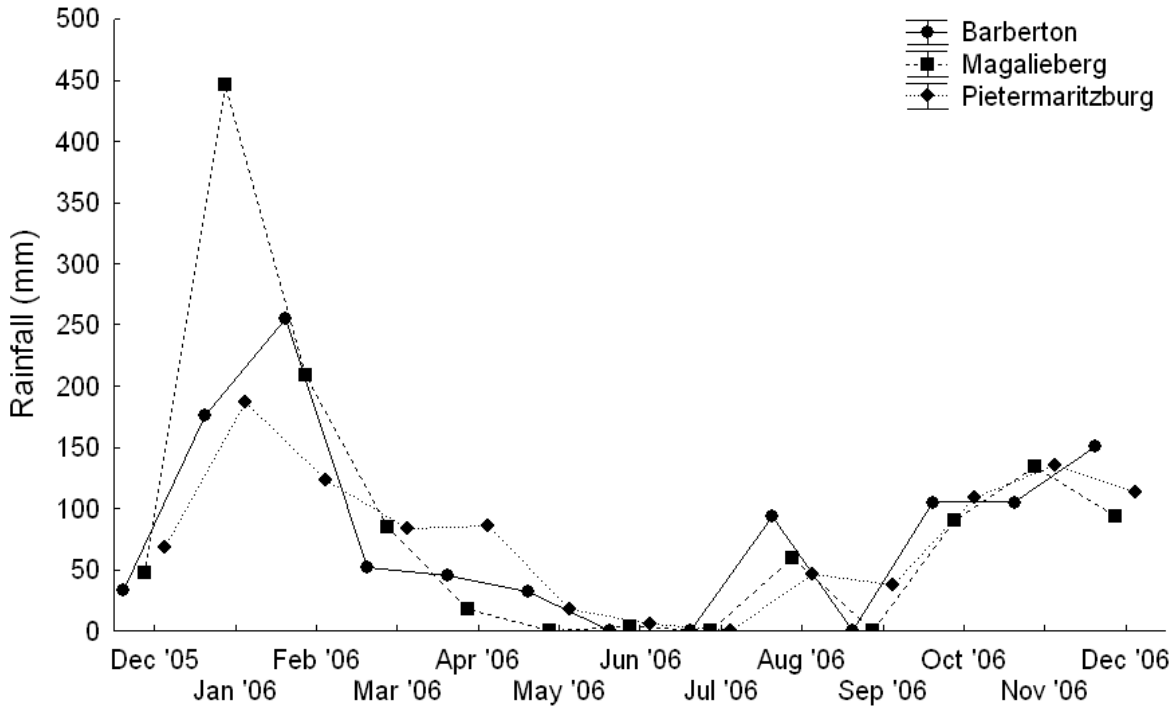


Figure 6.2: Mean monthly rainfall (mm) at the three field sites surveyed from December 2005 to December 2006. (Data provided by the South African Weather Service).

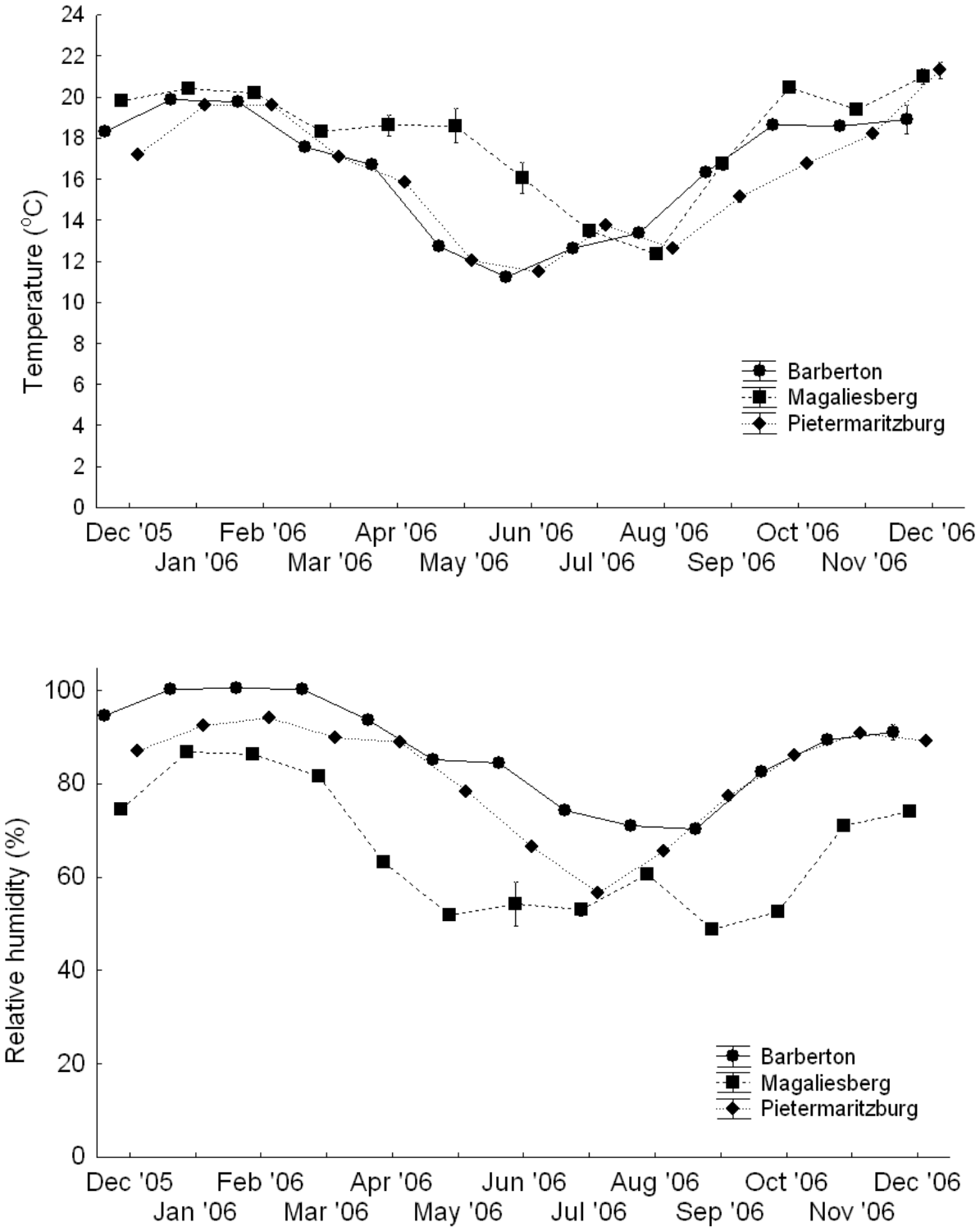


Figure 6.3: Monthly temperature and relative humidity at the three field sites surveyed from December 2005 to December 2006. (Means ± SE).

6.4.2 Density and size distribution of A. adenophora *populations*

Stem density differed significantly between the sites over the year ($F_{2,27}$=13.70, P<0.001) (Fig. 6.4). In December 2005 ($F_{2,27}$=4.66, P=0.018), October ($F_{2,27}$=9.30, P<0.001) and December 2006 ($F_{2,27}$=9.29, P<0.001) Pietermaritzburg had a significantly higher number of stems per m^2. Magaliesberg stem density decreased in July 2006 as a result of manual clearing in June 2006. Seedling density differed significantly between sites over the year ($F_{2,27}$=5.11, P=0.013) (Fig. 6.4). Pietermaritzburg had a significantly higher number of seedlings per m^2 in December 2005 ($F_{2,27}$=4.32, P=0.024) and December 2006 ($F_{2,27}$=4.86, P=0.016) after the flowering season.

The Kolmogorov-Smirnov tests showed that the size structure, based on stem height distribution, differed significantly (P<0.001) between each month for each site, and between each site, except for between April and July at Barberton (P>0.100) and April and October at Pietermaritzburg (P>0.100) (Fig. 6.5). Barberton had a population dominated by small stems in December 2005, and April and July 2006. These stems continued to make up the population in December 2006, with only a few smaller stems growing. Magaliesberg initially had a young population, after clearing in October 2005, which grew into taller stems in April 2006. By July 2006 the field site had been cleared again; however not all the stems were removed, as taller plants were still present. The Magaliesberg infestation recovered from clearing with an increase in young stems from October 2006. Pietermaritzburg had a recruiting population of young (shorter) stems in December 2005. This population continued to grow until October 2006, after which an increase in smaller plants occurred. These smaller plants may be a result of taller plants trailing along the ground, giving rise to new plants through sideshoot growth, and seedlings growing after the flowering period.

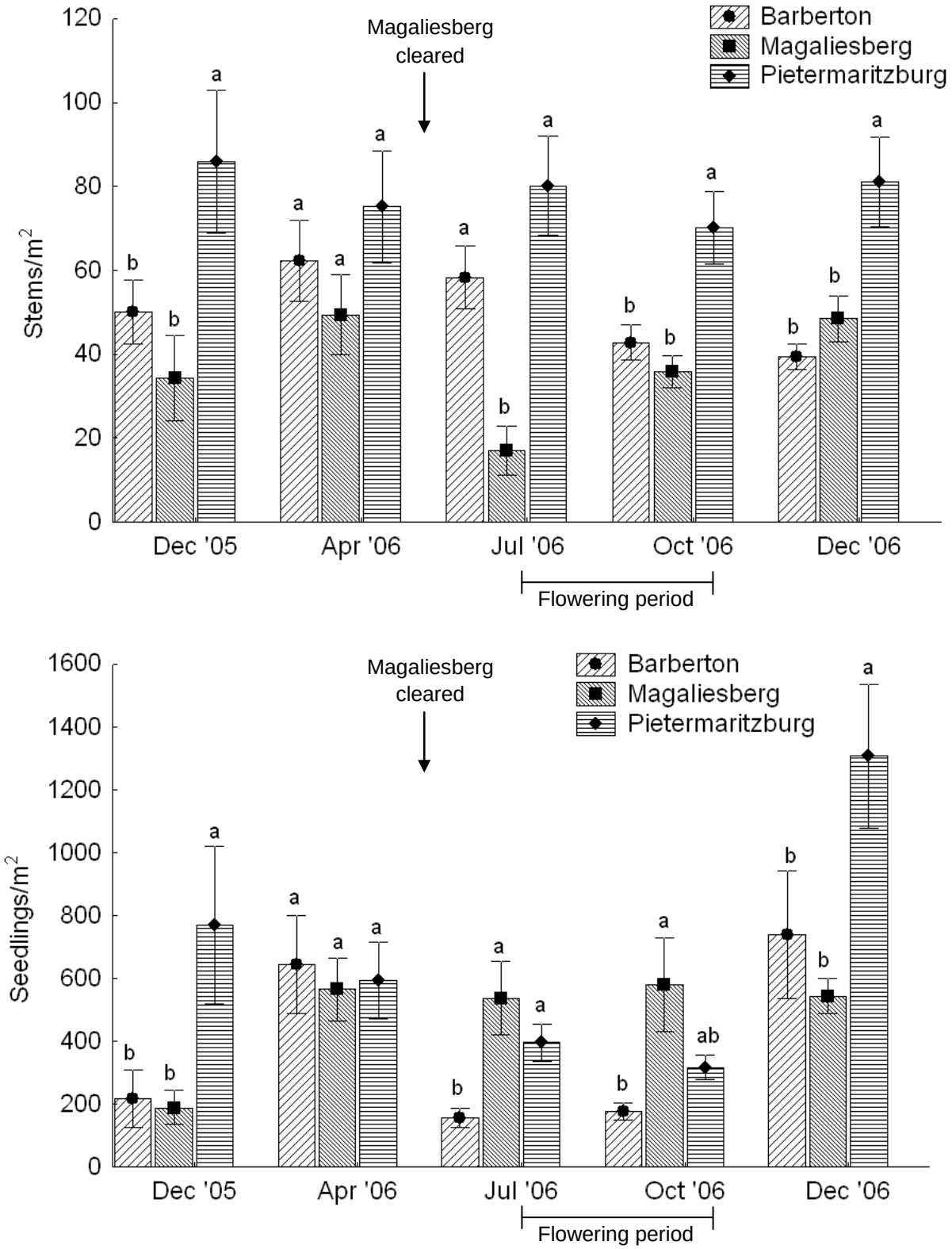


Figure 6.4: Density of *Ageratina adenophora* adult stems and seedlings per m^2 of invasion at three field sites from December 2005 to December 2006. For each month, means followed by different letters are significantly different, at $P<0.05$ (LSD). (Means ± SE).

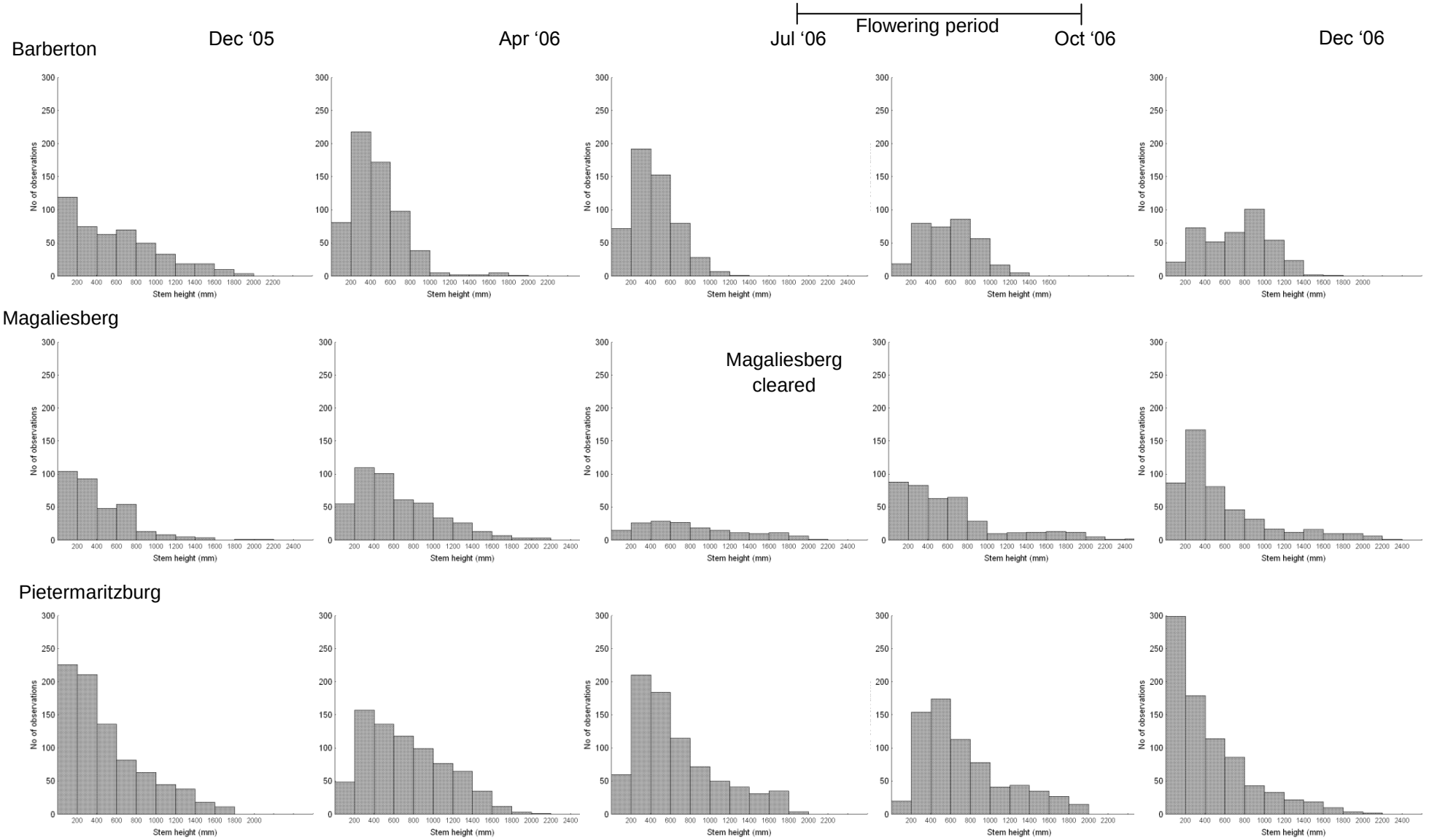
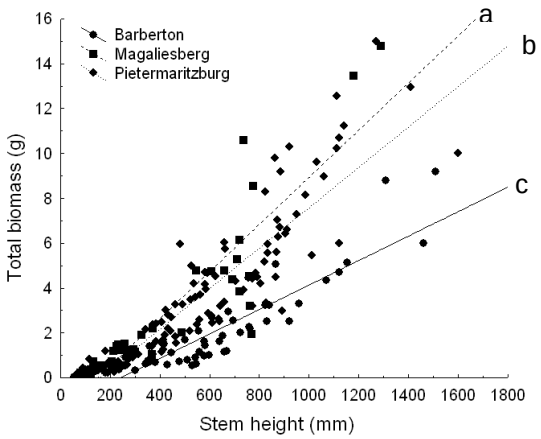


Figure 6.5: Size structure based on stem height distribution of *Ageratina adenophora* infestations at three field sites from December 2005 to December 2006.

6.4.3 Biomass of A. adenophora *populations*

The regressions between stem height and biomass differed significantly between sites in December 2005 ($F_{2,207}$=41.20, P<0.001), and April ($F_{2,260}$=45.36, P<0.001), July ($F_{2,256}$=46.59, P<0.001) and October 2006 ($F_{2,236}$=24.06, P<0.001) (Fig. 6.6). The best fit for all sites during all sample times was linear. During all sampled times stems in Magaliesberg had a higher biomass in relation to stem height, in comparison to the other two sites.

There was a significant difference in total above-ground biomass per m^2 between sites throughout the year ($F_{2,27}$=10.79, P<0.001) (Fig. 6.7). At the Barberton field site biomass per m^2 decreased from December 2005 to October 2006. Above-ground biomass per m^2 did not differ significantly between Pietermaritzburg and Magaliesberg except for July 2006, after clearing in the Magaliesberg.



December 2005

Barberton

Biomass = -1.32+0.0055*stemheight

$P < 0.001$; $R^2 = 0.821$

Magaliesberg

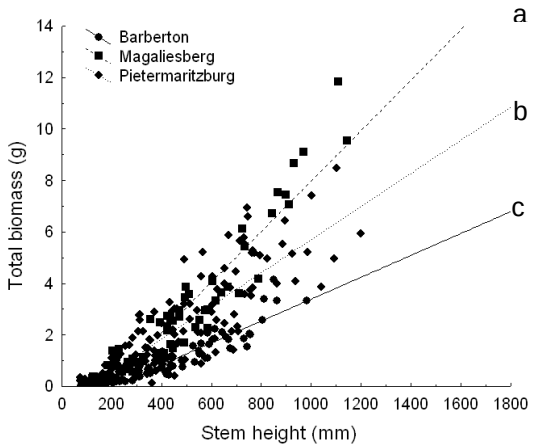
Biomass = -1.56+0.0105*stemheight

$P < 0.001$; $R^2 = 0.807$

Pietermaritzburg

Biomass = -1.43+0.0009*stemheight

$P < 0.001$; $R^2 = 0.819$



April 2006

Barberton

Biomass = -0.86+0.0043*stemheight

$P < 0.001$; $R^2 = 0.843$

Magaliesberg

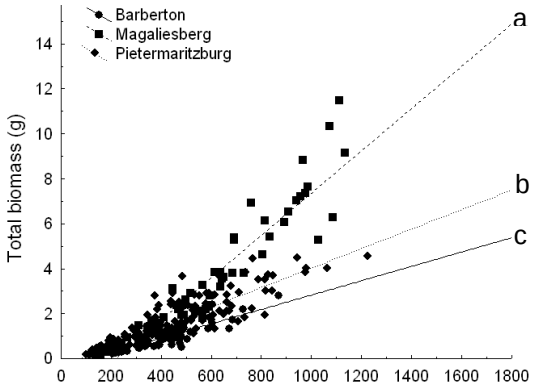
Biomass = -1.83+0.0098*stemheight

$P < 0.001$; $R^2 = 0.887$

Pietermaritzburg

Biomass = -0.73+0.0064*stemheight

$P < 0.001$; $R^2 = 0.737$



July 2006

Barberton

Biomass = -0.36+0.0032*stemheight

$P < 0.001$; $R^2 = 0.802$

Magaliesberg

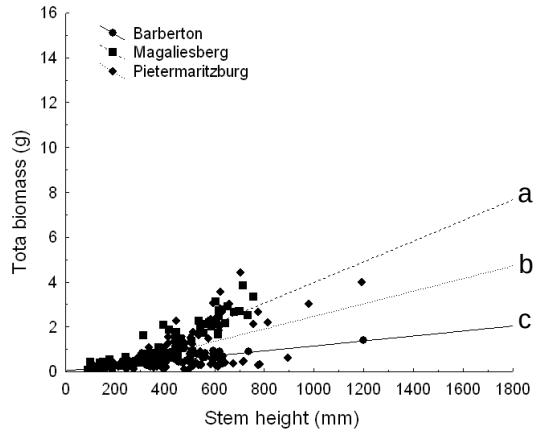
Biomass = -2.04+0.0094*stemheight

$P < 0.001$; $R^2 = 0.876$

Pietermaritzburg

Biomass = -0.31+0.0043*stemheight

$P < 0.001$; $R^2 = 0.773$



October 2006

Barberton

Biomass = 0.04+0.0011*stemheight

$P < 0.001$; $R^2 = 0.675$

Magaliesberg

Biomass = -0.62+0.0046*stemheight

$P < 0.001$; $R^2 = 0.782$

Pietermaritzburg

Biomass = -0.33+0.0028*stemheight

$P < 0.001$; $R^2 = 0.345$

Figure 6.6: The linear relationship between stem height and total above-ground biomass of *Ageratina adenophora* stems at three field sites from December 2005 to October 2006. For each month, lines followed by different letters have significantly different slopes, at $P<0.05$ (LSD).

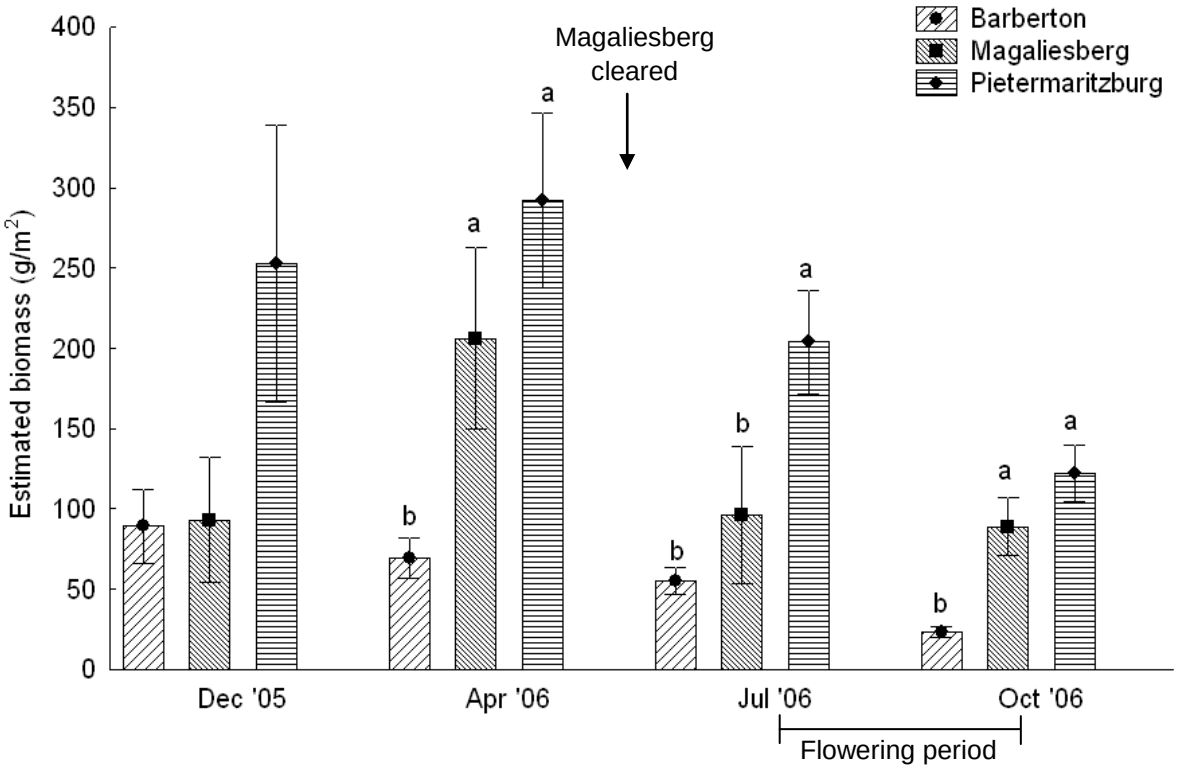


Figure 6.7: Estimated total above-ground biomass per m^2 of *Ageratina adenophora* stems within three invaded sites from December 2005 to October 2006. For each month separately means followed by different letters are significantly different, at $P<0.05$ (LSD). (Means ± SE).

6.4.4 Prevalence and severity of P. ageratinae *populations*

The pathogen was prevalent at Pietermaritzburg, 60% to 100% of stems had leaf-spots (Fig. 6.8). Pathogen-infected stems increased from 5% to 90% at Barberton. Pathogen prevalence was low at Magaliesberg after manual clearing, but recovered quickly. At all three sites no more than 50% of the leaves showed leaf-spots on pathogen-infected stems (Fig. 6.9). The severity of pathogen infection was generally low, with the majority of pathogen-infected stems only having 1-10% or 10-30% of their leaves infected.

6.4.5 Prevalence and severity of P. utilis *populations*

No more than 20% of stems were galled at Pietermaritzburg throughout the year (Fig.6.8), indicating a very low prevalence of the gall fly in crofton weed infestations. The severity of galling on galled stems was low (Fig.

6.10). Of the galled stems, 80-95% were only galled once throughout the year at Pietermaritzburg.

Levels of parasitism of the fly were low at Pietermaritzburg, 23% (April 2006), 33% (July 2006) and 34% (December 2006) of emerging adults from galls being parasitoids (Table 6.3). The emerging parasitoids have not yet been identified. There was no association between the level of parasitism and time of year (χ^2=3.52, df=5, *P*=0.620).

The percentage of new galls decreased as the percentage of flowering stems increased (Fig. 6.11), which may indicate asynchrony with the host-plant phenology.

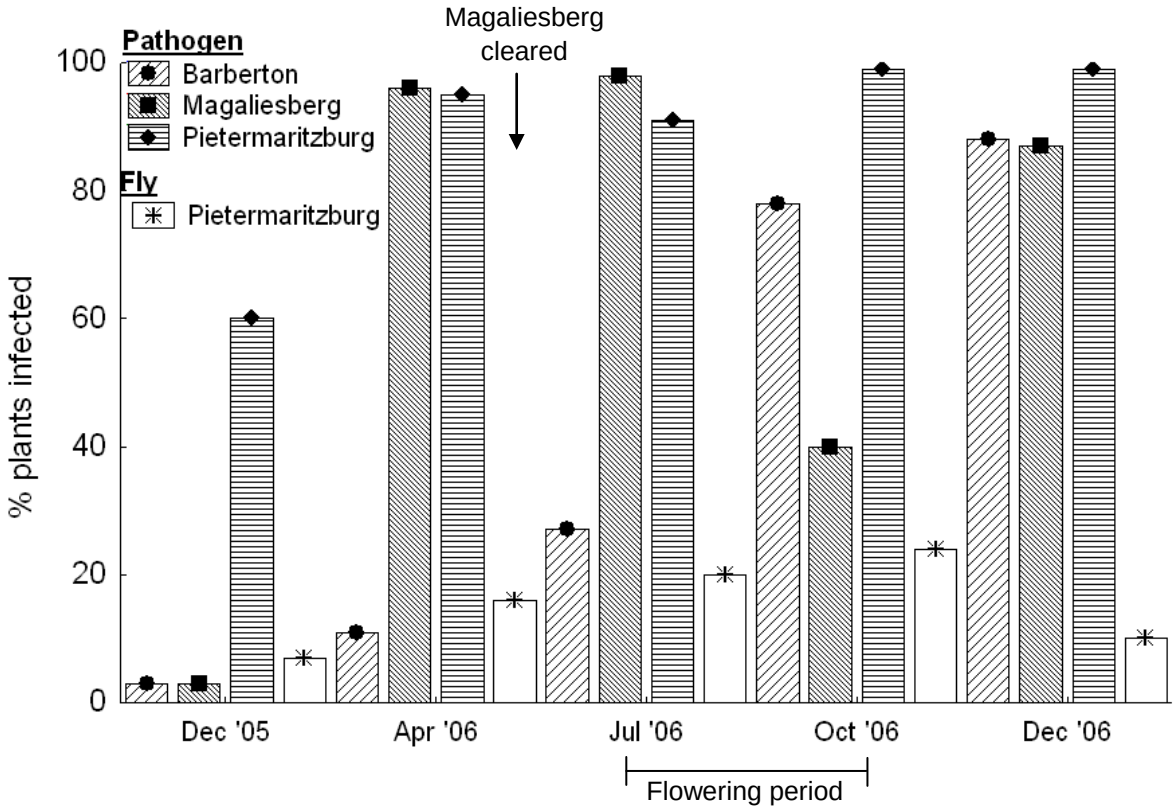


Figure 6.8: Percentage *Ageratina adenophora* stems infected with the fungal pathogen *Passalora ageratinae* or galled by the fly *Procecidochares utilis*, from December 2005 to December 2006, at three field sites.

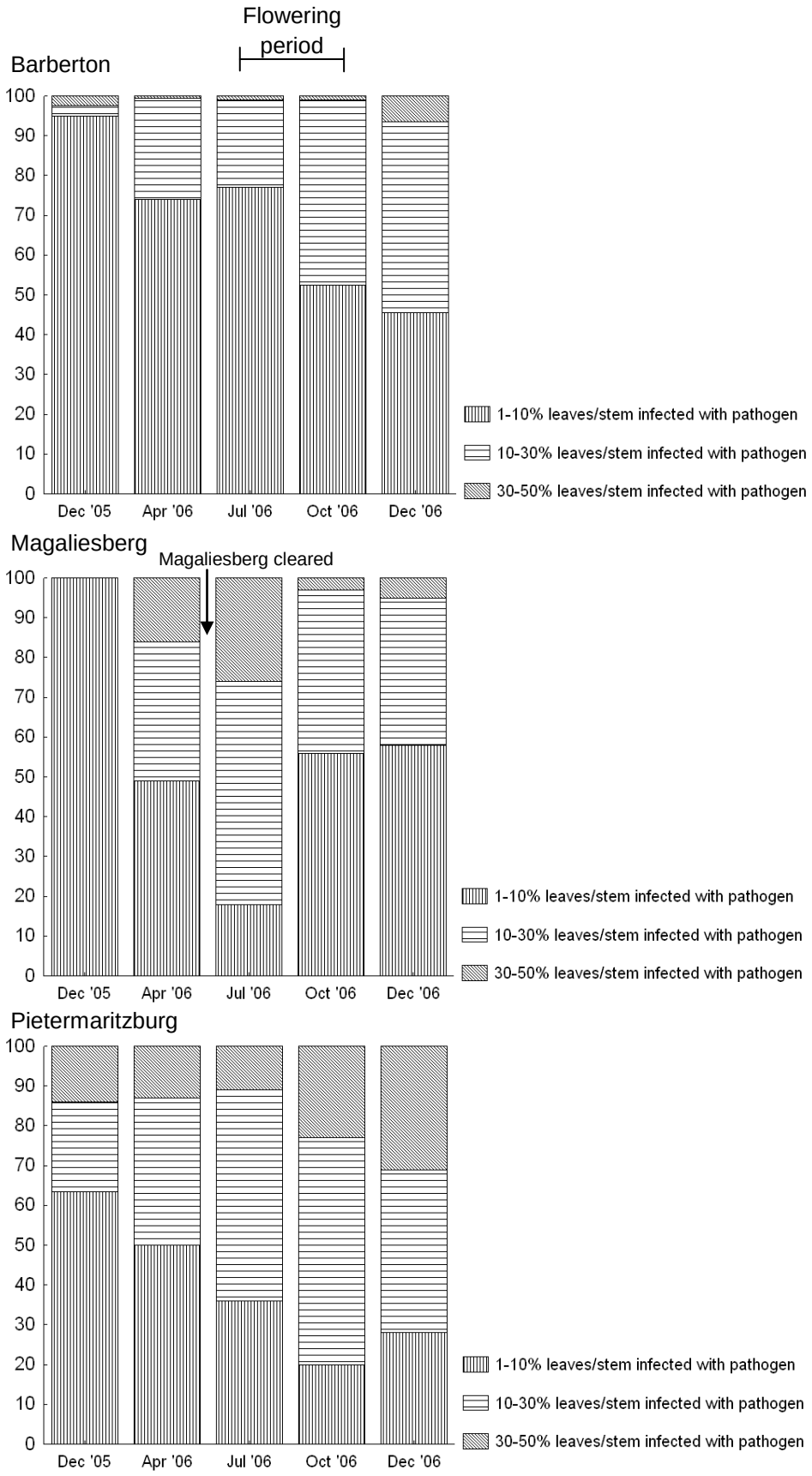


Figure 6.9: Percentage of *Ageratina adenophora* pathogen-infected stems with leaves infected with 1-10%, 10-30% or 30-50% by *Passalora ageratinae*, from December 2005 to December 2006, at three field sites.

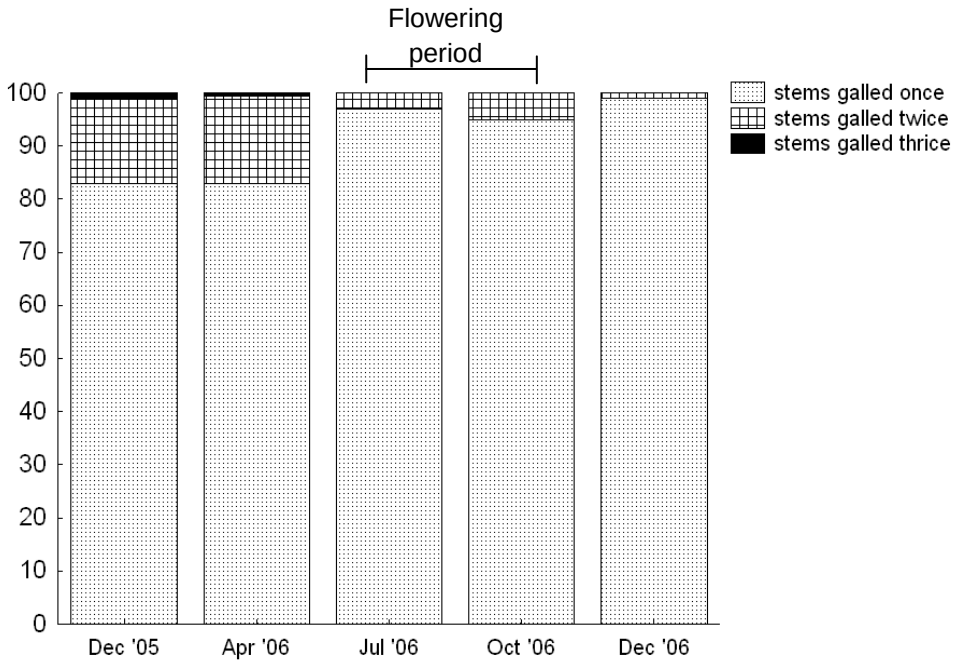


Figure 6.10: Percentage *Ageratina adenophora* galled stems with one, two or three *Procecidochares utilis* galls, from December 2005 to December 2006, at Pietermaritzburg.

Table 6.3: Percentage of *Procecidochares utilis* and parasitoid adults emerging from galls on *Ageratina adenophora* stems collected at Pietermaritzburg.

	% of adults emerging	
	Gall fly	Parasitoid
April 2006	77	23
July 2006	67	33
December 2006	66	34
Mean	**70**	**30**

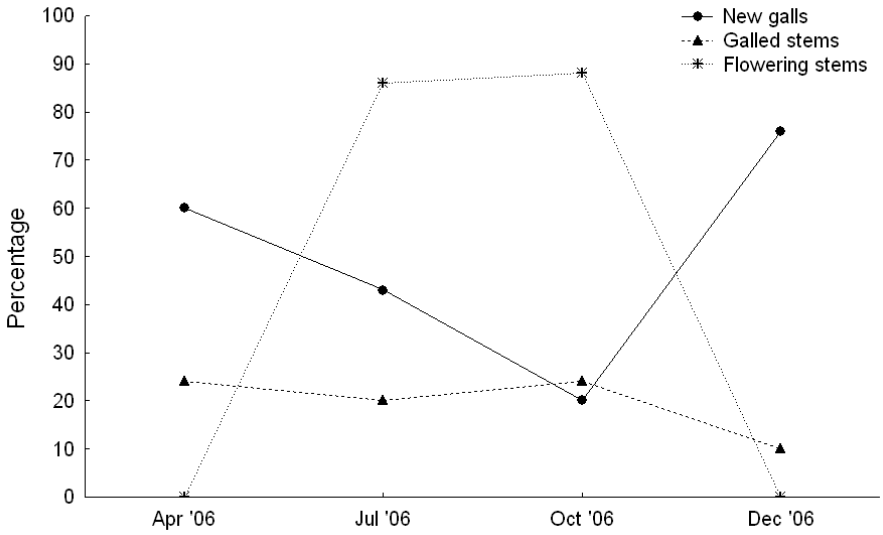


Figure 6.11: Percentage of flowering and galled *Ageratina adenophora* stems, and percentage of new *Procecidochares utilis* galls at Pietermaritzburg from April 2006 to December 2006.

6.4.6 Reproductive output of A. adenophora *populations*

The percentage of flowering stems was lowest at Barberton in July and October 2006 (Table 6.4). After stems were cleared from Magaliesberg, the percentage of flowering stems decreased from 86% to 39%. There is an association between the percentage of flowering stems and sampling time at the three field sites (χ^2=20.16, df=5, *P*=0.001).

Table 6.4: Percentage of *Ageratina adenophora* stems flowering at three field sites in July 2006 and October 2006.

	% flowering stems	
	July 2006	October 2006
Barberton	28	44
Magaliesberg	87	39
Pietermaritzburg	86	90

There were no flowering stems that were not infected with the pathogen and/or galled. There were significant differences between sites and level of biocontrol agent attack for number synflorescences/stem, number capitula/synflorescence, number filled achenes/capitula, number empty and aborted achenes/capitula and total number achene/capitula (Table 6.5, Table 6.6). The least number of synflorescences per stem was on pathogen infected stems at Barberton and galled stems with 30-50% pathogen infection at Pietermaritzburg. Pathogen infected stems at Barberton and Magaliesberg, and galled stems with 30-50% pathogen infection at Pietermaritzburg had the east number of capitula per synflorescence. The number of filled and total achenes per capitula was lowest on galled stems with 30-50% pathogen infection at Pietermaritburg. There was no significant difference between the germinability of achenes at the three sites (Table 6.5, Table 6.6). The number of filled achenes per stem (potential viable achene production) and the number of germinable achenes per stem (viable achene production) differed significantly between sites and levels of biocontrol agent attack (Table 6.5, Fig. 6.12).

Stems with only pathogen-infection, at Pietermaritzburg, had the highest number of filled achenes/stem and germinable achenes/stem, in comparison to the other sites and levels of biocontrol agent attack. Galled stems with 30-50% leaves infected with the pathogen had the lowest number of filled and germinable achenes per stem at Pietermaritzburg, and were similar to the stems at Barberton and Magaliesberg. The number of germinable achenes per m^2 did not differ between sites ($F_{2,84}$=2.59, P=0.083), but tended to be higher (P<0.1) at Pietermaritzburg (Fig. 6.13).

Table 6.5: Results of two-way ANOVA's assessing the site (df=2), level of biocontrol agent attack (df=5) and their interaction effect on the number of *Ageratina adenophora* synflorescences per stem, capitula per synflorescence, filled achenes per capitula, empty/aborted achenes per capitula, total number of achenes per capitula, percentage germination of filled achenes, filled achenes/stem and germinable achenes/stem. n= 120 stems. The corresponding data are presented in Table 6.6 and Figure 6.12. Significant *P*-values are in bold.

	Site		Biocontrol agent attack level		Site*Biocontrol agent attack level	
	F-ratio	*P*-value	F-ratio	*P*-value	F-ratio	*P*-value
Number synflorescences/stem	512.67	**<0.001**	153.94	**<0.001**	26.60	**<0.001**
Number capitula/ synflorescence	367.22	**<0.001**	100.95	**<0.001**	12.07	**<0.001**
Number filled achenes/capitula	865.78	**<0.001**	411.47	**<0.001**	68.89	**<0.001**
Number empty/aborted achenes/capitula	879.71	**<0.001**	411.11	**<0.001**	70.52	**<0.001**
Total number achenes/capitula	2673.98	**<0.001**	1172.32	**<0.001**	210.61	**<0.001**
Achene germination (%)	0.03	0.974	0.00	0.961	0.06	0.939
Filled achenes/stem	168.16	**<0.001**	40.81	**<0.001**	3.41	**<0.001**
Germinable achenes/stem	160.60	**<0.001**	38.63	**<0.001**	3.10	**<0.001**

Table 6.6: Number of *Ageratina adenophora* synflorescences per stem, capitula per synflorescence, filled achenes per capitula, empty/aborted achenes per capitula, total number of achenes per capitula and percentage germination of filled achenes at three field sites in October 2006. Different superscripts within rows are significantly different, at $P<0.05$ (LSD). (Means ± SE).

Sites	Barberton		Magaliesberg		Pietermaritzburg					
Level of biocontrol agent infection / Reproductive output	1-10% pathogen	10-30% pathogen	1-10% pathogen	10-30% pathogen	1-10% pathogen	10-30% pathogen	30-50% pathogen	1-10% pathogen 1 gall	10-30% pathogen 1 gall	30-50% pathogen 1 gall
Number synflorescences/stem	4.4±0.30^{e}	4.8±0.20^{de}	5.6±0.33^{abc}	5.4±0.38^{bc}	6.3±0.37^{a}	6.3±0.23^{a}	6.1±0.33^{ab}	5.9±0.42^{ab}	5.9±0.47^{ab}	4.9±0.38^{cde}
Number capitula/ synflorescence	7.8±0.56^{f}	8.9±0.58^{ef}	9.8±0.79^{cde}	8.7±0.72^{ef}	12.2±0.94^{ab}	13.8±1.02^{a}	13.3±0.89^{a}	10.7±0.82^{bcd}	11.2±0.92^{bc}	9.0±0.83^{def}
Number filled achenes/capitula	63.9±0.79^{abc}	43.9±1.10^{ab}	40.6±1.26^{bcd}	37.2±2.44^{d}	45.5±1.13^{a}	42.6±1.60^{ab}	38.8±2.58^{cde}	37.3±2.06^{de}	38.8±1.49^{ce}	36.2±1.61^{e}
Number empty/aborted achenes/capitula	21.6±0.97^{a}	19.8±0.77^{bcd}	18.2±0.80^{ab}	19.8±0.86^{bcd}	21.2±0.73^{ab}	21.6±0.95^{a}	20.2±0.83^{abc}	16.7±0.68^{e}	17.6±0.76^{e}	19.4±0.90^{cd}
Total number achenes/capitula	63.9±0.79^{a}	63.7±0.92^{a}	58.8±1.17^{a}	56.9±2.01^{b}	66.7±1.07^{a}	64.2±1.19^{a}	59.0±2.18^{b}	53.9±1.82^{c}	56.4±1.40^{bc}	55.6±1.35^{c}
Achene germination (%)	75.8±1.16	75.8±1.13	75.9±1.15	74.7±1.11	75.9±1.04	75.6±1.11	75.5±0.92	75.5±1.46	75.5±1.15	75.4±0.92

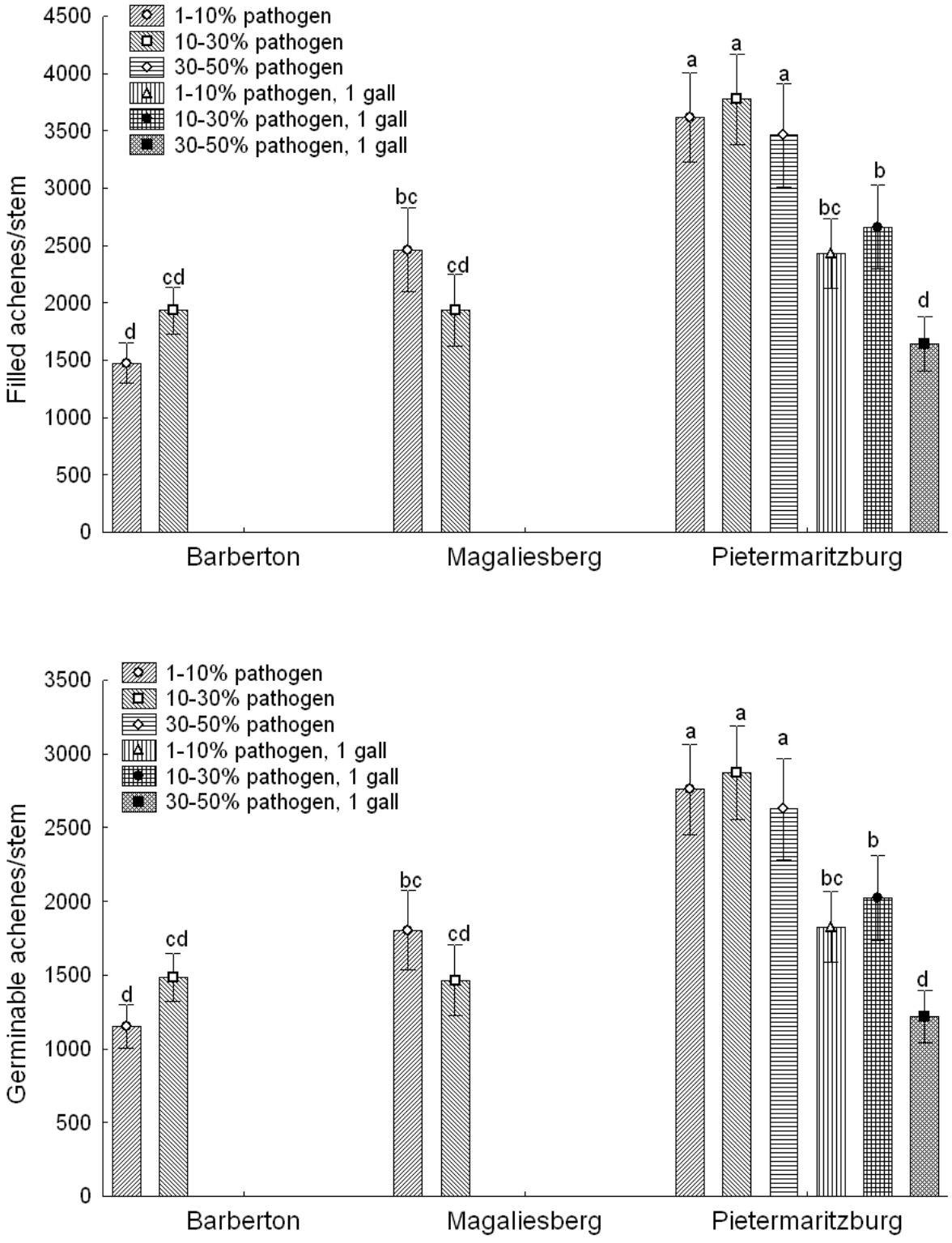


Figure 6.12: Number of filled achenes and germinable achenes per *Ageratina adenophora* stem in relation to six different attack levels, of pathogen infection and/or fly galling at three field sites in October 2006. Means followed by the same letter are not significantly different, at *P*<0.05 (LSD). (Means ± SE).

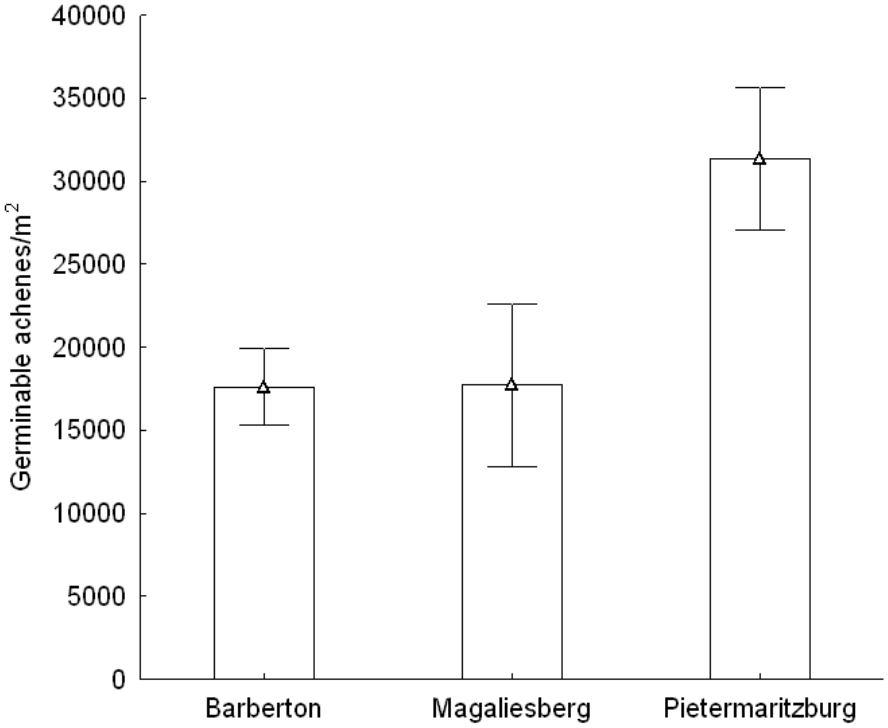


Figure 6.13: Mean total germinable achene production of *Ageratina adenophora* per m^2 of invasion at three field sites in October 2006. Means followed by the same letter are not significantly different, at $P<0.05$ (LSD). (Means ± SE).

6.5 Discussion

Controlled laboratory and field trials showed that the gall fly is able to restrict stem growth and the pathogen is able to inhibit sideshoot growth of crofton weed (Chapter 2 and 3), and both biocontrol agents reduce reproductive output and inflict stress on crofton weed at a ecophysiological level (Chapter 4 and 5). This study showed that the crofton weed infestations and the effect of the two agents differed at the three different localities in South Africa. In general Pietermaritzburg had the highest stem density and seedling recruitment in comparison to the Magaliesberg site, which was manually cleared during the study, and the Barberton field site, which was under a forest canopy. The pathogen was prevalent throughout the infestations but did not severely infect individual stems. In Pietermaritzburg the gall fly population was low, but did reduce the reproductive output of crofton weed.

Disturbance is a factor that influences the potential invasibility of an area (Lonsdale, 1999). An example of an invasive weed in South Africa is pompom weed, *Campuloclinium macrocephalum*, which favours disturbed lands such as abandoned agricultural fields, unutilised land and roadsides (Goodall *et al*., 2011). Mechanical clearing of crofton weed in Magaliesberg involves teams of people who manually pull the plant out of the ground, thereby disturbing the area. Clearing decreased the stem density of crofton weed infestations immediately. However, not all of the plants were removed in July and crofton weed was able to recover rapidly, with stem density doubling in five months. The infestation was renewed with new young plants, which may have emerged from soil-stored seed banks or from sideshoots rooting from trailing stems. Crofton weed seeds require light for germination (Zhang *et al*., 2008; Muniappan *et al*., 2009); therefore clearing taller plants may leave bare, open areas for seeds to germinate and seedlings to grow. Mechanical removal will not be effective on its own to control crofton weed in South Africa. Similar results have been found in China, where large labour-intensive and expensive chemical and mechanical control programmes have not been successful alone (Zhang *et al*., 2008). Therefore, for clearing to be effective plants should be cleared before the flowering season, and then follow-up clearing organised for soon after the flowering period.

One of the attributes that makes crofton weed a good invader is that it is invasive even in light-limited areas (Feng *et al*., 2007b). The crofton weed infestation at Barberton was under a pine forest canopy, with low light levels. The stems at this site were smaller than at the other two sites, therefore whilst crofton weed may be able to grow in low light areas; its growth may be inhibited. Even though crofton weed seeds require light for germination, a small percentage can germinate under low light levels (Lu *et al*., 2006). Seedling density was high in Barberton in December 2006, but the number of young plants was low after flowering compared to Magaliesberg and Pietermaritzburg; therefore the seedlings may not survive to adult stems, or their growth may be slower.

Stems in Magaliesberg had a higher biomass in relation to stem height in comparison to the other two sites. Barberton had the smallest biomass, this may be because the stems are smaller, and more resources are allocated to larger leaves to accommodate for low light levels rather than to stem growth (Muniappan *et al*., 2009). Above-ground biomass per m^2 did not differ significantly between Pietermaritzburg and Magaliesberg (except after clearing), therefore it may be possible that both the pathogen and gall fly have influenced the health of the crofton weed stems at Pietermaritzburg, therefore even though they are tall stems they may be weaker and thinner.

Pathogens can be very important in biocontrol programmes; however their ability to control the target weed depends on the pathogen's ability to inflict damage and the intensity or severity of the damage caused by the pathogen (Charudattan, 2005). Controlled field and laboratory experiments showed that the leaf-spot pathogen does not infect more than 20-50% of living leaves on crofton weed stems (Chapter 3 and 4). Weeds are able to outgrow pathogen infection by increasing uninfected vegetative growth (Charudattan, 2010). This is evident in the field surveys, where the pathogen infects up to 90% of crofton weed stems at a time, and was able to recover after manual clearing of crofton weed in a short time. However, on these infected stems no more than 50% of the living leaves are infected, and generally only up to 30% of leaves are infected at a time. This low severity of infection, or ability of crofton weed to outgrow pathogen infection, reduces the amount of control. In contrast, in Australia the majority of leaves are infected, leading to severe defoliation of whole stems (Dodd, 1961; Page & Lacey, 2006). These results in Australia were seen during the dry season, with less damage evident in damp shaded areas, and may be why the pathogen is less aggressive in the moist infestations of South Africa.

The gall fly has been very successful in Hawaii, killing crofton weed plants and clearing large infestations (Bess & Haramoto, 1959, 1972). Up to 100% of crofton weed stems were galled, with 1-7 galls per stem (Bess &

Haramoto, 1972). In Australia, the gall fly initially galled most stems, but now galls less than 50% of stems in crofton weed infestations (Page & Lacey, 2006). In China, galling levels are low with 10-37% of stems galled (Zhang *et al*., 2008). In this South African survey only up to 20% of crofton weed stems were galled at Pietermaritzburg, and of these stems most were only galled once. These levels are not high enough for the gall fly to be as prevalent and severe in its attack as is seen in Hawaii. In Australia low fly populations are explained by the 30-70% parasitism levels of the gall fly (Dodd, 1961, Page & Lacey, 2006). Parasitism of the gall fly is also seen in China (Li *et al*., 2006). However, in Hawaii parasitism levels of the gall fly reached 71-93% in the warmer months and 7-69% in the cooler months (Bess & Haramoto, 1972). When the gall fly was initially released in Pietermaritzburg, Bennett (1986) reported parasitism levels of 76%. However, this survey showed only 30% parasitism of the gall fly and a recent survey of the gall fly in the Magaliesberg also found 30% parasitism levels (Heystek *et al*., 2011). In Hawaii the gall fly was less successful in high rainfall areas (Bess & Haramoto, 1972); hence in the moist, high rainfall areas of South African crofton weed infestations the fly may be inhibited. An alternate explanation for low gall fly populations may be limits imposed by host-plant phenology. The female fly lays her eggs on or near the apical leaf bud at the tips of stems (Bennett & Van Staden, 1986), during the flowering season these oviposition sites are not available (Li *et al*., 2006). In Pietermaritzburg there was a decrease in the percentage of new galls when the percentage of flowering stems increased. As 90% of the crofton weed stems were flowering in October, the number of oviposition sites available for the gall fly may be very low.

In contrast, the younger plants at Barberton had a lower percentage of stems that flowered. Clearing at Magaliesberg did not stop the remaining stems from flowering but the number of flowering stems was reduced by 50%. Controlled laboratory and field trials showed that galling can reduce reproductive output by 53.8% (Erasmus *et al*., 1992; Chapter 4). Galled stems at Pietermaritzburg had a reduced reproductive output, in

comparison to stems infected with the pathogen only. However, because only 20% of stems were galled the number of germinable achenes per m^2 was highest at Pietermaritzburg compared to the other two sites. These results, along with laboratory and field trials, highlight the ability of the fly to reduce reproductive output. The effect is not seen at a population level as the fly population is low, suggesting that follow-up releases of the fly may be needed in Pietermaritzburg, and this should also be considered when releasing the fly at Magaliesberg and Barberton.

It is important to define what will be seen as successful biocontrol at the start of a biocontrol programme (Hoffmann & Moran, 2008; Müller-Schärer & Schaffner, 2008). Definitions of success can be at individual (e.g. plant growth), population (e.g. weed density) and community (e.g. native species composition) levels (van Klinken & Raghu, 2006). This study highlights the importance of defining success at the beginning of the programme. As the biocontrol of crofton weed in South Africa was a small opportunistic programme initiated in 1984 (Kluge, 1991), success was not defined, and baseline data on which to assess the success was not collected. This is not uncommon in biocontrol programmes as limited time and funding often restrict the amount of field data collected (Morin *et al*., 2009). The success of biocontrol programmes does not have to be limited to drastic reductions in the weed's density (Hoffmann & Moran, 2008). Based on field and laboratory experiments we do know that the agents, individually and together, affect crofton weed growth, reproduction and physiology at the individual plant level. Even though the area invaded by crofton weed in South Africa is increasing (Heystek *et al*., 2011), without previous infestation data it is not possible to say whether these two biocontrol agents have been unsuccessful, as we do not know what the situation would have been without them. Long term monitoring of weed infestations and their biocontrol agents is necessary to understand the efficacy of biocontrol agents (Morin *et al*., 2009). Monitoring the field sites in this study over a period of more than one year, may yield more information as to whether the infestations are increasing, if the biocontrol

agents are effective and if the effects of the agents at the individual level are carried forward to the population, community and ecosystem level.

6.6 Conclusion

Crofton weed infestations differ across South Africa, with factors such as disturbance (mechanical clearing) and light availability playing a role. Crofton weed can grow and withstand low levels of light; in addition mechanical clearing is not suitable as the only control option for crofton weed. Although the pathogen was prevalent throughout the sites it did not infect stems severely, as crofton weed was able to outgrow the infection. The gall fly population was low in Pietermaritzburg, with a low proportion of stems galled, and only galled once. Reasons for low fly population numbers could include parasitism, high rainfall and phenological asynchrony. Galling reduced reproductive output of crofton weed, but low galling levels means this does not influence the total number of germinable achenes at the site. It is not possible to say this biocontrol programme has been unsuccessful as we know the agents affect crofton weed at the individual stem level and we do not have baseline data prior to 1984 on which to make comparisons. Longer term monitoring of the sites may yield more information as to whether the invasions are growing or being controlled by the pathogen and fly.

Chapter 7: General discussion

7.1 Aims of this chapter

The aim of this chapter is to synthesise the overall findings of this thesis:

- Discuss the efficacy of the fly and pathogen as biocontrol agents of crofton weed.
- Discuss the use of field and laboratory trials in predicting the efficacy of biocontrol agents.
- Discuss the post-release evaluation of the fly and pathogen on crofton weed in South Africa, and possible future considerations when evaluating crofton weed.
- Considerations for the future of crofton weed biocontrol in South Africa.

7.2 Multiple biocontrol agents

This study evaluated the efficacy of the two biocontrol agents, a gall fly *Procecidochares utilis* and a leaf spot pathogen *Passalora ageratinae*, released against crofton weed in South Africa. Manipulative laboratory and field experiments were used to assess the agents, individually and in combination, in terms of the effect on vegetative growth (Chapter 2 and 3), reproductive output (Chapter 4) and ecophysiological variables (Chapter 5).

Individually both the fly and pathogen affect crofton weed growth and reproduction. The fly galls act as nutrient sinks inhibiting vertical growth of the plant, and reduce the reproductive output by 53.8%. These effects are particularly seen if the plant is galled more than once. However, crofton weed compensates for this inhibited vertical growth by producing sideshoots. These results correlate with the biomass of stems from the field trials, with galled stems allocating more biomass to sideshoots than bare stems and live leaves. The pathogen affects the lower leaves of the plant, which compensates by dropping the infected leaves and producing healthy new leaves upwardly. However, as the plant is assigning resources to new leaf growth there are less sideshoots produced. The

pathogen reduces reproductive output of crofton weed stems by 26.7%. In terms of the effect on crofton weed ecophysiology, the pathogen is the more influential biocontrol agent, reducing the photosynthetic rate and functioning of photosystem II of the lower (older) leaves. Neither agent influenced the ecophysiology of the top (younger) leaves. This study did not take a detailed look at the effect of the agents on the roots of crofton weed plants. Initial studies, in the laboratory trials, indicated that the agents did not affect biomass allocation to the roots, and the results are not reported in this thesis. Future studies could examine the effect of the agents on the roots using root chambers with glass fronts.

These two agents have been released in combination at some sites in South Africa, therefore it is important to understand the effect of the interaction between the fly and pathogen; a negative interaction between the agents could hinder crofton weed biocontrol. One of the best ways to understand the interaction between biocontrol agents is to look at the combined effect the agents have on the target weed (Hatcher & Paul, 2001). This study used Hatcher's (1995) four categories of interactions between herbivores and plant pathogens to assess the efficacy of the fly and pathogen together on crofton weed. The laboratory trials showed an additive interaction between the agents in terms of vegetative growth, with the fly reducing vertical plant growth and the pathogen inhibiting sideshoot growth. In contrast, an equivalent interaction between the fly and pathogen was found in the field trials, with the pathogen reducing sideshoot growth, and the fly weakening stems. The reproductive output results indicated an equivalent interaction with the fly reducing reproductive output the most. The interaction between the agents on crofton weed leaf physiology was equivalent, with the pathogen causing the most damage. Therefore, the agents are compatible, and each has an influence, and should continue to be released together at all infestations.

This study highlights that measuring the effects of multiple agents on the biocontrol of a target weed may not always be straightforward. In order to accurately assess the impact of the interaction between biocontrol agents,

consideration needs to be given to what plant parameters are measured, and when these measurements are taken in terms of the plant's life-cycle. For example, if sideshoot growth was not measured, or if monthly measurements had not been taken in the field and laboratory trials, the conclusion about the efficacy of the agents in combination might be different. In this case, the pathogen would not be seen to play a role in crofton weed biocontrol. In addition, evaluating the influence of the agents on the ecophysiology of crofton weed provided a better understanding of the effect of the agents. For example, the field and laboratory trials suggested that the pathogen does not inhibit crofton weed vertical growth. However, the ecophysiology results shows that the vertical growth is probably compensatory, as the pathogen affects the photosynthetic ability of crofton weed's lower leaves, and therefore crofton weed compensates for this with vertical plant growth with new healthy leaves.

The fly and pathogen do not inhibit each other in the control of crofton weed, instead they complement each other. Overall the interaction between the agents is equivalent, with both the agents influencing crofton weed. The control of crofton weed by these two agents is better explained by the cumulative model (Harris, 1985), rather than by the lottery model (Myers, 1985, 2008). The degree to which the agents may be effective in the field could depend on their prevalence and severity of agent infection at crofton weed infestations, as well as the influence of environmental conditions. The efficacy of the agents may differ during different times of the year and at different crofton weed infestations across South Africa. In Hawaii the fly has been very successful as it repeatedly galled, up to seven galls per stem, and up to 100% of the stems (Bess & Haramoto, 1972). However, the fly was less successful in high rainfall areas (Bess & Haramoto, 1959, 1972). In Australia the pathogen was more successful during the drier months of the year and defoliated whole stems (Dodd, 1961; Page & Lacey, 2006). Therefore, both agents should be released at all crofton weed infestations in South Africa, as they have been shown to work together under a variety of conditions.

7.3 Field and laboratory trials

Very often the efficacy of biocontrol agents is evaluated under greenhouse or laboratory conditions, rather than in the field (Raghu *et al*., 2006; Morin *et al*., 2009). However, greenhouse conditions are not necessarily representative of field conditions, and agent infection and efficacy can be under- or over-estimated. This study shows how the growth of the plant can differ between greenhouse and field conditions. The effect of the fly and pathogen on crofton weed reproductive output was similar between the greenhouse and field; however the vegetative growth was less in the field trials, in comparison to predictions from the greenhouse trials. In both trials plants were cut back to soil level, in the greenhouse stem regrowth was over 1m in a 10 month period, and in the field stem regrowth was 65cm over 14 months. This suggests that the favourable greenhouse conditions promote faster stem growth than seen in the field and that field trials may need to be run for a longer period than greenhouse trials in order to see the efficacy of biocontrol agents. In addition, once plants were over 1m tall in the greenhouse they were difficult to maintain within plant bags and prevent stems from breaking and trailing along the ground. In this situation field trials are beneficial as the trials can be run for longer and plants do not need to be maintained within bags within a limited area of space. This is also valuable in understanding the proliferation of crofton weed through sideshoot rooting. However, greenhouse trials can be useful for identifying plant variables to measure within the field trials, helping to reduce the work needed for field trials. Greenhouse trials can also be useful at the pre-release stage of biocontrol, such as understanding the interaction between multiple agents on a weed, and the ecophysiological response of weeds to the biocontrol agents, and therefore have an important role to play in biocontrol programmes.

The field trials in this study were one-sided as it was no possible to remove the pathogen from field plants. Sine these field trials were run the pathogen has been renamed and there may now be a fungicide available that can be applied from an early stage, allowing for control and galled-

only stems to be studied in the field. Alternatively plants grown in the greenhouse could be transplanted into the field. The field trials could then formally assess the interaction between the fly and pathogen under field conditions.

7.4 Post-release evaluations

The focus and funding for biocontrol programmes has predominantly been to find biocontrol agents, test for host-specificity and release the agents, with little attention given to the efficacy of the released agents in the field (Morin *et al*., 2009). However, in order to determine the success of biocontrol agents post-release evaluations are essential. In addition, the efficacy of biocontrol agents is not always as drastic as was seen with the release of the frond-feeding weevil, *Stenopelmus rufinasus*, against red waterfern, *Azolla filiculoides* (McConnachie *et al*., 2004). The efficacy of biocontrol agents may be more subtle as with the biocontrol of *Opuntia stricta* with the cactus moth, *Cactoblastis cactorum* and the cochineal insect, *Dactylopius opuntiae* (Hoffmann & Moran, 2008). Therefore, it is very important to have pre-release baseline data of the weed infestations and a pre-defined set of performance targets or definition of success. These definitions and targets allow for comparison and evaluation post-release of the agents and to define pre-release what plant parameters to measure (Müller-Schärer & Schaffner, 2008; Morin *et al*., 2009). This study highlights the limitation of defining a biocontrol programme as successful without pre-release evaluations and targets. We now know the agents negatively affect crofton weed, however we cannot ascertain clearly from post-release evaluations if this effect is seen at the population level in crofton weed infestations in South Africa. However, the laboratory and field results together suggest that the invasion of crofton weed has been curtailed by a small release effort.

This study shows that crofton weed and the two biocontrol agents are established in South Africa (Chapter 6). Crofton weed infestations can

reach densities of 30-50 stems per m^2 in Barberton, 20-50 stems per m^2 in Magaliesberg and 80 stems per m^2 in Pietermaritzburg. The pathogen is prevalent throughout the infestations but the severity of infection at the stem level is low (up to 30% of leaves are infected). Fly populations are low, 20% of stems are galled in Pietermaritzburg and stems are generally only galled once. Parasitism levels are approximately 30% in Pietermaritzburg and may play a role in the low fly population numbers. Surveys in the Magaliesberg will provide better understanding if the fly is more prevalent and severe in a lower rainfall area. Manual clearing of crofton weed is not an effective control method on its own, as stem density doubled in five months in the Magaliesberg after clearing. This study only looked at one infestation within three Provinces of South Africa; future studies could evaluate more infestations within the Provinces. Longer term monitoring is also essential to collect reliable data about the expansion of infestations and to assess the status of the surrounding communities and ecosystem (Morin *et al*., 2009).

In china, a recent study has shown how biotic and abiotic factors play an important role in the invasiveness of crofton weed (Wang *et al*., 2011). Seed dispersal by wind and water currents, as well as by human activities along transport routes play a large role in crofton weed invading new areas and long-distance dispersal. Invasion within these areas is further influenced by sideshoot growth from lateral stem nodes. This study is aided by long term monitoring and information of the invaded areas.

Additional information, which was not gathered in this study, which could help better understand the crofton weed infestations in South Africa include soil seed bank viability and seed limitation. We know that the fly and pathogen reduce the reproductive output of crofton weed, however whether this extends to a population level effect is unknown as we do not know how long crofton weed soil seed banks are viable and if crofton weed is seed-limited. New growth could be distinguished as growth from seeds, or sideshoots (identifiable by a rudimentary rhizome from the base of the new ‘seedling’). This will help with understanding whether the

recruitment of new plants in crofton weed infestations is through seeds or sideshoot growth of older trailing stems.

7.5 Future of *Ageratina adenophora* biocontrol in South Africa

There is a call to reduce the number of biocontrol agents released in biocontrol programmes to try and reduce the risks associated with releasing ineffective organisms (Raghu *et al*., 2006). It has been proposed that agents be released based on their possible efficacy versus the ease of rearing and host-specificity testing (van Klinken & Raghu, 2006). In order to assess the efficacy of possible agents it is necessary to understand plant demography, ecology and response to herbivory, this data can then be used with modelling to predict the efficacy of possible biocontrol agents (Briese, 2006; Raghu *et al*., 2006). Raghu *et al*. (2006) propose four “filters” to use in assessing and choosing possible biocontrol agents. These are to determine the weak link in a plant’s life-cycle, the type of damage this stage is susceptible to, agents that will affect this stage and the host range of these agents.

Whilst the fly and pathogen may exert some control over crofton weed, the infestations of crofton weed are expanding in South Africa, but surveys for new biocontrol agents have been undertaken, and potential biocontrol agents include a stem-boring moth and rust fungus (Heystek *et al*., 2011). Understanding if recruitment of new crofton weed plants is through seeds or sideshoots will help with the selection of a new biocontrol agent. Based on the success of the fly and pathogen on crofton weed in Hawaii and Australia, death or severe weakening of crofton weed stems can lead to effective control. However, the new agent will need to be more severe in its attack, in comparison to the fly and pathogen, in order to achieve control of crofton weed and inhibit compensatory growth. In addition, it is important to take into account the interaction with the already established fly and pathogen when selecting the new agents.

Until a new biocontrol agent is found it is important to increase the efforts of the current biocontrol against crofton weed in South Africa. No follow-up releases of the fly have occurred in Pietermaritzburg since its initial release in the 1980's, further releases of the fly may increase the fly population in Pietermaritzburg, which could improve the control of crofton weed. In addition, the fly has recently been released in the Magaliesberg area, from the findings in Pietermaritzburg consideration should be given to further release efforts of the fly in this area. The fly is also not present in the Barberton area and releases should be made there. The severity of the pathogen infection on individual stems is low in the field. Future studies may look into spraying an inoculum of the pathogen onto the field infestations to investigate if it is possible to increase the severity of the pathogen infection. This study has shown that the agents complement each other and do affect crofton weed providing some control, therefore until a new agent has been found the current programme should be continued with renewed efforts.

References

Ainsworth, N. (2003). Integration of herbicides with arthropod biocontrol agents for weed control. *Biocontrol Science and Technology, 13,* 547-570.

Anderson, W.P. (1996). *Weed science: Principles and applications 3rd ed*. West Publishing Company: New York.

Barton, J., Fowler, S.V., Gianotti, A.F., Winks, C.J., de Beurs, M., Arnold, G.C. and Forrester, G. (2007). Successful biological control of mist flower (*Ageratina riparia*) in New Zealand: Agent establishment, impact and benefits to the native flora. *Biological Control, 40*, 370-385.

Bennett, P.H. (1986). An investigation into the biological control of crofton weed, *Ageratina adenophora (K. & R.) (Syn. Eupatorium adenophorum Spreng.) by the gall fly Procecidochares utilis Stone.* MSc Thesis, University of Natal, South Africa.

Bennett, P.H. and Van Staden, J. (1986). Gall formation in crofton weed, *Eupatorium adenophorum* Spreng. (syn. *Ageratina adenophora*), by the *Eupatorium* gall fly *Procecidochares utilis* Stone (Diptera: Trypetidae). *Australian Journal of Botany, 34*, 473-480.

Bess, H.A. and Haramoto, F.H. (1959). Biological control of pamakani, *Eupatorium adenophorum*, in Hawaii by a tephritid gall fly, *Procecidochares utilis*, 2. Population studies of the weed, the fly, and the parasites of the fly. *Ecology, 40*, 244-249.

Bess, H.A. and Haramoto, F.H. (1972). Biological control of pamakani, *Eupatorium adenophorum*, in Hawaii by a tephritid gall fly, *Procecidochares utilis*, 3.Status of the weed, fly and parasites of the fly in 1966-71 versus 1950-57. *Proceedings of the Hawaiian Entomological Society, 21,* 165-178.

Briese, D.T. (2006). Can an *a priori* strategy be developed for biological control? The case of *Onopordum* spp. thistles in Australia. *Australian Journal of Entomology, 45,* 317-323.

Broughton, S. (2000). Commentary: Review and evaluation of Lantana biocontrol programs. *Biological Control, 17*, 272-286.

Buccellato, L. (2004). *A post-release evaluation of the biocontrol agent, a leaf-spot fungus, Phaeoramularia eupatorii-odorati, released against crofton weed, Ageratina adenophora, in the Magaliesberg region of South Africa.* Unpublished Honours Dissertation, University of the Witwatersrand, Johannesburg.

Buntin, D.G., Gilbertz, D.A. and Oetting, R.D. (1993). Chlorophyll loss and gas exchange in tomato leaves after feeding injury by *Bemisa tabaci* (Homoptera: Aleyrodidae). *Journal of Economic Entomology, 86,* 517-522.

Caesar, A.J. (2000). Insect – pathogen synergisms are the foundation of weed biocontrol. In Spencer, N.R. (Ed.), *Proceedings of the X International Symposium on Biological Control of Weeds, July 4-14, 1999*. Montana State University: Montana. pp 793-798.

Caesar, A.J. (2003). Synergistic interaction of soilborne plant pathogens and root-attacking insects in classical biological control of an exotic rangeland weed. *Biological Control, 28*, 144-153.

Campanella, D.M., McEvoy, P.B. and Mundt, C.C. (2009). Interaction effects of two biological control organisms on resistant and susceptible weed biotypes of *Chondrilla juncea* in western North America. *Biological Control, 50*, 50-59.

Charudattan, R., Linda, S.B., Kluepfel, M. and Osman, Y.A. (1985). Biocontrol efficacy of *Cercospora rodmanii* on water hyacinth. *Phytopathology, 75*, 1263-1269.

Charudattan, R. (2005). Ecological, practical, and political inputs into selection of weed targets: What makes a good biological control target? *Biological Control, 35*, 183-196.

Charudattan, R. (2010). A reflection on my research in weed biological control: using what we have learned for future applications. *Weed Technology, 24*, 208-217.

Clark, C.J., Poulsen, J.R., Levey, D.J. and Osenberg, C.W. (2007). Are plant populations seed limited? A critique and meta-analysis of seed addition experiments. *The American Naturalist, 170*, 128-142.

Crous, P.W., Schoch, C.L., Hyde, K.D., Wood, A.R., Gueidan, C., de Hoog, G.S. and Groenewald, J.Z. (2009). Phylogenetic lineages in the *Capnodiales*. *Studies in Mycology, 64*, 17-47.

Davis, A.S. (2006). When does it make sense to target the weed seed bank? *Weed Science, 54*, 558-565.

Day, M.D., Broughton, S. and Hannan-Jones, M.A. (2003). Current distribution and status of *Lantana camara* and its biological control agents in Australia, with recommendations for further biocontrol introductions into other countries. *Biocontrol News and Information, 24,* 63N-76N.

Denoth, M., Frid, L. and Myers, J.H. (2002). Multiple agents in biological control: improving the odds? *Biological Control, 24*, 20-30.

Dodd, A.P. (1961). Biological control of *Eupatorium adenophorum* in Queensland. *Australian Journal of Science, 23,* 356-365.

Dorchin, N., Cramer, M.D. and Hoffmann, J.H. (2006). Photosynthesis and sink activity of wasp-induced galls in *Acacia pycnantha*. *Ecology, 87*, 1781-1791.

Erasmus, D.J., Bennett, P.H. and Van Staden, J. (1992). The effect of galls induced by the gall fly *Procecidochares utilis* on vegetative growth and reproductive potential of crofton weed, *Ageratina adenophora*. *Annals of Applied Biology*, *120,* 173-181.

Feng, Y.L., Wang, J.F. and Sang, W.G. (2007a). Irradiance acclimation, capture ability, and efficiency in invasive and non-invasive alien plant species. *Photosynthetica*, *45*, 245-253.

Feng, Y.L., Wang, J.F. and Sang, W.G. (2007b). Biomass allocation, morphology and photosynthesis of invasive and noninvasive exotic species grown at four irradiance levels. *Acta oecologia, 31*, 40-47.

Field, C., Merino, J. and Mooney, H.A. (1983). Compromises between water-use efficiency and nitrogen-use efficiency in five species of California evergreens. *Oecologia*, *60*, 384-389.

Florentine, S.K., Raman, A. and Dhileepan, K. (2005). Effects of gall induction by *Epiblema strenuana* on gas exchange, nutrients, and energetics in *Parthenium hysterophorus*. *BioControl, 50,* 787-801.

Fowler, S.V., Syrett, P. and Hill, R.L. (2000). Success and safety in the biological control of environmental weeds in New Zealand. *Austral Ecology, 25,* 553-562.

Gardner, D.E. and Davis, C.J. (1982). *The prospects for biological control of nonnative plants in Hawaiian National Parks*, Technical Report 45, University of Hawaii at Manoa.

Goodall, J., Witkowski, E.T.F., Morris, C.D. and Henderson, L. (2011). Are environmental factors important facilitators of pompom weed (*Campuloclinium macrocephalum*) invasion in South African rangelands? *Biological Invasions*, *13*, 2217-2231.

Govender, M., Dye, P.J., Weiersbye, I.M., Witkowski, E.T.F. and Ahmed, F. (2009). Review of commonly used remote sensing and ground-based technologies to measure plant water stress. *Water SA*, *35*, 741-752.

Harris, P. (1985). Biological control of weeds: bureaucrats, botanists, beekeepers and other bottlenecks. In Delfosse, E.S. (Ed.), *Proceedings of the VI International Symposium on Biological Control of Weeds, August 19-25, 1984.* Agriculture Canada: Ottawa. pp 3-12.

Harris, P. and Shorthouse, J.D. (1996). Effectiveness of gall inducers in weed biological control. *The Canadian Entomologist, 128,* 1021-1055.

Haseler, B.W. (1965). Life-history and behaviour of the crofton weed gall fly *Procecidochares utilis* Stone (Diptera: Trypetidae). *Australian Journal of Entomology, 4,* 27-32.

Hatcher, P. E. (1995). Three-way interactions between plant pathogenic fungi, herbivorous insects and their host plants. *Biological Reviews, 70*, 639-694.

Hatcher, P. E. and Melander, B. (2003). Combining physical, cultural and biological methods: Prospects for integrated non-chemical weed management strategies. *Weed Research, 43,* 303-322.

Hatcher, P. E. and Paul, N.D. (2001). Plant pathogen-herbivore interactions and their effects on weeds. In Jeger, M.J., Spence, N.J. (Eds.), *Biotic interactions in plant-pathogen associations.* CABI Publishing: Wallingford, UK. pp193-225.

Henderson, L. (2001). *Alien weeds and invasive plants: A complete guide to declared weeds and invaders in South Africa.* ARC-PPRI, PPRI Handbook No. 12: Pretoria.

Herrera, L.P. and Laterra, P. (2009). Do seed and microsite limitation interact with seed size in determining invasion patterns in flooding Pampa grasslands? *Plant Ecology, 201*, 457-469.

Heystek, F. (2008). Renewed biocontrol research on crofton weed (*Ageratina adenophora*). *Plant Protection News, 77,* 15.

Heystek, F., Wood, A.R., Neser, S. and Kistensamy, Y. (2011). Biological control of two *Ageratina* species (Asteraceae: Eupatorieae) in South Africa. *African Entomology, 19*, 208-216.

Hilliard, O.M. (1977). *Compositae in Natal*. University of Natal Press: Pietermaritzburg.

Hoffmann, J.H. and Moran, V.C. (1998). The population dynamics of an introduced tree, *Sesbania punicea*, in South Africa, in response to long-

term damage caused by different combinations of three species of biological control agents. *Oecologia*, *114*, 343-348.

Hoffmann, J.H. and Moran, V.C. (2008). Assigning success in biological weed control: What do we really mean? In Julien, M.H., Sforza, R., Bon, M.C., Hatcher, P.E., Hinz, H.L. and Rector, B.G. (Eds.), *Proceedings of the XII International Symposium on Biological Control of Weeds*. CAB International: Wallingford, UK. pp. 687-692.

Julien, M.H. and Griffiths, M.W. (1998). *Biological control of weeds: A world catalogue of agents and their target weeds, 4th ed.* CSIRO Entomology. CABI Publishing: Oxon.

King, A.M., Brudvig, R. and Byrne, M.J. (2011). Biological control of dense-thorned bitter apple, *Solanum sisymbriifolium* Lam. (Solanaceae), in South Africa. *African Entomology, 19*, 427-433.

Klein, H. (2011). A catalogue of the insects, mites and pathogens that have been used or rejected, or are under consideration, for the biological control of invasive alien plants in South Africa. *African Entomology, 19*, 515-549.

Kluge, R.L. (1991). Biological control of crofton weed, *Ageratina adenophora* (Asteraceae), in South Africa. *Agriculture, Ecosystems and Environment, 37,* 187-191.

Kluge, R.L. (2000). The future of biological control of weeds with insects: No more ‘paranoia’, no more ‘honeymoon’. In Spencer, N.R. (Ed.), *Proceedings of the X International Symposium on Biological Control of Weeds, July 4-14, 1999*. Montana State University: Montana. pp 459-467.

Lambers, H., Chapin, F.S. and Pons, T.L. (2008). *Plant physiological ecology. 2nd ed*. Springer: New York, USA.

Land Protection. (2001). *NRM Facts: Ageratina adenophora*. Queensland Government, Natural Resources and Mines: Queensland.

Larson, K.C. and Whitman, T.G. (1997). Competition between gall aphids and natural plant sinks: plant architecture affects resistance to galling. *Oecologia, 109,* 575-582.

Li, A.F., Gao, X.M., Dang, W.G., Huang, R.X., Deng, Z.P. and Tang, H.C. (2006). Parasitism of *Procecidochares utilis* and its effect on growth and reproduction of *Eupatorium adenophorum. Journal of Plant Ecology, 30,* 496-503.

Li, H., Qiang, S. and Qian, Y. (2008). Physiological response of different croftonweed (*Eupatorium adenophorum*) populations to low temperature. *Weed Science*, *56*, 196-202.

Lonsdale, W.M. (1999). Global patterns of plant invasions and the concept of invasibility. *Ecology, 80,* 1522-1536.

Louda, S.M., Kendall, D., Connor, J. and Simberloff, D. (1997). Ecological effects of an insect introduced for the biological control of weeds. *Science, 277,* 1088-1090.

Lu, P., Sang, W. and Ma, K. (2006). Effects of environmental factors on germination and emergence of Crofton weed (*Eupatorium adenophorum*). *Weed Science, 54*, 452-457.

Macedo, T.B., Weaver, D.K. and Peterson, R.K.D. (2007). Photosynthesis in wheat at the grain filling stage is altered by larval wheat stem sawfly (Hymenoptera: Cephidae) injury and reduced water availability. *Journal of Entomological Science, 42,* 228-238.

Mack, R.N., Simberloff, D., Lonsdale, W.M., Evans, H., Clout, M. and Bazzaz, F. (2000). Biotic invasions: Causes, epidemiology, global consequences and control. *Issues in Ecology, 5*, 1-18.

McConnachie, A.J., Hill, M.P and Byrne, M.J. (2004). Field assessment of a frond-feeding weevil, a successful biological control agent of red waterfern, *Azolla filiculoides*, in southern Africa. *Biological Control, 29,* 326-331.

McDowell, S.C.L. (2002). Photosynthetic characteristics of invasive and noninvasive species of *Rubus* (Rosaceae). *American Journal of Botany*, *89*, 1431-1438.

McEvoy, P.B. and Coombs, E.M. (2000). Why things bite back: Unintended consequences of biological weed control. In Follett, P.A. and Duan, J.J. (Eds.), *Non-target effects of biological control*. Kluwer Academic. pp 167-194.

McFadyen, R.E.C. (1998). Biological control of weeds. *Annual review of Entomology, 43,* 369-393.

Meir, P., Levy, P.E., Grace, J. and Jarvis, P.G. (2007). Photosynthetic parameters from two contrasting woody vegetation types in West Africa. *Plant Ecology*, *192*, 277-287.

Moran, P.J. (2005). Leaf scarring by the weevils *Neochetina eichhorniae* and *N. bruchi* enhances infection by the fungus *Cercospora piaropi* on waterhyacinth, *Eichhornia crassipes*. *BioControl*, *50*, 511-524.

Morin, L., Hill, R.L. and Matayoshi, S. (1997). Hawaii's successful biological control strategy for mist flower (*Ageratina riparia*) – can it be transferred to New Zealand? *BiocontrolNews and Information, 18,* 77-88.

Morin, L., Evans, K.J. and Sheppard, A.W. (2006). Selection of pathogen agents in weed biological control: critical issues and peculiarities in relation to arthropod agents. *Australian Journal of Entomology, 45*, 349-365.

Morin, L., Reid, A.M., Sims-Chilton, N.M., Buckley, Y.M., Dhileepan, K., Hastwell, G.T., Nordblom, T.L. and Raghu, S. (2009). Review of approaches to evaluate the effectiveness of weed biological control agents. *Biological Control, 51*, 1-15.

Morris, M.J. (1989). Host specificity studies of a leaf spot fungus, *Phaeoramularia* sp., for the biological control of crofton weed (*Ageratina adenophora*) in South Africa. *Phytophylactica*, *21,* 281-283.

Morris, M.J. (1991). The use of plant pathogens for biological weed control in South Africa. *Agriculture, Ecosystems and Environment, 37,* 239-255.

Moseley, C.T., Cramer, M.D., Kleinjan, C.A. and Hoffmann, J.H. (2009). Why does *Dasineura dielsi*-induced galling of *Acacia Cyclops* not impede vegetative growth? *Journal of Applied Ecology, 46*, 214-222.

Mucina, L., Rutherford, M.C. and Powrie, L.W. (2006a). Vegetation atlas of South Africa, Lesotho and Swaziland. In Mucina, L. and Rutherford, M.C. (Eds.), *The vegetation of South Africa, Lesotho and Swaziland.* South African National Biodiversity Institute: Pretoria. pp 748-790.

Mucina, L., Hoare., D.B., Lotter, M.C., du Preez, P.J., Rutherford, M.C., Scott-Shaw, C.R., Bredenkamp, G.J., Powrie, L.W., Scott, L., Camp, K.G.T, Cilliers, S.S., Bezuidenhout, H., Mostert, T.H., Siebert, S.J., Winter, P.J.D., Burrows, J.E. Dobson, L., Ward, R.A., Stalmans, M., Oliver, E.G.H., Siebert, F., Schmidt, E., Kobisi, K. and Kose, L. (2006b). Grassland Biome. In Mucina, L. and Rutherford,M.C. (Eds.), *The vegetation of South Africa, Lesotho and Swaziland.* South African National Biodiversity Institute: Pretoria. pp 348-436.

Mucina, L. and Geldenhuys, C.J. (2006). Afrotemperate, Subtropical and Azonal Forests. In Mucina, L. and Rutherford,M.C. (Eds.), *The vegetation of South Africa, Lesotho and Swaziland.* South African National Biodiversity Institute: Pretoria. pp 584-614.

Müller-Schärer, H. and Schaffner, U (2008). Classical biological control: exploiting enemy escape to manage plant invasions. *Biological Invasions, 10,* 859-874.

Muniappan, R. and McFadyen, R. (2005). Gall-inducing arthropods in the biological control of weeds. In Raman, A., Schaefer, C.W. and Withers, T.M. (Eds.), *Biology, Ecology, and Evolution of Gall-Inducing Arthropods.* Science Publishers Inc.: New Hampshire. Volume 1, pp 709-730.

Muniappan, R., Raman, A. and Reddy, G.V.P. (2009). *Ageratina adenophora* (Sprengel) King and Robinson (Asteraceae). In Muniappan,

R., Reddy, G.V.P. and Raman, A. (Eds.), *Biological control of tropical weeds using arthropods.* Cambridge University Press: Cambridge. pp 63-73.

Myers, J.H. (1985). How many insect species are necessary for successful biocontrol of weeds? In Delfosse, E.S. (Ed.), *Proceedings of the VI International Symposium on Biological Control of Weeds, August 19-25, 1984.* Agriculture Canada: Ottawa. pp 77-82.

Myers, J.H. and Risley, C. (2000). Why reduced seed production is not necessarily translated into successful biological weed control. In Spencer, N.R. (Ed.), *Proceedings of the X International Symposium on Biological Control of Weeds, July 4-14, 1999*. Montana State University: Montana. pp. 569-581.

Myers, J.H. (2008). One agent is usually sufficient for successful biological control of weeds. In Julien, M.H., Sforza, R., Bon, M.C., Hatcher, P.E., Hinz, H.L. and Rector, B.G. (Eds.), *Proceedings of the XII International Symposium on Biological Control of Weeds*. CAB International: Wallingford, UK. pp. 601-606.

Nabity, P.D., Zavala, J.A., and DeLucia, E.H. (2009). Indirect suppression of photosynthesis on individual leaves by arthropod herbivory. *Annals of Botany, 103,* 655-663.

Nel, J.L., Richardson, D.M., Rouget, M., Mgidi, T.N., Mdzeke, N., Le Maitre, D.C., van Wilgen, B.W., Schonegevel, L., Henderson, L. and Neser, S. (2004). A proposed classification of invasive alien plant species in South Africa: Towards prioritising species and areas for management action. *South African Journal of Science, 100*, 53-64.

Newete, S.W., Oberprieler, R.G., and Byrne, M.J. (2011). The host range of the Eucalyptus Weevil, *Gonipterus "scutellatus"* Gylenhal (Coleoptera: Curculionide) in South Africa. *Annals of Forest Science, 68*, 1005-1013.

Niu, H., Liu, W., Wan, F. and Liu, B. (2007). An invasive aster (*Ageratina adenophora*) invades and dominates forest understories in China: altered

soil microbial communities facilitate the invader and inhibit natives. *Plant Soil, 294*, 73-85.

Olckers, T. (2004). Targeting emerging weeds for biological control in South Africa: the benefits of halting the spread of alien plants at an early stage of their invasion. *South African Journal of Science, 100*, 64-68.

Page, A.R. and Lacey, K.L. (2006). *Economic impact assessment of Australian weed biological control.* CRC for Australian weed management Technical Series #10, pp39.

Pearson, D.E. and Callaway, R.M. (2003). Indirect effects of host-specific biological control agents. *TREE, 18*, 456-461.

Pemberton, R.W. (2000). Predictable risk to native plants in weed biological control. *Oecologia, 125,* 489-494.

Peng, C., Chung, K. and Leu, W. (1998). Notes on three newly naturalised plants (Asteraceae) in Taiwan. *Taiwania, 43*, 320-329.

Plant Protection News. (1987a). Crofton Weed. Plant Protection News, 7. The Plant Protection Research Institute: Pretoria.

Plant Protection News. (1987b). Fungus against Port Jackson “Willow”. Plant Protection News, 7. The Plant Protection Research Institute: Pretoria.

Plant Protection News. (1988.) Fungus released against crofton weed. Plant Protection News, 11. The Plant Protection Research Institute Pretoria.

Pyšěk, P. and Richardson, D.M. (2007). Traits associated with invasiveness in alien plants: Where do we stand? In Nentwig, W. (Ed.), *Biological Invasions*. Springer-Verlag: Berlin. pp 97-125.

Raghu, S. and Dhileepan, K. (2005). The value of simulating herbivory in selecting effective weed biological control agents. *Biological Control, 34,* 265-273.

Raghu, S., Wilson, J.R. and Dhileepan, K. (2006). Refining the process of agent selection through understanding plant demography and plant response to herbivory. *Australian Journal of Entomology, 45,* 308-316.

Rambuda, T.D. and Johnson, S.D. (2004). Breeding systems of invasive alien plants in South Africa: Does Baker's rule apply? *Diversity and Distributions, 10*, 409-416.

Rayamajhi, M.B., Pratt, P.D., Center, T.D. and Van, T.K. (2010). Insects and a pathogen suppress *Melaleuca quinquenervia* cut-stump regrowth in Florida. *Biological Control, 53*, 1-8.

Rejmánek, M. and Richardson, D.M. (1996). What attributes make some plant species more invasive? *Ecology, 77,* 1655-1661.

Resco, V., Ignace, D.D., Sun, W., Huxman, T.E., Weltzin, J.F. and Williams, D.G. (2008). Chlorophyll fluorescence, predawn water potential and photosynthesis in precipitation pulse-driven ecosystems – implications for ecological studies. *Functional Ecology*, 22, 479-483.

Rieder, J.P., Evans, E.W. and Durham, S.L. (2001). Distribution of insect attacks in biological control of weeds: infestation of *Centaurea virgata* flowerheads by a gall fly. *Biological Control, 20*, 254-260.

Ripley, B.S., Muller, E., Behenna, M., Whittington-Jones, G.M. and Hill, M.P. (2006). Biomass and photosynthetic productivity of water hyacinth (*Eichhornia crassipes*) as affected by nutrient supply and mirid (*Eccritotarus catarinensis*) biocontrol. *Biological Control*, *39*, 392-400.

Rosskopf, E.N., Charudattan, R. and Kadir, J.B. (1999). Use of plant pathogens in weed control. In Bellows, T.S. and Fisher, T.W. (Eds.), *Handbook of biological control.* Academic Press: London. pp 891-916.

Rutherford, M.C. Mucina, L., Lötter, M.C., Bredenkamp, G.J., Smit, J.H.L., Scott-Shaw, C.R., Hoare, D.B., Goodman, P.S., Bezuidenhout, H., Scott, L., Ellis, F., Powrie, L.W., Siebert, F., Mostert, T.H., Henning, B.J., Venter, C.E., Camp, K.G.T., Siebert, S.J., Matthews, W.S., Burrows, J.E., Dobson, L., van Rooyen, N., Schmidt, E., Winter, P.J.D., du Preez, P.J.,

Ward, R.A., Williamson, S. and Hurter, P.J.H. (2006). Savanna biome. In Mucina, L. and Rutherford,M.C. (Eds.), *The vegetation of South Africa, Lesotho and Swaziland.* South African National Biodiversity Institute: Pretoria. pp 438-539.

Santiago, L.S. and Wright, S.J. (2007). Leaf functional traits of tropical forest plants in relation to growth form. *Functional Ecology*, *21*, 19-27.

Simelane, D.O. (2006). Effect of herbivory by *Teleonemia scrupulosa* on the performance of *Longitarsus bethae* on their shared host, *Lantana camara*. *Biological Control, 39*, 385-391.

StatSoft, Inc. (2007). STATISTICA (data analysis software system), version 8.0. www.statsoft.com

Stiling, P. and Cornelissen, T. (2005). What makes a successful biocontrol agent? A meta-analysis of biological control agent performance. *Biological Control, 34*, 236-246.

Thomas, M.B. and Willis, A.J. (1998). Biocontrol – risky but necessary? *TREE, 13*, 325-329.

Trounce, B. (2003). Crofton weed. *Agfact P7.6.36,* 2nd ed. New South Wales Agriculture.

Trujillo, E.E. (2005). History and success of plant pathogens for biological control of introduced weeds in Hawaii. *Biological Control, 33*, 113-122.

Turnbull, L.A., Crawley, M.J. and Rees, M. (2000). Are plant populations seed-limited? A review of seed sowing experiments. *Oikos, 88*, 225-238.

Turner, P.J., Morin, L., Williams, D.G. and Kriticos, D.J. (2010). Interactions between a leafhopper and rust fungus on the invasive plant Asparagus asparagoides in Australia: A case study of two agents being better than one for biological control. *Biological Control, 54*, 322-330.

Urban, A.J., Simelane, D.O., Retief, E., Heystek, F., Williams, H.E. and Madire, L.G. (2011). The invasive '*Lantana camara* L.' hybrid complex

(Verbenaceae): a review of research into its identity and biological control in South Africa. *African Entomology, 19*, 315-348.

van Klinken, R.D., Kriticos, D., Wilson, J. and Hoffmann, J. (2004). Agents that reduce seed production – essential ingredient or fools folly? In Cullen, J.M., Briese, D.T., Kriticos, D.J., Lonsdale, W.M., Morin, L. and Scott, J.K. (Eds.), *Proceedings of the XI International Symposium on Biological Control of Weeds*. CSIRO Entomology: Australia. pp. 621-622.

van Klinken, R.D. and Raghu, S. (2006). A scientific approach to agent selection. *Australian Journal of Entomology, 45,* 253-258.

van Wilgen, B.W., de Wit, M.P., Anderson, H.J., Le Maitre, D.C., Kotze, I.M., Ndala, S., Brown, B. and Rapholo, M.B. (2004). Costs and benefits of biological control of invasive alien plants: Case studies from South Africa. *South African Journal of Science, 100,* 19-20.

van Wilgen, B.W. and De Lange, W.J. (2011). The costs and benefits of biological control of invasive alien plants in South Africa. *African Entomology, 19*, 504-514.

Wang, F., Summerell, B.A., Marshall, D. and Auld, B.A. (1997). Biology and pathology of a species of *Phaeoramularia* causing a leaf spot of crofton weed. *Australian Plant Pathology, 26*, 165-172.

Wang, R., Wang, J., Qiu, Z., Meng, B., Wan, F. and Wang, Y. (2011). Multiple mechanisms underlie rapid expansion of an invasive alien plant. *New Phytologist, 191,* 828-839.

Witkowski, E.T.F. (1991). Effects of invasive alien Acacias on nutrient cycling in the coastal lowlands of the Cape Fynbos. *Journal of Applied Ecology, 28,* 1-15.

Witkowski, E.T.F., Lamont, B.B., Walton, C.S. and Radford, S. (1992). Leaf demography, sclerophyll and ecophysiology of two Banksias with contrasting leaf life spans. *Australian Journal of Botany, 40*, 849-862.

Witkowski, E.T.F. and Wilson, M. (2001). Changes in density, biomass, seed production and soil seed banks of the non-native invasive plant,

Chromolaena odorata, along a 15 year chronosequence. *Plant Ecology, 152*, 13-27.

Wittenberg, R. and Cock, M.J.W. (2001). *Invasive alien species: A toolkit of best prevention and management practices.* CABI Publishing: Oxon.

Woods, D.M., Pitcairn, M.J., Joley, D.B. and Turner, C.E. (2008). Seasonal phenology and impact of *Urophora sirunaseva* on yellow starthistle seed production in California. *Biological Control, 47,* 172-179.

Zachariades, C., Strathie, L.W., Retief, E. and Dube, N. (2011). Progress towards the biological control of *Chromolaena odorata* (l.) R.M. King & H. Rob.(Asteraceae) in South Africa. *African Entomology, 19*, 282-302.

Zhang, F., Liu, W.X., Wan, F.H. and Ellison, C.A. (2008). Sustainable management based on biological control and ecological restoration of an alien invasive weed, *Ageratina adenophora* (Asteraceae) in China. In Julien, M.H., Sforza, R., Bon, M.C., Hatcher, P.E., Hinz, H.L. and Rector, B.G. (Eds.), *Proceedings of the XII International Symposium on Biological Control of Weeds*. CABI International: Wallingford, UK. pp. 699-703.

Zimmermann, H.G. and Neser, S. (1999). Trends and prospects for biological control of weeds in South Africa. *African Entomology Memoir No.1*, 165-173.

Zimmermann, H.G., Moran, V.C. and Hoffmann, J.H. (2004). Biological control in the management of invasive alien plants in South Africa, and the role of the Working for Water programme. *South African Journal of Science, 100*, 34-40.

Zucchini, W. and Nenadić, O. (2006). A web-based rainfall atlas for Southern Africa. *Environmetrics, 17*, 269-283.