UNDERSTANDING THE FITNESS, PREFERENCE AND PERFORMANCE OF SPECIALIST HERBIVORES OF THE SOUTHERN AFRICAN BIOTYPE OF *CHROMOLAENA ODORATA* (ASTERACEAE), AND IMPACTS ON PHYTOCHEMISTRY AND GROWTH RATE OF THE PLANT

By

NONTEMBEKO DUBE

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College of Agriculture, Engineering and Science
University of KwaZulu-Natal
Pietermaritzburg
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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Entomology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg campus, South Africa, under the supervision of Dr Caswell Munyai, Dr Costas Zachariades, Dr Osariyekemwen Uyi and the guidance of Prof Fanie van Heerden. The research was financially supported by the Natural Resource Management Programmes of the Department of Environmental Affairs, and Plant Health and Protection of the Agricultural Research Council.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: N. Dube (Candidate)
Date: 08 August 2019

Signed: C. Munyai (Supervisor)
Date: 08August 8, 2019

Signed: C. Zachariades (Co-supervisor)
Date: 08 August 2019

Signed: O. Uyi (Co-supervisor)
Date: 08 August 2019

DECLARATION 1: PLAGIARISM

I, Nontembeko Dube, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;
b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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DECLARATION 2: PUBLICATIONS

For the six experimental chapters (Chapters 3-8) in this thesis, Chapters 3 and 4 are published and Chapters 5-8 are in preparation for submission for publication to an appropriate scientific journal. My three or sometimes 4 co-authors have been included as my supervisors or because of their significant scientific input. My role in each paper and presentation is indicated. The * indicates corresponding author.

Chapter 3

Dube N*, Zachariades C, Uyi OO, Munyai TC. 2017**.** Laboratory studies on the biology and host range of *Dichrorampha odorata* (Lepidoptera: Tortricidae), a biological control agent for *Chromolaena odorata* (Asteraceae). *Biocontrol Science and Technology* 27: 222-236.

Dube N, Zachariades C, Munyai T, Uyi O. 2017. Biology and host range of *Dichrorampha odorata* (Lepidoptera: Tortricidae), a biocontrol agent of *Chromolaena odorata* (Asteraceae). Poster Presentation. University of KwaZulu Natal Research Day, 26 October 2017, Westville, Durban, South Africa.

Chapter 4

Dube N*, Uyi O, Zachariades C, Munyai TC, Whitwell M. 2019. Impact of the shoot-boring moth *Dichrorampha odorata* (Lepidoptera: Tortricidae) on growth and reproductive potential of *Chromolaena odorata* (Asteraceae) in the laboratory. *Biocontrol Science and Technology* 29: 350-364.

Chapter 5

Dube N**,** Zachariades C. 2016. Host suitability testing of a gall forming fly *Polymorphomyia basilica* (Diptera: Tephritidae), a potential biological control agent of Siam weed *Chromolaena odorata* (Asteraceae) in South Africa. 20th Australasian Weeds Conference 11-15 September 2016-Perth, Western Australia.

Chapter 6-8

Dube N, Zachariades C, Uyi OO, van Heerden F, Munyai TC. 2017. Comparison of secondary compounds as influenced by *Pareuchaetes insulata* (Lepidoptera: Arctiidae; Erebidae) on *Chromolaena odorata*. 44th Annual Workshop on Biological Control of Weeds Research and Implementation, 30 October -01 November 2017, Grahamstown, South Africa.

Dube N, Zachariades C, Uyi OO, van Heerden F, Munyai TC. 2018. Impact of *Pareuchaetes insulata* on *Chromolaena odorata* respective to EICA hypothesis. 45th Annual Workshop on Biological Control of Weeds Research and Implementation, 29-31 October 2018, Salt Rock, Dolphin Coast, South Africa.

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ABSTRACT

The invasiveness and negative impacts of the alien shrub, *Chromolaena odorata* (L.) (Asteraceae) in South Africa resulted in the initiation of a biological control programme against the weed in the late 1980s. After the release of seven biocontrol agents, only two have successfully established to date *viz*. a leaf mining fly, *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae) and a moth with defoliating larvae, *Pareuchaetes insulata* Walker (Lepidoptera: Erebidae: Arctiinae). Surveys conducted suggested that *C. odorata* densities seem to be low in KwaZulu-Natal (KZN) province where *P. insulata* is present whilst its infestation is increasing in other places such as Limpopo province where the moth is absent. This study aimed to examine the life history traits, preference and performance of two biocontrol agents, *viz*. *Dichrorampha odorata* (Lepidoptera: Tortricidae) and *Polymorphomyia basilica* (Diptera: Tephritidae). A further objective of the study was to measure the effects of one of the established agents, *P. insulata*, on the competitive ability and defence mechanism of *C. odorata* by indirectly testing the predictions of the Evolution of Increased Competitive Ability (EICA).

Studies of life history traits of *D. odorata* in the laboratory indicated that the moth possesses good biological control attributes such as short-lived adults with high mating success, fecundity and egg hatchability. Of the 34 asteraceous plants subjected to larval no-choice tests, only *C. odorata* could sustain complete development of *D. odorata* to adulthood, although there was slight initial boring on 14 test species (plus *C. odorata*). Adult no-choice tests using seven test-plant species that were damaged in larval no-choice tests were consistent with the earlier trials, with larval damage, pupae and adults of *D. odorata* recorded from only *C. odorata*. This demonstrated that only *C. odorata* is a suitable host for *D. odorata* in South Africa and permission for the release of this first shoot-tip attacking agent was granted for biocontrol of *C. odorata* in South Africa.

To predict the efficacy of *D. odorata* as a biological control of *C. odorata*, a 9-month laboratory study was carried out. Plant growth metrics were compared across three treatments i.e. 0, 50 and 100% where newly hatched *D. odorata* larvae were inoculated

onto the shoot tips of *C. odorata*. At all treatment levels, the basal stem diameter of *C. odorata* was not affected by *D. odorata* larval feeding whilst the height of the main shoot and flower production of *C. odorata* were reduced at 50 and 100% relative to the control treatment. In general, the impacts of *D. odorata* on the weed were relatively small even though statistically significant, suggesting that the moth will modestly reduce the height and flower production of *C. odorata*.

Positive biological characteristics of *P. basilica* include a high rate of increase, long-lived and mobile adults, the ability of females to produce viable offspring without repeated mating, the ability of adults to eclose from galls on dry stems and the production of several generations per year. Thirty-two asteraceous plants were investigated to determine host specificity of *P. basilica* in single-choice adult tests and using single pairs of adults in no-choice tests, under laboratory conditions. Oviposition and larval development through to adulthood occurred on three other South American and on two South African species; one in the same tribe Eupatorieae, closely related to *C. odorata* and another one in the Astereae, less closely related to the weed, but both at a lower and slower rate. Females tended to retain their eggs under no-choice conditions in the presence of an unsuitable host, and to compensate by ovipositing at a higher rate when presented later with a *C. odorata* plant. Overall, this study predicts the ability of *P. basilica* to stretch to areas where *P. insulata* has failed to establish and supports the suitability of *P. basilica* for release in South Africa.

To determine the mechanism behind the decrease of *C. odorata* densities in KZN province, where the specialist herbivore *P. insulata* is present, compared to Limpopo province, where the weed is increasing and the moth is absent, the Evolution of Increased Competitive Ability (EICA) hypothesis was indirectly tested on plant defence and growth rate metrics. Inconsistent with EICA, total phenolics and tannins were generally higher in Thohoyandou (Limpopo province) (without *P. insulata*) and Komatipoort (Mpumalanga province) (with *P. insulata*) and lower in Pietermaritzburg (KZN province) (without *P. insulata*) and Umkomaas (KZN province) (with *P. insulata*). Flavonoids varied between the four locations, with higher concentrations in Komatipoort compared to Thohoyandou and Umkomaas, but not different to Pietermaritzburg. Growth parameters such as stem

diameter, number of shoots and number of flowering shoots from the garden experiment, supported the prediction of EICA, as plants from the Thohoyandou and Pietermaritzburg sites, where *P. insulata* is absent, showed stronger growth and reproductive potential. This study demonstrates the possible role of *P. insulata* in the decrease in population of *C. odorata* where the moth has persisted and suggests that other biotic and abiotic factors could be responsible for the unpredicted results for phytochemistry assays.

The second part of the EICA hypothesis posits that "specialist herbivores will demonstrate improved performance on plant individuals originating from an area where plants have been introduced". Consistent with EICA, *Pareuchaetes insulata* immature stages (newly hatched larvae-adult eclosion) that fed on leaves from Umkomaas, had prolonged development compared to larvae that were fed on leaves from Thohoyandou and Pietermaritzburg, and Komatipoort. Larvae and pupae that fed on the leaves from shade from Komatipoort had developmental trends intermediate between larvae feeding on the leaves from the shade from Thohoyandou and Umkomaas. Overall survival was lowest on leaves of plants obtained from Komatipoort. Contrarily, location did not appear to influence pupal mass but this variable was higher in plants in the full sun. In sum, the existing reassociation time may not be enough for evolutionary changes to have occurred in *C. odorata* defence and *P. insulata* response to plant evolution, and could explain the inconsistency in some *P. insulata* performance parameters on infested and uninfested populations of *C. odorata*.

The roles of pyrrolizidine alkaloids (PAs) as plant defences and in *P. insulata* mating behaviour are well known. The PAs in the roots of the southern African biotype (SAB) of *C. odorata* were therefore examined. Two PAs, rinderine and its stereoisomer *N*-oxide intermidine, were isolated from the roots of the SAB of *C. odorata* using GC-MS. The structures and configuration were confirmed by chemical and spectroscopic methods, especially one- ^{1}H dimensional NMR analysis. Therefore, confirmation of rinderine and intermidine in *C. odorata* in this study substantiate the establishment and spread of *P. insulata* in southern Africa due to, among other factors, reduced predation through defence by sequestered PAs.

This study demonstrated positive biological characteristics and high preference and performance of both the moth with shoot-tip boring larvae *D. odorata* and the stem-galling fly *P. basilica* on *C. odorata* compared to non-target plants, which highlights positive prospects for the biological control programme of *C. odorata* in South Africa. This study reports for the first time two pyrrolizidine alkaloids *viz.* intermidine and rinderine in southern African *C. odorata*. Aspects of EICA were not straightforward; however, this study showed the contribution of *P. insulata* to the reduction of *C. odorata* where the moth is present and further provides direction for future research for the biological control of *C. odorata* in South Africa.

Key words: *Chromolaena odorata*, biological control, *P. insulata*, EICA, pyrrolizidine alkaloids

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CHAPTER 1: GENERAL INTRODUCTION AND OVERVIEW

1.1 Study background motivation

Chromolaena odorata (L.) King and Robinson (Asteraceae) or Isandanezwe (in IsiZulu), is a shrub which is native to the Neotropics, from the southern Unites States of America to northern Argentina (Holm et al. 1977). It has become a serious pest in the humid tropics and subtropics of Asia, Africa and Oceania (Gautier 1992; Kriticos et al. 2005), and is regarded as one of the world's worst weeds (Holm et al. 1977; Zheng et al. 2018). Biological control research on *C. odorata* was initiated in 1988 in South Africa, and thereafter several biological control agents were imported for pre-release evaluation (Zachariades et al. 1999, 2011). Host-suitability testing is a key step in the process of restoring natural enemies in the country of introduction for biological control. It provides basic information regarding the safety of the biological control agent in question (Heard 2000) and answers to several ecological questions (Barone 1998). Pre-release research is also crucial to understand the efficacy of the biological control agent under assessment, to avoid releasing ineffective agents and to calculate the potential contribution of an agent to biological control (McClay and Balciunas 2005), while an understanding of the biology of an agent assists in estimating establishment chances and in selecting appropriate life stages for efficient release.

Similar to numerous other invasive alien plants in a new environment, *C. odorata* tends to form denser populations and to be more vigorous, larger, and to produce more seeds in its adventive range than in its native distribution (Zachariades et al. 2009). The release of alien species from natural enemies in their non-native ranges may lead to an increase in growth and reproduction not only due to a release from its natural enemies (the Enemy Release Hypothesis) (Keane and Crawley 2002) but also due to decreased allocation to defence and a simultaneous increase in allocation to growth, thus allowing increased competitive ability (the Evolution of Increased Competitive Ability (EICA) hypothesis proposed by Blossey and Notzöld (1995) and supported by Qin et al. (2013)). Anecdotal reports suggest that *C. odorata* abundance varies in South Africa, with the south coast of KwaZulu-Natal (KZN) province showing much lower infestation levels compared to 15 years ago. This was prior

to the establishment of a moth with defoliating larvae, *Pareuchaetes insulata* Walker (Lepidoptera: Erebidae: Arctiinae), first released in 2001 only in KZN (Zachariades et al. 2011; Zachariades et al. 2016). In contrast, surveys conducted in Limpopo province indicated increasing infestation by *C. odorata*. Although several insect agents, including *Pareuchaetes pseudoinsulata* Rego Barros (Lepidoptera: Erebidae: Arctiinae) and *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae), were released in Limpopo province, none of these *C. odorata* biological control agents have been recorded as established, thus motivating research on additional agents against *C. odorata*. *Pareuchaetes insulata* was recently recorded in Mpumalanga and eSwatini (formerly Swaziland) but the duration of its presence here is not known (probably short, having spread north from KZN). The decrease in infestation levels of *C. odorata* on the KZN south coast seemed to be greater than could simply be explained by direct herbivory by *P. insulata* (and the second established biocontrol agent, *C. eupatorivora*). Variation of *C. odorata* abundance could be explained through response of the plant (i.e. growth rate decrease) to herbivory by *P. insulata* and its response to induced secondary compounds of *C. odorata*, where the herbivore is expected to perform better on plants from which herbivory was excluded (Uesugi and Kessler 2013).

Therefore, host specificity of two insect herbivores *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae) and *Polymophormyia basilica* Snow (Diptera: Tephritidae) from the native range of the southern African biotype of *C. odorata* and additional mechanisms for the observed decline in *C. odorata* were investigated.

1.2 Aims

The broad aims of this study are (i) to determine the fitness, preference and performance of *D. odorata* and *P. basilica* on *C. odorata* compared to other host plants, for the biological control of *C. odorata*. Furthermore, (ii) to determine the impact of the established moth *P. insulata* on *C. odorata* growth and defence mechanisms.

1.3 Objectives

The specific objectives of this thesis are:

1. To determine the suitability of *D. odorata* for biological control of *C. odorata* in South Africa by examining life-history traits and fitness of the moth in the laboratory, anticipating that *D. odorata* will have a high survival rate only on *C. odorata* compared to that on other closely related plant species.
2. To determine the efficacy of *D. odorata* as a biological control agent of *C. odorata* by examining the weed's overall growth in response to different levels of infestation of *D. odorata* in the laboratory, anticipating that *D. odorata* will reduce the competitiveness of *C. odorata* in South Africa.
3. To determine the safety of *P. basilica* for release against *C. odorata* in South Africa by examining different life history traits and the selectivity of the fly on a number of plants in the Asteraceae family, anticipating high fecundity and offspring survival only on *C. odorata*.
4. To determine factors contributing to the difference in infestation by *C. odorata* in KZN (sites at Umkomaas and Pietermaritzburg), Limpopo (site at Thohoyandou) and Mpumalanga (site at Komatipoort) provinces by examining secondary compounds and growth rate of plants collected from these provinces and thereafter grown under uniform conditions, anticipating high levels of secondary compounds (flavonoids, total phenolics and tannins) in plants collected from the site with established *P. insulata* in KZN, and enhanced growth in plants collected from Limpopo.
5. To determine the longer-term impact of *P. insulata* on biological control of *C. odorata*, inferred by examining its performance on plants collected from KZN in comparison to plants collected from Limpopo province, anticipating better performance on plants collected from Limpopo and Pietermaritzburg.
6. To determine levels of pyrrolizidine alkaloids in the southern African biotype of *C. odorata* in locations with and without *P. insulata*.

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CHAPTER 2: LITERATURE REVIEW

2.0 Introduction

2.1 Invasive alien plants

Pollution is among the top global issues for which humans are the driving force through a variety of activities, changing the environment on local and global scales that lead to species invasions and extinctions (Müller-Schärer et al. 2004; Hooper et al. 2005; Miller et al. 2010). Invasive alien species, including plants, are among the major pollutants, and contribute substantially to the weakening of ecological reliability through reduced biodiversity, disturbance of native plant communities, increased soil erosion, and degradation of wildlife habitation (Muller and Martens 2005; te Beest 2010). Invasive alien plants (IAPs) contribute not only to biodiversity loss but also to economic loss, considering the expensive measures implemented in controlling them, including chemical, mechanical and biological control. Invasive alien plants also cause major economic losses through the damage they cause in terms of losses to agriculture (cropping and pastoral), silviculture, water loss, fisheries loss, transportation problems, ecotourism, and so forth (van Wilgen and Wilson 2018). Consequently, invasive alien weeds of agriculture have cost the economy of the USA, Australia, Brazil, the UK, India and South Africa US$ 37 billion per year for all countries combined (Pimentel et al. 2000; Pimentel e al. 2001; Briese et al. 2004).

Of the 2033 alien species (including animals and plants) found in South Africa today, some were deliberately introduced many years ago, either with the goal of establishing populations in nature, or for horticulture, agriculture, forestry or the pet trade, from where some escaped to become invasive (van Wilgen and Wilson 2018). The rest were introduced accidentally as commodity pollutants or as escapers on transport vectors (van Wilgen and Wilson 2018). Currently there are more than 700 invasive alien plants that are subject to legislation in South Africa, including trees and shrubs, grasses and reeds, climbers, terrestrial herbs and aquatics (Henderson and Wilson 2017). Van Wilgen and Wilson

(2018) highlight the impact of invasive alien plants in South Africa on surface runoff and groundwater, rangeland productivity and biodiversity intactness.

In an attempt to explain why alien plants become invasive, several hypotheses have been derived and debated (Jeschke 2014). These include the Enemy Release hypothesis (ERH) which posits that invasive alien plants benefit from the direct release from natural enemies (Keane and Crawley 2002). The ERH emphasizes that on introduction to an exotic region, a plant species should experience a decrease in top-down regulation by herbivores and other natural enemies, resulting in an increase in growth rates and/or reproductive output and consequently in distribution and abundance (Muller and Martens 2005). The ERH was extended into the evolutionary sphere by the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Notzöld 1995; Keane and Crawley 2002). The EICA hypothesis proposes that the absence of specialist herbivores for non-indigenous plants in the introduction range can lead to decreased allocation to defence and a simultaneous increase in allocation to growth, and consequently to increased competitive ability (Blossey and Notzöld 1995). According to EICA, plants in the invasive range should grow more vigorously and/or have higher reproductive output, and have lower levels of defensive metabolites, than plants of the same species in their native range. Studies that investigated the mechanism of EICA showed that evolutionary shifts in nitrogen allocation from cell walls (defence) to photosynthesis in invasive alien plant populations, resulted in faster growth and reduced structural and chemical defences (Feng et al. 2009, 2011). Joshi and Vrieling (2005) elaborated on EICA by proposing that the absence of specialist herbivores in invasive populations of a plant species could result in the evolution of lower protection against specialist herbivores through decreased production of quantitative chemical defences (expensive to produce), and reallocation of these resources to increased growth and reproduction, while retaining or increasing qualitative chemical defences (cheaper to produce) against generalist herbivores.

These hypotheses therefore contain the fundamental principles of biological control, which seeks to restore natural enemies (such as mites, pathogens and insects) of invasive alien

plants in the invasive range to achieve control. Biological control has been practiced for more than a century in South Africa (Mack 1995; Moran et al. 2013).

2.2 Biological control

Insect herbivores are well known for their significant role in prompting responses in their host plants in terms of architecture, growth and reproductive capacity (Miller et al. 2010). Herbivore attacks may delay seed ripening, lessen seed production and individual mass, lessen the growth rate of roots and shoots, lower the resistance of plants to diseases, and lessen the competitive ability of plants in comparison to their un-attacked neighbours (Crawley 1989a). It is no surprise that classical biological control (the introduction of a natural enemy of exotic origin to control a pest, usually also exotic, with the purpose of perpetual control of the pest) of invasive alien plants relies largely on the use of host-specific (usually monophagous or oligophagous) insect herbivores, together with some mite species and fungal pathogens (host specificity determined through a series of tests). These natural enemies suppress and restrict the densities, seed production and dispersal of invasive alien plants (Isaacson et al. 1996; Kenis et al. 2017). From hereon I will only discuss biological control of IAPs using insects.

2.3 Plant defences and insect herbivory

A number of selection pressures affect the feeding modes of phytophagous insects, including diversity of plant species within a community, the intra- and interspecific interactions among distinct plants, the likelihood of plant resource in space and time, the nutritional levels of plant tissues of different growth forms, and the diversity of mechanical and chemical defences contained in the plant tissues (Cates 1980). Of these, the chemical defensive mechanisms produced by plants, along with the apparency or availability and predictability of the food resources to herbivores, arise as vital for any analysis of plant-herbivore relationships (Cates 1980). Plant chemical defence against phytophagous insects is usually in the form of secondary metabolites which can be mostly assembled into qualitative and quantitative defences (Cates 1980). Qualitative defences are plant secondary compounds that occur in low concentrations, not expensive to produce and lethal to many polyphagous (generalist) insects but attract monophagous or oligophagous (specialist) insects which have

co-evolved with their host plants and can cope with and even use them e.g. alkaloids (including pyrrolizidine alkaloids, PAs) and glucolinates (van Dam et al. 1995; Müller-Schärer et al. 2004). Specialist herbivores (mono- and oligophagous insects) are often able to sequester qualitative defences and use them as host-finding cues, and oviposition and feeding stimulants (Hartmann et al. 1997; Klitzke and Trigo 2000) whilst some polyphagous insects such as *Zonocerus* grasshoppers overcome and sequester PAs for their defence (Boppré and Ficher 1994). Quantitative defences are mostly plant chemical compounds that function as digestibility reducers and are effective against mono-, oligo- and polyphagous insects, e.g. tannins and resins (Cates 1980; Müller-Schärer et al. 2004). The effectiveness of these defences is proportional to their concentration in the plant's tissues, and therefore they are often expensive to produce in adequate quantities. Monophagous herbivores are defined as those feeding on one or more plant species within a genus. Oligophagous herbivores are restricted to feeding on two or more genera in a family or closely related families, and polyphagous herbivores are defined as feeding on species from two or more plant families. In sum, it can be suggested that mono- or oligophagous insects are herbivores with restricted diets that will prefer the nutritious and highly toxic plant tissues, while polyphagous insects are herbivores with diets composed of several unrelated plant taxa and will often prefer the less nutritious plant tissues, that are low in toxin concentration (Cates 1980). The degree of specificity of insects governs the outcomes of host-specificity testing which is key in ensuring the safety of weed biocontrol (McFadyen 1998).

2.4 Pre-release studies (life history traits, host-specificity testing and laboratory impact trials)

2.4.1 Life history traits

Knowledge of life-history traits, genetics, and behaviours, among other biological factors, of both the agent and target plant species, all contribute to better predictions of the ecological host range and efficacy of the biological control candidate (Schaffner 2001) and could assist in making a decision about which life stage(s) will be most appropriate for host-specificity tests and even for releases (personal observations). Insects that have multiple generations a year, or attack multiple plant parts, and/or those plant parts that are most important for the growth and spread of the target plant, are often chosen above other specialist insects in order to improve effectiveness (McClay and Balciunas 2005).

2.4.2 Principles of host-specificity testing

Host-specificity testing is one of the fundamental pre-release studies in classical weed biological control, used as a measure to assess and predict the likelihood and consequences of non-target effects. Host-range testing seeks to prove if a candidate biological control agent is sufficiently host specific, typically through a series of tests under quarantine conditions in the country of introduction. Open-field host range studies, carried out within the native range of the plant (or where a biocontrol agent has previously established) are also useful in predicting the likelihood of non-target effects, since they reveal the host selection of herbivores displaying the whole array of pre- and post-alightment behaviours (Shaffner et al. 2018). Oftentimes, quarantine/laboratory host-range tests manifest false positives, which occur when an insect feeds on a plant in that it would not otherwise attack in the field (Marohasy 1998). Nevertheless, with proper application and interpretation of the results of trials to determine an insect's fundamental host range, quarantine/laboratory tests can identify which candidates may be appropriate host-specific biological control agents.

Host-specificity testing of classical agents (agents to be used in classical weed biological control) is often a multi-year, research-intensive process. The process varies depending on the target species investigated and is often initiated during the search for classical biological control agents in the region of origin of the invasive alien plant (Schaffner et al. 2018). During surveys in the native region, records on surrounding vegetation, supplemented by host collection, can show if insects found feeding on the plant are highly host specific and worth further consideration or instead are generalists that should be excluded from further investigation (Mason et al. 2017).

The best candidate agents are then exposed to laboratory-based host-range tests, using the target plant and non-target plants from a thorough plant list (Mason et al. 2017; Sheppard et al. 2005). This list includes plant species in the recommended area of introduction that are closely related phylogenetically to the target weed (Wapshere 1974; Briese 2005) or

are of special economic or cultural importance (Sheppard et al. 2005; Mason et al. 2017). The centrifugal phylogenetic approach posits that test plants more related to the weed in question are more likely to be attacked than more distantly related test plants. This is because, through their close phylogenetic relationship, they share traits important for the host selection and acceptance behavior of specialized phytophagous insects (Hinz et al. 2019). Conservative 'no-choice' tests, where the insect has only the option of feeding on the test-plant species provided or starving, define the insect's fundamental host range (also termed its physiological host range) or broadest range of plant acceptance. Choice tests, on the other hand, offer the candidate insect a choice between potential host plants in a shared pot and/or cage. This can either be a single choice between two plant species or a multiple choice between more than two species, often with a number of replicates of each species. Choice tests are closer towards testing the realized/ecological host range, although this can only be truly known in the field (i.e. one may still get some false-positive results in laboratory-based choice tests). No-choice tests help determine the range of hosts biologically accepted by the insect, whereas choice tests determine which of these hosts are preferred, and thereby are at greatest risk for damage (van Klinken 2000; Sheppard et al. 2005).

In some cases, field trials may be set up in the region of the insect's origin. The goal of these trials is to mimic natural processes as much as possible, to obtain a clearer understanding of host specificity (Mason et al. 2017). Such trials are usually used in combination with laboratory trials. Once all the above tests have been concluded and the risk of the agent forming preference and performance is deemed acceptably low, a host-specific agent is permitted, by the country's competent authority, to be released (Sheppard et al. 2005).

2.4.3 Laboratory impact trials

Although our knowledge and prediction of the impact of natural enemies against the target weed is key to the success of any biocontrol programme, it remains a less developed part of the science of biological control (Shea and Possingham 2000; Wratten and Gurr 2000). This is so because, globally, for every biological control agent introduced, host-specificity

clearance is mandatory whilst assessment of potential impact caused by candidate agents prior to release remains optional. Impact studies are important in the prioritisation and selection process in biological control programmes, to limit the introduction of inefficient biocontrol agents, as this carries both costs and risks (McClay and Balciunas 2005). Impact trials also assist in understanding agent performance and the reasons for success and failure of agents (Conrad and Dhileepan 2007). Nevertheless, it should be noted that laboratory-based trials may underestimate the impact of a natural enemy because they run for a relatively short period, on plants which are generally not stressed (Dube et al. 2019); and also because natural enemies may act synergistically with one another (the total effect may be greater than the sum of their individual effects) (e.g. Hoffmann and Moran 1998).

A number of biological control programmes have undertaken assessment of impact of a candidate biocontrol agent on plant architecture and biomass prior to release (e.g. Briese 1996; Conrad and Dhileepan 2007; Fay and Throop 2005; Frye and Hough-Goldstein 2013; Goolsby et al. 2004; Kloppel et al. 2003; Weed and Cassagrande 2011). The order Lepidoptera are among the successful biological control agents, following Hymenoptera, Diptera, Hemiptera and Coleoptera (Crawley 1989b; Winston et al. 2014). Among these insect groups, gall formers are widely known for their limited host range and injurious effects on the growth and fitness of their host plants, and thus have contributed substantially to success in biological control programmes globally (Harris and Shorthouse 1996; Goolsby et al. 2000; Diaz et al. 2014; Mukwevho et al. 2017). For example, a bud-galling wasp, *Trichilogaster acaciaelongifoliae* Froggatt (Hymenoptera: Pteromalidae) significantly reduced the reproductive potential of *Acacia longifolia* (Andrews) Willd. (Fabaceae) in South Africa (Dennill and Donnelly 1991). Also a univoltine shoot-galling weevil, *Rhinusa pilosa* Gyllenhal (Coleoptera: Curculionidae) investigated as a potential biological control agent in North America, was found to be host specific to *Linaria vulgaris* Mill (Plantaginaceae), native to Europe, and significantly reduced plant height, dry below-ground biomass, dry above-ground biomass and number of shoots produced (Gassmann et al. 2014). Within the Diptera, the fruit fly family, Tephritidae, is the second largest group of gall formers following Cecidomyiidae (Freidberg 1984). Most tephritids form galls on plants of the family Asteraceae (e.g. Dodson and George 1986; Fernandes et al. 1996;

Balciunas and Mehelis 2010; Buccellato et al. 2012), on roots, leaves or flower heads, and most widespread and commonly on stems (Freidberg 1984; Headrick and Goeden 1998).

2.5 *Chromolaena odorata*

Of the invasive alien shrubs present and under biological control in South Africa, *Chromolaena odorata* (L.) King and Robinson (Asteraceae) is well known as one of the world's worst weeds (Holm et al. 1977). *Chromolaena odorata* has a wide native distribution, ranging from the southern United States of America to northern Argentina, and including Central America and the Caribbean islands (Gautier 1992; Kriticos et al. 2005; Paterson and Zachariades 2013). This distribution is mirrored by the wide introduction range, with the plant being invasive in Central, West and southern Africa, India, Southeast Asia, southern China and parts of Oceania (Kriticos et al. 2005; Zachariades et al. 2009). Invasive populations have also recently been recorded in East Africa (Zachariades et al. 2013; Shackleton et al. 2017) and *C. odorata* is also one of the most common invasive plants in western Angola (Rejmanek et al. 2017). The high morphological and genetic variability of *C. odorata* in its native distribution partly illuminates the presence of two invasive biotypes of *C. odorata* known in its invasive range of distribution *viz* the dominant Asian/West African biotype (AWAB), possibly originating from Trinidad and Tobago (Yu et al. 2014), and the southern African biotype (SAB), originating from Jamaica or Cuba (Paterson and Zachariades 2013; Shao et al. 2018). Both AWAB and SAB *C. odorata* are invasive in Africa.

2.5.1 *East and Central Africa*

Recent studies (Rejmanek et al. 2017; Shackleton et al. 2017) are revealing rapidly increasing records of AWAB *C. odorata* in East and Central Africa. *Chromolaena odorata* was initially recorded in the mid-1970s in the central parts of the Democratic Republic of Congo (Gautier 1992; Hoevers and M'boob 1996). Its presence was first recorded in Kenya in 2006, in the eastern part of Rwanda in 2003, and in Tanzania between 2009 and 2010 near the eastern shores of Lake Victoria, in the western parts of the country (Zachariades et al. 2013). *Chromolaena odorata* is also present in the eastern parts of Tanzania, Rwanda,

Uganda, Kenya, Burundi, Uganda, Cameroon and Chad (Zebeyou 1991; Hoevers and M'boob 1996; Timbilla 1998; Zachariades et al. 2013; Rejmanek et al. 2017).

2.5.2 *Southern Africa and West Africa*

The earliest records of *C. odorata* in Africa were in South Africa and Nigeria. The SAB *C. odorata* was first recorded as naturalised at a location east of Ndwedwe (29° 30° S, 30°56° E) near Durban (Uyi, 2014) in KwaZulu-Natal province (KZN), South Africa in 1947, and was said to have been imported earlier in that decade (Zachariades et al. 1999). However, Zachariades et al. (2004) argued that its abundance throughout KZN at that time suggested that *C. odorata* might have been introduced earlier than was assumed. In addition, the plant was recorded growing in the Cape Town Botanical Garden in the mid-1800s, indicating that it was introduced into South Africa at least a century before it was recorded as being naturalised, although no link has been found between the two occurrences thus far (Zachariades et al. 2004). Because of its copious seed production and high growth rate, within South Africa the weed spread rapidly along the KZN coastal belt and now occurs from the Transkei region of the Eastern Cape to as far north as Kosi Bay in northern KZN, Mpumalanga and in Limpopo province (Zachariades et al. 2011) (Fig. 2.1). From the 1980s on, it was now considered to be one of the worst invasive alien plants in the subtropical eastern parts of southern Africa, including eSwatini (formerly Swaziland) and southern Mozambique (Liggitt 1983; Kluge 1990; Kluge and Caldwell 1993a; Zachariades et al. 1999).

A specimen of AWAB *C. odorata* was collected in Zimbabwe in the late 1960s (Gautier 1992), but has not been found there since (Sheppard et al. 2012). There are unverified reports of *C. odorata* from north-western Mozambique and southern Malawi (biotype unknown) (Zachariades et al. 2013). The AWAB has also been recorded from Mauritius (Zachariades et al. 2009) and Madagascar (Kull et al. 2012). The AWAB *C. odorata* was first recorded in West Africa in south-eastern Nigeria in 1942 (Ivens 1974) and rapidly spread across the neighbouring countries, including Ghana, southern Benin Republic and Togo around the 1970s and 1980s, the southern parts of Côte d'Ivoire; to the Gambia, Liberia, Burkina Faso, Guinea and Sierra Leone (Timbilla and Braimah 1996; Yehouenou

1996; Hoevers and M'boob 1996; Timbilla et al. 2003). Invasion success of *C. odorata* is partly attributed to release from natural enemies, proven chemical properties with allelopathic effects and genotypes with stronger competitive abilities i.e. more invasive than other plant species (Thoden et al. 2007; Qin et al. 2013; Yu et al. 2014) and this thesis focuses on the SAB *C. odorata*.

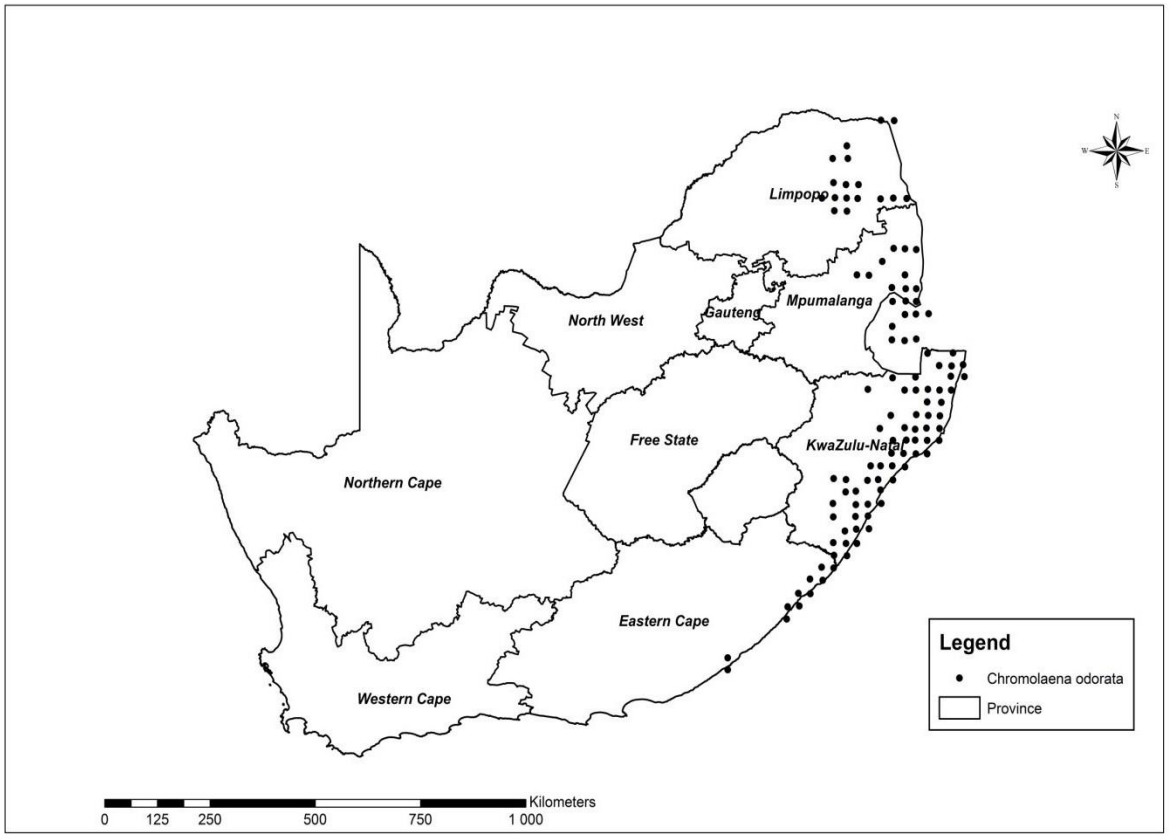

Figure 2.1: Distribution of *Chromolaena odorata* in South Africa and eSwatini. (Originally drawn by L. Henderson; data source: SAPIA database, ARC-Plant Protection Research Institute, Pretoria; modified by Uyi 2014).

2.5.3 Morphology and phytochemistry of *C. odorata* in South Africa

The SAB *C. odorata* is characterised by leaves that are small and smooth relative to AWAB, with a dark-green colour when growing in semi-shade, but yellow-green in the

sun and red when young. The stems are largely smooth and yellow-green in colour, with flowers that are narrower than those of AWAB, with a whitish colour (AWAB has pale lilac flowers); SAB floral bracts have round tips and are arranged tightly around the flower-head, compared to the AWAB of *C. odorata* which has pointed bracts which are more lax (Fig. 2.2). It has more rigid, upright branches, and may be more cold-tolerant and more susceptible to fire (Zachariades et al. 2009).

Figure 2.2: Difference between the flowers of SAB (on the left) and AWAB (on the right) of *C. odorata* (source C. Zachariades, ARC-PHP, South Africa, unpubl. data). Photographs of AWAB courtesy of C. Wilson.

There is ample knowledge on phytochemistry of the AWAB *C. odorata* (Biller et al. 1994; Thoden et al. 2007; Ngozi et al. 2009; Qin et al. 2013) whilst the SAB *C. odorata* has received little attention. Recent studies (Omokhua et al. 2017; Omokhua 2018) demonstrated that SAB *C. odorata* possesses good fungicidal properties for antifungal activity against *Candida albicans* (Robin) Berkhout (Saccharomycetaceae) and fungistatic activity. The outcomes of an additional analysis of the antimicrobial activities of the different growth stages of *C. odorata* revealed that all growth stages have some level of

activity against the tested bacterial and fungal strains, although young and mature non-flowering plants presented enhanced activities (Omokhua et al. 2017; Omokhua 2018). Phytochemical analysis of leaf extracts of *C. odorata* showed the presence of saponins, total phenolics, flavonoids and condensed tannins, with mature non-flowering plants containing higher amounts of phenolics, flavonoids and tannins compared to the young and flowering plants (Omokhua et al. 2017), and these compounds play a significant role in plant defence against insect herbivory (Robins et al. 1987; Clausen et al. 1992; Close and McArthur 2002; Treutter 2005; Barbehenn and Constabel 2011).

2.5.4 Negative impacts of *C. odorata* in South Africa

Chromolaena odorata has contributed tremendously to a reduction in biodiversity and carrying capacity of native ecosystems (Kluge 1990; Luwum 2002; te Beest 2010). For example, Leslie and Spotila (2000) showed that in KZN province, Lake St. Lucia's nesting Nile crocodiles *Crocodylus niloticus* Laurenti (Reptilia: Crocodilidae) require open sunny, sandy areas in which to deposit their eggs. However, *C. odorata* plants overrunning the nesting sites interfered with the egg laying potential of *C. niloticus* leading to a female-biased sex ratio and crocodiles later abandoned nesting at these sites. In Hluhluwe-Imfolozi Park alone, *C. odorata* has negatively impacted diversity and abundance of spider communities (Mgobhozi et al. 2008) and mammals (Dumalisile and Somers 2017); it has adversely impacted utilisation of forage species, fuelled canopy fires (te Beest et al. 2012), has led to a reshuffling of the population of the black rhinoceros, *Diceros bicornis* L. (Perissodactyla: Rhinocerotidae), and is partly responsible for the population decline of this species (Howison 2009).

2.5.5 Control measures against *C. odorata* in South Africa

Different control measures have been used against *C. odorata* (Goodall and Erasmus 1996; Luwum 2002; Klein 2002). Several foliar- and stump-treatment herbicides (Goodall and Erasmus 1996) were tested for the control of *C. odorata* in South Africa and for some, application in summer resulted in 90% weed reduction. However, registration of herbicides for specific weeds is compulsory in South Africa, and many of these herbicides were not registered for chromolaena. In addition, some of these herbicides were restricted

internationally and were therefore not considered for use (Goodall and Erasmus 1996); others were not sufficiently effective or damaged plantation trees and crops. Herbicides including tebuthiuron, glyphosate and triclopyr are registered for use against *C. odorata* and are effective in recommended concentrations (van Zyl 2012). Although chemical control of *C. odorata* is effective, the rapid growth rate and the spread of the plant made it difficult to control chemically in the long term and over the large areas of often low-value or inaccessible land that the weed invades (Goodall and Erasmus 1996; Zachariades et al. 1999).

Mechanical control that involves manual slashing with brush cutters, mattocks, hoes or tractor-drawn implements was also applied to control *C. odorata* (Goodall and Erasmus 1996). However, slashing causes regeneration and therefore needs to be followed by chemical control to be effective, manual weeding is labour intensive, and the use of tractor-drawn equipment is limited to accessible areas (Goodall and Erasmus 1996). Mechanical control methods may also lead to soil disturbance and erosion, require repeated follow-up operations and may damage untargeted species that are mistakenly cleared in dense infestations of the weed (Luwum 2002). Use of fire in grassland and savanna is an effective tool and strongly associated with *C odorata* reductions only when combined with cut-stump treatments (Goodall 2000; Dew et al. 2017). However, this kind of clearing programme is labour intensive and expensive. For example, over a decade Hluhluwe-Imfolozi Park has spent R103 million in a successful clearing programme, but for a relatively small area of 35,000 ha. Furthermore, *C. odorata* adult plants may re-sprout even after intense fires (Dew et al. 2017; te Beest et al. 2017), and if budget for keeping the infestation at maintenance levels (5%) is lost, the weed will return. These factors additionally substantiate the need for biological control.

Biological control is the only viable method of control when large areas are invaded and repetitious chemical or mechanical control becomes prohibitively expensive (Seibert 1989; Mack 1995), which is the case for *C. odorata*. *Chromolaena odorata* was considered a good target for biocontrol in South Africa because there were plenty of potential agents available, it was morphologically homogenous throughout its southern African invasive

range, no conflict of interest existed and it had susceptible stages in its biology (Kluge 1990).

None of the methods described above is applicable, on its own, to all areas of *C. odorata* infestation and at all times: a combination of methods ('integrated control') into an integrated management plan is usually necessary (Zachariades et al. 2011).

2.6 Biological control of *C. odorata*, with emphasis on South Africa

The global biological control programme against *C. odorata* was started in the 1960s, with a survey of natural enemies in Trinidad, and host-range testing of the most promising of these (Zachariades et al. 2009). This resulted in the release of *Pareuchaetes pseudoinsulata* Rego Barros (Lepidoptera: Erebidae) and *Apion brunneonigrum* Beguin-Billecoq (Coleoptera: Apionidae), but only *P. pseudoinsulata* established. The stem-galling fly *Cecidochares connexa* Macquart (Diptera: Tephritidae) was released in the 1990s and established widely. This fly and *P. pseudoinsulata* have resulted in a high level of control of the AWAB *C. odorata* in many parts of its invasive range.

A biological control programme has been in progress in South Africa since 1988 for long-term suppression of *C. odorata*, and several insect candidate agents have been assessed for host specificity (Zachariades et al. 1999; Zachariades et al. 2011). *Chromolaena odorata* is in the asteraceous tribe Eupatorieae and there are few closely related indigenous plants or crop plants to consider for host specificity tests. There are only three indigenous genera containing five species within the Eupatorieae in South Africa (Retief 2002). These include two *Mikania* and two *Adenostemma* species, *Stomatanthes africanus* (Oliv. & Hiern) R.M. King & H. Rob. which together with *C. odorata*, was previously in the genus *Eupatorium*. Funk et al. (2009) extensively revised the relationship between tribes of the Asteraceae based on molecular studies. The Eupatorieae and 11-12 closely related tribes such as Heliantheae were grouped into the 'Heliantheae Alliance', largely confined to the Americas; therefore, there are no speciose tribes closely related to the Eupatorieae in Africa. Briese (2005) used the term 'degree of phylogenetic separation' in relation to weed biological control to give an indication of relatedness, and this concept has been used to

determine the relatedness of each test plant at tribe level in this thesis. According to the phylogenetic centrifugal model, the non-target species that are most closely related to the target plant are most likely to be attacked by the candidate agent, and thus have to be sampled most intensively as test plants (i.e. all species within the Eupatorieae must be tested); while for those with a higher degree of phylogenetic separation, a smaller proportion can be sampled (e.g. 10% of *Senecio* species). Although there are numerous Asteraceae plants in South Africa, most of them are in more distantly related tribes and can thus be sampled less intensively.

Although several biological control agents have been considered against *C. odorata* in South Africa (Klein 2011, updated 2016), only seven have been released and only two of those released have definitely established. The failure of some agents to establish was attributed to climatic incompatibility, given that much of the native range of *C. odorata* lies within the tropics and has higher rainfall (Robertson et al. 2008). Differences in the SAB *C. odorata* and the AWAB invading other parts of the world (Paterson and Zachariades 2013; Shao et al. 2018) were also believed to result in the failure to rear or establish biocontrol agents. The origin of SAB could not be found for many years after the biocontrol programme had been initiated, and therefore natural enemies were of necessity collected from other morphotypes/genotypes of *C. odorata*. For insects and pathogens with narrow host ranges, this resulted in incompatibility between the candidate agent and the host plant (e.g. *C. connexa*, collected from Colombia, and some of the pathogens, collected from South America, did not develop well on SAB (Zachariades et al., 1999, 2011). The two agents established against *C. odorata* are a leaf mining fly, *C. eupatorivora*, and a moth with defoliating larvae, *P. insulata* Walker (Lepidoptera: Erebidae: Arctiinae). The biology of *P. insulata* is detailed in Dube (2008) and Uyi et al. (2014).

2.6.1 Possible factors affecting the establishment and spread of *Pareuchaetes insulata*

Three populations of *P. insulata*, collected from Florida (USA), Cuba and Jamaica, were released at 30 sites in KZN province between 2001 and 2009 (Zachariades et al. 2011), with the initial (2001-2003) releases being of the Florida population. *Pareuchaetes pseudoinsulata* had failed to establish in South Africa, possibly due to poor climatic

matching (Zachariades et al. 2011). A population of *P. insulata* from southern Florida (Fort Lauderdale) was subsequently released in South Africa because of the closer climate match between the two regions (Kluge and Caldwell 1993b; Parasram 2003). However, after releases of about 781,000 insects at 17 sites around KZN over the initial two-year period (Zachariades et al. 2011), initial post-release monitoring indicated poor or no establishment.

Establishment of *P. insulata* was initially confirmed in 2004 at only one release site at which the Florida population had been released. This was a coastal site 50km south-west of Durban, close to the town of Umkomaas, in the Cannonbrae eucalyptus plantation. It was followed by an outbreak in 2006 and a subsequent rapid population decline and another outbreak in 2014 in northern KZN (Zachariades et al. 2016). This was not surprising because *P. pseudoinsulata* has shown a similar trend of being an outbreak species in many other countries where it has established (Zachariades et al. 2009). Between 2006 and 2013, *P. insulata* was discovered along a 100 km stretch of the coastline surrounding the original establishment site, and up to 15km inland from it, but generally at low population levels (Zachariades et al. 2016).

It is likely that the Floridian and Jamaican populations of *P. insulata* came into contact in the field. Molecular analysis and investigation of the cross-breeding of the Cuban, Floridian and Jamaican populations of *P. insulata* showed no mating barrier between them (Dube et al. 2014), so there was probably successful interbreeding in the field. A comparative performance study in the laboratory, using the established *P. insulata* population, on *C. odorata* from Florida and South Africa, showed that the insect's performance was not affected by the host plant genotype on which it fed (Uyi et al. 2014). However, *P. insulata* generally showed better performance on shaded foliage relative to *C. odorata* foliage growing in full-sun conditions (Uyi et al. 2015), and it performed better on autumn foliage compared to that collected from the plant in late winter (Uyi et al. 2018). Low temperatures in winter at the established release site reduced locomotion activities of *P. insulata*, putting it at risk of predation and starvation (Uyi et al. 2017; Uyi et al. 2018). Additionally, chemoecological studies have revealed that males of various lepidopterans,

including of *Pareuchaetes* species on *Chromolaena*, produce sex pheromones from pyrrolizidine alkaloids (PAs) sequestered from the plant (Schneider et al. 1992; Conner 2009). The function of such male pheromones in arctiine behaviour is known to be the induction of sexual acceptance by the female for protection of females and eggs (Boppré 1990: Schneider et al. 1992; Conner 2009).

The above studies indicate that the erratic performance of *P. insulata* on *C. odorata* in South Africa is caused by a number of factors such as low temperatures as well as spatio-temporal variations and phytochemical characteristics of the leaves of *C. odorata*. Nevertheless, in general the moth has had a significant impact on *C. odorata*: annual monitoring of the originally established release site at Umkomaas since 2001 has documented a continuing decline in the *C. odorata* population, several smaller outbreaks of *P. insulata*, and anecdotally the restoration of indigenous flora. There has also been tremendous spread from the release point to the province of Mpumalanga (550km) and to neighbouring countries including eSwatini (Zachariades et al. 2016) and Mozambique (personal observations). On the other hand, no *P. insulata* larvae or damage were found during monitoring of 10 sites conducted in the northerly Limpopo province in May 2016, indicating that the moth may not yet have reached the isolated populations of *C. odorata* there. Inland areas of KZN such as Pietermaritzburg are thought to be climatically unsuitable for the moth, and it has not been recorded there (Zachariades et al. 2016; personal observations), even though these areas are close to (80km) the originally established site. Although *C. eupatorivora* is widespread, the damage posed by the fly is generally insignificant (Nzama et al. 2014).

Due to the incomplete control of *C. odorata* by the two established agents, it was desirable to consider additional insect species as potential biological control agents in South Africa, *viz.* the stem-boring weevil *Lixus aemulus* Petri (Coleoptera: Curculionidae); a long-horn beetle *Recchia parvula* Lane (Coleoptera: Cerambycidae), a moth with shoot-boring larvae, *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae); and a stem-galling fly, *Polymorphomyia basilica* Snow (Diptera: Tephritidae). The pre-release assessment of damage conducted on *L. aemulus*, first released in 2011, showed that the

larvae caused high mortality of the stems, as well as reducing the dry mass of infested stems and the number of achenes produced on the infested branches (Kluge and Zachariades 2006). However, no signs of definite establishment thus far are reported (C. Zachariades pers. comm.). *Recchia parvula* did not establish at one of the sites, but there was considerable larval damage at the second site, with mature larvae entering root crowns, additionally the long horn beetle is likely to be restricted to areas that experience a relatively cold winter because larval diapause is broken by low temperatures (C. Zachariades ARC-PHP unpublished). The other two agents form part of the current thesis and are discussed below.

2.6.2 *Dichrorampha odorata*

Currently the tortricid tribe Grapholitini, into which the genus *Dichrorampha* falls, is comprised of more than 1698 species occurring worldwide (Brown et al. 2013; Rota and Brown 2009). In the main, members of the subfamily Tortricinae incline to be polyphagous whereas most of moths in subfamily of Olethreutinae, into which Grapholitini falls, have narrower host ranges (e.g. Brown et al. 2008). While grapholitines are commonly known to be pests of economic importance in forests, ornamentals and crops, the narrower host ranges demonstrated in some species supports their efficacious use as biological control agents (Roe et al. 2009). The genus *Dichrorampha* has a zoogeographical origin consisting of Europe, Africa north of the Sahara, and most of Asia north of the Himalayas, with 31 described species. This genus is characterised by a male forewing with a well-developed costal fold, dark dots along the termen of the forewing, and female genitalia with sterigma, seventh sternite, and sclerotised posterior portion of the ductus bursa fused (Brown and Zachariades 2007). The plant family Asteraceae is host to the majority (about 25) of these species (Brown and Zachariades 2007).

The yellow larvae of an undescribed shoot-boring tortricid moth were first collected from *C. odorata* in Jamaica in 1999. A larger number were collected in November 2005, and imported into quarantine in South Africa, where a culture was easily established. The species was later described as *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae) (Brown and Zachariades 2007). A supplementary culture was

collected and imported in November 2012. In Jamaica, within its native range, *C. odorata* is a minor weed, which is not surprising as a single branch on a plant can host up to five natural enemies, viz. *C. eupatorivora*, the shoot-boring moth, *Phestinia costella* Hampson (Lepidoptera: Pyralidae), the stem-galling fly *P. basilica*, the shoot-mining fly, *Melanagromyza eupatoriella* Spencer (Diptera: Agromyzidae) and the shoot-boring moth *D. odorata* feeding on the plant simultaneously (personal observations). Although research had been conducted previously on both *P. costella* and *M. eupatoriella* (see Zachariades et al. 2009, 2011), both proved very difficult to culture in the laboratory, as did *P. basilica* until 2012. The ease with which *D. odorata* was cultured in the laboratory, together with other considerations (the moth is widespread in Jamaica, and fairly commonly encountered (Robinson 2012); it inflicts a similar level of damage as the other shoot-tip borers), resulted in it being considered an acceptable candidate biocontrol agent.

The adult *D. odorata* has cream and pale tan scales with a brown dorsum and grey-brown forewing. It is a small species with a length of 5 mm. *Dichrorampha odorata* is recorded as having a Caribbean distribution, with holotype and paratypes from Jamaica (Brown and Zachariades 2007). It has also been collected on *C. odorata* in Cuba (Strathie and Zachariades 2004) and a very similar looking pupa was found on *C. odorata* on mainland America but was never reared out (ARC-PHP, unpublished).

2.6.3 *Polymorphomyia basilica*

Polymorphomyia basilica was considered as a replacement for *C. connexa*, which has been triumphant in controlling the AWA *C. odorata* biotype in parts South-East Asia (e.g. Day et al. 2013) and which has established in West Africa (Paterson and Akpabey 2014; Aigbedion-Atalor et al. 2018). However, a culture of *C. connexa* could not be sustained on the SAB *C. odorata* in the laboratory (Zachariades et al. 1999), probably because of the high level of host-specificity of the fly, which was originally collected on *C. odorata* from the Caribbean coast of Colombia. *Polymorphomyia basilica* was imported from Cuba and Jamaica into South African quarantine several times (see Zachariades et al. 2011), but initially the insect could not be cultured.

After being shelved for some years (Zachariades et al. 2011), *P. basilica* was again collected in Jamaica and imported into quarantine in South Africa in November 2012, in a further attempt to culture it. Rooted stems were placed into individual small pots in a large emergence box with glass top and handling sleeves, in a glasshouse of ARC-PHP's Cedara, KZN, South Africa quarantine facility. Out of galls that were rooted, adult flies were obtained. Upon eclosion, adults were placed onto SAB *C. odorata* plants in the quarantine laboratory at ARC-PHP, Cedara and an F1 generation was successfully reared. Thereafter the fly was easily cultured, and used in experiments.

2.7 Conclusions

The importance of pursuing the biological control of invasive alien plants in South Africa has been realized and records shows the significant impact of biological control agents released over the past century (Moran et al. 2013). Although frequent fires followed by stump treatment seem to be promising for the control of *C. odorata* in South Africa, this method remains insufficient on its own (Dew et al. 2017), whilst biocontrol, once the agent is established in the field, is sustainable i.e. permanent, and incurs few other costs (Kenis et al. 2017). Large-scale funding for chemical clearing in South Africa, as has been provided by the *Working for Water* programme since 1995, may end, and without biological control in place, *C. odorata* would rebound to previous levels in a few years. Therefore, this supports additional research on its biological control, which established agents such as *P. insulata* seem to have contributed to where it is present (Zachariades et al. 2016). This thesis aims to fill the following gaps: (i) Evaluation of new potential agents for locations not reached by, or restrictive for the establishment of, the moth where *C. odorata* is still problematic. (ii) Extensively quantify the impact of *P. insulata* in the field where it has established, using various aspects of already available data such as progress in phytochemical properties of SAB *C. odorata* (e.g. Omokhua et al., 2017). Finally, (iii) determine PAs in SAB *C. odorata*; PAs are well known for their contribution in the mating behaviour of *Pareuchaetes species* (Schneider et al. 1992; Conner 2009) but are only documented in the AWAB *C. odorata* (Biller et al. 1994).

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CHAPTER 3: LABORATORY STUDIES ON THE BIOLOGY AND HOST RANGE OF *DICHRORAMPHA ODORATA* (LEPIDOPTERA: TORTRICIDAE), A BIOLOGICAL CONTROL AGENT FOR *CHROMOLAENA ODORATA* (ASTERACEAE)

3.1 Abstract

Dichrorampha odorata (Lepidoptera: Tortricidae) is a moth from Jamaica whose larvae bore into, and kill, the shoot tips of the invasive alien plant, *Chromolaena odorata* (L.) King and Robinson (Asteraceae). This study reports aspects of the biology of *D. odorata*, and also determined the host specificity (larval and adult no-choice trials) of the moth. Adults were short lived (ranging from 2 to 7 days), with females laying a mean of 15.4 eggs. Eggs took 9 days to hatch, larvae 20–23 days to develop and the pupal stage lasted 11–12 days, giving an overall lifecycle period of 41–45 days. Larval no-choice tests using 34 asteraceous test species indicated that only *C. odorata* could sustain complete development of *D. odorata* to adulthood, although there was slight initial boring 14 test species (plus *C. odorata*). Results from the adult no-choice trials, in which seven test-plant species were exposed to *D. odorata*, were consistent with those from larval trials, with larval damage, pupae and adults of *D. odorata* recorded from only *C. odorata*. This confirmed that only *C. odorata* is a suitable host for *D. odorata* in South Africa. Permission has subsequently been granted for the release of *D. odorata* in South Africa, thus making it the first shoot-tip attacking agent to be released against *C. odorata*. It is hoped that in the field, high levels of damage by the moth will reduce the height and therefore competitiveness of *C. odorata*, thereby contributing to the success of biological control of this plant.

Key words: Invasive alien plant, weed biological control, shoot-tip borer, tortricidae, biology and lifecycle, host specificity

3.2 Introduction

Chromolaena odorata (L.) King and Robinson (Asteraceae) is a scrambling shrub native to the Neotropics, from the southern USA to northern Argentina, including the islands of the Caribbean (Holm et al. 1977) that has become a serious pest in the humid tropics and subtropics of Asia, Africa and Oceania (Gautier 1992; Kriticos et al. 2005). Africa is invaded by both the Asian West African biotype (AWAB) of *C. odorata* (sensu Robertson et al. 2008), which is also found in India, Southeast Asia, China and Oceania, and the southern African biotype (SAB). These two biotypes are morphologically and genetically disparate from one another but display high within-biotype homogeneity (Paterson and Zachariades 2013; Yu et al. 2014; Zachariades et al. 2009). The SAB originates from one of the Caribbean Islands, and particularly Jamaica or Cuba (Paterson and Zachariades 2013). *Chromolaena odorata* was first recorded as naturalised in South Africa in the late 1940s, when it was found near Ndwedwe, KwaZulu-Natal (KZN) (Zachariades et al. 2011). From KZN it spread rapidly into the Eastern Cape, Mpumalanga and Limpopo provinces, as well as into the neighbouring countries of Mozambique and Swaziland (Goodall and Erasmus 1996). It continues to spread through these areas and to increase in density where already present. The most recent estimate of the area invaded by *C. odorata* in South Africa is 1,444,336 ha or 101,179 'condensed' ha (Zachariades et al. 2011).

While a considerable level of biological control of the AWAB of *C. odorata* has been achieved in Southeast Asia and Oceania (Zachariades et al. 2009), the success of biological control in South Africa has been limited (Zachariades et al. 2011). Incompatibility between the SA biotype of *C. odorata* and many of the earlier candidate agents, collected from South and Central America types of *C. odorata* plants dissimilar to the SAB, is believed to be a factor contributing to this inadequate level of biocontrol. In order to avoid such incompatibility issues, in more recent years biological control agents from the Caribbean region, mainly Jamaica, have been targeted for release in South Africa. These include the leaf-mining fly *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae) and a moth with defoliating larvae, *Pareuchaetes insulata* (Walker) (Lepidoptera: Erebidae). Cultures

of this last species were collected from Cuba and Jamaica after the apparent failure of establishment of a population of the same species, collected in Florida, USA from *C. odorata* dissimilar to the SAB (ultimately the *P. insulata* population from Florida did establish in South Africa, with likely genetic input from Jamaican and possibly Cuban releases (Dube et al. 2014)). Neither *C. eupatorivora* (Nzama et al. 2014) nor *P. insulata* (Zachariades et al. 2011) have had a major impact on the weed in South Africa, particularly in seasonally drier inland areas where *C. odorata* is at the margins of its climatic tolerance (te Beest et al. 2013) (although it is now considered possible that the efficacy and distribution of *P. insulata* may have been underestimated). Consequently, *C. odorata* still poses a threat to native biodiversity (Howison 2009; Purdon 2011; Tantsi 2012; te Beest 2010). Therefore, further biological control agents were deemed necessary, in particular those that attack other plant parts rather than just the leaves.

In Jamaica, within its native range, *C. odorata* is a minor weed, which is not surprising as a single branch on a plant can host up to five species of natural enemies, viz. *C. eupatorivora*, the shoot-boring moth *Phestinia costella* Hampson (Lepidoptera: Pyralidae), the stem-galling fly *Polymorphomyia basilica* Snow (Diptera: Tephritidae), the shoot-mining fly *Melanagromyza eupatoriella* Spencer (Diptera: Agromyzidae) and the shoot-boring moth *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae) feeding on the plant simultaneously (personal observations). Although research had been conducted previously on both *P. costella* and *M. eupatoriella* (see Zachariades et al. 2009, 2011), both proved very difficult to culture in the laboratory, as did *P. basilica* until 2012. *D. odorata* was first collected on *C. odorata* in Jamaica in 1999 and easily cultured in South African quarantine in 2005 (Brown and Zachariades 2007). The moth is widespread in Jamaica, and fairly commonly encountered. It inflicts a similar level of damage as the other shoot-tip borers, and was thus considered an acceptable candidate biocontrol agent.

Currently the tribe Grapholitini, into which the genus *Dichrorampha* falls, is comprised of more than 1698 species occurring worldwide (Brown et al. 2013; Rota and Brown 2009). In the main, members of subfamily Tortricinae incline to be polyphagous whereas most of moths in the subfamily of Olethreutinae, into which Grapholitini falls, have narrower host

ranges (e.g. Brown et al. 2008). While grapholitines are commonly known to be pests of economic importance in forests, ornamentals and crops, the narrower host ranges demonstrated in some species supports their efficacious use as biological control agents (Roe et al. 2009). The genus *Dichrorampha* has a zoogeographical origin consisting of Europe, Africa north of the Sahara, and most of Asia north of the Himalayas, with 31 described species. This genus is characterised by a male forewing with a well-developed costal fold, dark dots along the termen of the forewing, and female genitalia with sterigma, seventh sternite, and sclerotised posterior portion of the ductus bursa fused (Brown and Zachariades 2007). The plant family Asteraceae is host to the majority (about 25) of these species (Brown and Zachariades 2007).

The adult *D. odorata* has cream and pale tan scales with a brown dorsum and greybrown forewing. It is a small species with a length of 5 mm. *D. odorata* is recorded as having a Caribbean distribution with holotype and paratypes from Jamaica (Brown and Zachariades 2007). It has also been collected on *C. odorata* in Cuba (Strathie and Zachariades 2004) and a very similar looking pupa was found on *C. odorata* on mainland America but was never reared out (ARC-PHP, unpubl.). This paper outlines detailed host-specificity tests and biology of *D. odorata* on *C. odorata* in South Africa.

3.3 Materials and methods

3.3.1 Culturing methods and quarantine conditions

Both culturing of and trials on *D. odorata* were carried out in the quarantine laboratory and glasshouse of the Agricultural Research Council, Plant Health and Protection (ARC-PHP), Cedara (29.54153° S, 30.26764° E) near Pietermaritzburg, KZN, South Africa. Laboratories and glasshouses were kept within a temperature range of 22–28°C, 40–70% relative humidity and either a 12-hour photoperiod using growth lights (adults) or natural lighting (larvae).

In order to culture *D. odorata*, between 15 and 25 newly eclosed adults were placed into a steel-framed cage of 0.9 × 0.5 × 0.5 m with gauze panels, over several days depending on the availability of adults. Four *C. odorata* plants, potted in 18 cm diameter pots in a medium

consisting of a 1:1 ratio of river sand and 'Gromor' potting medium (Gromor, Cato Ridge, South Africa), and selected for their large number of growing shoot tips, were placed into the cage prior to introduction of the moths. The plants were propagated from soft shoot cuttings taken from the field and rooted in a mistbed. No attempt was made to sex adults when culturing the insect. Cages were sprayed with water daily, but no food was provided for the moths. Plants were removed from the cage about 10 days after the last moth had been introduced, to allow for egg hatching and larval development. These plants were placed into a walk-in-cage (3.3 × 2.3 × 1.9 m) in the glasshouse. Larvae bored into the shoot tip and down the stem for 2–3 cm, forming a characteristic slight, discoloured swelling. The mature larva exited the stem tip and pupated on the leaf, after cutting a crescent shaped flap from the edge of the leaf, folding it over itself and tying it shut with silk. Leaves with pupae were removed from plants and placed into Petri dishes on a slightly dampened piece of filter paper, to allow for adult eclosion.

3.3.2 Biology of *D. odorata*

In the laboratory, aspects of the biology of *D. odorata* were recorded, including adult longevity, adult fecundity (numbers of eggs laid and hatched) and larval development. Pupae of *D. odorata* were placed in petri dishes for eclosion and adult pairs selected while mating or using size dichotomy, as female moths are usually bigger than males (e.g. Dube 2008). One pair was placed into a steel-framed, gauze-panelled cage (0.9 × 0.4 × 0.4 m) containing one *C. odorata* plant until both adults died. Cages were inspected daily and the presence of dead adults was recorded. The number of eggs laid was recorded on the ninth day of the trial. The number of eggs hatching was recorded from day 10 (as the culturing routine showed that eggs hatched about 10 days after they were laid). Out of 12 adult pairs that were exposed to plants, only 5 pairs with viable eggs were considered for the results.

Four potted *C. odorata* plants in each of four 0.9 × 0.5 × 0.5 m cages were exposed to 25 adult moths in the laboratory for 48 hours to allow for similar-aged larvae to be used in larval developmental trials. To maximise the number of larvae obtained, exposed plants were checked on the 12th day after the start of the exposure period for hatched larvae. Fifty newly hatched 1–2-day-old larvae dissected from shoot tips were inoculated onto 5 plants

at a rate of 10 larvae per plant; the head-capsule width (HCW) of 5 of these larvae was measured on the same day. On the third day following inoculation, five larvae, one collected from each of the five plants, were dissected from their shoot tips and put into the freezer for five minutes to slow their movement, after which the HCW of each larva was measured. These larvae were then placed into the culture, i.e. they were not measured again. Thereafter, HCWs were measured non-destructively twice a week (i.e. days 3, 7, 10, 14, 17, 21, 24, 28, etc.), using five larvae each time, until pupation occurred, to determine the number of larval instars and the larval development period. This was repeated twice. In the third set, 4 potted plants were exposed to 25 adults as above but HCW were measured non-destructively every day except on the weekends, using between 5 and 10 larvae, until pupation occurred. Larval development trials were carried out in January 2013 and were repeated in January 2015 and June 2015.

3.3.3 Host-specificity testing

3.3.3.1 Choice of test plants

The family Asteraceae is one of the largest angiosperm families worldwide, with an estimated 23,000–30,000 species (Funk et al. 2009). Only a few species of Asteraceae have economic value: these include sunflower (*Helianthus annuus* L.), lettuce (*Lactuca sativa* L.), chicory (*Cichorium intybus* L.), safflower (*Carthamus tinctorius* L.) and a few other minor crop, medicinal and ornamental species (Simpson 2009). The family is represented by about 4000 species in sub-Saharan Africa excluding Madagascar (African Plant Database (version 3.4.0)). According to Funk et al. (2009), the subfamily Asteroideae, into which *C. odorata* falls, is strongly supported as monophyletic and now contains 20 tribes. A grouping of 13 of these tribes, known as the ‘Heliantheae Alliance’ and which includes the Eupatorieae, into which Chromolaena falls, is largely confined to the Americas (Baldwin 2009; Funk et al. 2009). There are only a few Eupatorieae, and a relatively small number of species within the other tribes within the Heliantheae Alliance, that are indigenous to sub-Saharan Africa and the rest of the Old World. This makes the test-plant species list much shorter for members of the Heliantheae Alliance invading the Old World.

Test plants were selected according to the proposed centrifugal testing criteria of Wapshere (1974), bearing in mind advances in both the phylogeny of the Asteraceae (Funk et al. 2009) and in host-plant selection approaches (Briese 2005). The main taxonomic level at which species were ranked was Tribe. None of the five Eupatorieae indigenous to South Africa (Retief 2002) are in the same subtribe as *C. odorata*, although this is disputed for one of the indigenous species, *Stomatanthes africanus* (Oliv. & Hiern) R.M. King & H. Rob. (Anderberg et al. 2007), which was previously placed within the same genus (Eupatorium) as *C. odorata*. Several other alien species of Eupatorieae, all of American origin, are invasive in South Africa, and these were included in the host specificity tests in order to obtain a better idea of the host range of *D. odorata*, rather than because an attack on these species in South Africa would be considered in a negative light. The closely related Tribe Heliantheae sensu stricto contains the major crop species *H. annuus* (sunflower) and a number of indigenous species, and was therefore also tested fairly intensively. Other tribes of the Asteraceae were less intensively tested, because they are phylogenetically more distant to *C. odorata* (see section 3.4.2 for the list of test plants).

3.3.3.2 Larval no-choice trials

In the laboratory in South Africa, a preliminary host-specificity trial for *D. odorata*, using 10 test-plant species (1 replicate each) was conducted in 2006, to get an indication of host range (Brown and Zachariades 2007). Mid-instar larvae were used in these larval no-choice tests. The favourable results obtained from this trial prompted further host-range testing in 2009. During these trials, the host range of *D. odorata* was examined by comparing the larval feeding response to 34 species of Asteraceae, including 3 cultivars of 1 species as well as *C. odorata* (in section 3.4.2). This was achieved through the conservative larval no-choice tests, similar to those conducted by Cruttwell (1977) on *P. costella* (at that time identified as *Mescinia nr parvula* (Zeller)) from Trinidad. These tests were considered appropriate both because the larvae are easily dissected from stem tips into which they have bored, and because larvae are highly mobile, thus boring readily into the *C. odorata* shoot tip onto which they have been placed, and being unlikely to bore into plants that they find unsuitable as hosts.

Larval no-choice tests were conducted between September 2009 and December 2011 in the quarantine glasshouse, using five 1–2-day-old, first-instar larvae per replicate. Trials were conducted during the spring, summer and autumn seasons (viz. September to May) when plants were actively growing. Five larvae were placed on each of five vegetative, growing, terminal shoot tips of a single plant, each larva on a separate shoot tip, in a steel-framed cage with gauze panels, of dimensions 0.9 × 0.4 × 0.4 m. Shoot tips on which larvae were placed were marked using short lengths of wool. If the plant species to be tested had only one shoot tip (e.g. *H. annuus* L., Asteraceae), five plants of that species were regarded as one replicate.

On the fourth day after inoculation, all shoots on the plants were inspected for boring by the inoculated larvae, as the larvae are mobile and may have moved to shoots other than those onto which they were placed. The presence or absence of boring on each shoot was recorded. After nine days, all shoots on all plants were again inspected, and the level of damage for each shoot scored as follows: 0 = no damage, 1 = slight damage, 2 = some boring and 3 = considerable damage (i.e. equivalent to the typical level of damage on control plants). The trial was monitored until larvae on the control plants had pupated and eclosed. The larval no-choice trial period was divided into 21 'runs' each of which consisted of a control plant (*C. odorata*) plus several test plants, usually each a different species, onto which larvae were placed simultaneously. The selection and number of test plants for each of these runs was limited by the availability of actively growing shoot tips on the different test species, space in the quarantine as well as the availability of larvae.

3.3.3.4 Adult no-choice oviposition trials

These tests were conducted to demonstrate that the use of non-neonate larvae in larval no-choice trials gave a similar indication of host range as trials in which eggs were laid on plants. Seven test-plant species, selected because preliminary larval boring was recorded in larval no-choice trials, were used, together with a *C. odorata* plant as a control. Three replicates were conducted for each species. Five pairs of newly eclosed *D. odorata* adults were placed onto each plant, which was housed in a 0.9 × 0.4 × 0.4 m cage as described earlier. Plants were exposed to adults for 11 days (greater than or equal to the expected

adult moth lifespan) and the number of eggs laid and hatched was counted on the 11th day (eggs had started hatching at this time). The presence of larval boring on plants was recorded each day except weekends; thereafter the intensity of damage was recorded, as per the larval no-choice trials, 9 days after (on day 20 after exposure to adults) and pupation was observed from the 15th day (day 26 after exposure).

3.3.4 Statistical analysis

For larval no-choice trials, the control (*C. odorata*) was compared separately to each species of test plant using a Mann–Whitney Unpaired comparison, for the number of damaged shoot tips on day 4, the feeding score on day 9, and the number of pupae and adults obtained. For adult no-choice trials, data were transformed using $\sqrt{(x + 1)}$ and a one-way ANOVA was performed. Post hoc comparisons were performed using Tukey's HSD test. Statistica® was used to perform the analyses.

3.4 Results

3.4.1 Biology of *D. odorata*

In the laboratory, *D. odorata* completed its lifecycle (from egg laying to adult eclosion) in about 41–45 days (n = 10). The eggs are deposited singly onto the upper surface of the leaf, towards the centre of the leaf and generally between the central vein and one of the other two main veins. The scale-like egg is flattened laterally against the leaf, to which it is firmly attached, and is circular in appearance when viewed from above, with diameter of 0.60 ± 0.03 mm (mean ± SE, n = 20). It is initially transparent but becomes pale orange as the larva develops (Fig. 3.1(a, b)). Following an incubation period of 9 ± 0.00 days (mean ± SE) (n = 50), on the 10th ± 0.00 day (mean ± SE) (n = 50) the pale yellow larva hatches, moves to the nearest shoot tip and bores into it. Within four days a small black spot appears on the shoot tip as a result of damage and frass. The larva becomes a stronger yellow colour as it matures. From about the ninth day onwards the damage caused by the larva is extensive, leading to the death of the shoot tip.

In the laboratory, four to eight eggs were often laid on each suitable leaf. This resulted in a shortage of terminal shoot tips, with the result that larvae often migrated down to axillary

shoots on the same stem. However, this may be an artefact of high numbers of moths in a confined space, because in the field in Jamaica only terminal shoot tips were observed to be damaged; larvae in the laboratory were unable to complete development in short (<2 cm) axillary tips. In the laboratory, larvae may be present in almost every growing shoot tip on each plant and inflict substantial damage to the plant. The larva eventually bores 2–3 cm down the stem. The final instar larva vacates the shoot tip in which it developed, moves onto a nearby leaf, cuts and rolls a section of the leaf, seals the roll with silk and pupates inside. During the pre-pupal stage, the larva becomes dark yellow, shorter and fatter just before it emerges from the shoot tip.

Adults mate and females oviposit from the first day after eclosion. In the laboratory, *D. odorata* females laid 15.4 ± 6.14 eggs (mean ± SE, n = 5), with oviposition ranging from 5 to 39 per female, of which 96% (14.8 ± 6.18, mean ± SE, n = 5) hatched. Adults had a short lifespan of 4.8 ± 0.59 (mean ± SE, n = 10) days (ranging from 2 to 7 days), larvae pupated between 20 and 23 days (n = 10) after hatching and adults eclosed 11–14 days (n = 10) after pupation. Based on HCWs, *D. odorata* larvae usually developed through five instars, although six instars were recorded for a few larvae (n = 227) (Table 3.1). HCW ranges for each instar (Table 3.1) were delimited using a frequency plot (Fig. 3.2).

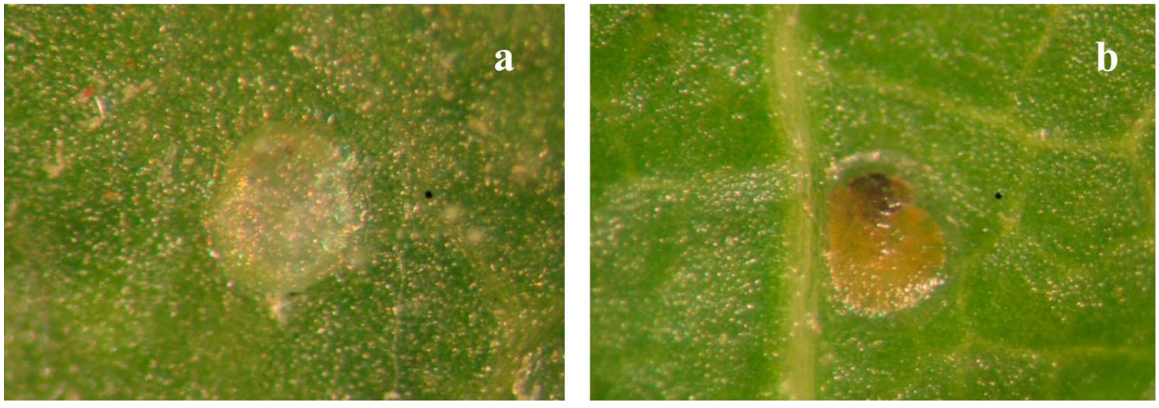

Figure 3.1: *D. odorata* (a) newly laid egg; (b) mature egg. Note the yellow body of the larva curled up inside the egg, together with its brown head-capsule. Photographs of other developmental stages are available in Brown and Zachariades (2007).

Table 3.1: Mean HCW indicating five to six larval instars of *D. odorata* as per Dyar's Law (Berg and Merritt, 2009).

Instar no.	N larvae	Mean ± SE per instar (mm)	Range (mm)	Ratio[a]
1	41	0.17 ± 0.001	0.16 ± 0.18	n/a[b]
2	35	0.24 ± 0.003	0.22 ± 0.28	1.42
3	60	0.34 ± 0.004	0.29 ± 0.41	1.38
4	64	0.47 ± 0.003	0.42 ± 0.54	1.41
5	26	0.62 ± 0.006	0.58 ± 0.70	1.31
6	1	0.78	n/a	1.26

[a]Mean head-capsule of current instar divided by that of previous instar.
[b]n/a-not available

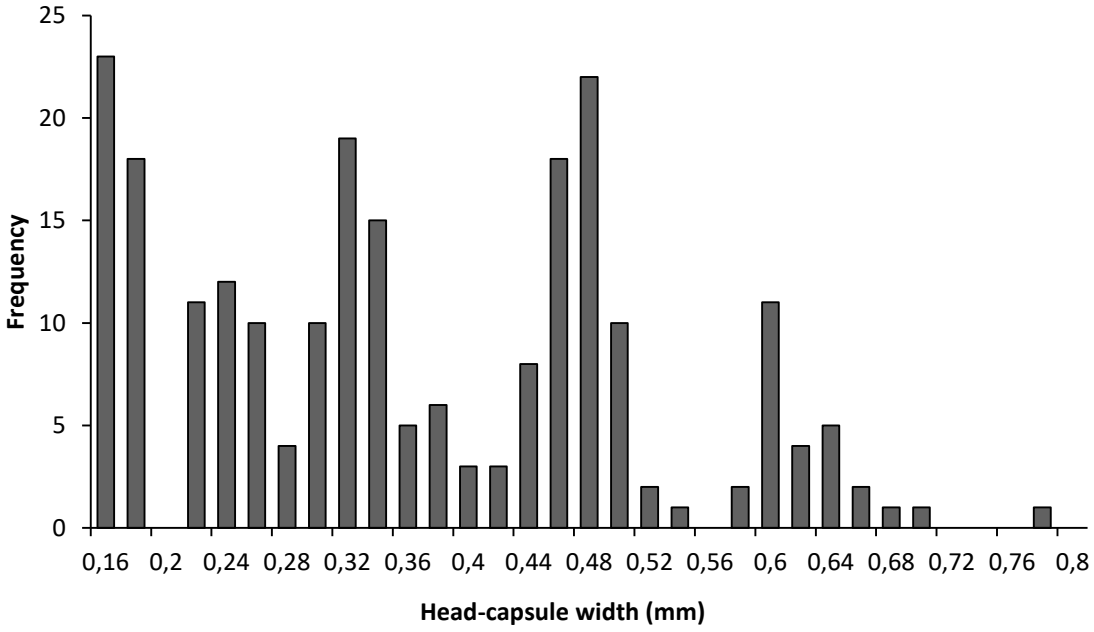

Figure 3.2: Frequency of larvae against HCW, measured at 50× magnification using an ocular micrometer. N = 227 larvae.

3.4.2 Host-specificity testing

3.4.2.1 Larval no-choice tests

Initial larval boring (4 days after inoculation) was recorded on 14 of the test species (+ chromolaena), distributed through the subfamilies and tribes of the Asteraceae that were tested (Table 3.2). However, inspection of plants on day 9 indicated that none except *C. odorata* and *S. africanus* supported any further larval development, and none of the test species which were undamaged on day 4 displayed any damage on day 9. For *S. africanus*, only one shoot tip out of a total of 15 (3 replicates) was scored as a 3, with all the others scored as 0. No further larval development was observed on any plants except *C. odorata*, where considerable damage was seen and larvae developed through to adulthood. Larval feeding damage on *C. odorata* was largely scored as a 3 (Table 3.2). On 2 of the 21 *C. odorata* control plants, no pupation occurred, but even in this case the damage caused by larvae was scored as a 3.

Table 3.2: Larval no-choice host-range tests for *Dichrorampha odorata*: test plant species and varieties, larval feeding response on the fourth and ninth day after inoculation, and resulting numbers of pupae and adults. Five larvae were inoculated onto five shoot tips for each replicate of each species.

SUBFAMILY Tribe[1]	Subtribe[1]	Degree of phylogenetic separation[2]	Plant species	No. replicates	Status[4]	No. damaged shoots: day 4 (SE)[6]	Feeding score: day 9 (SE)[5,6]	No. pupae (SE)[6]	No. adults (SE)[6]
ASTERIODEAE									
Eupatorieae	Praxelinae	0	*Chromolaena odorata*[3]	21	A,I	4.57 (0.15)a	2.54 (0.10)a	3.48 (0.31)a	3.33 (0.30)a
Eupatorieae	Oxylobinae	0	*Ageratina adenophora*[3]	3	A,I	2.33 (1.45)a	0.67 (0.57)b	0b	0b
Eupatorieae	Oxylobinae	0	*Ageratina riparia*[3]	3	A,I	1.0 (0.58)b	0.20 (0.12)b	0b	0b
Eupatorieae	Eupatoriinae	0	*Stomatanthes africanus*[3]	3	N	0.33 (0.33)b	0.20 (0.20)b	0b	0b
Eupatorieae	Adenostemmatinae	0	*Adenostemma caffrum*	3	N	0.33 (0.33)b	0.07 (0.07)b	0b	0b
Eupatorieae	Ageratinae	0	*Ageratum conyzoides*	3	A,I	3.33 (1.20)a	0.40 (0.20)b	0b	0b
Eupatorieae	Mikaniinae	0	*Mikania capensis* ex KZN	3	N	3.67 (0.67)a	0.40 (0.12)b	0b	0b
Eupatorieae	Gyptidinae	0	*Campuloclinium macrocephalum*[3]	3	A,I	n/a[7]	n/a[7]	n/a[7]	n/a [7]
Eupatorieae	Adenostemmatinae	0	*Adenostemma viscosum*	3	N	n/a	n/a	n/a	n/a
Heliantheae	Ecliptinae	3	*Blainvillea gayana*	3	N	0.67 (0.67)b	0.13 (0.13)b	0b	0b
Heliantheae	Helianthinae	3	*Helianthus annuus (AGSUN 8251)*	3	A,C	n/a	n/a	n/a	n/a
Heliantheae	Helianthinae	3	*Helianthus annuus (HYSUN 333)*	3	A,C	n/a	n/a	n/a	n/a
Heliantheae	Helianthinae	3	*Helianthus annuus (PAN 7094)*	3	A,C	n/a	n/a	n/a	n/a
Heliantheae	Helianthinae	3	*Helianthus tuberosus*	3	A,C	n/a	n/a	n/a	n/a
Heliantheae	Spilanthinae	3	*Spilanthes mauritiana*	3	N	n/a	n/a	n/a	n/a
Heliantheae	Ecliptinae	3	*Wedelia natalensis*	3	N	n/a	n/a	n/a	n/a
Tageteae	Pectidinae	5	*Tagetes erecta*	3	A,O	n/a	n/a	n/a	n/a
Coreopsidae	Coreopsidinae	6	*Bidens schimperi*	3	N	n/a	n/a	n/a	n/a
Anthemidae	Artemisia Group	11	*Artemisia afra*	3	N	n/a	n/a	n/a	n/a
Anthemidae	Phymaspermum Group	11	*Schistostephium heptalobum*	3	N	0.33 (0.33)b	0.07 (0.07)b	0b	0b
Astereae	Unplaced Genus	11	*Microglossa mespilifolia*	3	N	2.33 (0.88)a	0.67 (0.48)b	0b	0b
Astereae	Homochrominae	11	*Felicia amelloides*	3	N	0.33 (0.33)b	0.07 (0.07)b	0b	0b
Calenduleae	n/a	11	*Osteospermum muricatum*	3	N	n/a	n/a	n/a	n/a
Calenduleae	n/a	11	*Chrysanthemoides monilifera*	3	N	n/a	n/a	n/a	n/a
Calenduleae	n/a	11	*Garuleum sonchifolium*	3	N	0.67(0.67)b	0.07(0.07)b	0b	0b
Gnaphalieae	n/a	11	*Callilepis laureola*	3	N	2.33 (0.67)a	0.47 (0.13)b	0b	0b
Senecioneae	n/a	11	*Delairea odorata*	3	N	1.67 (1.20)b	0.07 (0.07)b	0b	0b
Senecioneae	n/a	11	*Senecio deltoideus*	3	N	n/a	n/a	n/a	n/a
Senecioneae	n/a	11	*Senecio angulatus*	3	N	n/a	n/a	n/a	n/a
CICHORIOIDEAE									
Vernonieae	Gymnantheminae	13	*Distephanus anisochaetoides*	3	N	2.0 (1.15)a	0.40 (0.20)b	0b	0b
Arctoteae	Arctotidinae	13	*Arctotis arctotoides*	3	N	n/a	n/a	n/a	n/a
Cichorieae	Cichoriinae	13	*Cichorium intybus*	3	A,C	n/a	n/a	n/a	n/a

Cichorieae	Lactucinae	13	*Lactuca sativa*	3	A,C	n/a	n/a	n/a	n/a
CARDUOIDEAE									
Cardueae	Carduinae	16	*Cynara scolymus*	3	A,C	n/a	n/a	n/a	n/a

[1]From Anderberg *et al.* (2007)
[2]At Tribe level, based on Funk *et al.* (2009) and Briese (2005).
[3]Previously all in the genus *Eupatorium.*
[4]A = alien, C = crop, I = invasive, N = native, O = ornamental.
[5]Each inoculated shoot was scored as follows: 0 = no boring; 1 = initial boring but larva did not develop further; 2 = some boring and larval development; 3 = considerable boring, normal larval development. A mean score for each replicate was then calculated.
[6]Within the same column, different letters following Mean (SE) indicate a significant difference ($p < 0.05$) between the control and the test species. Mann-Whitney U comparison.
[7]n/a- not available from number of shoots damaged through to adults eclosion

3.4.2.2 Adult no-choice oviposition trials

Of the eight plant species that were tested, *D. odorata* laid fertile eggs on four species (Table 3.3), all alien to South Africa and in the tribe Eupatorieae, namely the target weed (*C. odorata*), *Ageratina adenophora* (Spreng.) R.M. King & H. Rob., *A. riparia* (Regel). R.M. King & H. Rob. and *Ageratum conyzoides* L. *C. odorata* was by far the most suitable host compared to the other species on which the moth laid significantly fewer fertile eggs (Table 3.3). None of the other four plant species were selected for oviposition by the moth. Although some of the eggs laid on test plants hatched, no larval boring was observed. Larval damage, pupae and adults of *D. odorata* were recorded from only the *C. odorata* controls (Table 3.3).

Table 3.3: Host selection of *D. odorata* adults as determined by oviposition, egg hatching, larval mining and development during adult no-choice tests (mean ± SE).

Plant species	No. of replicates	No. of eggs[a]	Eggs hatched[a]	No. of shoots damaged[a]	No. of pupae[a]	No. of adults[a]
Chromolaena odorata	4	79.00 ± 19.53a	78.80 ± 19.67a	48.75 ± 10.66a	31.25 ± 3.15a	30.00 ± 2.80a
Adenostemma caffrum	3	0b	0b	0b	0b	0b
Ageratina adenophora	3	7.60 ± 2.19b	5.30 ± 3.53b	0b	0b	0b
Ageratina riparia	3	1.33 ± 0.88b	1.33 ± 0.88b	0b	0b	0b
Ageratum conyzoides	3	6.00 ± 4.16b	5.00 ± 4.51b	0b	0b	0b
Mikania capensis ex KZN	3	0b	0b	0b	0b	0b
Stomatanthes africanus	3	0.67 ± 0.67b	0b	0b	0b	0b
Microglossa mespilifolia	3	0b	0b	0b	0b	0b

[a]one-way ANOVA on $\sqrt{(x + 1)}$ transformed data. Within a column, significant differences between species are indicated by different letters (Tukey HSD).

3.5 Discussion

Dichrorampha odorata could contribute positively to the biocontrol of *C. odorata* in South Africa. The study on the biology of *D. odorata* has highlighted attributes which suggest that it has good prospects as a biocontrol agent (e.g. Madire 2013). The moth is multivoltine with a short lifecycle and fairly high reproductive potential, all key to enhance rapid population increases in the field. Rapid population increases and sustained agent densities are generally crucial for biocontrol success (Grassmann 1996).

3.5.1 Safety of *D. odorata* as a biological control agent

The fact that *D. odorata* was an undescribed species until recently suggests that it is not a significant crop pest where it occurs. Since its description by Brown and Zachariades (2007), no host records have been found to indicate that it feeds on other species of plants in its native range. Host-suitability tests described here suggest that *D. odorata* is highly host specific and will not pose any threats to non-target Asteraceae species that are native or of commercial value in South Africa. In no-choice trials in quarantine, first-instar *D. odorata* larvae initially bored into 14 test species other than the control, but intense damage was observed only on *C. odorata*, as was subsequent development to pupation and adulthood. One shoot tip of one replicate of *S. africanus*, indigenous to South Africa and closely related to *C. odorata*, experienced initial intense damage but could not support larval development of *D. odorata*. The high level of host specificity of *D. odorata* was even more evident in adult no-choice trials where newly hatched larvae only accepted *C. odorata* for feeding and development.

Oviposition by *D. odorata* was induced on four non-target plants within the tribe Eupatorieae, although a strong oviposition preference for *C. odorata* was recorded. A high percentage of egg hatching was seen on all plants on which oviposition occurred, except for *S. africanus*. However, only two eggs were laid on this species, possibly because it has very small leaves. No larval boring into the shoot tips of any of the four non-target species selected for oviposition was recorded. Only *C. odorata* received considerable damage and completely supported development of *D. odorata*, indicating that none of the test plants can be considered to be at risk. Limited adult no-choice trials were undertaken because of concerns that the use of non-naïve larvae in the larval no-choice trials may have biased the results of these trials (it was not practical to use naïve larvae). However, this does not appear to be the case. The minimal damage and oviposition recorded on some non-target species are most often attributed to cage artefacts and they infrequently happen under field conditions (McFadyen et al. 2002; Simelane 2005). Failure of *D. odorata* to complete development on test plants, with minimal damage and oviposition, suggests that a population of the moth would not be sustained on these plants in the field.

3.5.2 Potential for establishment of a field population

Jamaica, where the *D. odorata* culture held in South African quarantine was collected, has been shown to be a likely origin of the SA biotype of *C. odorata*. Therefore, no mismatch is expected

between the biocontrol agent and its host plant, which was a problem earlier in the South African *C. odorata* biocontrol programme (Zachariades et al. 2011). Given that the lifecycle of *D. odorata* is less dependent on leaves than the lifecycles of the two currently established agents (*P. insulata* and *C. eupatorivora*), it is hoped that *D. odorata* will establish in areas where *C. odorata* leaves wilt and die in the dry season. Robinson (2012) did not find any significant patterns in Jamaica with regards to the altitudinal distribution of *D. odorata*, nor with its preference for degree of shading or its seasonal distribution. However, the lack of an obvious diapause period in *D. odorata* and the dissimilarity of the Jamaican climate to that of South Africa (Robertson et al. 2008), may act negatively in determining distribution and population levels of *D. odorata* in South Africa.

Tortricidae, mainly pest species, appear to be susceptible to parasitism in Europe, Australia and Turkey (Aydogdu and Beyarslan 2007; Brockerhoff and Kenis 1996; Paull and Austin 2006). Torgersen and Beckwith (1974) reported that 24 species of parasitoids were found associated with the large aspen tortrix in Alaska, USA. Nor are all leaf rolling tortricids protected from parasitoids by their behaviour (Berndt et al. 2002). Post-release evaluations will determine whether *D. odorata* efficacy will be negatively affected by native parasitoids or predators in South Africa.

Permission to release *D. odorata* for biocontrol of *C. odorata* in South Africa was granted by the regulatory authorities in June 2013 and releases were initiated shortly thereafter. Up to now over 9000 insects, 87% (over 8000) of which were pupae, but also including 247 adults and 934 larvae, have been released at 15 sites in KZN, Mpumalanga and Limpopo provinces. The moth seems to be persisting at only one of the sites (which has thus far received more insects (1972) than any of the other sites). This phenomenon is similar to that of *P. insulata* on *C. odorata* which initially persisted in low numbers at one release site in South Africa (Zachariades et al. 2011) but eventually established and has now spread as far as Swaziland (Zachariades et al. 2016).

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CHAPTER 4: IMPACT OF THE SHOOT-BORING MOTH *DICHRORAMPHA ODORATA* (LEPIDOPTERA: TORTRICIDAE) ON GROWTH AND REPRODUCTIVE POTENTIAL OF *CHROMOLAENA ODORATA* (ASTERACEAE) IN THE LABORATORY

4.1 Abstract

A 9-month laboratory study was undertaken to determine the impact of herbivory by a moth with shoot-boring larvae, *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae) on growth and reproductive ability of its host plant, *Chromolaena odorata* (L.) King and Robinson (Asteraceae), a major invasive alien plant species in southern Africa. Newly hatched *D. odorata* larvae were inoculated onto 0 (control), 50 and 100% of the shoot tips of *C. odorata* in the laboratory. At all treatment levels, the basal stem diameter of *C. odorata* was not affected by *D. odorata* larval feeding. Larval feeding by *D. odorata* significantly reduced the height of the main shoot and flower production in *C. odorata* relative to the control treatment but promoted branching by increasing the number of shoot tips. However, the differences in plant height and number of flowers between the 50 and 100% inoculation levels were not statistically significant. *Dichrorampha odorata* is the first shoot-tip attacking agent that is being released as a biological control agent against *C. odorata* in South Africa. In general, the impacts of *D. odorata* on the weed were relatively small even though statistically significant. The findings of this study suggest that high levels of damage by the moth will modestly reduce the height, flower production, and the competitiveness of *C. odorata*, thereby contributing to the biological control of the weed in South Africa.

Key words: Invasive alien weed, biological control, shoot-tip borer, Tortricidae, efficacy

4.2 Introduction

The adverse impacts of invasive alien plants on agriculture, forestry, biodiversity of natural environments, human health, water supplies and the economy of South Africa are well documented (Henderson and Wells 1986; Olckers et al. 2005; Zachariades et al. 2016). Alien plants invading South Africa range from trees and shrubs, grasses and reeds, climbers, to terrestrial herbs and aquatics (Henderson 2001). Of these, *Chromolaena odorata* (L.) King and Robinson (Asteraceae) with international notoriety as one of the world's worst shrubs (Holm et al. 1977) has contributed tremendously to a reduction in biodiversity and carrying capacity of native ecosystems in South Africa (Kluge 1990; Luwum 2002; te Beest 2010). For example,

Leslie and Spotila (2000) showed that in KwaZulu-Natal (KZN) province, Lake St. Lucia's nesting Nile crocodiles *Crocodylus niloticus* Laurenti (Reptilia: Crocodilidae) require open sunny, sandy areas in which to deposit their eggs. However, *C. odorata* plants overrunning the nesting sites created fibrous root mats unsuitable for egg-chamber and nest construction. Shading by this invasive alien plant led to a female-biased sex ratio and crocodiles later abandoned nesting at these sites. In Hluhluwe-Imfolozi Park alone, *C. odorata* has negatively impacted diversity and abundance of spider communities (Mgobhozi et al. 2008) and mammals (Dumalisile 2008); it has adversely impacted utilisation of forage species, has led to a reshuffling of the population of black rhino and is partly responsible for the population decline of this species (Howison 2009). Chemical, mechanical and other conventional methods of controlling the weed have proven not to be sustainable (Zachariades et al. 2011). A biological control programme has been in development since 1988 for long-term suppression of *C. odorata*, and several insect candidate agents have been assessed (Zachariades et al. 1999; Zachariades et al. 2011).

Insect herbivores are notorious for prompting unpredictable responses on their host plant's performance in terms of architecture, growth and reproductive capacity (Miller et al. 2009). Herbivore attacks may delay seed ripening, lessen seed production and individual mass, lessen the growth rate of roots and shoots, lower the resistance of plants to diseases, and lessen the competitive ability of plants in comparison to their un-attacked neighbours (Crawley 1989a). It is no surprise that classical biological control relies on the use of insect herbivores in the form of natural enemies, in addition to mites and pathogens, to suppress and restrict the densities, seed production and dispersal of invasive alien plants (Isaacson et al. 1996). Of the insect herbivores, Lepidoptera are among the successful biological control agents following Diptera, Hemiptera and Coleoptera (Crawley 1989b; Winston et al. 2014).

Our knowledge of, and prediction of the impact of natural enemies against the target weed is key to the success of any biocontrol programme, but remains a less developed part of the science of biological control (Shea and Possingham 2000; Wratten and Gurr 2000). This is so because, globally, for every biological control agent introduced, host specificity clearance is mandatory whilst assessment of potential impact caused by candidate agents prior to release remains optional. Studies conducted on the latter are as important in the prioritisation and selection process in biological control programmes, to limit the introduction of inefficient biocontrol agents, and to understand agent performance and the reasons for success or failure

of agents in biological control of weeds (Conrad and Dhileepan 2007). In addition, pre-release efficacy studies reduce the costs and risks associated with releasing inefficient biological control agents (McClay and Balciunas 2005).

A number of biological control programmes have undertaken assessment of impact of a candidate biocontrol agent on plant architecture and biomass prior to release (e.g. Briese 1996; Conrad and Dhileepan 2007; Fay and Throop 2005; Frye and Hough-Goldstein 2013; Goolsby et al. 2004; Kloppel et al. 2003; Weed and Cassagrande 2011). Although several biological control agents have been released against *C. odorata* in South Africa (Klein 2011, updated 2016), only two have definitely established. The failure of some agents was attributed to the difference in biotype invading southern Africa in relation to the biotype invading other parts of the world (Paterson and Zachariades 2013; Shao et al. 2018), resulting in incompatibility between the agent and the host plant. The two agents established against *C. odorata* are a leaf mining fly *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae) and a moth with defoliating larvae, *Pareuchaetes insulata* (Walker) (Lepidoptera: Erebidae). Monitoring conducted on *P. insulata* showed restoration of indigenous flora where the moth had persisted, and tremendous spread from the release points (Zachariades et al. 2016). The prerelease assessment of damage conducted on the stem-boring weevil *Lixus aemulus* Petri (Coleoptera: Curculionidae), first released in 2011 for the biological control of *C. odorata*, showed that the larvae of *L. aemulus* caused high mortality of the stems, as well as reducing the dry mass of infested stems and the number of achenes produced on the infested branches (Kluge and Zachariades 2006).

Larvae of a shoot-boring moth, *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae), were collected from *C. odorata* in Jamaica in November 2005, and imported into quarantine in South Africa; a supplementary culture was collected in November 2012. *Dichrorampha odorata* is multivoltine, easy to rear and is highly specific to *C. odorata* (Dube et al. 2017). Following host-range trials, permission to release *D. odorata* was obtained in June 2013 and releases were initiated soon thereafter. Over 20,000 insects, mainly pupae but also larvae and adults, have been released at 17 sites thus far. Although initial persistence of about 7 months has been recorded at one of these sites, *D. odorata* has not yet established. The reasons for this are uncertain and could include climate incompatibility between Jamaica and South Africa (Robertson et al. 2008), or the biology of the insect. Many lepidopterans have proved difficult to establish; for example, at the only site at which *P. insulata* is known to have

established, 335 000 larvae were released (Zachariades et al. 2011). This study reports on the pre-release impact of *D. odorata* (Dube et al. 2017) at different densities on *C. odorata* in South Africa. Studies such as this have far-reaching implications for biocontrol programmes going forward, and may be used to evaluate the desirability for additional releases of *D. odorata*, especially in light of the initial failure of the agent to establish.

4.3 Materials and methods

4.3.1 Culturing methods and quarantine conditions

Both culturing of, and trials on, *D. odorata* were carried out in the quarantine laboratory and glasshouse at the Agricultural Research Council, Plant Health and Protection (ARC-PHP), Cedara (29.54153° S, 30.26764° E), near Pietermaritzburg, KZN province, South Africa. Laboratories and glasshouses were kept within a temperature range of 22–28°C, 40–70% RH and either a 12-hour photoperiod using growth lights (adults) or natural lighting (larvae). In order to culture *D. odorata*, between 15 and 25 newly eclosed adults were placed into a steel-framed cage of 0.9 × 0.5 × 0.5 m with gauze panels, over several days depending on the availability of adults. Four *C. odorata* plants, potted in 18 cm diameter pots in a medium consisting of a 1:1 ratio of Umgeni river sand and 'Gromor' potting medium (Gromor, Cato Ridge, South Africa), and selected for their large number of growing shoot tips, were placed into the cage prior to introduction of the moths. The plants were propagated from soft shoot cuttings taken from the field and rooted in a heated mist-bed with rooting hormone (Seradix® No. 1). Cages were sprayed with water daily, but no food was provided for the moths. Plants were removed from the cage about 10 days after the last moth had been introduced, to allow for egg hatching and larval development. These plants were placed into a walk-in-cage (3.3 × 2.3 × 1.9 m) in the glasshouse. Larvae bored into the shoot tip and down the stem for 2–3 cm, forming a characteristic slight, discoloured swelling. Only one larva could develop per shoot tip. The mature larva exited the stem tip and pupated on the leaf, after cutting a crescent shaped flap from the edge of the leaf, folding it over itself and tying it shut with silk. Leaves with pupae were removed from plants and placed into Petri dishes on a slightly dampened piece of filter paper, to allow for adult eclosion (Dube et al. 2017).

4.3.2 Impact trial

This trial was initiated in November 2011, coinciding with early summer, in order to encompass an entire summer growing season and the flowering period thereafter. About 80 similar-sized shoot cuttings of *C. odorata*, each with one terminal growth point, were collected

from the field in Pietermaritzburg, South Africa and propagated as described (above section 4.3.1 page 65), whereafter they were planted into 18 cm diameter pots in standard medium as above. At that time the terminal shoot was removed, with the result that each young plant developed two growth points, of similar size, from the node closest to the terminal shoot. Once these 2 side shoots had developed sufficiently (each about 5 cm long), four plants of about 25 cm in height were selected from the *C. odorata* stock plants (used for culturing biocontrol agents) and placed in a standard insect-rearing cage with 30 *D. odorata* adults for 10 days, to allow the adults to mate and lay eggs. After ten days (the egg incubation period), 48 of the small plants with two shoots, of similar condition and size, were selected and haphazardly assigned to three groups of sixteen plants each. These plants were all kept in a walk-in cage in the quarantine glasshouse, to prevent attack by extraneous *D. odorata* or other insects. These were inoculated with *D. odorata* larvae of about one-day old, dissected from shoots on the plants on which adults had laid eggs (larvae are mobile and will tunnel into a shoot if they are placed nearby). Based on the behaviour of ovipositing females, which tend to lay eggs on the tallest shoots on a plant (C. Zachariades pers. communications), larvae were always placed on the tallest shoot tips of treatment plants throughout the trial, in order that they tunnel into the tips. Three treatment levels were used, and plants were re-inoculated once a month. Sixteen plants were subjected to a 'low' infestation rate with 50% of their shoots inoculated with larvae, and sixteen plants to a 'high' infestation rate, with 100% of their shoots also inoculated with larvae. For controls (N = 0), no shoot tips were inoculated. Ten additional plants, planted at the same time as the 48 used for the trials, were destructively sampled at the start of the trial to measure biomass. Each inoculated shoot tip was marked with a short piece of wool to prevent inoculating each tip with more than one larva, and to allow monitoring. In order to achieve the correct level of inoculation, on the fourth day after inoculation the shoot tips were checked, and if the larvae had not bored in, the shoot tips were re-inoculated with 5-day old larvae, until February 2012. After 4 months (February), there was not enough time to confirm if larvae had truly bored into shoot tips after inoculation. For the purpose of both counting and inoculating, shoot tips were defined by having two or more pairs of leaves that were all more than 1 cm long. After each month all pupae were harvested, and any newly-sprouted shoot tips of greater than 1 cm or flowering buds that could sustain a larva were re-inoculated with *D. odorata* to maintain a consistent percentage infestation rate until the end of the trial.

Several plant growth parameters were measured once a month: basal stem diameter, the height of the tallest shoot (=plant height) and the number of shoots greater than 1 cm per plant. In

order to obtain an estimate of the total number of branches on each plant on each sampling occasion, the number of undamaged shoot tips counted on that occasion was added to the total number of larvae previously inoculated onto the plant. This rests on the assumption that each larva successfully inoculated resulted in the death of that shoot tip, resulting in the formation of a discrete branch from which new branches would form. During flowering season, only vegetative shoot tips that could sustain larval development were counted and inoculated.

The trial continued until after the plants had flowered (June 2012) and set seed to determine the impact of *D. odorata* on reproduction. Between January and April 2012, plants were treated once a month with a preventive mixed soil drench of Previcur® and Benlate®, against root pathogens (Pythium and Phytophthora species). As a result of root pathogens and/or overheating in the quarantine glasshouse caused by power cuts, of the 48 plants at the start of the experiment, only 22 had survived by the end of it (control: 7 plants, 50%: 6 plants, and 100%: 9 plants). Initially all plants were watered daily with 500 ml tap water but at 3 months Blumat® automatic waterers were inserted into all the plant pots. At the end of the trial all the surviving plants were destructively sampled to measure their dry biomass (stems, leaves and roots separately).

4.3.4 Statistical analyses

The effects of *D. odorata* inoculation levels on stem diameter, plant height, leaf biomass, stem biomass and root biomass of *C. odorata* plants were analysed using Generalized Linear Model (GLM) assuming a normal distribution with an identity link function. The effects of *D. odorata* inoculation levels on the numbers of shoot tips and flowers produced by the *C. odorata* plants were analysed using GLM assuming a Poisson distribution with a loglinear link function. When the overall results were significant, the differences among the treatments were compared using the sequential Bonferroni's test. The relationships between *C. odorata* growth parameters (number of shoot tips, stem diameter and plant height) and duration of plant growth (in months) for the different treatments were determined using simple linear regression analyses. With the exception of the regression analyses that was performed using Microsoft Excel and Genstat 12.0 (VSN International, Hemel Hempstead, UK), all other analyses were performed using SPSS statistical software version 16.0 (SPSS, Chicago, USA).

4.4 Results

Over a period of 9 months, larval feeding by *D. odorata* did not significantly influence the basal stem diameter of *C. odorata* (Table 4.1, Figure 4.1(a)), but significantly reduced plant height (Table 4.1, Figure 4,1(b)) in plants exposed to *D. odorata* compared to the control treatment. However, there was no difference between the 50% and the 100% treatments. The number of shoot tips of *C. odorata* plants increased as a function of *D. odorata* infestation (Table 4.1, Figure 4.1(c)) and the total number of flowers produced was significantly influenced by *D. odorata* infestation (Table 4.1, Figure 4.1(d)). Uninfested (=control) plants produced more flowers compared to *D. odorata*-infested plants, although there was no difference between the 50% and 100% treatments. *Dichrorampha odorata* larval feeding significantly reduced leaf biomass of *C. odorata* plants (Table 4.1, Figure 4.2(a)). Uninfested plants had greater biomass compared to treated plants (50% and 100%); however, there was no difference between the 50% and 100% treatments. Larval feeding by *D. odorata* significantly increased stem and root biomass compared to the control treatment (Table 4.1, Figure 4.2(b and c)). The leaf, stem and root biomass of *C. odorata* plants at the start of the trial (i.e. Time Zero) were significantly lower than the biomass (leaf, stem and root) of the three treatment plants at the end of the trial (Figure 4.2(a–c)).

Regression analyses showed significant positive relationships between numbers of shoot tips and duration (months) of plant growth for the various treatments (apart from the control) (R^2 = 0.443, $F_{2,7}$ = 3.54, P = 0.109; 50% treatment: R^2 = 0.962, $F_{2,7}$ = 161.51, P = 0.001; 100% treatment: R^2 = 0.964, $F_{2,7}$ = 161.75, P = 0.001) (Figure 4.3(a)). Irrespective of treatment types, stem diameter (control: R^2 = 0.829, $F_{1,7}$ = 35.04, P = 0.001; 50% treatment: R^2 = 0.739, $F_{1,7}$ = 20.84, P = 0.004; 100% treatment: R^2 = 0.746, $F_{1,7}$ = 21.54, P = 0.003) and plant height (control: R^2 = 0.833, $F_{1,7}$ = 35.89, P = 0.001; 50% treatment: R^2 = 0.883, $F_{1,7}$ = 54.02, P = 0.001; 100% treatment: R^2 = 0.916, $F_{1,7}$ = 76.94, P = 0.001) increased with plant growing durations (Figure 4.3(b and c)).

Table 4.1: Generalized linear model (GLZ) results for effects of *Dichrorampha odorata* inoculation levels on plant parameters of *Chromolaena odorata*.

Effect	d.f.	Wald χ^2	P
Stem diameter			
Intercept	1	1278.504	**0.0001**
Treatment	2	0.001	0.900
Plant height			
Intercept	1	143126.543	**0.0001**
Treatment	2	130.678	**0.0001**
Number of shoot tips			
Intercept	1	13658.408	**0.0001**
Treatment	2	436.138	**0.0001**
Number of flowers			
Intercept	1	710686.759	**0.0001**
Treatment	2	149.134	**0.0001**
Leaf biomass			
Intercept	1	8048.598	**0.0001**
Treatment	2	2946.634	**0.0001**
Stem biomass			
Intercept	1	7885.398	**0.0001**
Treatment	2	3093.855	**0.0001**
Root biomass			
Intercept	1	1132.495	**0.0001**
Treatment	2	427.937	**0.0001**

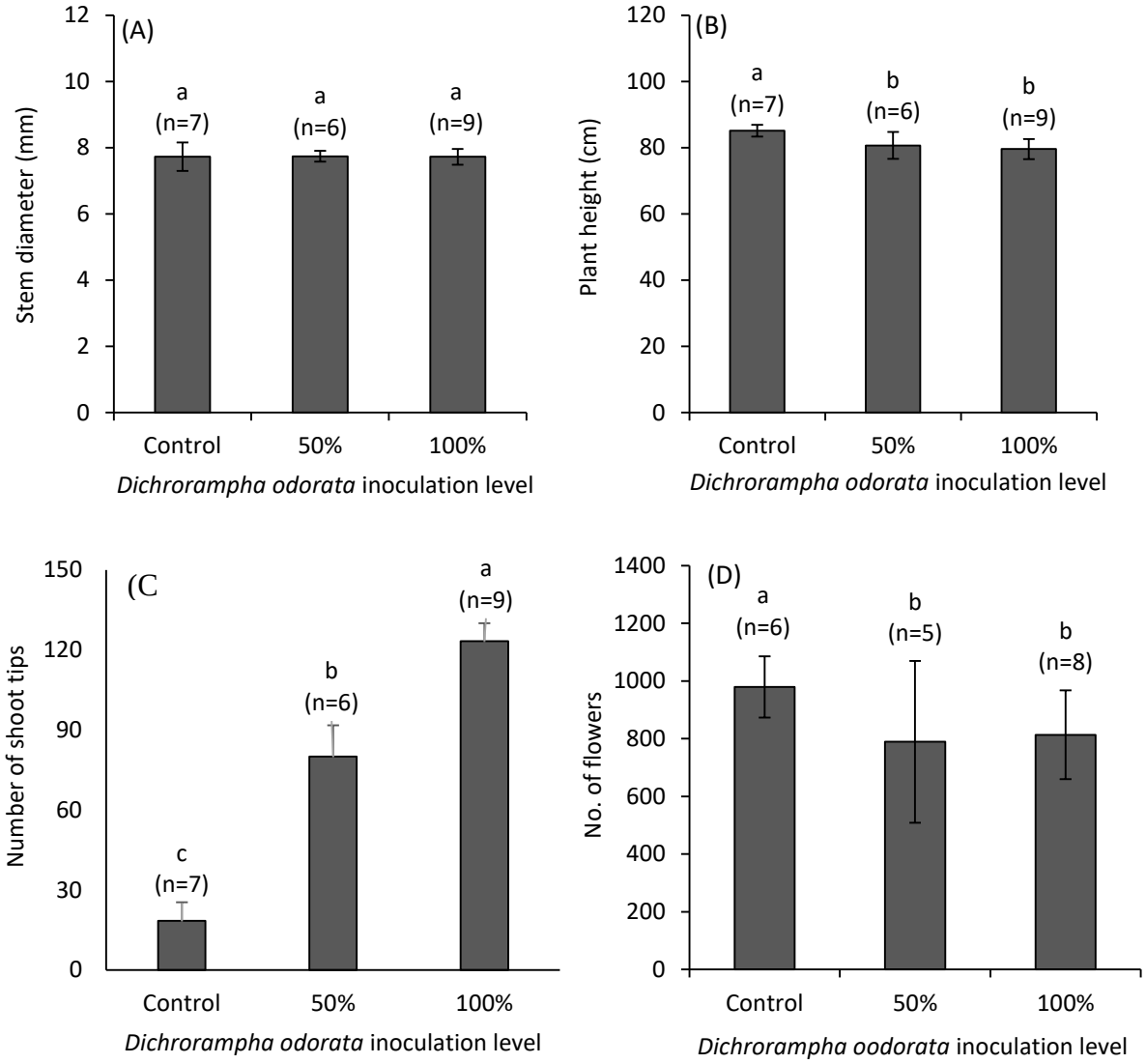

Figure 4.1: Effect of *Dichrorampha odorata* inoculation levels on basal stem diameter (A), Height of main shoot (B), number of shoot tips (C) and the number of flowers (D) of *Chromolaena odorata* plants after nine (9) months of inoculation with varying levels of *D. odorata* larvae. Means (after Generalized Linear Model analysis (GLM)) with the same letters above the bars are not significantly different (sequential Bonferroni test: $P > 0.05$). Sample sizes are given in parentheses.

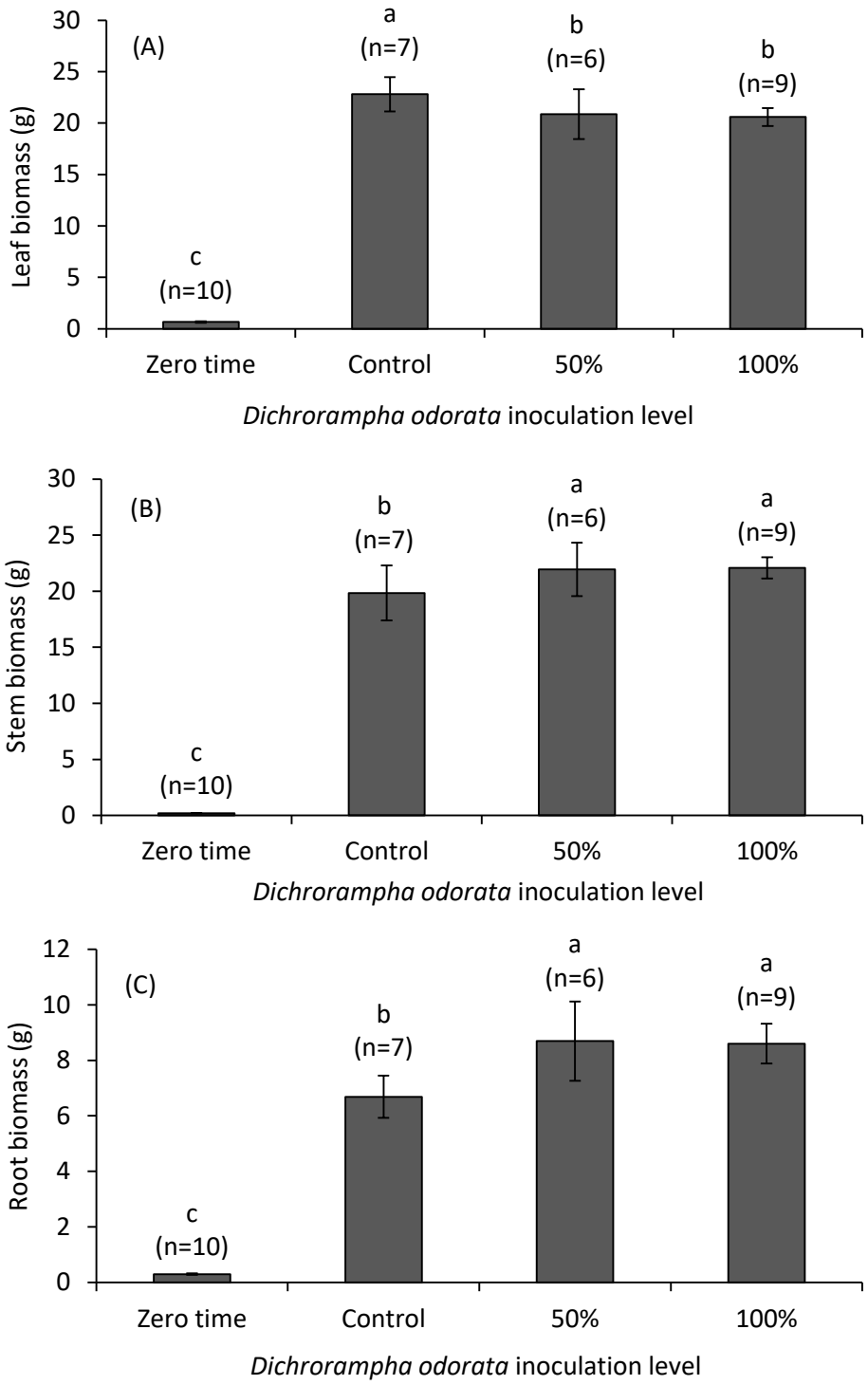

Figure 4.2: Effect of *Dichrorampha odorata* inoculation levels on leaf biomass (A), stem biomass (B) and root biomass (C) of *Chromolaena odorata* plants after nine (9) months of inoculation with varying levels of *D. odorata* larvae. Means (after Generalized Linear Model analysis (GLM)) with different letters above the bars are significantly different (sequential Bonferroni test: $P<0.05$). Sample sizes are given in parentheses.

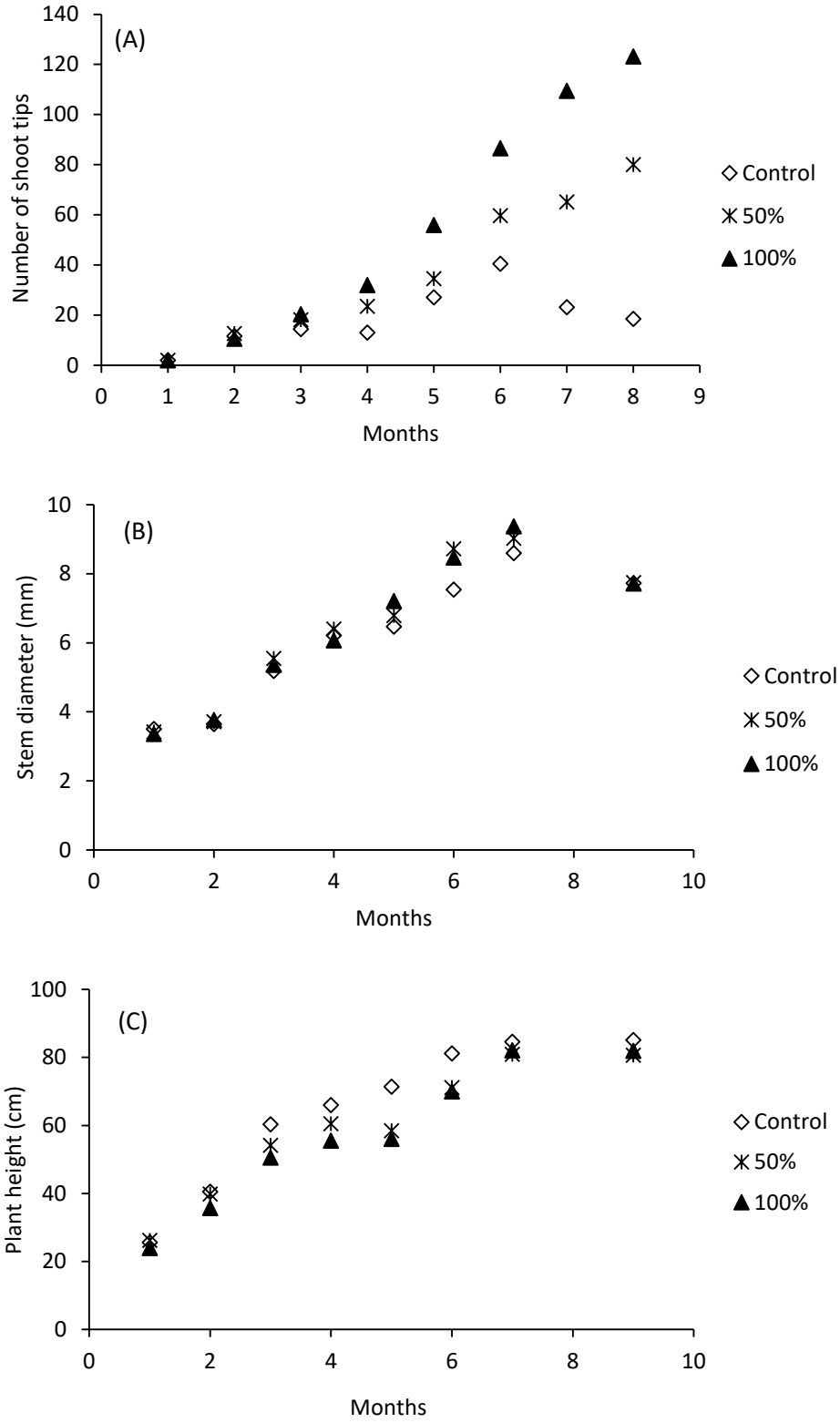

Figure 4.3: Relationships between *Chromolaena odorata* growth parameters [number of vegetative shoot tips (A), stem diameter (B) and plant height (C)] and duration (in months) of plant growth in the control (plain squares), 50% (asterisks) and 100% (triangles) *Dichrorampha odorata* infestation levels.

4.5 Discussion

Pre-release studies quantifying the impacts of biological control agents on the performance of invasive plant species are increasingly receiving attention (Balciunas and Smith 2006; Grevstad et al. 2013; Milbrath and Biazzo 2016; Reddy and Mehelis 2015) because data from such studies help to inform the prioritisation of agents for further study or to estimate plant impact post-release (e.g. Balciunas and Smith 2006; Reddy and Mehelis 2015). In this study, we demonstrated that the shoot-boring activities of larvae of *D. odorata* significantly reduced plant height, number of flowers and leaf biomass in *C. odorata* plants. In general, the impacts of herbivory were relatively small even though statistically significant. This is similar to what has been seen in other impact studies (e.g. Reddy and Mehelis 2015), and so not unusual, but it indicates that in the field the herbivory impacts will be fairly subtle.

The reduction of plant height due to the feeding activities of the larvae of *D. odorata* could decrease the fitness and competitive ability of *C. odorata* in the field. A reduction in plant height due to herbivory has been recorded in other systems (e.g. Simelane and Phenye 2005; Wilbur et al. 2013) and is a desired result of implementing biological control programme. The reduced flower production in treated plants (caused by *D. odorata*) in this study is analogous to the findings of other authors who reported that flower production can be indirectly affected by insect feeding through various kinds of damage that reduces bud production, bud burst or sexual reproduction (Crawley 1989a; Wise and Sacchi 1996). The reduced leaf biomass caused by *D. odorata* will certainly have negative effects on photosynthetic rate. It is plausible that the reduction in leaf biomass of *C. odorata* might have influenced the reduced flower production in *C. odorata* infested plants in this study. In contrast, root and stem biomasses increased in the presence of *D. odorata* larvae, whilst stem diameter was unaffected. Roots play a vital role in plant responses to above-ground herbivory by storing photoassimilates and synthesising secondary metabolites involved in leaf defences (Erb et al. 2009) to enable future regrowth; and increase of root biomass in response to herbivory is well documented (Nalam et al. 2013; Paige and Whitham 1987). Several studies have demonstrated increased exportation of carbon from the damage site into the storage organs (stems and roots) after herbivory (Gomez et al. 2012). Similar to other studies (e.g. Schat and Blossey 2005), the increased stem and root biomass in *C. odorata* could be attributed to the excessive production of carbon (unused during photosynthesis) that is stored in the stem and root, consequent upon attack by *D. odorata* on the stem tips of the plant. In addition, several years of damage may be necessary to observe depletions of roots and stem biomass in long-lived perennial species such as *C.*

odorata (e.g. Ringselle et al. 2015). Overall, this study and others demonstrate that plant herbivory results in a decrease in reproductive output such as leaves and flowers rather than in root and stem biomasses (Maschinski and Whitham 1989; Strauss and Agrawal 1999).

Results in this chapter showed that shoot herbivory by *D. odorata* resulted in increased production of shoot tips and damaged the apical meristems in *C. odorata*, which shortened the stem length (or plant height, Table 4.1) and tended to increase the production of axillary branches. The number of shoot tips (cumulative number of branches), as calculated using the sum of the number of undamaged vegetative shoot tips and previously inoculated larvae, reached a maximum for controls on sampling occasion 6 (Figure 4.2 (a)), after which they declined. This could possibly be explained by the development of flowering shoot tips towards the end of the trial, which were not counted. The positive effects on lateral growth (increased branching) and negative effect on leader growth (plant height) resulted in a change in *C. odorata* plant architecture. Other studies have also observed a similar pattern. For example, the destruction of the lead shoot of Pinyon pine by the moth, *Diorytria albovitella* (Hust) (Lepidoptera: Pyralidae), stimulates the lateral buds and the plant changes from a tree to a dense shrub (Whitham and Mopper 1985). Increased branching is not only a vital mechanism involved in increased tolerance of herbivory, but a key mechanism of plant compensation to damage that is commonly observed (Schat and Blossey 2005; Strauss and Agrawal 1999; Trumbule et al. 1993). According to Trumbule et al. (1993), increased branching due to herbivory can reduce plant height thus affecting competition for light and seed dispersal.

The lack of a significant difference in all plant performance metrics between the 50 and 100% inoculation treatment suggests that shoot herbivory of half of the total shoots of individual plants of *C. odorata* may be sufficient to reduce plant height and flower production. It is also not impossible that 100% larval infestation of the shoots may cause a reduction in plant nutrients (especially nitrogen, water content) and these nutrient reductions can consequently have negative effects on the performance (survival, growth and development) of *D. odorata*, thereby limiting its impact on *C. odorata*. Despite the feeding activities of the larvae of *D. odorata*, the significant positive relationships between some plant performance metrics (number of shoot tips and stem diameter) and duration of plant growth suggests that the moth is unable to cause plant mortality, at least in our 9-month experiment. The effect of herbivore damage can be influenced by environmental conditions such as variation in light intensity in the plant's growing environment (Berg et al. 2015; Milbrath and Biazzo 2016). For example,

low light is a stress to plants and can enhance the effect of plant damage on perennial species (such as *C. odorata*), including causing plant mortality if herbivory levels are severe (Baraza et al. 2004; Lentz and Cipollini 1998; Norghauer et al. 2008). Our data indicate that *D. odorata* has desired attributes as a biological control agent. The moth has been released since June 2013 and because of the relatively low numbers that have been released since then (over 20 000) and that it is a lepidopteran, it would be premature to conclude that it is a failure in the field.

To conclude, our study showed that larval feeding damage by the shoot-boring moth *D. odorata* has the capacity to reduce flower production and plant height in *C. odorata* in a laboratory experiment. Whether such individual-level damage has the potential of imposing negative effects on the population dynamics of *C. odorata,* especially in combination with damage by other established biocontrol agents, remains to the seen. During our exploration in Jamaica, we could not estimate the impact of *D. odorata* on *C. odorata* as it often co-existed with other insect herbivores such as *Phestinia costella* Hampson (Lepidoptera: Phycitinae), *Melanagromyza eupatoriella* Spencer and/or *Polymorphomyia basilica* Snow (Diptera: Tephritidae). However, the negative effects of *D. odorata* on leaf biomass, plant height and reproduction suggests that it plays a role in abundance and population dynamics of *C. odorata*, at least in part, in its native range. This co-existence of this moth with other insect herbivores in its native range suggests that its impact will probably be complementary in South Africa and that it can utilise *C. odorata* as a host plant without being detrimental to established biocontrol agents such as *P. insulata* and *C. eupatorivora*. This study suggests that, if it becomes established, *D. odorata* may contribute modestly to reduce the menace caused by *C. odorata* in South Africa but recommends more biocontrol agents as a complement for areas where the moth does not establish.

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CHAPTER 5: LIFE HISTORY TRAITS AND HOST SUITABILITY OF A GALL-FORMING FLY, *POLYMORPHOMYIA BASILICA* (DIPTERA: TEPHRITIDAE) FOR THE BIOLOGICAL CONTROL OF *CHROMOLAENA ODORATA* (ASTERACEAE) IN SOUTH AFRICA

5.1 Abstract

Gall formers are well known for their narrow host range and injurious effects on the growth and fitness of their host plants. The tephritid *Cecidochares connexa* has been used to good effect as a biological control agent on the Asian/West African biotype of *Chromolaena odorata*, but does not develop well on the different, southern African *C. odorata* biotype. A stem-galling tephritid fly, *Polymorphomyia basilica*, from the northern Caribbean islands, was considered as a potential biological control agent for the invasive alien shrub, *Chromolaena odorata* in South Africa. Life history traits and host range on 32 asteraceous plants were investigated in single-choice adult tests and using single pairs of adults in no-choice tests, under laboratory conditions. Genetic and morphological similarity of *C. odorata* between the Caribbean Islands and southern Africa indicates that establishment of *P. basilica* in South Africa is likely. Positive biological characteristics of *P. basilica* include a high rate of increase, long-lived and mobile adults, the ability of females to produce viable offspring without repeated mating, the ability of adults to eclose from galls on dry stems and the production of several generations per year. Use of a single pair of adults for no choice tests proved to be efficient. Oviposition and larval development through to adulthood occurred on three other South American and on two South African species; one in the same tribe Eupatoreae, closely related to- and another one on Astereae less closely related *C. odorata*, but both at a lower and slower rate. Females tended to retain their eggs under no-choice conditions in the presence of an unsuitable host, and to compensate by ovipositing at a higher rate when presented later with a *C. odorata* plant. The ability of *P. basilica* to develop on indigenous species triggers concern; nevertheless, false positive results are common under quarantine conditions. The poor offspring survival on non-target plants tested in this study confirms the suitability of *P. basilica* for release in South Africa.

Key words Gall formers, Tephritid fly, Invasive alien plant, Asteraceae, *Polymorphomyia basilica*, biology, host range

5.2 Introduction

Gall insect-plant interactions have been the subject of numerous studies yet remain difficult to understand (Fay et al. 1996; Shorthouse et al. 2005). Nevertheless, the fundamental role of galls is outlined through a number of hypotheses, including the nutrition, micro-environment and enemy hypotheses, which partly explain these interactions (Price et al. 1987). The nutrition hypothesis posits that galls are a source of enriched nutrition over other feeding modes and has been supported by both morphological and developmental evidence. Changes occurring in cell structure from galling, entail the reduction of chemical defences such as phenolics whilst increasing nutrients; these changes are beneficial to the feeding and development of the galler (Stone and Schonrogge 2003). Although there is limited knowledge about the impact of variation in gall microclimate, the microenvironment hypothesis posits that gall tissues are for the protection of the galler from unfavourable physical conditions such as desiccation (Price et al. 1987; Stone and Schonrogge 2003). Unlike free-living organisms, gallers are in a concealed feeding place and the expectation is that plant galls provide protection from natural enemies such as parasitoids (Price et al. 1987). However, records showed that gall protection is only limited to generalist predators, and that when analyses are made over a broader taxonomy, gallers often have more specialist predators than free-living organisms do. Therefore, the enemy hypothesis, which predicts that galls protect gallers from attack by natural enemies, is not widely accepted, and neither is the mutual benefit hypothesis, as even with plant-pollinating gall formers, parasitism remains the rule. The reduction in plant growth and reproduction caused by the galling insect (e.g. seed-feeding pollinating wasps in Machado et al. 2001) in contrast to the effective reproduction and rapid proliferation of the insects themselves, casts doubt on the sustainability of the mutual benefit hypothesis (Price et. al. 1987). These hypotheses give a glimpse on the importance of galls, which is to provide nourishment, shelter and protection to the gall former and its offspring (Shorthouse et al. 2005), to understand the impact of gall formation on plants and how plant species respond to gall formation.

The ability to form galls is present in a number of life forms including fungi, nematodes, mites and insects (Muniappan and McFadyen 2005; Subbotin et al. 2004; Sagliocco et al. 2011). Gall formers are found in more than 13 000 insect orders including Diptera, Hemiptera, Coleoptera and Hymenoptera (Dennill and Donnelly 1991; Crespi et al. 1997; Adair 2005; Gassmann et al. 2014). Insect gall-formers are widely known for their limited host range and injurious effects on the growth and fitness of their host plants, and thus have largely contributed substantially

to success in biological control programmes globally (Harris and Shorthouse 1996; Goolsby et al. 2000; Diaz et al. 2015; Mukwevho et al. 2017). For stem galls, in a typical/common lifecycle, the young larva of a gall inducer tunnels downwards in the pith of the stem; with time, it closes the upper part of the cavity with a small plug and the presence of the larva is revealed by a moderate swelling of the plant tissue (Friedberg 1984). In some insects that pupate inside the gall, the gall then grows in response to the development of the larva, and pupation is completed inside the gall (e.g. Gassmann et al. 2014). Before pupation or diapause, the larva scrapes a certain spot in the wall of the gall, leaving only a thin layer which the emerging adult easily breaks through upon exit (Friedberg 1984). The anatomy and physiology of the gall varies between species of gall inducers (Shorthouse et al. 2005). Several studies reported on the success of gall inducers in weed biological control; for example, a bud-galling wasp, *Trichilogaster acaciaelongifoliae* Froggatt (Hymenoptera: Pteromalidae) significantly reduced the reproduction potential of *Acacia longifolia* (Fabaceae) in South Africa (Dennill and Donnelly 1991); and a univoltine shoot-galling weevil *Rhinusa pilosa* Gyllenhal (Coleoptera: Curculionidae) investigated as a potential biological control agent in North America , was found host specific to *Linaria vulgaris* Mill (Plantaginaceae) native in Europe, significantly reduced plant height, dry below-ground biomass, dry above-ground biomass and number of shoots produced (Gassmann et al. 2014). Within the Diptera, the fruit fly family, Tephritidae, is the second largest group of gall formers following Cecidomyiidae (Freidberg 1984). Most tephritids form galls on plants of the family Asteraceae (e.g. Dodson and George 1986; Fernandes et al. 1996; Balciunas and Mehelis 2010; Buccellato et al. 2012), on roots, leaves or flower heads and most widespread and commonly on stems (Freidberg 1984; Headrick and Goeden 1998).

Several tephritids have been considered or are known for their significant success in biological control of invasive alien plants in South Africa and globally (e.g. Harris and Shorthouse 1996; Balciunas and Mehelis 2010; Buccellato et al. 2012; Winston et al. 2014). Among invasive alien plants present in South Africa, the southern African biotype (SAB) of a scrambling shrub *Chromolaena odorata* (L.) R.M. King and Robinson (Asteraceae), with an origin in the northern Caribbean islands, and particularly Jamaica or Cuba (Paterson and Zachariades 2013; Shao et al. 2018), has been targeted for biological control since 1988. Of the host-specific biocontrol agents released in South Africa, only *Pareuchaetes insulata* Walker (Lepidoptera: Erebidae: Arctiinae), a moth with defoliating larvae, and *Calycomyza eupatorivora* Spencer, a leaf-mining fly (Diptera: Agromyzidae) are known to have established successfully (in 2004

and 2003 respectively), and are widely dispersed in KwaZulu-Natal (KZN) and Mpumalanga provinces in South Africa, and in Swaziland and Mozambique (Zachariades et al. 2016; ARC-PHP, unpubl.). Nevertheless, *C. odorata* remains a significant weed in South Africa, particularly in seasonally drier inland areas where neither *C. eupatorivora* nor *P. insulata* have had a major impact (te Beest et al. 2013).

In order to complement these two leaf-feeding biocontrol agents, a moth with shoot-boring larvae, *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae), from Jamaica, a stem-boring weevil, *Lixus aemulus* Petri (Coleoptera: Curculionidae), from Brazil, and a long-horn beetle, *Recchia parvula* (Lane) (Coleoptera: Cerambyciidae), from Argentina, were screened for host specificity and more recently released in South Africa. Although possessing an origin in the Caribbean islands and thus being compatible with the SA biotype of *C. odorata*, attempts to establish *D. odorata* in South Africa appear to have failed thus far. The most likely explanation is poor climatic matching, but predation may also play a role (Nqayi 2019; ARC-PHP unpubl. data). *Lixus aemulus* has a long lifecycle and slow rate of population increase, and was collected from a genotype of *C. odorata* different to the SA biotype in a tropical area with high rainfall. Permission for the release of *R. parvula* was only granted in 2016, and the insect was collected from *Chromolaena hookeriana* (Griseb.) R.M. King & H. Rob. and is univoltine (Zachariades et al. 2011). Hence the forecast of establishment for these two biocontrol agents is also still unclear, and if they do establish, it will take many years for them to become widespread and abundant. However, *C. odorata* remains untouched in provinces like Limpopo in South Africa and continues to pose a threat to native biodiversity (Dube et al., 2017; personal observations). Therefore, it was desirable to examine the life history traits and host specificity of a further candidate, the stem-galling fly *Polymorphomyia basilica* Snow (Diptera: Tephritidae) from Jamaica, for release as a biocontrol agent against *C. odorata* in South Africa.

5.3 Materials and methods

5.3.1 Collection of cultures imported into South Africa

Polymorphomyia basilica was considered as a replacement for *Cecidochares connexa* Macquart (Diptera: Tephritidae), which has been triumphant in controlling the Asian West African biotype AWAB of *C. odorata* in South-East Asia (e.g. Day et al. 2013) and established in West Africa (Paterson and Akpabey 2014; Aigbedion-Atalor et al. 2019). However, a culture of *C. connexa* could not be sustained on the SAB *C. odorata* in the laboratory (Zachariades et

al. 1999), probably because of the high level of host-specificity of the fly, which was originally collected from the Caribbean coast of Colombia. *Polymorphomyia basilica* was imported from Cuba and Jamaica into South African quarantine several times (see Zachariades et al. 2011), but initially the insect could not be cultured. This was because (i) too few adults eclosed to start a culture, due to a high parasitism rate and/or because the galls with larvae and pupae were difficult to keep in good condition in the laboratory. Unlike *C. connexa,* where galls are woody and hardy, and can be dissected and pupae removed, *P. basilica* have softer, smaller galls that shrivel quickly. In addition, the larvae push their spiracles through the gall "window" before pupating and thus pupae cannot be dissected out of galls; or (ii), once a technique had been developed to maintain galls in a good condition until adults eclosed (through rooting the cut stems with galls in a mistbed), although a good number of flies eclosed at the same time, no F1 generation that was obtained, presumably because females did not lay fertile eggs (Zachariades et al. 2007).

After being shelved for some years, *P. basilica* was again collected in Jamaica and imported into quarantine in South Africa in November 2012, in a further attempt to culture it. About 100 galls containing pupae and/or larvae were collected and a culture was successfully reared from this batch. This could be attributed to improved quarantine conditions e.g. space and light that was now available, and/or the use of enzymatic yeast hydrolase (see below section 5.3.2 page 86). The culture of *P. basilica* imported into South Africa was collected at 24 sites in Jamaica, on plants of *C. odorata*. A collection and export permit was issued by the National Environment and Planning Agency on 27 November 2012, Reference No. 18/27. Only galls without exit holes made by either flies or parasitoids were collected. Galls were collected together with a 5 cm length of stem below the gall, in order to root the stem which would keep the gall and its contents alive. The galled stems were dipped into rooting hormone, placed in seedling trays containing damp vermiculite, and the seedling tray was placed into a transparent plastic bag to maintain humidity, in an area with plentiful light but no direct sun. By the end of the field trip most of the stems had rooted, and stems were packaged for return to South Africa by removing them from the vermiculite and covering the roots and stem bases with damp tissue paper. Cuttings with galls were then placed in a plastic aerated tub.

5.3.2 Culturing methods in quarantine

Rooted stems were placed into individual small pots in a large emergence box with glass top and handling sleeves, in a glasshouse of ARC-PHP's Cedara, KZN, South Africa quarantine

facility. Galls where there was no "window" indicating pupation (before pupating, the larva chews a tunnel to the exterior of the gall, leaving only a thin epidermal through which it can escape as a newly eclosed adult) and no roots were placed in the mistbed for a few days, but returned to high quarantine if they pupated. Out of 77 galls that were rooted, 40 adult flies were obtained (n = 21 females, 16 males, 3 not sexed), as well as about 20 unidentified hymenopteran parasitoids. These included a small orange larval parasitoid (n = 13) that bored out through the wall of the gall, and a larger, black hymenopteran (n = 6) that emerged through the pupal window and was thus presumably a larval-pupal parasitoid. A few other hymenopterans also emerged from the galls.

Upon eclosion, adults were placed onto SAB *C. odorata* plants in the quarantine laboratory at ARC-PHP, Cedara. These plants were grown from field-collected (southern African biotype) shoot-tip cuttings rooted in a heated mistbed, and then transferred into 18cm diameter pots in a mixture of river sand and Gromor^{TM} potting medium at a ratio of 1:1. Plants were fertilized using either Osmocote^{TM} or a fertigation dripper system. Six standard insect cages (0.5 x 0.5 x 0.9m with a steel frame and gauze panels) were used as breeding cages. Each cage had a transparent plastic curtain covering the entrance to prevent the vagile adults from escaping. Four plants were placed into each cage, together with 10 pairs of adults (females are easily distinguished from males by the presence of a prominent ovipositor). After two weeks, adults were captured using glass vials, and plants were replaced. Plants on which eggs had been laid were placed in a large walk-in cage (2 x 4 x 2m) to allow for larval development. Eclosing adults were captured and used in culturing and experiments.

Enzymatic yeast hydrolase, mixed with sugar in a ratio of 1:3, was dispensed dry in small containers in each cage containing adults, as the adult females of some tephritid species require the nutrients contained in such foods to develop their ovules (M.P. Hill, Rhodes University, B. Barnes, retired ARC-Infruitec, pers. comm. 2012). This technique may have contributed towards the success of culturing the fly, as oviposition and galls were obtained, and subsequently many generations of flies used in both biology and host-specificity studies, and it continues to be used.

5.3.3 Life history traits of *P. basilica*

To determine the biology of *P. basilica*, a pair of newly emerged adults was exposed to one *C. odorata* plant with more than 10 shoot tips in a 0.4 x 0.4 x 0.9m cage. Plants were watered

using a Blumat^{TM} permeable clay cone inserted into the soil of the pot, and replenished via capillary action from 2 litre bottles which were filled 1-2 times a week. This obviated the need for manual watering and thus decreased the chances that flies would escape. Enzymatic yeast hydrolase was prepared in 1:3 enzyme:sugar ratio as a nutrient source. Cages were inspected 5 times a week to determine adult longevity, pre-oviposition period, shoot-tip probing (i.e. oviposition attempts), gall formation, gall maturation and adult eclosion. Plants were replaced after 80% of the shoots had been probed, to allow the females more oviposition resources. To determine if eggs of *P. basilica* are oviposited singly or in clusters, four *C. odorata* plants with more than 15 growing shoots were exposed to 5 pairs of *P. basilica* for 2 days. Twenty-five probed shoots were collected from the 4 plants and dissected and inspected under the microscope at 12x magnification in the laboratory.

5.3.4 Host-range trials

5.3.4.1 Test-plant list

Choice of test plants was as per Dube et al. 2017/Chapter 3 but in this chapter *Distephanus anisochaetoides* was replaced by *Distephanus angulifolius*. Basically, test plants were selected according to the proposed centrifugal testing criteria of Wapshere (1974), bearing in mind advances in both the phylogeny of the Asteraceae (Funk et al., 2009) and in host-plant selection approaches (Briese 2005). The main taxonomic level at which species were ranked was Tribe (Table 5.1). None of the five Eupatorieae indigenous to South Africa (Retief 2002) are in the same subtribe as *C. odorata*, although this is disputed for one of the indigenous species, *Stomatanthes africanus* (Oliv. & Hiern) R.M. King & H. Rob. (Anderberg et al. 2007), which was previously placed within the same genus (*Eupatorium*) as *C. odorata*. Several other alien species of Eupatorieae, all of American origin, are invasive in South Africa (Table 5.1), and these were included in the host specificity tests in order to obtain a better idea of the host range of *P. basilica*, rather than because an attack on these species in South Africa would be considered in a negative light. The closely related Tribe Heliantheae sensu stricto contains the major crop species *H. annuus* (sunflower) and a number of indigenous species, and was therefore also tested fairly intensively (Table 5.1). Other tribes of the Asteraceae were less intensively tested, because they are phylogenetically more distant to *C. odorata*.

Table 5.1: Test plants list used for *P. basilica* and their degree of separation between *C. odorata* and test plant species.

Subfamily Tribe[a]	Subtribe[a]	Degree of phylogenetic separation[b]	Plant species	Status[d]
Asteriodeae				
Eupatorieae	Praxelinae	0	*Chromolaena odorata*[c]	A,I
Eupatorieae	Oxylobinae	0	*Ageratina adenophora*[c]	A,I
Eupatorieae	Oxylobinae	0	*Ageratina riparia*[c]	A,I
Eupatorieae	Eupatoriina	0	*Stomatanthes africanus*[c]	N
Eupatorieae	Adenostemmatinae	0	*Adenostemma caffrum*	A,I
Eupatorieae	Adenostemmatinae	0	*Adenostemma viscosum*	N
Eupatorieae	Ageratinae	0	*Ageratum conyzoides*	A,I
Eupatorieae	Mikaniinae	0	*Mikania capensis ex* KZN	N
Eupatorieae	Gyptidinae	0	*Campuloclinium macrocephalum*[c]	A,I
Heliantheae	Helianthinae	3	*Helianthus annuus* PAN 7095 CL	A,C
Heliantheae	Helianthinae	3	*Helianthus annuus* AGSUN 8251	A,C
Heliantheae	Helianthinae	3	*Helianthus annuus* P65LC54	A,C
Heliantheae	Helianthinae	3	*Helianthus tuberosus*	A,C
Heliantheae	Spilanthinae	3	*Spilanthes mauritianum*	N
Heliantheae	Ecliptinae	3	*Wedelia natalensis*	N
Tageteae	Pectidinae	5	*Tagetes erecta*	A,O
Coreopsidae	Creopsidinae	6	*Bidens schimperi*	N
Anthemidae	Artemisia Group	11	*Artemisia afra*	N
Anthemidae	Phymaspermum Group	11	*Schistostephium heptalobum*	N
Astereae	Unplaced Genus	11	*Microglossa mespilifolia*	N
Astereae	Homochrominae	11	*Felicia amelloides*	N
Calenduleae	n/a	11	*Osteospermum muricatum*	N
Calenduleae	n/a	11	*Chrysanthemoides monilifera*	N
Senecioneae	n/a	11	*Delarea odorata*	N
Senecioneae	n/a	11	*Senecio tamoides*	N
Senecioneae	n/a	11	*Senecio deltoides*	N
Cichorioideae				
Vernonieae	Gymnantheminae	13	*Distephenus angulifolius*	N
Arctoteae	Arctotidinae	13	*Arctotis arctotoides*	N
Cichorieae	Cichoriinae	13	*Cichorium intybus*	A,C
Cichorieae	Lactucinae	13	*Lactuca sativa*	A,C
Carduoideae				
Cardueae	Carduinae	16	*Cynara scolymus*	A,C

[a]From Anderberg et al. (2007).
[b]At Tribe level, based on Funk et al. (2009) and Briese (2005).
[c]Previously all in the genus Eupatorium.
[d]A = alien, C = crop, I = invasive, N = native, O = ornamental.

5.3.4.2 Paired-choice trials

Preliminary paired-choice trials were conducted using adult *P. basilica* and nine species of Asteraceae, in order to obtain some idea of the fly's host range. A similar method was used for *R. parvula* host-range trials (Zachariades 2015, unpublished report): one control plant (*C. odorata*) and one test plant were placed diagonally opposite one another in a cage of the same type as the breeding cages. The position of the plants was determined using a random number system. Three pairs of adults, between 7 and 10 days old, were introduced into the cage, into which a container with a piece of wet Oasis™ floral foam was placed, to provide drinking water to the adults. A bottle cap containing enzymatic yeast hydrolase was provided. The top half of each cage was wrapped in a transparent plastic sheet to decrease airflow and increase humidity. The plants were rotated clockwise by 90° every two days, and the trial was terminated 2 days after the final rotation (usually 9-10 days because plants were not rotated at the weekend). During each rotation, any dead flies were recorded. If the dead fly was male, it was replaced, and if female, it was not replaced. The total number of live flies and their gender, and of dead and missing flies, was recorded when the trial was terminated. Plants were removed from cages, labelled, and set aside to record gall development.

Plants were inspected 7-20 days after removal from the cages for the presence of galls; at this time the number of shoot tips considered suitable for oviposition were also counted. For some of the test plants, a few shoot tips were dissected to check for oviposition. All plants were inspected again after 42 days, with the following parameters recorded: number of galls without pupation windows, those with pupation windows but from which adults had not yet exited; and those from which adults had exited. A few plants were inspected on an *ad hoc* basis thereafter. Only one replicate per test plant species was conducted, therefore no statistical analysis was carried out.

5.3.4.3 No-choice trials using adults

Thirty-two test plant taxa possessing at least 25 shoot tips per plant were exposed to newly emerged pairs of *P. basilica* in 0.4 x 0.4 x 0.9m cages. Because adults do not feed on plant tissues, the tests were narrowed to record only oviposition response and to follow larval survival to adulthood. Enzymatic yeast hydrolase was prepared in a 1:3 enzyme: sugar ratio as a nutrient source, particularly to allow females to develop their ovules. Plants were exposed to *P. basilica* adults for 25 days. Cages were inspected daily (from Monday to Friday) to confirm the presence of adults, and adults were replaced by newly eclosed ones if they escaped or died

before ten days (approximate pre-oviposition period) and with the adults from the culture if they died after 10 days. The numbers of probes and galls present were counted on the last day of exposure to adults, and thereafter the plants were inspected once a week, first for gall formation and then adult eclosion. Gall diameters were measured after the adults had eclosed.

Adults sometimes failed to mate irrespective of suitable conditions e.g. light, humidity or food. Because only one pair per test plant per replicate was used in these trials, to ensure that the pair used consisted of fertile adults, the experimental design was modified by, after exposing test plants to *P. basilica*, exposing the same pair to *C. odorata* as a "second control" for 10 days. These plants were inspected for gall formation and survival of progeny to adulthood.

For adult no-choice trials, the control (*C. odorata*) was compared separately to each test species using a Mann-Whitney Unpaired comparison, for the number of probes, galls formed, galls with pupated larvae, adults eclosed and gall sizes of the eclosed adults. For comparison of a second control with its test plant, Wilcoxon Matched Pairs test was used.

5.4 Results

5.4.1 Life history traits of *P. basilica*

Adults (Fig 5.1A) of *P. basilica* are diurnal and are strong fliers. After a pre-oviposition period of 11.4 ± 0.64 (range 5-16) days, females probed and inserted eggs into the tissue of young leaves within the shoot tip, visible through a scar (Fig 5.1B (i)). The egg hatches and the larva tunnels into the stem and the internodal stem below the shoot tips start swelling into a helical gall after a period of 9-11 days (Fig 5.1B (ii)), as the young larva moved down the stem. To assess the average number of eggs laid would have required physical disruption of plant tissue. Instead, female fecundity was measured by the number of galls formed per plant and per female. In 25 shoots that were dissected it was found that *P. basilica* female lays one egg, 0.73 ± 0.01mm in length and 0.28 ± 0.02mm width (mean ± SE) (n=15), in the tissue of a young leaf whereby every scar contains an egg. There can be more than 1 scar in one shoot, eggs being deposited in each young leaf opposite to one another, as a result on several occasions, more than one larva was recorded from the same stem (Fig. 5.1C). Nevertheless, each gall always contained only one larva. In the laboratory, the *P. basilica* female probed and deposited eggs in the shoot tips of the plant throughout the year.

Adults were long-lived (females: 48.8 ± 8.03 days (mean ± SE) (n = 17), up to 109 days; males: 36.2 ± 10.72 days (n = 14), up to 126 days). Females of *P. basilica* probed 46.8 ± 12.37 (mean ± SE) (n = 18) shoots and these developed into 42.9 ± 13.0 (n = 17) galls, with up to 159 galls per female. Newly eclosed females which spent 4-11 days with a male (i.e. during their preoviposition period) did not produce galls (n = 4), but those that spent between 18-97 days paired with males continued laying viable eggs even in the absence of the male (n = 6).

Pupation windows (Fig. 5.1D) developed in 36.8 ± 11.6 (mean ± SE) (86%) of the galls, with a maximum of 130 per adult female, and adults eclosed (Fig. 5.1E (i)) from 83% of these. In plants where galled stems died and galls shrivelled as a consequence, adults were still able to eclose from galls with a window (n=21) (Fig. 5.1E (ii)). In the laboratory, *P. basilica* completed its lifecycle (from egg laying to adult eclosion) in about 5-8 weeks (49.08 ± 1.97 days) (mean ± SE) (range: 38-60 days) (n=54). After initial gall formation, larval development took around 3 weeks (21.9 ± 2.01) (mean ± SE) (n=59) before the appearance of a window. Pupation lasted 13-22 days (16.84 ± 0.50) (mean ± SE) (n=54) before adults eclosed. No diapause period was noted in the laboratory.

Some predation was observed in the laboratory. Galls were partly eaten from the exterior in order to access and feed on the larva or pupa inside. The predator was not seen. *Dichrorampha odorata* larvae, which sometimes infected the *P. basilica* culture because both were being reared in the laboratory, were also recorded tunnelling into *P. basilica* galls and feeding on the fly larvae. This was particularly the case where *P. basilica* hatched first and *D. odorata* followed, however, in the field and because of their co-evolution, *P. basilica* may not lay eggs in a shoot tip already attacked by a *D. odorata* larvae or vice versa.

It was observed that most windowed dried galls eclosed whilst most to all pupal mortality was observed from the green galls. In addition, predation and other mortality (rotten larvae or pupae) were observed only for green galls (with both larvae and pupae), whilst the dry galls were not predated.

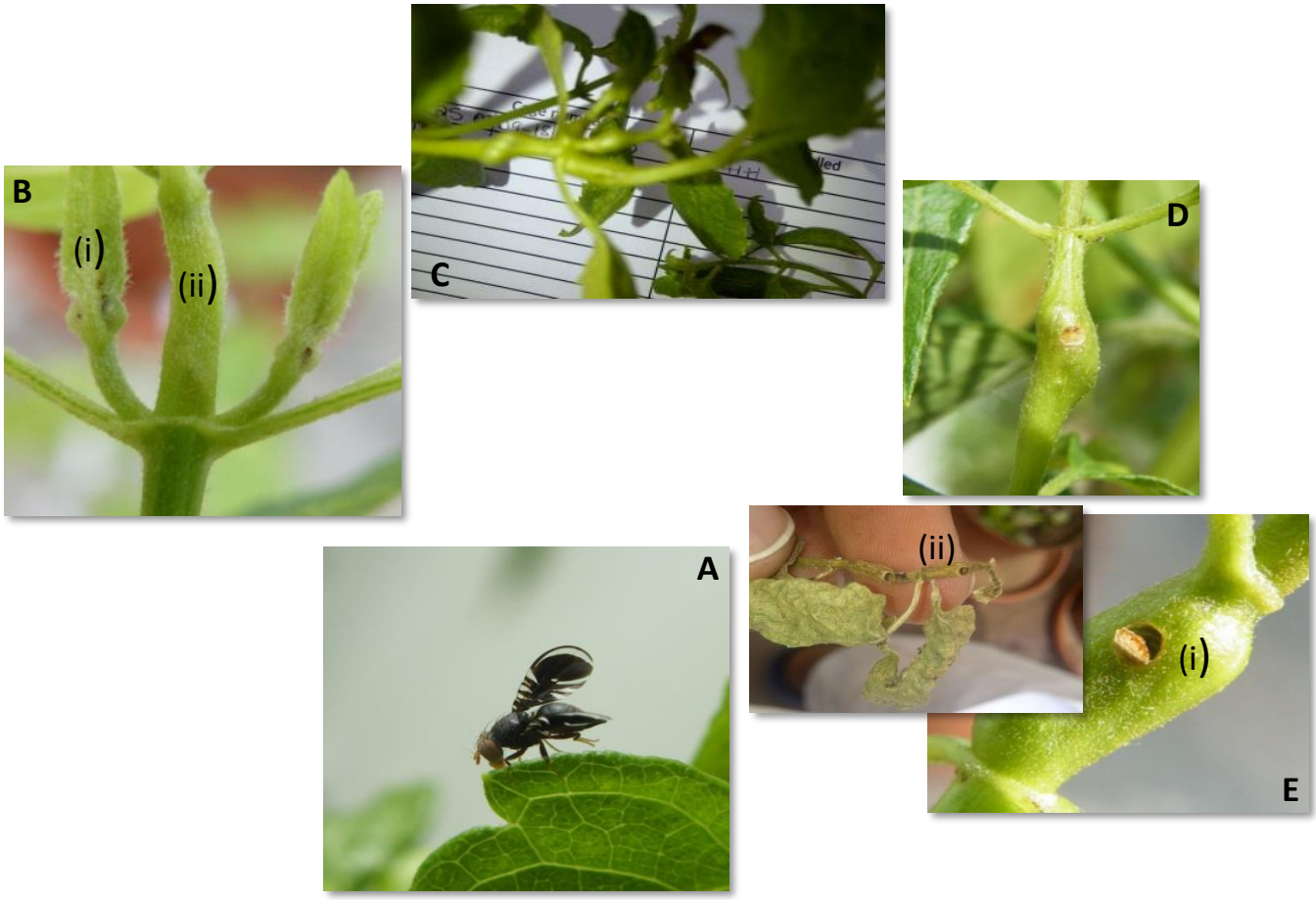

Figure 5.1: Indicating life stages of *P. basilica*, A an adult, B (i) female probe with egg, (ii) swelling of *C. odorata* as a result of growing larva, C galls continue to grow as a result of growing larvae, D window formed by the larvae before pupating and E (i) open gall cases indicating that an adult has eclosed (ii) including eclosion on dry galls.

5.4.2 Host specificity of *P. basilica*

5.4.2.1 Preliminary paired-choice trials using adults

In general, the methodology used appeared successful and appropriate, although, given what is now known about the fecundity of females, there were probably too many adults per plant. Many adults survived throughout the trial period, and many galls were formed on controls (Table 5.2). Galls were also recorded on three test species. No eggs were found in shoot tips of *Mikania capensis* DC. ex KZN or *Ageratina adenophora* (Spreng.) King & H. Rob. that were dissected. Galls were present on stems supporting flowers or flowerbuds on *Campuloclinium macrocephalum* (Less.) DC. (pompom weed), *Adenostemma viscosum* J.R. Forst. & G. Forst., *Ageratum conyzoides* L. and *C. odorata*.

Forty-two days after plants were removed from the trials, larvae in the majority of galls on control plants had pupated and exited (Table 5.3). Larvae in the majority of galls on *A. conyzoides* had also pupated, as had half the larvae on *C. macrocephalum*. However, no adults had exited from galls on these two species. Galls on *A. viscosum* remained very small, with none of the larvae pupated, or split open. At 56 days after termination of trials, adults had eclosed from five galls on *A. conyzoides*, while one had eclosed on *C. macrocephalum*. At this time, some parts of these plants had died, so these numbers may be conservative.

5.4.2.2 Adult no-choice trials

Probing activity was recorded only on *C. odorata* during trials (Table 5.4). However, females also laid fertile eggs on *Stomatanthes africanus* (Oliv. & Hiern) R.M. King & H. Rob., *Felicia amelloides* (L.) Voss and *A. viscosum* (indigenous), and on *Ageratina riparia* (Regel) R.M. King & H. Rob., *C. macrocephalum* and *A. conyzoides* (all invasive alien species), indicating that probe marks were difficult to see on test plant species. Galls on *S. africanus* and *F. amelloides* were low in number and with small diameters, and the only equivalent plants to *C. odorata* were (i) *A. conyzoides*, and (ii) *C. macrocephalum*. Both species equally supported the development of *P. basilica* and the flies produced galls equivalent in diameter to those on *C. odorata* (although the paired-choice trials indicated slower development of the larvae on these species). The only indigenous species which was previously in *Eupatorium* is *S. africanus*. A few galls were recorded on *A. viscosum* but could not be sustained through to pupation. Six galls were found on one replicate of *F. amelloides* and one adult eclosed whilst the other five did not pupate or eclose. For both *S. africanus* and *F. amelloides*, the gall from which the adult eclosed was very small compared to normal galls found on *C. odorata* (Table 5.4), and the adult itself was smaller than those developing on *C. odorata*, and had a short lifespan. Overall, *P. basilica* formed significantly more galls, with higher larval survival and adult eclosion compared to the other test species (Table 5.4).

For *P. basilica* exposed to a second control, gall formation and survival of progeny to adulthood was high, even though the nonparametric Wilcoxon Matched Pairs test did not indicate any significant difference between test plants and the second control (Table 5.4). In some cases, gall formation was significantly higher on the second controls of test plants than on the second controls of *C. odorata* (Table 5.4), possibly because the females had more eggs available, as they had not laid on the test plants.

Table 5.2: Numbers of galls on test and control plants after 7-20 days, and the percentage of shoot tips galled on these plants in paired-choice trials.

Test plant species	No. galls		% shoot-tips galled	
	Control	Test species	Control	Test species
Mikania capensis ex KZN	37	0	102.8	0
Ageratina adenophora	31	0	40.3	0
Ageratum conyzoides	21	18	35.6	34.6
Campuloclinium macrocephalum	20	7	48.8	33.3
Adenostemma viscosum	14	9	41.2	18.0
Ageratina riparia	42	0	68.9	0
Melanthera scandens	19	0	59.4	0
Cineraria saxifraga	38	0	48.7	0
Schistostephium flabelliforme	28	0	77.8	0

Table 5.3: Percentage of galls in which larvae had pupated (including those exited) and percentage from which adults had eclosed, 42 days after termination of adult paired-choice trials.

Test plant species	% galls pupated		% galls exited	
	Control	Test species	Control	Test species
Mikania capensis ex KZN	76.2	n/a	38.1	n/a
Ageratina adenophora	90.0	n/a	53.3	n/a
Ageratum conyzoides	88.0	60.0	68.0	0
Campuloclinium macrocephalum	69.6	50.0	56.5	0
Adenostemma viscosum	68.8	0	56.3	0
Ageratina riparia	67.5	n/a	52.5	n/a
Melanthera scandens	82.4	n/a	58.8	n/a
Cineraria saxifraga	91.7	n/a	88.9	n/a
Schistostephium flabelliforme	97.2	n/a	77.8	n/a

Table 5.4: No-choice trials using *Polymorphomyia basilica* adults and species of Asteraceae, conducted in the quarantine laboratory at ARC-PHP Cedara.

	Test plant						*Chromolaena odorata* afterwards			
Plant species	N	No. probes (SE)[a]	No. galls formed (SE)[a]	No. galls pupated (SE)[a]	No. adults eclosed (SE)[a]	Gall sizes of eclosed adults (mm) (SE)[a]	N	No. galls formed (SE)[a,b]	No. galls pupated (SE)[a,b]	No. adults eclosed (SE)[a,b]
Chromolaena odorata	34	20.53 (1.67)a	25.18 (2.29)a	22.32 (2.25)a	19.03 (2.38)a	3.6 (0.3)a (N=646)	17	20.0 (2.29)a	17.18 (2.38)a	15.35 (2.51)a
Adenostemma caffrum	6	0b	0b	0b	0b	-	3	14.67 (2.19)a	9.67 (1.08)a	8.0 (1.41)a
Adenostemma viscosum	6	0b	1.5 (0.56)b	0b	0b	-	3	13.0 (7.57)a	11.0 (7.37)a	10.33 (6.74)a
Mikania capensis ex KZN	6	0b	0b	0b	0b	-	3	12.67 (5.49)a	12.67 (4.91)a	8.0 (4.04)a
Stomatanthes africanus	6	0b	3.0 (1.86)b	0.17 (0.17)b	0.17 (0.17)b	1.8 (N=1)	3	12.33 (2.85)a	10.33 (2.96)a	7.00 (2.65)a
Ageratina adenophora	6	0b	0.33 (0.21)b	0b	0b	-	3	18.67 (8.95)a	18.0 (8.96)a	16.67 (9.56)a
Ageratina riparia	6	0b	1.83 (0.75)b	1.00 (0.45)b	0.33 (0.33)b	3.3 (0.1) (N=2)	3	14.33 (3.84)a	13.33 (3.28)a	8.33 (4.91)a
Campuloclinium macrocephalum	6	0b	7.33 (1.90)b	3.17b (1.83)b	3.17b (1.83)b	3.8 (0.2)a (N=19)	3	12.0 (5.51)a	6.33 (5.84)a	6.0 (5.51)a
Ageratum conyzoides	6	0b	6.67 (3.89)b	4.17 (3.80)b	4.0b (3.74)b	3.6 (0.1)a (N=23)	3	14.33 (4.81)a	11.33 (4.06)a	10.33 (3.28)a
Wedelia natalensis	6	0b	0b	0b	0b	-	3	24.0 (7.50)a	22.0 (8.54)a	21.33 (8.95)a
Spilanthes mauritianum	6	0b	0b	0b	0b	-	3	16.67 (4.67)a	15.67 (4.33)a	14.33 (3.28)a
Helianthus annuus PAN 7095 CL	6	0b	0b	0b	0b	-	3	29.33 (0.88)b	26.67 (1.45)a	24.33 (1.76)a
Helianthus annuus AGSUN 8251	6	0b	0b	0b	0b	-	3	27.67 (2.19)a	25.67 (2.19)a	24.33 (1.86)a
Helianthus annuus P65LC54	6	0b	0b	0b	0b	-	3	23.00 (2.65)a	19.00 (2.08)a	13.00 (2.31)a
Helianthus tuberosus	6	0b	0b	0b	0b	-	3	32.33 (3.84)b	30.33 (4.48)b	29.67 (3.84)b
Tagetes erecta	6	0b	0b	0b	0b	-	3	9.00 (1.15)b	8.00 (1.52)a	6.33 (1.86)a
Bidens schimperi	6	0b	0b	0b	0b	-	3	29.67 (1.45)b	27.00 (1.00)b	25.00 (0.58)a
Delairea odorata	6	0b	0b	0b	0b	-	3	17.33 (5.86)a	16.00 (6.50)a	7.33 (3.28)a
Senecio tamoides	6	0b	0b	0b	0b	-	3	13.33 (9.94)a	13.00 (9.61)a	12.33 (9.87)a
Senecio deltoideus	6	0b	0b	0b	0b	-	3	14.67 (0.88)a	12.67 (0.33)a	11.67 (0.33)a
Artemisia afra	6	0b	0b	0b	0b	-	3	17.00 (2.87)a	15.33 (2.33)a	13.00 (4.58)a
Schistostephium heptalobum	6	0b	0b	0b	0b	-	3	27.67 (4.10)a	24.33 (3.48)a	18.33 (3.76)a
Microglossa mespilifolia	6	0b	0b	0b	0b	-	3	24.33 (5.24)a	21.67 (5.21)a	21.67 (5.21)a
Felicia amelloides	6	0b	1.00 (1.00)b	0.17 (0.17)b	0.17 (0.17)b	2.2 (N=1)	3	19.67 (6.39)a	17.00 (4.93)a	15.33 (5.40)a
Chrysanthemoides monilifera	6	0b	0b	0b	0b	-	3	15.33 (6.64)a	13.33 (6.06)a	10.00 (5.13)a
Osteospermum muricatum	6	0b	0b	0b	0b	-	3	12.00 (6.66)a	10.67 (8.64)a	9.00 (9.25)a
Garuleum sonchifolium	6	0b	0b	0b	0b	-	3	11.67 (4.81)a	10.67 (4.41)a	10.67 (4.41)a
Distephanus angulifolius	6	0b	0b	0b	0b	-	3	22.33 (3.18)a	17.67 (4.41)a	13.33 (5.36)a
Lactuca sativa	6	0b	0b	0b	0b	-	3	11.67 (0.88)b	11.33 (0.88)a	10.0 (0.58)a
Cichorium intybus	6	0b	0b	0b	0b	-	3	20.0 (2.31)a	17.67 (1.45)a	15.67 (1.76)a
Arctotis arctotoides	6	0b	0b	0b	0b	-	3	35.67 (1.76)b	29.33 (4.10)b	28.67 (4.26)a
Cynara scolymus	6	0b	0b	0b	0b	-	3	36.33 (2.19)b	35.00 (1.73)b	33.67 (0.88)b

[a]Within the same column, different letters following mean (SE) indicate a significant difference ($p < 0.05$) between the control (*C. odorata*) and the test species. Mann-Whitney U comparison.
[b]Comparison of control 2 with its test plant (no. galls, no. windows, no. adults): Wilcoxon Matched Pairs test, $p > 0.05$ for all comparisons.

5.5 Discussion

Polymorphomyia basilica exhibited positive life history traits and high levels of host specificity, similar to several other gall formers (Harris and Shorthouse 1996; Goolbsy et al. 2000; Diaz et al. 2014; Mukwevho et al. 2017), particularly tephritids. Adults of *P. basilica* are diurnal and are strong fliers (see Aluja and Norrbom 2001) and sexes look similar but are differentiated by the presence of a prominent ovipositor at the posterior of the female's abdomen (as in Balciunas and Mehelis 2010). The pre-oviposition period of *P. basilica* ranged from 5-16 days; the end of this period was confirmed by the presence of visible scars (that developed into stem galls), formed by a female inserting her ovipositor into the tissue of young leaves within the shoot tip in an attempt to insert eggs. The ovipositors of *C. connexa* females form similar probes in growing *C. odorata* shoot tips as they lay eggs in these shoots (McFadyen et al. 2003). *Cecidochares connexa* differs from *P. basilica* in that the female lays a cluster of eggs in each shoot tip (McFadyen et al. 2003).

In the laboratory, female *P. basilica* probed and deposited a good number of 0.73 ± 0.01 mm eggs (with high percentage of survival to adulthood) in the shoot tips of the plant throughout the year. Shoots that were dissected indicated that there can be more than 1 scar in one shoot and eggs can be deposited in each young leaf opposite to one another, as a result on several occasions, more than one larva was recorded from the same stem. More than one egg/gall in the shoot could be because of multiple events caused by limited plant shoots in the cage. For example, eggs of *Tephritis dilacerate* Loew (Diptera: Tephritidae) measured 2.6-5.7 mm in diameter (Peschken 1979) whilst eggs of *Parafreutreta regalis* Munro (Diptera: Tephritidae) measured 0.58 ± 0.02 mm (mean ± SE) (Balciunas and Mehelis 2010). This variation in egg sizes could be attributed to variation in location (stems, roots or flower heads) and structure (simple ovules or complex) of tephritid galls (Friedberg 1984).

The internodal stem below the shoot tips started swelling into a helical gall after a period of 9-11 days indicating egg hatching. By comparison, *T. dilacerata* hatched in 4-5 days (Peschken 1979) whilst the galls of *Urophora cardui* (L.) (Diptera: Tephritidae) began to form 15 days after oviposition (Peschken and Harris 1975) and 8-13 days for *P. regalis*

(Balciunas and Mehelis 2010). This study did not assess larval instars of *P. basilica* but records of tephritid flies show three larval instars (Headrick and Goeden 1998) (e.g. Peschken 1979 and Balciunas and Mehelis 2010). The larva feeds and develops on the enriched contents of the gall, causing it to compete with the plant organs for nutrients and photosynthate, and reducing chemical defences such as phenolics, while the larva remains protected from adverse abiotic conditions such as desiccation (Stone and Schonrogge 2003). *Polymorphomyia basilica* pupation is completed inside the gall, and before pupation the larva chews a tunnel through the wall of the gall, leaving only a thin epidermal layer or "window" which the emerging adult easily breaks through upon exit (as in Friedberg 1984; Gassmann et al. 2014). In *C. odorata* plants where galled stems died and galls shrivelled as a consequence, adults were still able to eclose, if the gall already had a pupation window, signalling that *P. basilica* may be able to establish in relatively dry areas like northern KZN.

Adults of *P. basilica* were long-lived, ranging from one to four months but multivoltine. Newly eclosed females which spent 4-11 days with a male (i.e. during their preoviposition period) did not produce galls, but those that spent between 18-97 days paired with males continued laying viable eggs even in the absence of the male. This is a positive attribute that could permit release of fertile females that can establish a population. While multiple mating is common in insect species, either with different males or with the same male, a single or a few matings can be sufficient for females to fertilize their eggs in some insect species (Li et al. 2014).

Polymorphomyia basilica galls, which form in the stem internodes, are monothalamous and contain only one larva per cavity, unlike some other species of tephritids with polythalamous galls (Friedberg 1984). Balciunas and Mehelis (2010) reported a similar life history to that of *P. basilica* for the monothalamous *P. regalis* on *Delairea odorata* Lamaire (Asteraceae). Contrarily, *C. connexa* forms much larger communal galls that contain multiple larvae, at the stem nodes (McFadyen et al. 2003). The galls of the closely related *Procecidochares australis* Aldrich on *Heterotheca subaxillaris* (Lam.) Britt. and Rusby (Asteraceae) are also polythalamous (Friedberg 1984).

In laboratory trials, *P. basilica* accepted *C. odorata* as a main host. *Ageratum conyzoides* and *C. macrocephalum* were fairly acceptable for oviposition (27% and 29% the number of galls, respectively, compared to controls under no-choice conditions), although adults took longer to eclose under paired-choice conditions. Relatively few adults eclosed from *A. conyzoides* and *C. macrocephalum* during these tests (21% and 17% eclosion of adult progeny respectively, compared to the controls under no-choice conditions). Additionally, under same tests (no-choice trials) adults took longer to eclose on these plants than on controls. *Adenostemma viscosum*, *A. riparia*, *S. africanus* and *F. amelloides* were also accepted but this was minimal. *Adenostemma viscosum* could not sustain larval development whilst in *S. africanus* only 1 adult (which lived for less than 1 day) eclosed, out of 18 galls that were formed (94% mortality) across the six replicates. Similarly, *F. amelloides* had only 6 galls formed, all in 1 replicate and from these, only 1 adult eclosed (83% mortality). Two adults in total eclosed from *A. riparia*, from 11 galls formed (82% mortality). The diameter of normal galls of *C. odorata* on average were 3.6 mm whilst those of *S. africanus* and *F. amelloides* were 1.8 and 2.2 mm, respectively (50% and 61% of the diameter of the control), and the corresponding adults were small. Only 0.9% of the number of adults eclosing from the *C. odorata* controls eclosed from *S. africanus* and *F. amelloides*, and 1.7% from *A. riparia*. *Felicia amelloides* (tribe Astereae) was the only species outside the tribe Eupatorieae on which gall formation was recorded. Females tended to retain their eggs under no-choice conditions in the presence of an unsuitable host, and to compensate by ovipositing at a higher rate when presented later with a *C. odorata* plant.

Insecta as a group feed upon a highly diverse range of organic constituents, so it is remarkable that most species exhibit a high level of host specificity in their food selection. This is hypothesised to be driven by competition and natural selection, enabling each species to utilise a defined set of resources more efficiently than any of its competitors (Waldbauer 1968). Although true monophagy is reported among non-fruit-eating Tephritidae, many species are rather monophagous or narrowly oligophagous (Headrick and Goeden 1998). *Polymorphomyia* species and a number of other tephritids such as

Urophora solstitialis (L.) (Diptera: Tephritidae) are known to be gallers of asteraceous plants (Korytkowski 1971; Friedberg 1984; Woodburn 1993), and plants in this family usually possess multiple secondary compounds which are used in the defence of the plant from natural enemies. For example, *Lactuca serriola* L. (Asteraceae) and *C. odorata* contain flavonoids, terpenoids, and so forth (Elsharkawy et al. 2014; Omukhua et al. 2017). These chemical compounds often differ in their absolute and relative concentration and composition between plant species, as in *L. serriola* compared to *Achillea fragrantissima* (Forssk). Sch. Bip. (Asteraceae) (Elsharkawy et al. 2014). Insects with narrow host ranges ('specialists') have developed mechanisms to overcome specific secondary chemicals; this enables them to feed and develop on a single plant species (monophagy), or a group of closely related (and thus chemically similar) plant species (oligophagy). Some are even known to be attracted to secondary compounds such as pyrrolizidine alkaloids, which they sequester as defence chemicals or sex pheromones (Biller et al. 1994). Although *P. basilica* has generally manifested a high degree of host specificity, evident in the lack of oviposition and/or high larval mortality recorded from most test plants, it is not surprising that limited oviposition and larval development was recorded in some asteraceous plants other than *C. odorata*. This was inescapable especially in the eat-or-die conditions of no-choice trials, and very low survival of the progeny on a few selected non-target plants further attests to the specificity of this tephritid. Although, adults of *P. basilica* do not feed but females had a vital role of choosing whether to lay or not to lay on non-host plants in an "oviposit or leave no progeny" scenario (Jaenike 1990; Gripenberg et al. 2007; Rigsby et al. 2014). During larval no-choice trials of *D. odorata, S. africanus, A. riparia* and *A. conyzoides* were also nibbled but could not sustain survival of the moth (Dube et. al. 2017). The suitability of a plant species as a host is affected not only by the presence or absence of defensive chemicals but also of those which act to stimulate the insect into eating it. Waldbauer (1968) illustrated that poor growth in insects is attributed to a low rate of intake due to the absence of a non-nutrient phagostimulant; this might be the case in the plants that were occasionally selected by the female for oviposition but could not sustain significant development of *P. basilica* larvae. The fly completely avoided Senecioneae species; this is interesting as, along with *C. odorata,* this tribe has pyrrolizidine alkaloids (e.g. Hartmann and Dierich 1998; Hartmann 2009) and several other species tested as

potential biocontrol agents against *C. odorata* have displayed slight feeding on Senecioneae. This further illustrates the level of host specificity *P. basilica* possesses.

van Klinken (2000) has discussed the extrapolation of laboratory trial results into the field, to predict field host range. Based on this, it is likely that *C. macrocephalum* and *A. conyzoides,* plants of South American origin that are declared invaders under National Environmental Management Biodiversity Act, No. 10 of 2004 under Category 1b nationally (Department of Environmental Affairs, 2014a, b) had galls almost equal to those of *C. odorata,* and have a distribution in South Africa which at least partly overlaps that of *C. odorata*, will receive some feeding and possible oviposition damage in the field, but this is of no concern in South Africa. *Campuloclinium macrocephalum* is in any case subject to a biological control programme (McConnachie et al. 2011), while one of the biocontrol agents on *C. odorata* (*P. insulata*) has been recorded using *A. conyzoides* as a secondary host in the field but not on other non-target plants (Zachariades et al. 2011).

Regarding the possible use of *S. africanus, A. riparia* and *F. amelloides* as host plants by *P. basilica* in the field: although these plants could receive eggs, because they fall within the 'physiological host range' of the fly, oviposition levels in the field would not be meaningful because adults are expected to be highly mobile and fly elsewhere to locate suitable host, in this case *C. odorata*. Furthermore, in South Africa, *S. africanus* grows in high altitude grasslands in Mpumalanga and does not overlap with that of *C. odorata* which grows only in the subtropical lower altitude areas, so adults feeding on *C. odorata* would not come across *S. africanus* readily. *Ageratina riparia*, a weed originating in Central America and the Caribbean, is under a biological control programme in South Africa (Morris 1991) and is of no concern. In conclusion, we are confident that *P. basilica* is sufficiently host specific for release as a biological control agent in South Africa.

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CHAPTER 6: IMPACT OF *PAREUCHAETES INSULATA* ON PHYTOCHEMISTRY AND GROWTH MATRICES OF *CHROMOLAENA ODORATA*: COMPARISON IN PLANTS WITH AND WITHOUT SPECIALIST HERBIVORE *PAREUCHAETES INSULATA* (LEPIDOPTERA: EREBIDAE: ARCTIINAE)

6.1 Abstract

The Evolution of Increased Competitive Ability (EICA) hypothesis proposed that the successful invasion by alien plants in their introduced ranges results from an evolutionary shift in resource allocation from defence to growth due to release from natural enemies. A moth with defoliating larvae, *Pareuchaetes insulata*, has been confirmed as established since 2004 (released from 2001-2003) on *Chromolaena odorata* on the south coast of KwaZulu-Natal (KZN) province in South Africa, and has spread to northern KZN, Mpumalanga province and neighbouring countries but the moth is not present in Limpopo province, or in some interior regions of KZN. This study aimed at testing EICA on *C. odorata* from locations with and without *P. insulata*. Leaf extracts of plants from Thohoyandou (Limpopo province), Komatipoort (Mpumalanga province), Umkomaas (KZN) and Pietermaritzburg (KZN) were examined for plant defences using standard methods that quantify total phenolics, flavonoids and tannins. Plants collected from full sun and from shade in these four locations were grown under common greenhouse conditions, and the number of vegetative and flowering shoots, the plant height and the basal stem diameter were measured as plant growth parameters. Inconsistent with EICA, total phenolics and tannins were generally higher in Thohoyandou and Komatipoort and lower in Pietermaritzburg and Umkomaas. Flavonoids varied between the four locations, with higher concentrations in Komatipoort compared to Thohoyandou and Umkomaas, but not different from Pietermaritzburg. Growth parameters such as stem diameter, number of shoots and number of flowering shoots, supported EICA, as plants from the Thohoyandou and Pietermaritzburg sites, where *P. insulata* is absent, showed stronger growth and reproductive potential. This study demonstrates the possible role of *P. insulata* on the decrease in population of *C. odorata* where the moth has persisted and suggests that other

biotic and abiotic factors could be responsible for unpredicted results for phytochemistry assays.

Key words: EICA, biological control *Chromolaena odorata*, *Pareuchaetes insulata*, plant defence, plant growth rate

6.2 Introduction

Pollution is among the top global issues of which humans have continued to be driving forces through a variety of activities, changing the environment on local and global scales in ways that lead to species invasions and extinctions (Müller-Schärer et al. 2004; Hooper et al. 2005; Miller et al. 2010). Invasive alien species, including plants, play a major part in this by remarkably weakening ecological resilience through reduced biodiversity, disturbance of native plant communities, increased soil erosion, and degradation of wildlife habitats (Muller and Martens 2005). In South Africa most alien species found today were deliberately introduced many years ago, either with the goal of establishing populations in nature, or for horticulture, agriculture, forestry or the pet trade (from where some escaped to become invasive) (van Wilgen and Wilson 2018). The rest were introduced accidentally as commodity pollutants or as escapees on transport vectors (van Wilgen and Wilson 2018).

In an attempt to explain the reasons for the success of alien plant invasiveness, several hypotheses, including the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Notzöld 1995) (which is the evolutionary extension of the Enemy Release Hypothesis (ERH) (Keane and Crawley 2002)), and the Shifting Defence Hypothesis (SDH) (Müller-Schärer et al. 2004; Joshi and Vrieling 2005) have been derived. The EICA hypothesis states that the absence of specialist herbivores for non-indigenous plants in the introduction range can lead to decreased allocation to defence and a simultaneous increase in allocation to growth, and consequently to increased competitive ability (Blossey and Notzöld 1995). The ERH posits that invasive alien plants benefit directly from the release from natural enemies (Keane and Crawley 2002), and is the foundational hypothesis for the success of classical biological control of weeds. Studies that investigated the

mechanism of EICA revealed that evolutionary shifts in nitrogen allocation from cell walls (defence) to photosynthesis in invasive alien plant populations, resulted in faster growth and reduced structural and chemical defenses (Qin et al. 2013). According to EICA, plants in the invasive range should grow more vigorously and/or have a higher reproductive output and have a lower levels of defensive metabolites than plants in the native range. The ERH emphasizes that on introduction to an exotic region, plant species should experience a decrease in top-down regulation by herbivores and other natural enemies, resulting in an increase in distribution and abundance (Muller and Martens 2005). Enemy release and EICA hypotheses explain the dominance of invasive plants in the non-native range and are therefore among the fundamental principles of a biological control programme, which seeks to restore natural enemies of the invasive alien plant in an invasive range to achieve control (Mack 1995; te Beest et al. 2009). Tied to ERH and EICA is SDH, which is an extension of EICA. It predicts that after invasive plants are introduced to new ranges, consequent to escape from specialist herbivores they will evolve reduced resistance to these by lowering their expensive digestibility ('quantitative') compounds. However, because they are often still attacked by generalist herbivores in their introduction range (Müller-Schärer et al. 2004), they will increase their cheap, toxic defence ('qualitative') compounds which are effective against generalists.

A number of experiments exist that support the predictions of EICA and/or ERH for some invasive alien plant species, but for other species the evidence is either not convincing for either one or both of the hypotheses, or is the opposite (Stastny et al. 2005; Qin et al. 2013; Shelby et al. 2016; Becerra et al. 2017; Wang et al. 2017; Rouifed et al. 2018; Davis et al. 2019). This shows that occasionally, susceptibility to invasion by alien plants can be strongly influenced by several other factors, such as plant community composition, propagule pressure, disturbance regime and resource availability (Herms and Mattson 1992; Hooper et al. 2005; Moles et al. 2011; Gruntman et al. 2016). For example, Callaway and Ridenour (2004) propose that some invasive alien plants transform from native weaklings to invasive bullies by exuding biochemicals that are highly inhibitory or allelopathic to plants or soil microbes in invaded communities, but relatively ineffective against natural neighbours in the native range that had adapted over time. The authors refer

to this as the Novel Weapon Hypothesis (NWH). In addition to EICA, the NWH suggests the role of plant chemistry as a displacement mechanism for successful invasion (e.g. Dai et al. 2016).

The southern African biotype (SAB) of the invasive *Chromolaena odorata* (L.) R.M. King and Robinson (Asteraceae) is a scrambling shrub native to the Caribbean islands (Paterson and Zachariades 2013; Shao et al. 2018) which was first recorded as naturalised in South Africa in the late 1940s, when it was found near Ndwedwe, KwaZulu-Natal (KZN) (Zachariades et al. 2011). From KZN it spread rapidly into the Eastern Cape, Mpumalanga and Limpopo provinces, as well as into the neighbouring countries of eSwatini (Swaziland) and Mozambique (Goodall and Erasmus 1996). It has contributed enormously to a reduction in biodiversity and carrying capacity of native ecosystems in South Africa (Kluge 1990; Luwum 2002; te Beest 2010; Dew et al. 2016). The South African biological control programme has been in place since 1988 for long-term suppression of *C. odorata*, and several potential biocontrol agents (mainly insects) and pathogens have been assessed (Zachariades et al. 1999; Zachariades et al. 2011; den Breeyen 2002; Zachariades et al. 2016). The two agents established on *C. odorata* are a moth with defoliating larvae, *Pareuchaetes insulata* (Walker) (Lepidoptera: Erebidae: Arctiinae), and a leaf-mining fly, *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae). Monitoring conducted on *P. insulata* showed restoration of indigenous flora where the moth had persisted, with remarkable spread from the release points (Zachariades et al. 2016). Adaptation to local conditions is one of the important forms of evolution in invasive plant populations; hence, if evolutionary changes occur, this chapter seeks to investigate their pace and direction(s), which may help to improve predictions of the impact of subsequent biological control agents (Müller-Schärer et al. 2004).

There is ample knowledge of the phytochemistry of the Asian/West African biotype (AWAB) of *C. odorata* (Biller 1994; Thoden et al. 2007; Ngozi et al. 2009; Qin et al. 2013) but little is known about that of the southern African biotype (SAB) (Omokhua et al. 2017). Phytochemicals including saponins, phenolics, flavonoids and tannins were detected in the southern African biotype (Omokhua 2017). Whilst alkaloids were not detected in SAB *C.*

odorata, this group of secondary compounds is known to deter generalist and attract specialist herbivores (Macel 2011). Among plant constituents, it is generally acknowledged that plant phenolics play a role in protecting plants from both insect and mammalian herbivory (Robins et al. 1987; Clausen et al. 1992; Close and McArthur 2002; Barbehenn and Constabel 2011) through lowering the digestibility of the plant. They are known as quantitative defensive chemicals because their effectiveness is correlated to their concentration in the plant tissues. In general, phenolics are described as a very large group of structurally dissimilar plant secondary compounds including terpenoids, flavonoids and tannins (Bray and Thorpe 1954; Bennett and Wallsgrove 1994; Brielmann et al. 2006; Bakhvalov et al. 2009). Tannins are known to have the potential to be the most vital class of secondary metabolites in plant defense against herbivory because of their dominance in nature. Most herbivores, and certainly all generalist herbivores, routinely encounter tannin-rich diets as invasive alien plants escape specialist herbivores from their native range but often need defence against generalist herbivores in the introduction range (Joshi and Vrieling 2005). No other class of secondary metabolite is satisfactorily abundant in nature to cover the defence of such a broad array of plants (Clausen et al. 1992). Terpenoids are similarly well known as feeding deterrents to different mammals and generalist insects and attractants for host plant localization (Lerdau et al. 1994) whilst flavonoids also play a vital role in plant defence against herbivores and environmental stress such as photodamage (Treutter 2005). Customarily, methods for indicating that constitutive phenolics take part in plant defence have depended on measuring the total phenolic content of plant tissues (Lattanzio et al. 2006).

All studies that have investigated EICA, ERH or SDH use populations from both the invasive and native locations (e.g. Hinz and Schwarzländer 2004; te Beest et al. 2009; Feng et al. 2011; Qin et al. 2013; Shelby et al. 2016; Egli 2017; Kwong et al. 2019). Similarly, few studies that considered re-association of the specialist herbivore and its impact on the coevolved host plant in the country of introduction included plants from the native range (Zangerl and Berenbaum 2005; Zangerl et al. 2008; Rapo et al. 2010; Jogesh et al. 2014; Wan et al. 2019). A thorough knowledge of the variation among introduced populations in terms of their biological control history constitutes an excellent but yet

underrated outline to study the evolutionary ecology of invasive plants (Rapo et al. 2010). It was suggested that another way to improve our understanding of evolutionary changes in introduced plant populations in response to different herbivore assemblages is to compare life-history traits of these invasive populations within the introduced range that have experienced successful biological control with those of populations that have not been exposed to classical biological control (Rapo et al. 2010). Here, the infestation and decrease of *C. odorata* where *P. insulata* has persisted and its prolific densities or invasiveness in locations where *P. insulata* is permanently absent is used as a model to test the prediction of the EICA hypothesis. Plants from a location where the specialist herbivore *P. insulata* was released, had established and persisted since 2001 (probably the only site, out of 30 release sites, at which it did establish; Zachariades et al. 2016) as representative of the native range, were compared to plants from locations where *P. insulata* has never been recorded, as representatives of the invasive range. We also included a location to which *P. insulata* has only recently (first recorded in 2016) spread, to determine how the results will compare to those from the infested and uninfested sites. It is recommended that measuring growth rates alone would be a poor predictor of the competitive ability of plants (Shelby et al. 2016). Therefore, plant growth parameters were measured, along with flavonoid, phenolic and tannin contents. The following predictions were made:

1. Plant parts of *C. odorata* in the established site of *P. insulata* will have higher concentrations of secondary compounds (quantitative defences), lower concentrations where the insect has recently been discovered and lowest concentrations at the sites where *P. insulata* is absent.
2. *Chromolaena odorata* collected from the site where *P. insulata* is absent will grow faster and will have high reproductive output (vegetative shoots and flowers/flowering shoots); these metrics will be lower where the insect has just been discovered, and the plant will grow slowest with lowest reproductive output where *P. insulata* was released, has established and persisted since 2001.

6.3 Materials and methods

6.3.1 Collection of plant material for phytochemistry/secondary compounds

Study locations and collection protocol

To prepare for testing of the EICA hypothesis, plant materials were collected from 3 provinces *viz*. KZN, Limpopo and Mpumalanga in South Africa. These locations were selected on the basis of *Pareuchaetes insulata* presence for more than 15 years for the establishment site, for unknown time (but recorded between 1-2 years at the time of study) and where the moth is absent (Table 6.1). To control for the latitude in influencing the phytochemistry and growth rate (Moles et al. 2011) of *C. odorata* where *P. insulata* is absent, we also sampled a second site in KZN without *P. insulata*. We surveyed for the presence of *P. insulata* and *C. eupatorivora* in Limpopo province at 10 sites in May 2016 and none of the biocontrol agents were recorded. The distance from the establishment site i.e. Cannonbrae Sappi plantation, Umkomaas, KZN to Latunandwa river bank, Thohoyandou, Limpopo where there are no records of *P. insulata,* is 798 km; to Komati River Chalets, Komatipoort, Mpumalanga where *P. insulata* has recently been discovered is 543km, and to Peter Brown Drive, Pietermaritzburg, KZN, a control site where none of *C. odorata* biocontrol agents established, is 83km. To have full representation of the sites we collected plant materials from both a full-sun and a shaded habitat at each site.

Location 1: Umkomaas, Cannonbrae (the release and establishment site), KZN province

Chromolaena odorata plant parts (leaves, stems and roots) were collected from Cannonbrae Sappi plantation (see Table 6.1 for co-ordinates) at Umkomaas on a sunny day on the 08th November 2016. The plantation was inspected for 2 hours to identify mature *C. odorata* with thick stems of about 1.5-3 cm diameter, at a site with full-sun and a site with shade. Plants in full sun were characterized by yellowish-green, smaller leaves with no trees shading *C. odorata* plants, while the shaded site was characterized by plants growing under tall trees, with dark green and broad leaves. For each transect, full-sun or shade, plant parts were collected within 24 m at 6m intervals and the first data collection was initiated at 0 m and consisted of 5 quadrats (i.e. 0, 6, 12, 18 and 24) of 2 x 2 m square (with a surface of 4 m^2). Plants were hand pulled from the soil within 2 m of the transect to obtain the roots, or a spade was used where the soil was harder. Roots were removed from the stems

using secateurs. For stem sampling, green succulent stems (i.e. current growing season) were collected using secateurs for analysis, rather than the thick, tough, woody stems; and the leaves were hand removed from the stems. Each of the plant parts were put into separate paper bags labelled with transect number, type and site (e.g. FSC 1 leaves represent leaves collected from full sun (FS), Cannonbrae (C) and quadrat 1, while ShC 1 leaves represents leaves collected from the shade (Sh), Cannonbrae (C) and quadrat 1 (and thereafter referred to as ShC)).

Location 2: Thohoyandou, Lutanandwa river banks, Limpopo province

Due to the idiosyncratic nature of the sites (shaded and full-sun sites) with respect to the abundance and distribution of the *C. odorata* at the sites, we employed a systematic random sampling method. At the shaded site ShL, sampling was done along a 60 m transect. Plant materials (roots, stems and leaves) were randomly collected at five sampling points (0, 15, 30, 45 and 60 m within a $4m^2$ quadrat) after approximately 10- to 15-m intervals along the transect. At the full-sun site FSL, plant materials (roots, stems and leaves) were randomly collected at five sampling points (0, 20, 40, 60 and 80 within a $4m^2$ quadrat) after approximately 20 m intervals along a 80-m transect. Like previously, each of the plant parts were put into separate paper bags labelled with transect number, type and site (e.g. FSL 1 leaves represent leaves collected from full sun (FS), Limpopo/Thohoyandou (L) and quadrat 1, while ShL 1 leaves represents leaves collected from the shade (Sh), Limpopo/Thohoyandou (L) and quadrat 1 (and thereafter referred to as ShL)).

Location 3: Komatipoort, Komati River Chalets, Mpumalanga province

At the shaded site, sampling was done along a 60 m transect. Plant materials (roots, stems and leaves) were randomly collected at 0, 15, 30, 45 and 60 m sampling points (within a 4 m^2 quadrat) after approximately 10 to 15 m intervals along the transect. At the full-sun site, plant materials (roots, stems and leaves) were randomly collected at five sampling points (0, 5, 10, 15 and 20 within a 4 m^2 quadrat) after approximately 6 m intervals along a 25-m transect. For Komatipoort, FSM represented full sun Mpumalanga whilst ShM represented shade Mpumalanga

Location 4: Peter Brown Drive, Pietermaritzburg, KZN province

At the shaded site, sampling was done along a 16 m transect. Plant materials (roots, stems and leaves) were randomly collected at five sampling points (within a 4 m^2 quadrat) after approximately 4-m intervals (i.e. 0, 4, 8, 12 and 16 m) along the transect. At the full-sun site, plant materials (roots, stems and leaves) were randomly collected at five sampling points (within a 4 m^2 quadrat) after approximately 6 m intervals along a 30-m transect (at 0, 6, 12, 16 and 24 m). For plants collected from Pietermaritzburg, FSP represented full sun Pietermaritzburg whilst ShP represented shade Pietermaritzburg.

6.3.2 Collection of cuttings for growth rate comparison studies of *Chromolaena odorata*

Cuttings of *C. odorata* used in this experiment were collected from the 4 sites and transects that were earlier used whilst collecting plant parts for phytochemistry analysis i.e. Thohoyandou FSL 1-5, ShL 1-5, Komatipoort FSM 1-5 & ShM 1-5, Umkomaas FSC 1-5 & ShC 1-5 and Pietermaritzburg FSP 1-5 and ShP 1-5. For the collection of cuttings, one bag was used per habitat i.e. cuttings from full sun were collected from transect 1-5 and put in one bag and cuttings from the shaded transects were collected from 1-5 and were put in a separate plastic bag.

During the time of sampling, Thohoyandou (03 October 2017 08:30-12:30) and Mpumalanga (04 October 2017 14:30-16:45) had not received much rain and the plants still had good numbers of flowers/seeds on them (seeds were collected for possible germination and use of these progeny in trials). Plants from the sunny site in Thohoyandou were growing vigorously whilst those from the shade were etiolated and less vigorous. At the time of collection, Komatipoort was very dry, with few plants and seeds present, and as a result, cuttings could only be collected from 3 transects within the previously used full-sun and/or shaded area. All cuttings were kept in plastic bags closed with pegs and placed in cooler boxes with ice. KZN had already received plentiful rain at the time of sampling and at the shaded area in Cannonbrae, most plants had very few flowers or seeds. The full-sun habitat contained healthy, robust plants and many seeds, despite the rains. Cuttings were collected on the 10/10/2017 09:01-10:55. Plants in Pietermaritzburg were

generally fewer, similar to the shaded area of Umkomaas, but they were growing vigorously.

Table 6.1: Locations, ecological conditions, coordinates and altitude of the sites where plant material was collected for phytochemistry (leaves, stems and roots) and growth rates (cuttings) representing 3 provinces, with and without *Pareuchaetes insulata* in South Africa.

Province	Site	*P. insulata* status	Habitat	Latitude (S)	Longitude (E)	Altitude (m)
Limpopo	Thohoyandou	Absent	Full sun	23° 03' 47.1"	30° 14' 53.4"	545
			Shade	23° 03' 43.5"	30° 14' 55.2"	545
Mpumalanga	Komatipoort	Present[b]	Full sun	25° 26' 47.6"	31° 57' 39.6"	134
			Shade	25° 26' 47.7"	31° 57' 40.5"	135
KwaZulu Natal 1	Umkomaas	Present[a]	Full sun	30° 13' 17.4"	30° 46' 57.5"	49
			Shade	30° 13' 13.6"	30° 46' 54.2"	50
KwaZulu Natal 2	Pietermaritzburg	Absent	Full sun	29° 34' 57.48"	30° 20' 36.48"	882
			Shade	29° 34' 57.84"	30° 20' 36.42"	882

[a]*Pareuchaetes insulata* present for more than 15 years
[b]*Pareuchaetes insulata* present for unknown number of years (probably 1-2)

6.3.4 Plant material processing

Leaves collected from Umkomaas, Thohoyandou, Komatipoort and Pietermaritzburg were spread on newspapers on the desks and dried at room temperature at the University of KwaZulu-Natal Chemistry department. Plant material of *C. odorata* was prepared by grinding the dried leaves with a mill into small pieces of about 0.01-1.0 mm and later stored at room temperature (25 °C).

6.3.5 Extraction of plant material for phytochemical determination

Leaf samples (0.1 g) from the different locations (Limpopo, Mpumalanga, Pietermaritzburg, Umkomaas) and habitats (full-sun vs shade) were weighed into centrifuge tubes; 10 ml of 50% methanol (MeOH) was added and the material was centrifuged at 300 × g for 10 min and filtered through Whatman No. 1 filter paper. The resultant extracts were immediately used for the phytochemical determination to prevent deterioration and decomposition of metabolites. Because alkaloids were not detected in the leaf extracts in Omokhua (2015), this chapter excluded alkaloids.

6.3.6 Quantitative determination of phytochemicals

Standard methods were used to quantitatively determine total phenolics, total flavonoids and condensed tannins using the freshly prepared 50% MeOH crude extracts. Total phenolic compositions of the plant extracts were evaluated using the Folin-Ciocalteu method (Makkar, 2003) with some modifications. Using gallic acid as the standard to determine total phenolic content, 50 µl of the 50% MeOH plant extracts was transferred into test tubes (5 test tubes replicates for each extract), 950 µl of sterile distilled water was added, followed by the addition of 500 µl of 1 N Folin-C reagent and 2.5 ml of 2% sodium carbonate ($NaCO_3$) in the dark. Similarly, blanks containing 50% MeOH in place of the plant extracts and different concentrations of gallic acid were prepared (concentration between 0 and 150 mg/ml). The test tubes containing the mixtures were incubated at room temperature for 40 min, and 200 µl of the reacted mixtures were immediately transferred into 96 well plates and absorbance was measured at 725 nm using a microplate reader. Total phenolics were expressed as gallic acid equivalents (GAE) per gram dry weight.

Total flavonoid content of the plant extracts was determined using the aluminium chloride method as described by Abdel-Hameed et al. (2009) with some modification. One hundred microlitre of plant extract was mixed with 100 µl of 20% $AlCl_3$ and 2 drops of glacial acetic acid. The mixture was diluted with 50% MeOH to 3000 µl. Blank samples were prepared with plant extracts without $AlCl_3$, and a standard curve was prepared using catechin (concentration between 0-150 mg/ml) in MeOH. After 40 min, absorbance was read at 415 nm using a microplate reader. The total flavonoid content was expressed as mg catechin equivalent (CAE) /g of dry plant material.

To determine condensed tannins, the butanol-HCl assay using cyanidine chloride as the standard was employed. In triplicate, 250 µl of 50% MeOH plant extracts were measured into test tubes, 3000 µl of butanol-HCl reagent and 100 µl of ferric reagent were added; a blank containing 50% MeOH and cyanidine chloride of different concentrations were also prepared. All test tubes containing the mixture were vortexed, covered tightly with a lid and incubated at 99^{o}C for 1 h. The mixtures were allowed to cool and absorbance was

measured at 550 nm using a microplate reader. Condensed tannins were expressed as cyanidine chloride equivalents (CCE) per dry weight.

6.3.7 Garden experiment

This trial was initiated in October 2017 and ran until the end of April 2018 (i.e. the duration of the growing season). The cuttings were planted 1-2 days after collection and were left for at least 2 weeks in the mistbed at the University of KwaZulu-Natal (Pietermaritzburg campus) before they were potted, to allow for the formation of reasonable root stocks. Cuttings from Mpumalanga (Komatipoort) and Limpopo (Thohoyandou), all of equivalent size, were planted with a rooting hormone on the 5th of October in 23 x 16.5 cm plastic trays containing moistened vermiculite, whilst cuttings from KZN 1 (Umkomaas) and KZN 2 (Pietermaritzburg) were similarly planted on the 10th October. For each site, cuttings were planted in 3 trays from the shaded area and 3 trays from the full-sun area. By the 27th October 2017, cuttings from all 4 sites had rooted. Four-hundred and fifty-eight rooted plant cuttings from full-sun and shaded plants from all 4 sites (56 each x 8) were planted into 22 cm diameter pots containing a fertilized soil mix of garden refuse decomposed for 18 months, then sieved and treated with methyl bromide. Before planting, the soil was mixed with superphosphate and 2:3:2 (14) fertilizer, both at a rate of 600g per cubic metre. The garden experiment was conducted in a shadehouse in the botanical gardens at the University of KwaZulu-Natal, Pietermaritzburg campus (29° 37′ 30.828" S, 30° 24′ 14.303" E).

All 458 potted plants were randomly positioned and were watered accordingly. After 6 weeks, all plants were fertilized with 10 ml of plantacote (9g of Plantacote, AGLUKON Spezialduenger GmbH & Co. KG, Germany: 14% nitrogen, 8% phosphorus pentaoxide, and 15% potassium oxide – all soluble in water). During this time there was an outbreak of *Zonocerus elegans* Thunberg (Orthoptera: Pyrgomorphidae), which were controlled with Malasol (active ingredient: malathion). By April 2018 the plants were all tall and most were flowering, and at the end of the month, several plant growth parameters were measured: the number of shoots per plant, the number of flowering shoots, basal stem diameter, and the height of the tallest shoot.

6.3.8 Statistical analysis

The effects of location and habitat on the concentrations of secondary metabolites (phenolics, flavonoids and tannins) were compared using a General Linear Model analysis of variance (GLM ANOVA). Furthermore, the effects of location and habitat on plant growth metrics *viz.* plant height, stem diameter, number of shoots and number of flowering shoots were compared using a General Linear Model analysis of variance (GLM ANOVA). When the overall results were significant, the differences among the treatments were compared using the Tukey's Honest Significant Difference (HSD) test. The analyses were performed using IBM SPSS Statistical software version 20.0 (SPSS, Chicago, IL, USA).

6.4 Results

6.4.1 Total phenolic contents

Total phenolic concentration differs as a function of location and habitat (Fig. 6.1, Table 6.2). Phenolic contents of full-sun leaves were greater than those from the shaded habitat, irrespective of location/site. Phenolic concentrations in the leaves from Thohoyandou and Komatipoort plants growing in full sun were higher than those on leaves from Umkomaas and Pietermaritzburg in full sun and there was no significant difference in tannin concentration in the leaves from the latter, or between leaves from the full-sun habitat in Komatipoort and in Thohoyandou (Fig. 6.1, Table 6.2). Leaves from the shaded habitat at the Pietermaritzburg site had a higher concentration of phenolics compared to leaves from Umkomaas, but were not different from leaves in the shade at Thohoyandou or Komatipoort (Fig. 6.1, Table 6.2). At all 4 sites, the leaves of plants growing in the full-sun habitat had significantly higher phenolic contents than those growing in the shade.

6.4.2 Flavonoid contents

Flavonoid content differed as a function of location and habitat (Fig. 6.2, Table 6.2), with the highest levels on leaves from Komatipoort and Pietermaritzburg plants growing in full-sun. Flavonoid contents of leaves was lower on plants collected from Umkomaas plants in full-sun habitat compared to all other locations (Fig. 6.2, Table 6.2). Flavonoid contents of

full-sun leaves were greater than those from shaded habitat, irrespective of location/site. Flavonoid contents of shaded leaves from Pietermaritzburg was higher compared to those obtained from the same habitat in Umkomaas, Komatipoort and Thohoyandou, which were similar (Fig. 6.2, Table 6.2). Interestingly, flavonoid content of shaded leaves from Pietermaritzburg equaled (or was slightly higher than) those of full-sun leaves from Umkomaas and Thohoyandou.

6.4.3 Tannin contents

Tannin concentrations differed as a function of location and habitat (Fig. 6.3, Table 6.2). Condensed tannin contents of full-sun leaves were greater than those from shaded habitat at all locations apart from Umkomaas, which had seemingly equal levels in shade and full-sun leaves. Tannin concentrations were higher in the leaves from Thohoyandou and Komatipoort plants in full-sun than those in the leaves from Umkomaas and Pietermaritzburg plants in a similar habitat (Fig. 6.3, Table 6.2). Irrespective of habitat, condensed tannin contents were lower in leaves of plants from Umkomaas. Tannin concentrations of shaded leaves did not differ across the 4 locations (Fig. 6.3, Table 6.2).

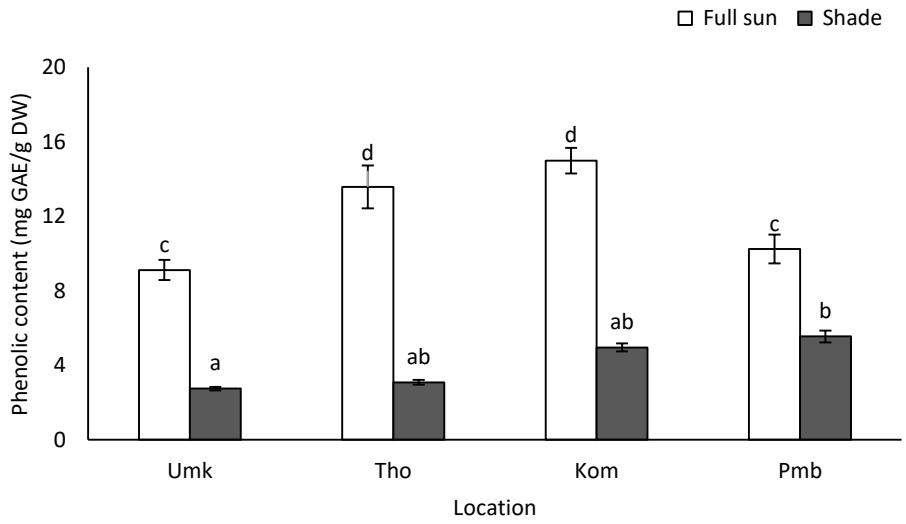


Figure 6.1: Total phenolic content, as gallic acid equivalents detected in *Chromolaena odorata* leaves from four locations (Umkomaas, Thohoyandou, Komatipoort, and Pietermaritzburg) and two habitats (shade versus full-sun) in South Africa. Values for each bar are means ± SEM. DW = dry weight; GAE = gallic acid equivalent; Umk = Umkomaas, south coast of KZN; Tho = Thohoyandou, Limpopo province, Kom = Komatipoort, Mpumalanga province; Pmb = Pietermaritzburg, Midlands of KZN.

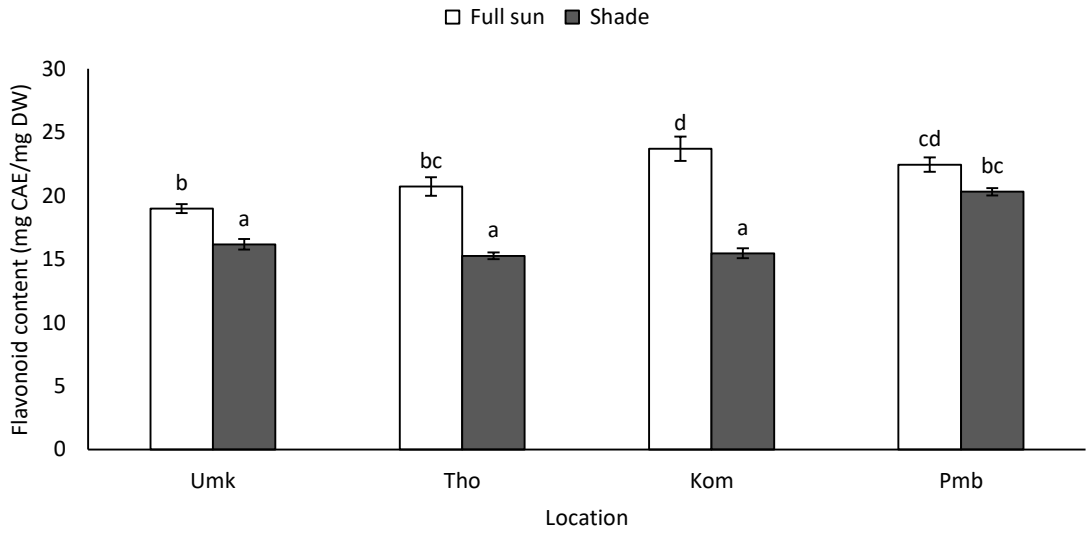


Figure 6.2: Flavonoid content as catechin equivalents detected in *Chromolaena odorata* leaves from four locations (Umkomaas, Thohoyandou, Komatipoort, and Pietermaritzburg) and two habitats (shade versus full-sun) in South Africa. Values for each bar are means ± SEM. DW = dry weight; CAE=catachin equivalents; Umk = Umkomaas, south coast of KZN; Tho = Thohoyandou, Limpopo province, Kom = Komatipoort, Mpumalanga province; Pmb = Pietermaritzburg, Midlands of KZN.

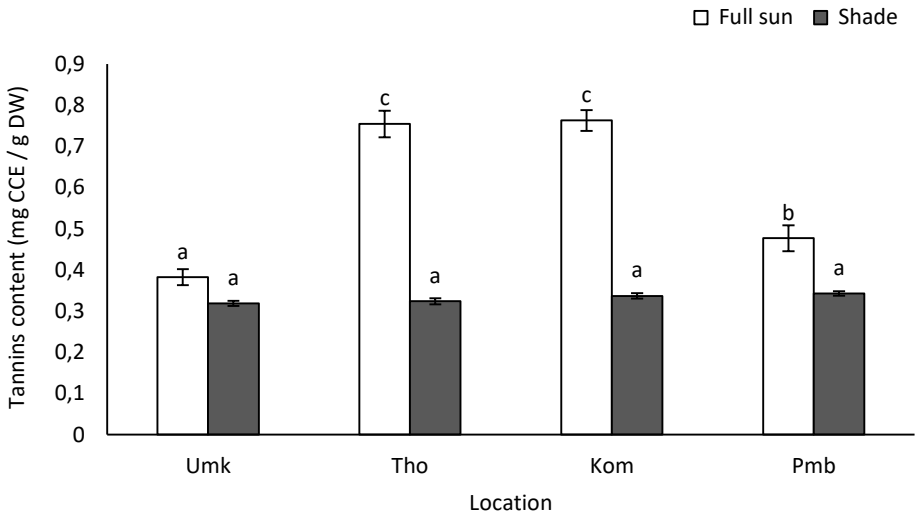


Figure 6.3: Condensed tannin content as cyanidine chloride equivalents detected in *Chromolaena odorata* leaves from four locations (Umkomaas, Thohoyandou, Komatipoort, and Pietermaritzburg) and two habitats (shade versus full-sun) in South Africa. Values for each bar are means ± SEM. DW = dry weight; CCE = cyanidine chloride equivalents; Umk = Umkomaas, south coast of KZN province; Tho = Thohoyandou, Limpopo province, Kom = Komatipoort, Mpumalanga province; Pmb = Pietermaritzburg, Midlands of KZN province.

Table 6.2: Results of GLM ANOVA for effects of *P. insulata* on phenolic, flavonoid and tannin contents of *C. odorata*.

Analysis	Source of variation	DF	MS	F-value	P-value
Phenolics	Location	3	27.692	15.59	**<0.001**
	Habitat	1	623.212	350.75	**<0.001**
	Loc.Hab	3	19.899	11.2	**<0.001**
	Total	39			
Flavonoids	Location	3	30.08	20.35	**<0.001**
	Habitat	1	217.285	146.98	**<0.001**
	Loc.Hab	3	19.391	13.12	**<0.001**
	Total	39			
Tannins	Location	3	0.096188	48.04	**<0.001**
	Habitat	1	0.695017	347.09	**<0.001**
	Loc.Hab	3	0.092557	46.22	**<0.001**
	Total	39			

6.4.4 Plant growth parameters

After six months (from November 2017 to end of April 2018), the growth parameters of *C. odorata* plants in the garden experiment differed as a function of location but not habitat (Fig. 6.4, Table 6.3). Plants from Thohoyandou and Pietermaritzburg generally reproduced better than plants from Umkomaas and Komatipoort. Plant height was not affected by the location and there was no significant difference among the two habitats in Umkomaas, Thohoyandou, Komatipoort or Pietermaritzburg (Fig. 6.4A, Table 6,3). Plants from Thohoyandou had significantly wider basal stem diameter than plants from Umkomaas or Komatipoort (Fig. 6.4B, Table 6.3). Stem diameters of plants from Pietermaritzburg were significantly broader than those at Komatipoort, generally bigger than those at Umkomaas (although there was no significant difference with the latter), but had significant smaller stem diameters than the plants from Thohoyandou (Fig. 6.4B, Table 6.3). Stem diameters of the plants from Umkomaas were not statistically different from Komatipoort plants (Fig. 6.4B, Table 6.3).

The reproductive potential (vegetative and flowering shoots) was significantly higher for plants from Thohoyandou and Pietermaritzburg compared to plants from Umkomaas and Komatipoort (Fig. 6.5, Table 6.3). Plants from Thohoyandou and Pietermaritzburg had significantly more shoots compared to plants from Umkomaas and Komatipoort (Fig. 6.5A, Table 6.3). Similarly, plants from Thohoyandou had significantly more shoot tips compared to plants from Pietermaritzburg. However, there was no significant difference in the number of shoot tips for plants from Umkomaas and Komatipoort (Fig. 6.5A, Table 6.3). Plants from Thohoyandou and Pietermaritzburg had significantly more flowering shoots compared to plants from Umkomaas and Komatipoort (Fig. 6.5B, Table 6.3). There was no statistical difference in the number of flowering shoots for plants from Thohoyandou and Pietermaritzburg or between the number of flowering shoots from Umkomaas and Komatipoort (Fig. 6.5B, Table 6.3).

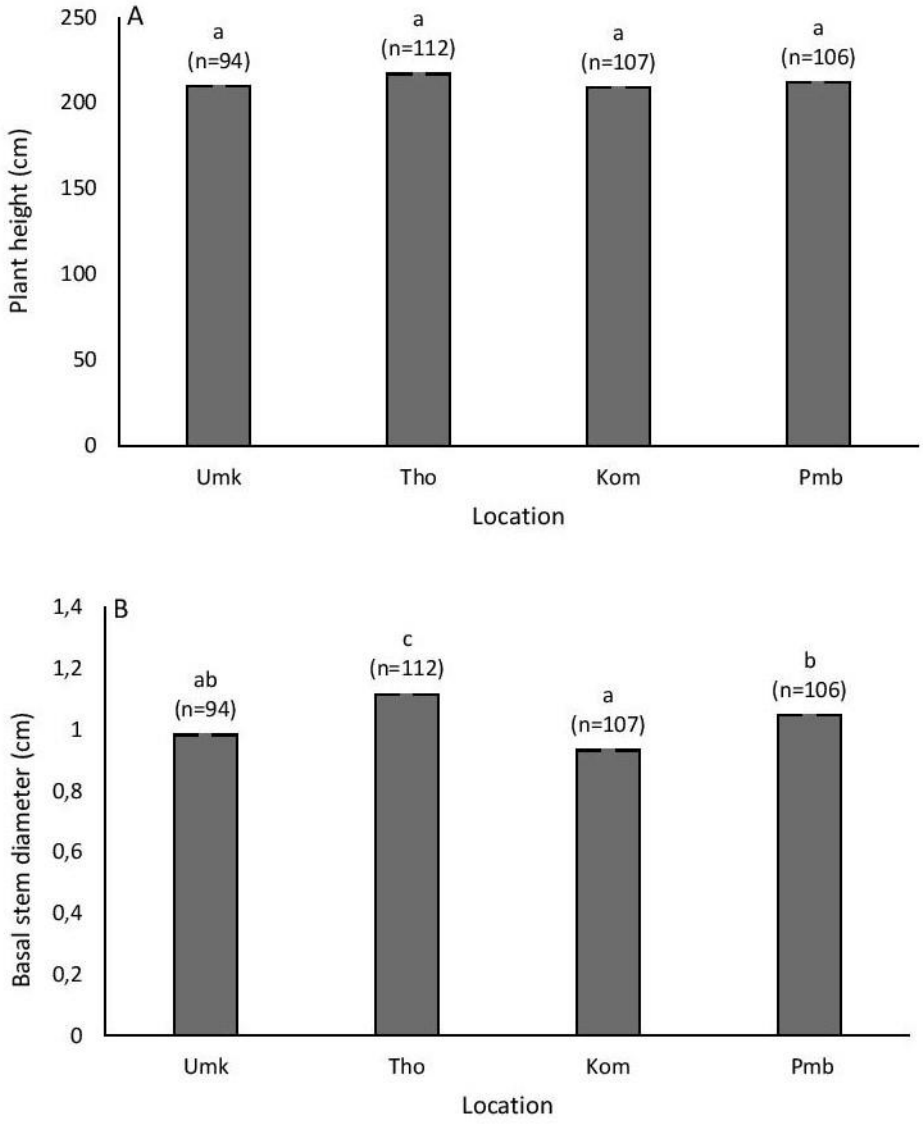


Figure 6.4: Effect of location and habitat on (A) plant height and (B) stem diameter of *Chromolaena odorata* after 5 months of growing without *P. insulata*. Means (after Generalized Linear Model analysis (GLM)) with the same letters above the bars are not significantly different (sequential Bonferroni test: $P > 0.05$). Sample sizes are given in parentheses.

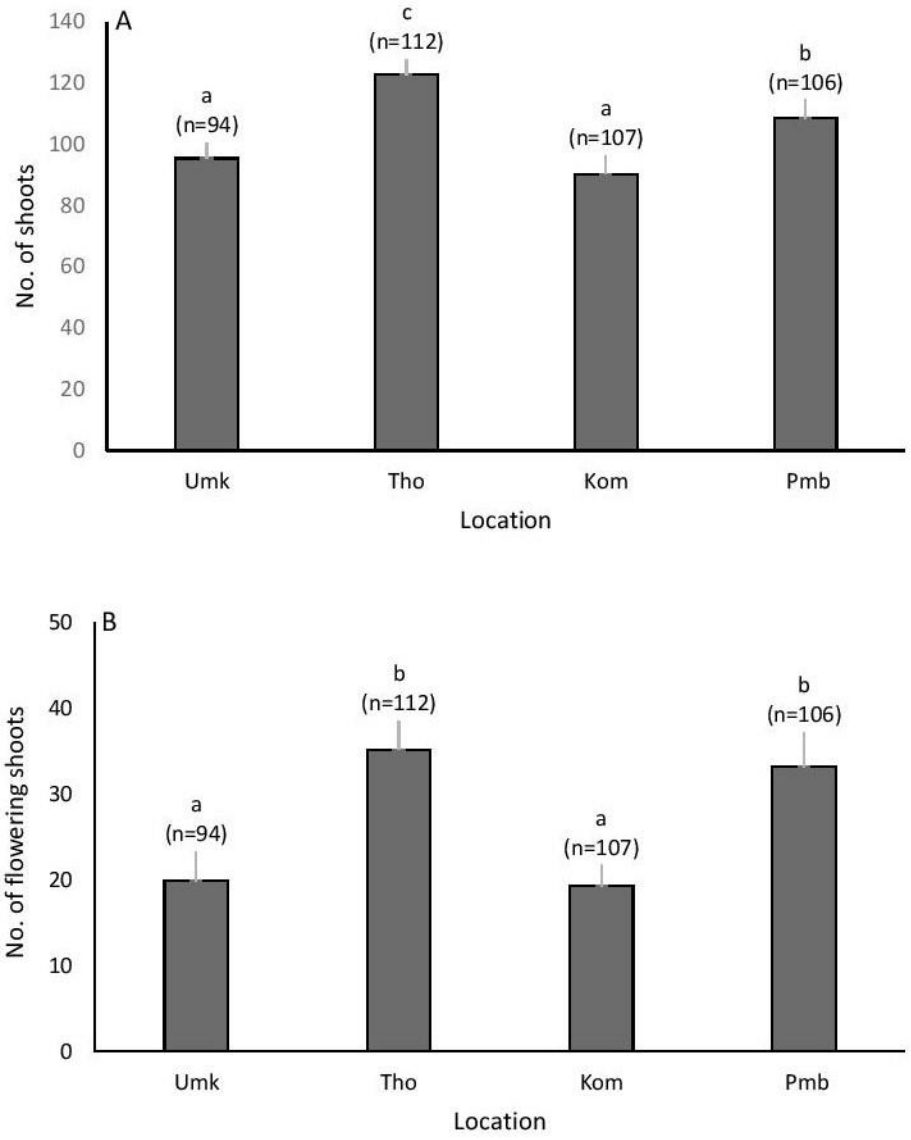


Figure 6.5: Effect of location on number of shoot tips (A) and number of flowering shoots (B) of *Chromolaena odorata* plants after five months of growing them under the garden experiment. Means (after Generalized Linear Model analysis (GLM)) with the same letters above the bars are not significantly different (sequential Bonferroni test: $P > 0.05$). Sample sizes are given in parentheses.

Table 6.3: Results of GLM ANOVA for effects of *P. insulata* on plant height, stem diameter, number of shoots and number of flowering shoots on *C. odorata*.

Analysis	Source of variation	DF	MS	F-value	P-value
Plant height	Location	3	1395	1.13	0.338
	Habitat	1	81	0.07	0.798
	Loc.Hab	3	42	0.34	0.793
	Total	418			
Stem diameter	Location	3	0.67632	13.08	<0.001
	Habitat	1	0.02788	0.54	0.463
	Loc.Hab	3	0.05306	1.03	0.381
	Total	418			
Number of shoots	Location	3	22703	16.02	<0.001
	Habitat	1	453	0.32	0.572
	Loc.Hab	3	483	0.34	0.796
	Total	418			
Number of flowering shoots	Location	3	7558.4	10.16	<0.001
	Habitat	1	150.5	0.2	0.653
	Loc.Hab	3	454.3	0.61	0.608
	Total	418			

6.5 Discussion

6.5.1 Secondary chemicals

A number of hypotheses have been proposed to explain the processes involved in the successful invasion by alien plants, including reallocation of resources from defence to growth and reproduction and possession of allelopathic properties that inhibit unrestricted growth of native plants (Blossey and Notzöld 1995; Keane and Crawley 2002; Müller-Schärer et al. 2004; Callaway and Ridenour 2004). Studies that tested EICA or ERH in different regions of the invasive range without data from the native range are rare, but the principles of EICA and ERH were used to interpret data in this chapter. The few studies that have tested EICA mainly investigated the comparative vigour of a plant species using individuals from its native and invasive range under homogenous environments without measuring chemical defence levels (e.g. Muller and Martens 2005; Franks et al. 2008; Qin et al. 2013). Other theories (e.g. SDH) are considered because EICA alone does not

integrate the basic difference between specialist and generalist herbivores (Muller and Martens, 2005). The first prediction, that *C. odorata* from Umkomaas and Komatipoort will have higher concentrations of secondary metabolites (quantitative) and is chemically well defended, was not supported in the results presented here. However, the second prediction, that *C. odorata* plants from Thohoyandou and Pietermaritzburg will grow more vigorously and have higher reproductive potential, was partly supported.

Several studies have shown evidence of one, both or none of the two main theories behind invasion success of alien plants. In China, Qin et al. (2013) showed that *C. odorata* plants grown at high nutrient concentration from the invasive range demonstrated superior competitive responses compared with *C. odorata* plants from the native range. This was consistent with the hypothesis that the stronger competitive ability of some invasive species is consequent to evolution of decreased allocation to costly chemical defences. In contrast, a review by Doorduin and Vrieling (2011) reported (i) no difference in the level of pyrrolizidine alkaloids (qualitative defence) between native and invasive individuals of *Cynoglossum officinale* L. (Boraginaceae), and (ii) no difference in the concentration of diterpenes (quantitative defence) between native and invasive individuals of *Solidago gigantea* Ait. (Asteraceae), regardless that the plants in the country of introduction experienced less herbivory compared to the native range. (iii) However, the level of hypericin (quantitative defence) was lower in invasive *S. gigantea* individuals compared to native individuals. (iv) Seedlings of *Lepidium draba* (L.) Brassicaceae from the invaded range contained a higher concentration of glucosinolates (qualitative defence) than those from the native range, whilst plants of 3 months old showed no difference. Myrosinase activity (enzyme responsible for the hydrolysis of glucosinolates (Botti et al. 1995)) was significantly higher in invasive *L. draba* individuals compared to native individuals. Egli (2017) showed that total alkaloid concentration within the leaves of *Senecio madagascariensis* Poir (Asteraceae) was significantly higher in Australian (invasive range) plants than South African (native range) plants, possibly because of signicantly higher total abundance of insect herbivores on Australian plants than in South Africa. Similarly, stem samples displayed a significant difference in alkaloid concentrations, with concentrations

in Australian populations being nearly double that of South African populations, but showing no significant differences among regions in South Africa or Australia.

Phenolic contents in this study were higher in the leaves from Thohoyandou and Komatipoort plants growing in full-sun compared to those from the same habitat in Umkomaas and Pietermaritzburg (KZN), and thus not consistent with predictions. In general, phenolics are described as a very large group of structurally dissimilar plant secondary compounds including terpenoids, flavonoids and tannins (Bray and Thorpe 1954; Bennett and Wallsgrove 1994; Brielmann et al. 2006; Bakhvalov et al. 2009). Phenolics serve a dual function of both resisting and attracting different life entities in the vicinity of plants (Baidez et al. 2007; Battacharya et al. 2010). Among plant constituents, it is generally acknowledged that plant phenolics play a role in plant development or and in protecting plants from both insect and mammalian herbivory and fungal pathogens (Close and McArthur 2002). A study on aphids that were presented with 4 wheat cultivars containing different concentrations of phenolics showed that aphids preferentially fed on the cultivar with lowest concentration (Bennett and Wallsgrove 1994). The concentration of leaf phenolics has frequently been shown to increase in plants grown under conditions of high light or nutrient limitation (Close and McArthur 2002). For example, Dudt and Shure (1994) showed that leaf phenolics generally increased with greater insolation from forest to field and when sunlight was greater within field or forest habitat. In addition, it was suggested that the level of many phenolics is low under some environmental conditions not because the resources to produce them are limited, but because the risk of light damage is low and they are not required (Close and McArthur 2002) and this explains the low levels of total phenolics in shaded *C. odorata* at all locations in this study. Furthermore, phenolic concentration within a plant tissue is dependent on season and may vary at different stages of growth and development. Several environmental factors such as trauma, wounding, drought and pathogen attack are also known to affect the synthesis of phenolics (Battacharya et al. 2010).

Flavonoid contents in this study were also not consistent with the predictions; rather, their concentrations were significantly lowest at Umkomaas and highest at Komatipoort in the

full-sun habitat; and were similar in the shaded habitat at all sites except Pietermaritzburg, where they were higher. They were consistently higher in the sun than shade, albeit not always significantly. Flavonoids are known to have allelopathic properties and to be beneficial for the plant, functioning as physiologically active compounds, as protective agents during environmental stress including absorption of UV radiation for protection of the internal tissues of leaves and stems, as attractants or as feeding deterrents and for their significant role in plant resistance to frost or drought (Treuter 2005, 2006). Plant flavonoids have been reported to affect the behaviour, development and growth of several phytophagous insects and to a certain extent to play a role in host selection (Lattanzio et al. 2006). Apart from biotic stress, several abiotic factors such as salt, drought, heavy metals, cold, light, temperature variations, nutrient and climate, affect concentrations of secondary compounds, including flavonoids (Akula and Ravishankar 2011). For example, it was shown that light (UV-B) increased flavonoid concentrations in barley, cucumber and *Picea abies* (L.) H. Karst (Pinaceae). In addition, elevated levels of trioxygen (or ozone), a gas found in two layers of the atmosphere, increased the concentration of quercetin aglycon, whilst elevated carbon dioxide reduced the concentrations of kaempferol aglycon (Akula and Ravishankar 2011). Therefore, varying levels of flavonoids in Thohoyandou, Komatipoort, Pietermaritzburg and Umkomaas were clearly influenced more by other factors than by whether *C. odorata* had been fed on previously by *P. insulata*.

Levels of condensed tannins in this study were higher in plants growing in full sun in Thohoyandou and Komatipoort and lower in plants in the same habitat from Umkomaas and Pietermaritzburg, whilst there was no difference in tannin levels in plants growing in the shade across all 4 locations. Therefore, tannin levels also did not support EICA. Tannins are well known for their role of defending plants against insects and large mammals (Robins et al. 1987; Clausen et al. 1992; Barbehenn and Constabel 2011). A study on tulip poplar leaves showed that they exhibited remarkable sensitivity to light changes, and that tannin levels were significantly lower in shaded areas of the forest (Dudt and Shure 1994). Furthermore, tannins are thought to play a major role in plants as a barrier to herbivory, and to have different levels between damaged and undamaged leaves (Hay and Brown 1992). Although high levels of tannins were not expected in Thohoyandou because no

specialist herbivores have established there yet, the site is not excluded from generalist herbivores (Clausen et al. 1992; van der Meijen 1996). Müller-Schärer et al. (2004) stated that quantitative defences or secondary metabolites such as tannins generally occur in high concentration and defend the plant against specialist herbivores. This would also apply to certain generalists that are adapted to qualitative plant toxins such as pyrrolizidine alkaloids, which are usually sufficient to defend the plant against generalists (see the example of *Zonocerus* species below). Furthermore, tannin contents may elongate insect development times, making them susceptible to predators and parasitoids (Coley and Barone 1996). Variation in tannin concentrations is known to be a highly plastic trait and is attributed to plant phenotype, tissue developmental stage, and environmental conditions, hence may explain the differences in Thohoyandou and Komatipoort versus Umkomaas and Pietermaritzburg (Barbehenn and Constabel 2011).

High concentrations of tannin, flavonoid and phenolic contents in plants growing in full sun compared to those growing in shade at all our sites (non-significant at Umkomaas) are in accordance with the Carbon Nutrient Balance hypothesis, which states that light intensity can affect the C/N balance within the plant and eventually affects secondary compounds. Shading has been shown to increase concentrations of N-based secondary compounds such as alkaloids and decrease concentrations of C-based secondary metabolites such as tannins (Herms and Mattson 1992; Crone and Jones 1999), so the full-sun or high-light conditions would lead to increased C-based defences (Coley and Barone 1996).

Contrary to the predictions, levels of phenolic and tannin contents were high in Thohoyandou and Komatipoort and lower at Umkomaas and Pietermaritzburg, whilst the expectation, was a similarity between Thohoyandou and Pietermaritzburg (with lower levels) and between Umkomaas and Komatipoort (with higher levels), considering the respective absence and presence of *P. insulata* at these two pairs of locations. Altitudes or latitude gradients are also known to influence the concentrations of phenolic contents, including tannins (Bennett and Wallsgrove 1994; Moles et al. 2011). Bennett and Wallsgrove (1994) showed that the populations of two *Inga* species (family) at higher altitudes contain significantly higher phenolic concentrations regardless of the leaf stage,

compared to the populations at lower altitudes. However, altitudinal variation does not explain the patterns seen in *C. odorata* (Table 6.1). In addition, whilst Moles et al. (2011) cited a few studies in which plants were shown to have higher levels of chemical defences at low latitudes, which agrees with the current study (Table 6.1), the majority of studies reviewed demonstrated that chemical defences were significantly higher in plants from higher latitudes. The Growth Rate Potential hypothesis predicts that the amount of resources such as water, nutrients and light available in the environment to support growth act together with herbivory to determine the quantitative patterns of defence (Herms and Mattsons 1992); and contrasting effects of generalist and specialist herbivores can explain the variation of levels of defence in plants (van der Meijden 1996). Therefore, these findings support data in this chapter and further illustrate that the concentration of secondary metabolites and biological invasions may be driven by several biotic and abiotic factors in the introduced ranges, in addition to enhanced fitness due to release from their specialist herbivores. The variation of flavonoid contents in my study could be explained by their availability as per requirement at a particular location (Close and McArthur 2002).

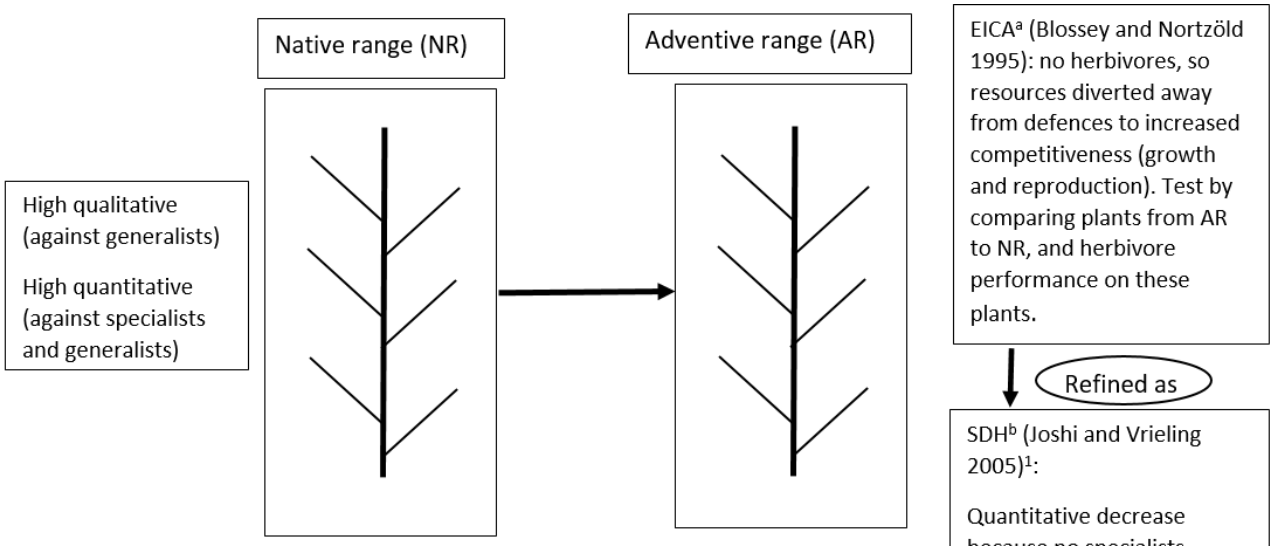
6.5.2 Plant growth metrics

In Thohoyandou and Pietermaritzburg there are no records of a specialist herbivore *P. insulata,* whilst Umkomaas has had establishment and persistence of this herbivore for over 15 years and Komatipoort has only recent records of the moth. The current study on *C. odorata* shows that the plants in locations where *P. insulata* is absent (Thohoyandou and Pietermaritzburg) grew more vigorously and had higher reproductive potential than those where *P. insulata* is present (Umkomaas and Komatipoort). As per the first part of EICA, clearly plants may have benefited from escaping their specialist enemy, implying that there has been some type of resource shift. Although plant height did not vary between locations, plants from the locations where *P. insulata* is absent developed thicker basal stem diameters, with higher numbers of shoot tips and flowering shoots compared to Umkomaas and Komatipoort where *P. insulata* is present. Several studies have demonstrated improved performance of plants when released from natural enemies in the introduction ranges, i.e. the plants from the native range vs invasive range were grown under identical conditions to compare them, without any specialist natural enemies being present. (e.g. Hinz and

Schwarzläender 2004; Zou et al. 2008; Feng et al. 2009; te Beest et al. 2009; Leishmann et al. 2014; including the AWAB *C. odorata* - Qin et al. 2013; Uesogi and Kessler 2016; Zheng et al. 2018). This suggests that escape from their specialist herbivores (Keane and Crawley 2002) contributed to the strong competitive ability and environmental adaptability demonstrated by both SAB and AWAB *C. odorata*, which facilitated their invasiveness in their non-native range (Yu et al. 2014; Shao et al. 2018).

Contradictory results are common in the studies conducted to test hypotheses of EICA and its extensions (e.g. Hinz and Schwarzländer 2004; Shelby et al. 2016) (see Fig. 6.6). Although some studies have demonstrated increased growth and reproductive output in the introduced range where the specialist natural enemies are absent, the same studies showed higher levels of secondary metabolites such as pyrrolizidine alkaloids in the introduction relative to the native locations (Stastny et al. 2005). This can be explained by the SDH hypothesis that predicts an increase of qualitative defences (useful against generalist herbivores and not specialist herbivores) such as PAs in invaded area (explained in Harvey et al. 2013, 2015 and Egli 2017). Similarly, this study showed that plants had life-history traits consistent with the assumptions of EICA (Blossey and Notzöld 1995) but did not show that the presence versus absence of a specialist herbivore had an impact on plant quantitative chemical defences. In addition, my study corroborated the studies that considered the impact caused by re-association of specialist herbivores with their host plants in the introduction country. For example, Jogesh et al. (2014) showed reduced pollination and higher fitness and reproductive effort of *Pastinaca sativa* L. (Apiaceae) in locations with its specialist herbivore *Depressaria pastinella* Duponchel (Lepidoptera: Oecophoridae) (and accounted evolution of large size as a component of florivore tolerance) in comparison to locations where the specialist herbivore was absent, but did not find evidence for the evolution of increased chemical defences. This variability in defence parameters measured emphasizes that several environmental factors, such as light intensity (Roberts and Paul 2006), plants' structural traits (Hanley et al. 2007), time (Harvey et al. 2013), generalist herbivores (Harvey et al. 2015) and allelopathy (Dai et al. 2016), play in concert to regulate the invasion of alien plants and adds that EICA may not fully explain the invasion success of *C. odorata.* Or maybe not enough time elapsed for evolution to

have occurred on *C. odorata* when considering studies looking at reassociation for 150 years (see Ch 7 for a detailed discussion on effects of time on both plants and insect responses). Nevertheless, these results should be interpreted with caution as this study only indirectly tested EICA.



Definitions (from Müller-Schärer et al. 2004):

Quantitative[2]: includes tannins, flavonoids, etc., decrease digestion, need to be produced in large amounts to be effective (dose-dependant). Effective against specialists, second line of defence against generalists[3]. May be expensive to produce (large amounts needed).

Qualitative: toxins, such as pyrrolizidine alkaloids, produced in small amounts, effective against (most) generalists but specialists have evolved to tolerate or take advantage of them. May be cheap to produce (small amounts).

Notes:

[a]Evolution of Increased Competitive Ability; [b]Shifting Defence Hypothesis

[1]Müller-Schärer et al. (2004) introduced this concept but did not present it as a named hypothesis. Rapo et al. (2010) made use of the combination of EICA and its refinement, SDH. Doorduin and Vrieling (2011) introduced the idea that EICA+SDH may need time to evolve. Fukano and Yahara (2012) study supported EICA. Wan et al. (2018) directly tested SDH by using generalist and specialist herbivores. Jogesh et al. (2014) found that a plant exposed to a specialist herbivore initially responded by increasing in size as a defence mechanism, and speculated that a change in chemical defences may take longer to evolve. This does not directly support EICA.

[2]There is a separate test for total phenolics, tannins, flavonoids etc. even though tannins and flavonoids both fall under the definition of phenolics.

[3]Unless the generalists can act as specialists in some cases, by sequestering these toxins such as PAs. This is the case for *Zonocerus* species for chromolaena. This presents an interesting case because chromolaena may then have had to produce quantitative defences even before any specialist herbivores were introduced.

Figure 6.6: A schematic representation of hypotheses, linked to top-down regulation by natural enemies, that explain the increased performance and invasiveness of alien plants in their invasive ranges.

6.5.3 Implications for biological control of *C. odorata*

Pareuchaetes insulata has had a positive impact on the management of *C. odorata*, and probably accounts for the restoration of indigenous flora, where it has persisted in southern Africa (Zachariades et al. 2016). Although this could be explained by the ERH alone, the substantial decrease in *C. odorata* in KZN over the past 15 years provided motivation to test the EICA hypothesis. Patterns of quantitative defensive chemicals did not support EICA, but some plant growth metrics did. Populations of both *P. insulata* and *P. pseudoinsulata* typically experience an initial major outbreak when introduced as a biocontrol agent into a new area, followed by smaller outbreaks every few years over the subsequent period (Zachariades et al. 2009). This pattern seems to hold where the two *Pareuchaetes* species spread away from the initial area of establishment: an initial major outbreak on the 'invasive front' of the moth occurs. In Ghana, the cover of *C. odorata* has decreased from an estimated 80% to 30% (Braimah et al. 2013) and this may be similar in other countries in which *P. pseudoinsulata* and *P. insulata* have established (R. McFadyen pers. comm.). Although the observed *Pareuchaetes* population dynamics can be explained by ERH (decreasing food resources for the biocontrol agent over time), EICA and its refinements could also be invoked: the plants may regain their long-term (evolutionary) defensive mechanisms after the first outbreak, resulting in reduced performance of the insect over time.

The mechanisms of EICA have largely not been investigated (but see Qin et al. 2013), but presumably involve genetic or epigenetic changes within or between generations. It is worthwhile noting that *C. odorata* reproduces apomictically (Rambuda and Johnson 2004), with no or very little gene recombination across generations – if EICA were to rely on directional selection through the latter mechanism, apomixis may prevent it. Several studies have been conducted on physiological responses by *C. odorata* to herbivory by *P. pseudoinsulata*. Marutani and Muniappan (1991) found that feeding by *P. pseudoinsulata* larvae caused yellowing of *C. odorata* leaves, and that tough yellow leaves contained high nitrate nitrogen which resulted in slow growth and high mortality of larvae. Mechanical defoliation did not achieve the same result. The yellowing of plants in response to *P. insulata* feeding has been recorded in South Africa. Raman et al. (2006) described the

mechanism at sub-cellular level but found that once feeding has ended, the cells revert to their former state.

Zonocerus elegans is a polyphagous grasshopper species frequently found feeding on *C. odorata* in South Africa; its congener *Zonocerus variegatus* (L.) in West Africa is also polyphagous and similarly frequently feeds on *C. odorata*. Boppré and Fischer (1994) demonstrated that *Z. variegatus* is attracted to *C. odorata* as a source of pyrrolizidine alkaloids, for protection of its eggs against predation and increased fitness. *Zonocerus elegans* in South Africa is also known to do the same (Boppré et al. 1984). This behaviour is known as pharmacophagy: the search for particular secondary metabolites directly, consumption of them independently of food uptake, and use of them for enhanced fitness, whereas a different plant is fed on to obtain nutrients. The case of *Zonocerus* grasshoppers overcoming and indeed sequestering PAs from chromolaena for their defence is interesting, because although they are generalists, they behave as specialists on *C. odorata*. They may have stimulated the plant into investing more in quantitative chemical defences even before *P. insulata* was released, and this may explain for example why defences such as flavonoids do not differ between Thohoyandou and Umkomaas (van der Meijden 1996).

If true, would EICA enhance biocontrol, reduce its effectiveness, or make no difference? Considering that the growth of the plant would slow down once re-association had occurred (given enough time) but that the performance of the agent also slows down. What happens to agents that are released subsequently (if EICA is true)? Presumably they would have reduced performance compared to if they were introduced as the initial species of agent. If e.g. *Polymorphomyia* was released onto chromolaena in Limpopo maybe its performance would be better than if released at Cannonbrae (controlling for variables like climate difference). The next chapter considers the second part of EICA, i.e. performance of the specialist herbivore *P. insulata* on SAB *C. odorata* collected from sites infested and uninfested by the moth in the country of introduction.

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CHAPTER 7: LARVAL PERFORMANCE OF A SPECIALIST HERBIVORE *PAREUCHAETES INSULATA* (LEPIDOPTERA: EREBIDAE: ARCTIINAE) ON *CHROMOLAENA ODORATA* PLANTS FROM DIFFERENT LOCATIONS IN SOUTH AFRICA

7.1 Abstract

The Evolution of Increased Competitive Ability (EICA) hypothesis posits that specialist herbivores will demonstrate improved performance on plant individuals originating from an area where plants have been introduced, compared to individuals of the same plant species from its native range, because the plants have diverted resources from defence against natural enemies, towards growth and reproduction. To test EICA, an experiment was conducted on newly hatched *Pareuchaetes insulata* larvae fed on leaves of *Chromolaena odorata* plants that were collected from locations where *P. insulata* is present (Umkomaas and Komatipoort) and locations where the moth has not been recorded (Thohoyandou and Pietermaritzburg). Insect performance metrics measured were larval development time, pupal development time, total development time, pupal weight, growth rate and overall survival. Consistent with EICA, immature stages (newly hatched larvae-adult eclosion) that fed on leaves from Umkomaas, a location with an exposure to *P. insulata* for 14-18 years, had prolonged development than larvae that were fed on leaves from Thohoyandou and Pietermaritzburg, locations without exposure to *P. insulata*, and Komatipoort, a location that has only recently been reassociated with *P. insulata*. Faster larval development was also evident on plant cuttings obtained from full-sun compared to those obtained from shaded habitat. Larvae that fed on the leaves from shade from Komatipoort had developmental time intermediate between larvae feeding on the leaves from the shade from Thohoyandou and Umkomaas. Overall survival was lowest on leaves of plants obtained from Komatipoort. Pupae of the larvae which fed on the leaves from full sun from Komatipoort showed intermediate trends of development between pupae of the larvae that fed on leaves from full sun from Umkomaas and Thohoyandou. Location did not appear to influence pupal mass but this variable was higher in full-sun plants from

Umkomaas, Thohoyandou and Pietermaritzburg. *Chromolaena odorata* was first recorded in KwaZulu-Natal, South Africa 72 years ago, while *P. insulata* was introduced 18 years ago. The existing reassociation time may not be enough for evolutionary changes to occur in *C. odorata* defence and *P. insulata* response to plant evolution, and could explain the inconsistency in some *P. insulata* performance parameters on infested and uninfested populations of *C. odorata*.

Key words: Evolution of increased competitive ability (EICA), *Chromolaena odorata* specialist herbivore, reassociation, *P. insulata*

7.2 Introduction

Among several invasive plant forms including trees, grasses and reeds, climbers, terrestrial herbs and aquatics, shrubs are as successful invaders because they also possess secondary metabolites, which in the area of introduction they may use as novel weapons to outcompete indigenous plants (Callaway and Ridenour 2004; Qin et al. 2013). Additionally, shrubs may become aggressive in a new range because they escape their specialist herbivores (Blossey and Notzöld 1995; Keane and Crawley 2002), although to some extent they will encounter generalist herbivores which may be deterred to some extent by a number of secondary metabolites found in the shrubs (Müller-Schärer et al. 2004; Joshi and Vrieling 2005). While the shrubs may become prolific in the absence of specialist herbivores in the country of introduction, oftentimes their native specialists may also invade exotic ranges where these shrubs are already naturalised, such as in biological programmes where the native specialists are intentionally introduced to control or suppress the invasive shrubs (Fukano and Yahara 2012). Biological control programmes are mostly successful because, when reassociated with the shrubs with reduced defence mechanisms in the introduction country, performance of native specialist herbivores become enhanced. This is proposed in the Evolution of Increased Competitive Ability (EICA) hypothesis, which further posits that specialist herbivores will demonstrate improved performance on plants from an area where the species has been introduced, compared to performance on plants from the native range, because the plants have diverted resources from defence against natural enemies, towards growth and reproduction (Blossey and Notzöld 1995).

Plants on the forest floor, including shrubs, comprise the majority of species diversity and play a vital role in forests, for the most part in forest monocultures (Karolewski et al. 2013). Shrubs are essential for biodiversity and contribute greatly to ecosystem function for a number of reasons i.e. the understory (a) protects the soil against erosion (b) it reduces evaporation from the soil surface, and improves the microclimate of the forest interior, by limiting the penetration of wind, (c) it warrants the establishment of structurally and chemically different forest litter, contributing to soil biodiversity and (d) it assembles mineral nutrients (Kumar et al. 2017; Yang et al. 2019). Therefore, shrubs inhibit soil degradation, improving soil structure and chemical composition and that is why they are more often considered in establishment of a forest in an area where there was no tree cover (Karolewski et al. 2013; Rice et al. 2018). Although understory plants appear capable of maintaining a positive carbon balance under the big trees, light remains the major environmental factor limiting or promoting their growth and reproduction (Chazdon and Pearcy 1991).

The effect of light on understory plants can be explained by the carbon-nutrient balance hypothesis which predicts that when plant carbon availability is restricted relative to nitrogen (e.g. low light at high soil nitrogen), concentrations of foliar carbon-based defensive chemicals (e.g. tannins and terpenoids) will decline relative to the concentration of nitrogen, and plants should be more edible to herbivores. Contrarily, when carbon availability is high relative to nitrogen (e.g. high light at low soil nitrogen), leaf nitrogen concentration should decline, concentrations of carbon-based defensive chemicals should increase, and plants should be less edible to herbivores (Herms and Mattson 1992; Moran and Showler 2005).

Light conditions significantly modify the structure, water content and concentration of metabolites in leaves. This is corroborated in the growth differentiation balance hypothesis which posits that plants growing under shaded habitats, with limited resource supply, should display inadequate growth and photosynthesis, and have reduced biomass and secondary metabolites compared with plants growing in sunny habitats with high levels of resource supply (Herms and Matsson 1992). Consequently, understory shrubs of some

species are severely defoliated by folivorous insects and differ significantly in the degree of leaf damage (Karolewski et al. 2007). Frequently, shrubs of some species may be nearly completely defoliated by folivorous insects depending on the light intensity of their habitat (Crone and Jones 1999; Karolewski et al. 2013). The reduced leaf damage in plants growing in high light conditions compared to those in shade, as well as the less frequent damage of sunlit leaves than shaded leaves of the same plant, may be due to higher levels of defense metabolites in leaves, such as tannins, flavonoids, or gluconates (Dudt and Shure 1994; Close and McArthur 2002) which deter and/or attract insect herbivores (Lankau 2007).

A number of studies have demonstrated the enhanced performance (measured by the susceptibility of host plant in response to the specialist herbivore, and better growth rate, fecundity and developmental times of the herbivore) of a specialist herbivore when reassociated with its host plant compared to its performance on individuals of the host plant from its native range (Wolfe et al. 2004; Meyer et al. 2005; Rapo et al. 2010; Fukano and Yahara 2012; Jogeshi et al. 2014; Wan et al. 2019). However, several others have showed that the improved performance of specialist herbivores can be influenced by other environmental factors, such as sunlight (Trumbule and Denno 1995; Crone and Jones 1999; Diaz et al. 2011; Uyi et al. 2015; Uyi et al. 2018). Such environmental factors have thus confounded the understanding of evolutionary changes – this is evident in the sometimes mixed results obtained from these kinds of studies. Furthermore, exotic plant species are introduced into a diverse environment harbouring diverse recipient communities, and different genotypes may arrive in different regions or habitats, which may eventually influence the trend and swiftness of evolutionary changes in introduced populations (Rapo et al. 2010).

A biological control programme was initiated against the invasive alien shrub, *Chromolaena odorata* (L.) R.M. King and Robinson (Asteraceae), in South Africa in the late 1980s. *Chromolaena odorata* was first recorded as naturalised in South Africa 72 years ago (Zachariades et al. 1999, 2011). One of the successful biological control agents, in terms of establishment and dispersal, is a moth with defoliating larvae, *Pareuchaetes*

insulata Walker (Lepidoptera: Erebidae), a specialist herbivore from Florida, USA, which lies within the native range of *C. odorata*. About 335 000 larvae were released at a site near Umkomaas on the south coast of KwaZulu-Natal province (KZN) in South Africa from 2001-2003 (over 15 years ago) (Zachariades et al. 2011). Out of the total of almost two million insects (including later introductions of the same species from Jamaica and Cuba) were released between 2001 and 2009, at 30 sites in KZN, this was the only confirmed establishment. An initial population outbreak of the moth was recorded at the Umkomaas site in 2004-2006, and *P. insulata* has since spread to northern KZN, was found in in eSwatini (Swaziland) in 2015, in Komatipoort in Mpumalanga province in 2016 (Zachariades et al. 2016) and in south-western Mozambique in 2017 (ARC-PHP, unpublished data). However, *P. insulata* has not yet been found in Limpopo province or in colder parts of KZN and we do not know how long it has been in Mpumalanga province before we recorded it, although it probably only arrived there 1-2 years prior. There has been a remarkable reduction of *C. odorata* in the Umkomaas area over the years, even though the moth population has fluctuated markedly (several smaller-scale outbreaks followed by crashes in the population). More broadly, the moth appears to have remarkably reduced the reproductive potential of *C. odorata* in many of the areas where it is present (13 years personal observations and unpublished studies). In general, the invasiveness of *C. odorata* in parts of KZN appears to have decreased, although this has not been linked directly to the moth, or to the other biocontrol agent established on *C. odorata*, the leaf-mining fly *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae).

There was an initial concern that a degree of incompatibility between the southern African biotype of *C. odorata*, originating from Jamaica or Cuba (Paterson and Zachariades 2013; Shao et al. 2018), and *P. insulata*, originating largely from Florida (USA), may have resulted in low populations and inconsistent establishment of the insect in the field in South Africa. However, Uyi et al. (2014) did not find much evidence of this in the laboratory. Uyi et al. (2015, 2016, 2017) outlined the role played by habitat (shaded vs full-sun) and temperature on the establishment and efficacy of this arctiine moth. This study was conducted because it seemed that the decline in *C. odorata* was greater than could be expected from defoliation under the Enemy Release Hypothesis model. After feeding by

Pareuchaetes pseudoinsulata Rego Barros (Lepidoptera: Erebidae: Arctiinae) larvae, *C odorata* leaves turn yellow (Marutani and Muniappan 1991), and this may have resulted in long-term changes to the plant populations. Also, populations of *P. insulata and P. pseudoinsulata*, introduced as biocontrol agents in several countries, have been characterised by an initial massive outbreak followed by smaller ones and lower populations, which could be explained by lower *C. odorata* densities available but also by long-term changes in plant defence at a population level.

To understand the evolutionary responses of *C. odorata* to the reassociation with its specialist herbivore, an experiment on plant phytochemistry and growth rates was conducted in the previous chapter using *C. odorata* plants collected from full sun and shade in locations with and without *P. insulata* infestation. This chapter then answers the following questions: do *C. odorata* plants from specialist enemy-free environments (i.e. Thohoyandou in Limpopo province and Pietermaritzburg in colder parts of KZN province) support better growth performance of *P. insulata* than the plants from environments that are reassociated with the specialist herbivore (i.e. Umkomaas release and establishment point in KZN province and Komatipoort in Mpumalanga province)? Will results from the *C. odorata* plants from a site that has recently become reassociated with *P. insulata* be intermediate between the sites where the specialist herbivore is present and absent?

7.3 Materials and methods

7.3.1 Study system: origin and maintenance of plants and moths

Plants used in this study were from the garden experiment conducted in a shadehouse in the botanical gardens at the University of KwaZulu-Natal, Pietermaritzburg campus in Chapter 6. After the completion of the garden experiment, plants were moved to the tunnels at the Agricultural Research Council, Plant Health and Protection (ARC-PHP), Cedara (29° 32’ S, 30° 16’ E) for this study, were placed randomly on plant-holding tables for about a week before use and were watered accordingly.

Pareuchaetes insulata larvae were collected from Umkomaas in the Sappi Cannonbrae plantation, KZN province, South Africa (30° 13' S, 30° 46' E), where the insect was recorded as established since 2004 (Zachariades and Strathie 2006). The larvae were maintained in the laboratory at ARC-PHP Cedara in 2l Freezette trays with Oasis blocks stalked with chromolaena bouquets, to give them enough space and food. Initially larvae were fed *C. odorata* from Cannonbrae and thereafter from Peter Brown Drive, Pietermaritzburg, and were changed to clean trays with fresh leaves as needed until they pupated, at 25 ± 2° C, 65 ± 10% relative humidity (RH), with a photoperiod of L12:D12. Pupae were transferred to separate 2l Freezette trays with Oasis blocks stalked with *C. odorata* bouquets and were monitored for adult eclosion and oviposition.

7.3.2 Larval performance trials

On the 4th day after oviposition (approximate duration from egg laying to hatching of *P. insulata*) newly hatched individual larvae from above (section 7.3.1) were transferred to 100 ml aerated plastic containers with a circular net screen window (25 mm diameter on the top for ventilation), lined with moistened filter paper at the bottom to maintain high relative humidity. Leaves used for larval feeding were obtained from plants in the tunnels at Cedara that were used in the garden experiment for comparison of *C. odorata* growth rates, collected from full sun and shady habitats in Thohoyandou, Komatipoort, Umkomaas and Pietermaritzburg in Chapter 6. For each site and habitat (full sun and shade), 240 larvae were transferred to 100 ml plastic containers with leaves, one larva per container, and these were placed in a growth chamber set at 25° C. As per Uyi (2014), this technique presented two main benefits: (i) feeding larvae in isolation prevented biases due to competition and consequent food deprivation and (ii) prevention of variations due to microhabitat effects. Initially (from 1st to 3rd instar larvae), the filter paper in the plant containers was moistened after 3 days. At this time, frass was removed and new leaves were added, and thereafter the same procedure was conducted after every second day until pupation. All leaf materials were obtained fresh from over eight plants per habitat on each collection day from the tunnel and replaced by new materials from different plants on subsequent visits. The daily use of new leaf tissues is consistent with field observations of *Pareuchaetes* species

preferentially feeding on undamaged leaves in the presence of an abundant food supply. Although the use of excised leaves in the determination of insect survival and performance has been a subject of debate (Olckers and Hulley 1994; Blossey and Notzöld 1995), a recent study found that egg and larval survival did not differ between leaves on intact plants and excised leaves in the specialist herbivore, *Pieris napi* (L.) (Lepidoptera: Pieridae, Pierini), whereas larval growth was slightly, but significantly, faster on leaf-cuttings (Friberg and Wiklund 2016). The use of excised leaves is a standard method for providing uniform materials in the laboratory-based feeding studies of this kind (see Uyi et al. 2015, 2017). During the trial, mortality was also recorded. Because of the limited plant material available from growth rate studies, leaves which had not been eaten were washed and were reused with the new leaves during each changeover.

To prevent reduced relative humidity in the growth chamber, all containers were kept inside a Ziploc^{TM} bag (600 x 450 mm). Previous studies have showed that larvae take approximately 10-11 days to develop from 1st to 3rd instar (Dube 2008; Uyi 2014); therefore, after 10 days, containers were inspected every day in order to follow individual larvae through to pre-pupation and pupation. After pupation, leaves were removed from the containers and the pupae were monitored after 3 days for eclosion. During the pupal stage, sex was determined as per Dube (2008) and larval performance was scrutinized based on the number of surviving pupae. The following parameters were measured: total larval duration (defined here as the number of days from hatching until pupation), total pupal duration (pre-pupal and pupal combined) and growth rate (pupal mass in mg/larval+pupal development duration).

7.3.3 Statistical analysis

Following arcsine square root transformation of the survival data, the effects of location and habitat on overall survival of *P. insulata* (neonate larva to adult eclosion) were analysed using a Generalized Linear Model (GLZ), assuming a normal distribution with an identity link function. When the result of the analysis was significant, the differences were separated using the sequential Bonferroni test. The effects of location and habitat on larval development time, pupal development time, total development time, pupal mass and

growth rate were compared using a two-way analysis of variance (ANOVA). Due to unequal sample sizes among treatments in the insect performance trials, means were compared using Tukey Kramer's test. The survival data was analysed using IBM SPSS Statistical software version 20.0 (SPSS, Chicago, IL, USA), while the insect performance data was performed using Genstat 12.0 (VSN International, Hemel Hempstead, UK).

7.4 Results

7.4.1 Larval development time

Larval development time differed as a function of location and habitat (Table 7.1, Fig. 7.1A). Larvae feeding on leaves from Umkomaas (infested) developed significantly faster than the larvae feeding on leaves from Komatipoort (infested), Thohoyandou (uninfested) and Pietermaritzburg (uninfested) (Table 7.1). Furthermore, larvae feeding on the leaves from full sun Umkomaas developed significantly faster than larvae feeding from the shade (Table 7.1, Fig. 7.1A). Interestingly, larvae feeding on the leaves from the shade from Komatipoort had developmental trends intermediate between larvae feeding on the leaves from the shade from Thohoyandou and Umkomaas and similarly were not significantly different to larvae feeding on the leaves from the shade from Pietermaritzburg (Table 7.1, Fig. 7.1A). The larvae feeding on the leaves from the full sun from Umkomaas and Thohoyandou developed significantly faster than larvae feeding on the leaves from the shade (Table 7.1, Fig. 7.1A).

7.4.2 Pupal development time

Pupal development time differed significantly as a function of location and habitat (Table 7.1, Fig. 7.1B). Pupal development time of larvae that fed on leaves from Umkomaas was significantly longer than that of the pupae from other 3 sites (Table 7.1, Fig. 7.1B). Pupae of the larvae that fed on leaves from the full sun Umkomaas developed for a significantly longer period than pupae from the larvae that fed on the leaves from full sun Thohoyandou and Pietermaritzburg, but did not differ significantly from pupae of the larvae that fed on leaves from Komatipoort full sun (Table 7.1, Fig. 7.1B). In fact, pupae of the larvae that

fed on the leaves from full sun Komatipoort showed intermediate trends of development time between pupae of the larvae that fed on leaves from full sun Umkomaas and those from Thohoyandou (Table 7.1, Fig. 7.1B).

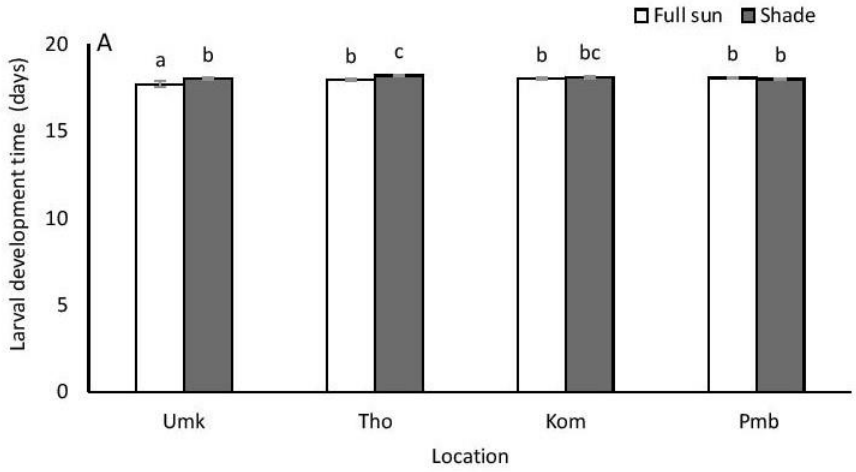
7.4.3 Total developmental time

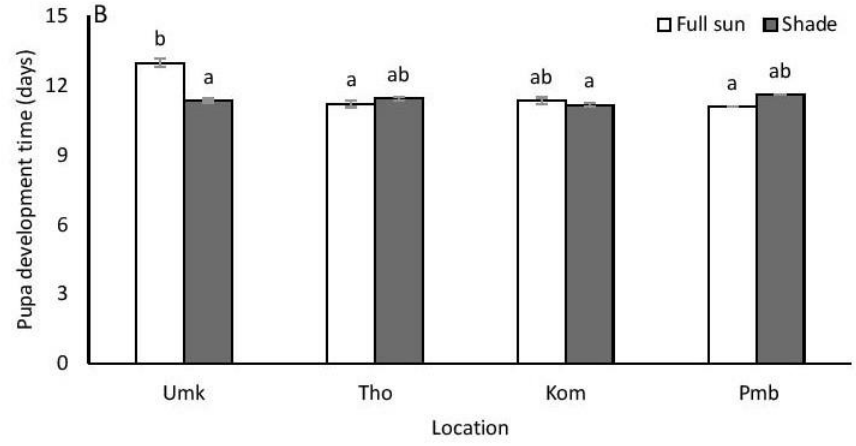
Total development time differed as a function of location and habitat (Table 7.1, Fig. 7.1C). Total development time was significantly longer on leaves from Umkomaas compared to that on the leaves from Thohoyandou, Komatipoort and Pietermaritzburg (Table 7.1, Fig. 7.1C). Total development time was significantly longer on the leaves of plants collected in full sun from Umkomaas compared to that on the leaves of shaded plants from Umkomaas. Total development time on the leaves from full sun Umkomaas was significantly longer than that on the leaves of full-sun plants from Thohoyandou, Komatipoort and Pietermaritzburg but there was no significant difference in total development time between habitat and location between these 3 sites (Table 7.1, Fig. 7.1C).

7.4.4 Pupal mass, growth rate and overall survival

Pupal mass and growth rate differed as a function of habitat but not as a function of location (Table 1, Fig. 7.2, 7.3). Pupal mass and growth rate of larvae that were fed on leaves from full sun were greater than those on the leaves from the shade in Umkomaas, Thohoyandou (pupal mass only) and Pietermaritzburg (Table 7.1, Fig. 7.2, 7.3). Contrarily, pupal mass of the larvae that fed on leaves from full sun Komatipoort was significantly lower than those from the shade, and from full sun Umkomaas, Thohoyandou and Pietermaritzburg (Fig. 7.2). Similarly, pupal mass of the larvae that fed from shade Komatipoort was significantly higher than those from the shade from the other 3 locations (Fig. 7.2). Generally, growth rate was significantly greater in full sun than shade, but as with pupal mass, growth rate was significantly lower on leaves from full sun Komatipoort than from shade and was contrary to Umkomaas, Thohoyandou and Pietermaritzburg (Fig. 7.3. Table 7.1). Although there was significantly lower survival in insects that were fed on leaves from Komatipoort compared to those from Umkomaas, Thohoyandou and

Pietermaritzburg (Fig. 7.4), generally the mortality was low, with a survival rate ranging from 88.3% for insects fed on leaves from Komatipoort to 98.3% for those fed on leaves from Pietermaritzburg (Fig. 7.4).


A
Full sun
Shade
Larval development time (days)
20
15
10
5
0
a
b
b
c
b
bc
b
b
Umk
Tho
Kom
Pmb
Location


B
Full sun
Shade
Pupa development time (days)
15
12
9
6
3
0
b
a
a
ab
ab
a
a
ab
Umk
Tho
Kom
Pmb
Location

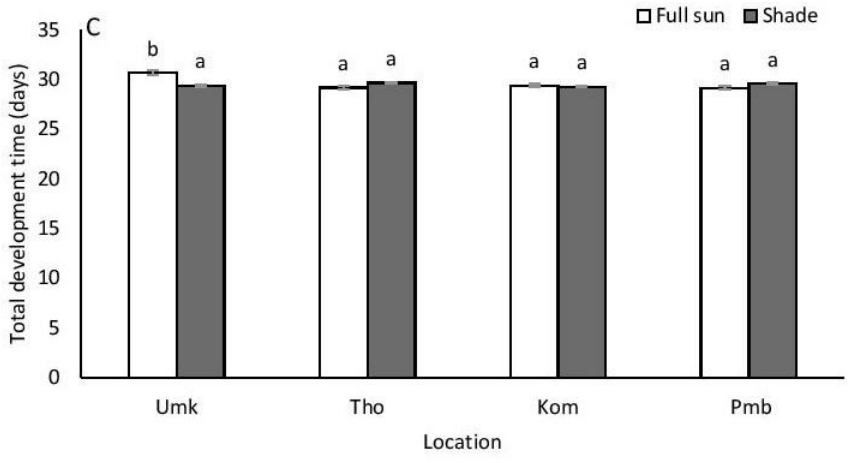

C
Full sun
Shade
Total development time (days)
35
30
25
20
15
10
5
0
b
a
a
a
a
a
a
a
Umk
Tho
Kom
Pmb
Location

Figure 7.1: Development times of *Pareuchaetes insulata* reared on *Chromolaena odorata* leaves collected from garden experiment of plants collected from shaded and full-sun habitats in locations where the moth is present or has not been recorded (mean ± SE) (A) Larval development time (days); (B) pupal development time (days); (C) total development time of *P. insulata* (days). Means with different letters above bars are significantly different ($p < 0.05$) after Tukey–Kramer test.

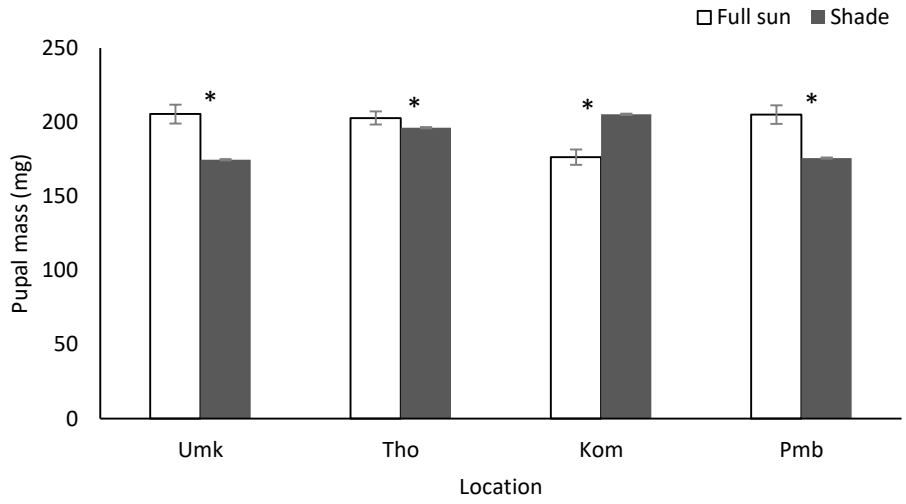


Figure 7.2: (mean ± SE) pupal mass (mg) of *Pareuchaetes insulata* reared on *Chromolaena odorata* leaves collected from garden experiment of plants collected from full-sun and shaded locations where the moth is present or has not been recorded. Asterisk denotes significant differences in pupal mass between shaded and full-sun habitat ($p < 0.05$) after student t-test.

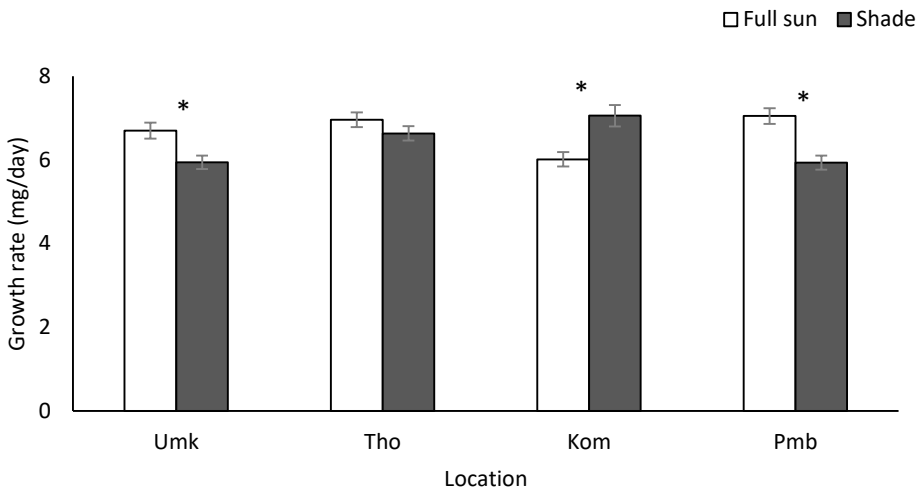


Figure 7.3: (mean ± SE) growth rate mg/day) of *Pareuchaetes insulata* reared on *Chromolaena odorata* leaves collected from garden experiment of plants collected from full-sun and shaded locations where the moth is present or has not been recorded. Asterisk denotes that growth rate was significantly different between shaded and full-sun habitat ($P < 0.05$) after student t-test.

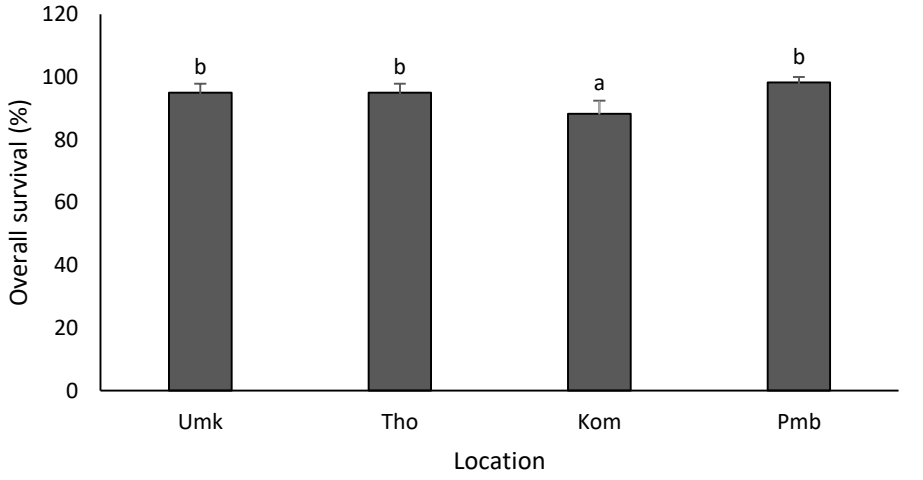


Figure 7.4: (mean ± SE) overall survival (%) of *Pareuchaetes insulata* reared on *Chromolaena odorata* leaves collected from garden experiment of plants collected from full sun and shaded locations where the moth is present or has not been recorded. Means capped with different letters are significantly different (sequential Bonferroni test: $P < 0.05$).

Table 7.1: Performance of *Pareuchaetes insulata* reared on *Chromolaena odorata* leaves collected from garden experiment of plants collected from full sun and shade of infested and uninfested locations over one generation at 25°C in the growth chamber in the laboratory.

Analysis	Source of variation	DF	MS	F-value	P-value
Larval dev. time	Location	3	0.5324	3.07	**0.029**
	Habitat	1	1.1786	6.78	**0.001**
	Loc.Hab	3	0.4368	2.52	0.059
	Total	225			
Pupal dev. time	Location	3	10.199	9.69	**<.001**
	Habitat	1	3.788	3.6	0.059
	Loc.Hab	3	12.893	12.25	**<.001**
	Total	225			
Total dev. time	Location	3	6.189	5.2	**0.002**
	Habitat	1	0.741	0.62	0.431
	Loc.Hab	3	9.873	8.3	**<.001**
	Total	225			
Pupal mass	Location	3	1222.2	1.46	0.227
	Habitat	1	6057.3	7.23	0.008
	Loc.Hab	3	10672.8	12.74	**<.001**
	Total	225			
Growth rate	Location	3	2.284	2.17	0.092
	Habitat	1	5.791	5.5	**0.02**
	Loc.Hab	3	12.236	11.63	**<.001**
	Total	225			

7.5 Discussion

The EICA hypothesis posits that specialist herbivores will demonstrate improved performance on plant individuals originating from the area into which the plant species has been introduced, compared to those from the native range, because the plants trade some of their defensive capacity for increased growth once they are in the new area that lacks specialist herbivores (Blossey and Notzöld 1995). The corollary of this is that once a specialist herbivore has been introduced into the adventive range (e.g. as a biocontrol agent) the process will reverse. Following this logic, this study examined the potential for the evolution of resistance to a specialist herbivore in the invasive alien shrub *C. odorata*, after reassociation with its native co-evolved herbivore *P. insulata* for over a decade. To

get a fuller representation of each location, we sampled from both shaded and full-sun habitats.

Leaving aside EICA predictions, studies that considered the responses of insect herbivores, in terms of performance metrics such as development, growth and fecundity, to vegetation grown in the sun or shade and have showed mixed results and these appear to be species-specific. For example, some studies have recorded improved immature survival, more rapid development, increased pupal mass and high fecundity in insects that were fed on vegetation from the shade (Trumbule and Denno 1995; Jansen and Stamp 1997; Sipura and Tahvanainen 2000; Crone and Jones 1999; Diaz et al. 2011), while others showed the same variables to be greater in a full-sun environment (Sipura and Tahvanainen 2000; Moran and Showler 2005; Osier and Jennings 2007). Still other studies showed no difference in the performance of insect herbivores between a shade and full sun environment (Moore et al. 1988; Horner and Abrahamson 1992; Potter 1992; Sipura and Tahvanainen 2000). Karowleski et al. (2013) found that leaves of all the species they examined that were growing in the sun had higher concentrations of defence metabolites than those in the shade; and leaves of the shrubs *Prunus serotine* Ehrh. (Rosaceae), *Sambucus nigra* (L.) B.L. Turner (Adoxaceae), *Cornus sanguinea* L. (Cornaceae) and *Frangula alnus* Mill. (Rhamnaceae) in full-sun were less injured than those on the shade. Similarly, previous chapter (Ch 6) generally showed higher concentrations of total phenolics, tannins and flavonoids in *C. odorata* leaves collected from full sun than those from the shade. Over the years, higher levels of *P. insulata* damage on *C. odorata* plants were observed (personal observations) growing in the shade compared to plants growing in the full sun. Uyi et al. (2015, 2018) demonstrated enhanced performance (faster development, higher pupal mass, increased growth rate and higher host index suitability score) in individuals of *P. insulata* that were reared on shaded leaves of *C. odorata* compared to high sunlight vegetation.

Results in this chapter showed mixed results relative to EICA, the carbon nutrient hypothesis or findings of Uyi et al. (2015, 2018); in some cases metrics did not show differences in *P. insulata* performance between two habitats or in different locations. This

could be attributed to the fact that the leaves used in this experiment were not collected direct from the field at the time of exposure to the larvae but were rather from plants that were collected from the two habitats and grown in a shade house where they received the same amount of light, water and nutrients. Additionally, inconsistency in host-plant preference is not new in *Pareuchaetes* species. For example, *P. pseudoinsulata* demonstrated greater preference for the leaves of *C. odorata* growing in full sun habitat than those from a shaded habitat, although better performance was found for some but not in others of these listed metrics in full sun vs shade habitats (Uyi et al. 2017).

Although there are studies that tested and did not support the EICA hypothesis (Bosdorff et al. 2005; Shelby et al. 2016), there is a good record of studies that confirmed enhanced plant growth in the absence of a specialist herbivore (Leishman et al. 2014; Rouifed et al. 2018) and enhanced performance of the specialist herbivore on plants in the introduction range compared to those in the native range (Blossey and Notzöld 1995; Meyer et al. 2005). To explain such contradictory evidence, Dietz and Edwards (2006) invoked the importance of time since invasion. Possibly, the use of plants grown from the *C. odorata* seeds that were collected together with other plant material from each of the four sites and represented generation N + 1, may have produced more distinctive results. Recently, a few studies (e.g. Zangerl et al. 2008; Cripps et al. 2009; Rapo et al. 2010; Jogesh et al. 2014; Wan et al. 2019) considered evolutionary changes of invasive weeds after reassociation with their specialist herbivores in the country of introduction.

It was predicted that the altered selection pressure on *C. odorata* after introduction of *P. insulata* will enhance the moth's performance when fed leaf material from *C. odorata* sites where the moth is absent, will show intermediate trends at a site where *C. odorata* is recently reassociated with *P. insulata*, and will slow its performance when fed on plant material from a site at which it has been present for more than 15 years. Results in this chapter showed mixed performance of the specialist herbivore when reunited with *C. odorata* in the country of introduction. Contrary to our expectation, immature stages (newly hatched to pupation) which were fed on leaves from Umkomaas (infested by *P. insulata* for over 15 years) developed faster than the larvae feeding on leaves from

Komatipoort where *P. insulata* was found in 2015, and on leaves from Thohoyandou and Pietermaritzburg (both enemy free). However, consistent with EICA prediction, pupal and total developmental time to adulthood was faster on *P. insulata* that fed on leaves from Komatipoort, Thohoyandou, and Pietermaritzburg compared to Umkomaas. Additionally, larvae fed on the leaves from the shaded habitat in Komatipoort had developmental trends intermediate between larvae feeding on the leaves from the shade from Thohoyandou and Umkomaas. Similarly, pupae of the larvae that fed on the leaves from full-sun Komatipoort showed intermediate trends of development between pupae of the larvae that fed on leaves from full-sun Umkomaas and Thohoyandou. Unexpectedly, and contrary to Uyi et al. (2015) pupal mass and growth rate of *P. insulata* fed on leaves from full sun Umkomaas, Thohoyandou and Pietermaritzburg were higher than those from the shade. This could be possible because Uyi et al. (2015) collected plants directly from the two habitats and fed insect larvae simultaneously, while plants used in this study were first grown in the garden experiment with uniform conditions for 8 months before being exposed to *P. insulata*. But pupal mass and growth rate were greater on *P. insulata* from shade Komatipoort compared to *P. insulata* from the full sun at the same site, and from shade Umkomaas, Thohoyandou and Pietermaritzburg. Lastly, overall survival was lower on *P. insulata* fed on leaves from Komatipoort compared to those on leaves from Umkomaas, Thohoyandou and Pietermaritzburg. The anomalous results from Komatipoort were consistent with EICA (rapid evolution caused by enemy reassociation) and the prediction that results from the *C. odorata* plants from a site that has recently become reassociated with *P. insulata* being intermediate between the sites where the specialist herbivore is present or absent.

Rapo et al. (2010) proposed that the introduction of biological control agents should reverse the increased allocation to competitive ability and defences against generalist herbivores, and select for plants with life-history traits that are more similar to those of plants in the native range, than those of plants in the introduced range that have not been exposed to biological control. Zangerl and Berenbaum (2005) and Zangerl et al. (2008) showed that an invasive European weed *Pastina sativa* L. (Apiaceae), demonstrated phytochemical shifts in response to the accidental introduction for over 152 years of its major herbivore *Depressaria pastinacella* Duponchel (Lepidoptera: Oecophoridae), such as increased

concentrations of the floral volatile used by *D. pastinacella* for orientation, which is counterproductive for the plant and illuminates a potential consequence of classical biological control. Furthermore, Zangerl et al. (2008) showed that populations of *P. sativa* newly infested by coevolved *D. pastinacella* experienced selection for phytochemical changes. Phytochemical changes including yellowing of *C. odorata* leaves caused by herbivory by *P. insulata* (as with *P. pseudoinsulata* in Marutani and Muniappan (1991)) and variation of chemical defences such as phenolics, flavonoids and tannin contents could be responsible for inconsistent performance of *P. insulata* in populations of *C. odorata* reassociated and without this specialist herbivore in the country of introduction. Reassociation of *Ambrosia artemisiifolia* L. (Asteraceae) with its specialist herbivore *Ophraella communa* Lesage (Coleoptera: Chrysomelidae) manifested better performance of the beetle on uninfested plants from more remote Japanese islands, with enhanced growth compared to that on plants infested by the beetle on other Japanese islands over 10-12 years previously (Fukano and Yukano 2012) and strongly supported EICA. A study on *A. artemisiifolia* in China that included the generalists *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) and *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae), the specialist herbivore *O. communa* and chemical defence between sites infested and uninfested by *O. communa* (whilst all 2 insect herbivores were found at all sites), demonstrated increased qualitative defence metabolites in populations of *A. artemisiifolia* infested by *O. communa* for 9-13 years, but no differences in quantitative defence traits in infested and uninfested *A. artemisiifolia*. In the same study, the specialist herbivore, *O. communa,* performed better on plant populations where it is not reassociated with the host plant *A. artemisiifolia* (Wan et al. 2019).

Evolutionary changes in plant defence traits and insect performance can be influenced by several other environmental factors such as temperature, light intensity, soil, water nutrients, etc. (Moloney et al. 2009; Bickford 2016). This study demonstrated some trends supporting EICA. However, the existing reassociation time (18 years since first release in 2001 and 15 years since initial outbreak in 2004) may not be enough for evolutionary changes to occur in *C. odorata* defence, and could explain inconsistency of *P. insulata* performance observed in some parameters on infested and uninfested population of *C.*

odorata. Although variation in host plant (genotypes from Florida and South Africa) showed no effect on the performance and fitness-related traits of *P. insulata* (Uyi et al. 2014), our study only examined reassociation of *C. odorata* with a population of *P. insulata* originally from Florida, on a *C. odorata* genotype dissimilar to the biotype invading southern Africa (i.e. a 'new association'), and excluded generalists such as *Zonocerus* species common in *C. odorata*. The use of a *P. insulata* population from Jamaica (from where the southern African biotype of *C. odorata* originates) may have yielded different results. Joshi and Vrieling (2005) suggested the modification of EICA to reflect that, in the introduced range, where specialist herbivores are largely absent, plants might be attacked by native generalist herbivores, with the expectation that plant toxins (= qualitative defence metabolites, effective against generalists and relatively cheap to produce) will increase in concentration. As discussed in previous chapter (Ch7 section 6.5.3 pg 138), high concentrations of quantitative secondary metabolites such as phenolics, tannins and flavonoids are known to deter both generalist herbivores and specialist herbivores, to attract or not to have an impact on some (Van der Meijden 1996; Müller-Schärer et al. 2004). Therefore, if *P. insulata* is negatively affected by high concentrations of quantitative defences, that could explain its higher damage and performance on leaves of *C. odorata* plants growing in shaded than in full-sun habitat (Herms and Mattson 1992; Coley and Barone 1996; Crone and Jones 1999). Given that this study was only conducted in the range of introduction of *C. odorata*, it is recommended that future studies of this nature include native range data. Additionally, it is possible that the indigenous grasshopper *Zonocerus elegans* (L) (Orthoptera: Pyrgomorphidae), acting as a specialist herbivore on *C. odorata* because of its ability to sequester pyrrolizidine alkaloids, prevented EICA mechanisms from taking place in *C. odorata* in South Africa after the introduction of the weed. Work done on the Asian/West African biotype of *C. odorata* demonstrated that *P. insulata* is also attracted to qualitative defences in the form of pyrrolizidine alkaloids (Schneider et al. 1992; Conner 2009). The next chapter considers the roots for determination of pyrrolizidine alkaloids from the southern African biotype of *C. odorata*.

7.6 References

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CHAPTER 8: PYRROLIZIDINE ALKALOIDS FROM THE SOUTHERN AFRICAN BIOTYPE OF *CHROMOLAENA ODORATA* (ASTERACEAE)

8.1 Abstract

Pyrrolizidine alkaloids (PAs) are ester alkaloids composed of an amino alcohol and mono- or dicarboxylic acids found in several plant families, including the Asteraceae. Pyrrolizidine alkaloids are used for plant defence against generalist invertebrate herbivores. However, specialist herbivores that are able to sequester PAs from their host plant species derive a number of benefits from these defence compounds. This study investigated PAs from the roots of the southern African biotype (SAB) of *Chromolaena odorata* in different regions and habitats in South Africa, with and without the specialist herbivore *Pareuchaetes insulata*. Alkaloids were initially detected by thin layer chromatography sprayed with acetic anhydride heated and resprayed with Ehrlich reagent. Two pyrrolizidine alkaloids, rinderine and its stereoisomer *N*-oxide intermidine, were isolated from the roots of the SAB *C. odorata* using GC-MS. The structures and configuration were confirmed by chemical and spectroscopic methods especially one-dimensional ^{1}H NMR analysis. Pyrrolizidine alkaloids are known to be used by arctiine moths to find their host-plant and sequestered for mating purposes, and furthermore these compounds make these lepidopterans unpalatable to their predators, relative to their palatable counterparts. Therefore, confirmation of rinderine and intermidine in *C. odorata* in this study substantiates the establishment and spread of *P. insulata* in southern Africa due to, among other factors, reduced predation.

Key words: Pyrrolizidine alkaloids, SAB *Chromolaena odorata*, *Pareuchaetes insulata*, GC-MS, NMR

8.2 Introduction

Of the secondary metabolites found in plants, pyrrolizidine alkaloids (PAs) have been well considered for their biosynthetic, chemical and ecological aspects (Klitzke and Trigo 2000). Pyrrolizidine alkaloids are N-based metabolites found in plants all over the world in the families of Asteraceae, Boraginaceae, Leguminosae, Orchidaceae, Apocynaceae,

Convolvulaceae and Ranunculaceae (Witte et al. 1993; Hartmann et al. 2001). Since plants are immobile, they are assumed to use secondary metabolites including PAs to preserve their fitness and make them resistant to adverse environments such as herbivory (Klitzke and Trigo 2000). Although PA-producing plants are usually avoided by generalist herbivores due to the toxic nature of these qualitative defensive compounds (van Dam et al. 1995; Gardner et al. 2006), some insect herbivores (usually specialist species with a narrow host-plant range) have overcome this chemical barrier by evolving adaptations to use PAs for their own benefit. For example, some insects (as both larvae and adults) store PAs obtained from plants for protection against predators and to synthesize pheromones necessary for courtship success (see review in Boppré 1990; Witte et al. 1993; Conner 2009; Macel 2011). Furthermore, males of such specialist insects are known to transfer a significant amount of PAs from their spermatophores to females during copulation. The females pass this gift, together with PAs that they themselves procured as larvae, to the eggs for defence against predators and parasitoids (Boppré 1990; Bezzerides et al. 2004; Conner 2009).

Chromolaena odorata (L.) King and Robinson (Asteraceae) is an invasive scrambling shrub with a wide native distribution ranging from the southern United States of America to northern Argentina, and including both Central America and the Caribbean islands (Gautier 1992; Kriticos et al. 2005; Paterson and Zachariades 2013). This wide native range distribution is mirrored by the wide range of introduction, with the plant being invasive through many parts of the humid tropics and subtropics of Africa, southern and Southeast Asia, China and parts of Oceania (Kriticos et al. 2005). The genetic and morphological variability of *C. odorata* in its native distribution partly illuminates the presence of two invasive biotypes of *C. odorata* known in its invasive range of distribution *viz.* the dominant Asian/West African biotype (AWAB), possibly originating from Trinidad and Tobago (Yu et al. 2014; Shao et al. 2018), which is the form invasive in all parts of the Old World other than southern Africa, and the southern African biotype (SAB) originating from Jamaica or Cuba (Paterson and Zachariades 2013; Shao et al. 2018) and invasive in southern Africa only. The invasion success of *C. odorata* is partly attributed to release from

natural enemies, proven strong chemical properties with allelopathic effects and genotypes with stronger competitive abilities (Thoden et al. 2007; Qin et al. 2013; Yu et al. 2014).

A recent study conducted on the phytochemical properties of SAB of *Chromolaena odorata* revealed several secondary compounds previously recorded as present in AWAB; however, this did not detect the presence of alkaloids (Omokhua et al. 2017). Biller et al. (1994) reported five PA monoesters that were abundant in alkaloid extract of AWAB *C. odorata* viz, 7-angeloylretronecine, 9- angeloylretronecine, intermidine, rinderine and 3'-acetylrinderine. Of these, the most dominant in the roots were rinderine and intermidine (Fig. 8.1). However, no study has reported the presence of alkaloids in SAB.

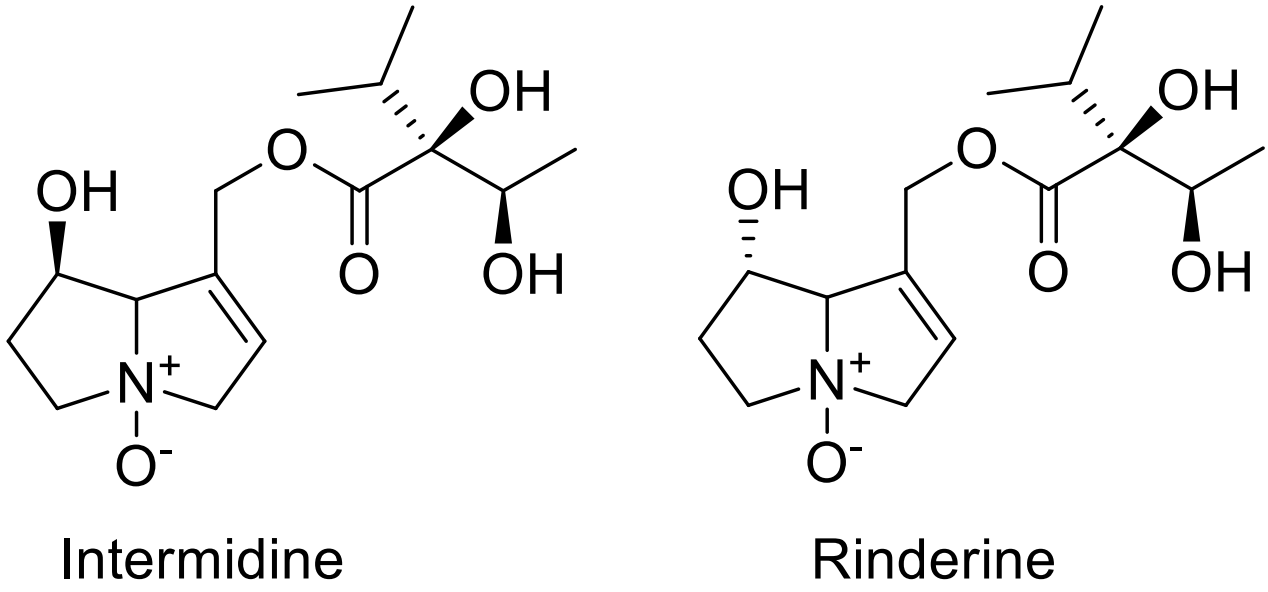

Figure 8.1: Structures of two of the most dominating pyrrolizidine alkaloid *N*-oxides, intermidine and rinderine, found in the roots of AWAB of *Chromolaena odorata* as reported by Biller et al. (1994).

Pyrrolizidine alkaloids of *C. odorata* exhibited nematicidal effects on the root-knot nematode *Meloidogyne incognita* Kofoid and White (Meloidogynidae) (Thoden et al. 2007). Vegetative shoots of *C. odorata* showed only trace amounts of PAs, whilst roots and inflorescences exhibited higher concentrations; the assumption was that the tiny

amounts of PAs in foliage indicated that avoidance of *C. odorata* by herbivores could be attributed to other secondary compounds, and that the high concentration in the inflorescence seemed to be vital during seed development (Biller et al. 1994). Furthermore, four PAs were found in all stages and in both sexes of the field-collected generalist herbivore *Zonocerus variegatus* (L) (Orthoptera: Pyrgomorphidae) from West Africa, after feeding on the AWAB *C. odorata*, and it is possible that it enjoyed a non-nutritional relationship with *C. odorata*, for fitness benefits (known as pharmacophagy) (Witte et al. 1993; Biller et al. 1994). Males of various lepidopterans are known to produce sex pheromones from PAs of plant origin (Boppré 1990; Witte et al. 1993; Klitzke and Trigo 2000). The function of such male pheromones in arctiine moth behaviour (e.g. *Pareuchaetes* species (Lepidoptera: Erebidae) on *C. odorata*) is known to be the induction of sexual acceptance by the females (Schneider et al. 1992). Additionally, because of their ability to sequester toxic PAs and being capital breeders (organisms in which reproduction is financed using stored capital), arctiine moths are known for being unpalatable to predators in all life stages and this enables them to occupy behavioural and ecological contexts not available to their pleasant-tasting peers (Uyi 2014). *Pareuchaetes insulata* Walker (Lepidoptera: Erebidae) a moth with leaf defoliating larvae from Florida was tested for host specificity in the early 1990s for the biological control of *C. odorata* in South Africa (Kluge and Caldwell 1993) and was released in KwaZulu-Natal (KZN) province in the early 2000s (Zachariades et al. 2011). Establishment of the moth was confirmed in 2004 at Umkomaas and from here it has spread to northern KZN, Mpumalanga province, eSwatini (formerly Swaziland) (Zachariades et al. 2016) and Mozambique, but is not found in Limpopo province or the colder Midlands of KZN. According to the Shifting Defence Hypothesis (an extension of the second part of the Evolution of Increased Competitive Ability), after invasive plants are introduced to new ranges, they will evolve reduced resistance to specialist herbivores, thereby allowing for an increase in their cheap, toxic defence compounds (such as PAs) (Müller-Schärer et al. 2004). This study seeks to isolate and identify PAs from SAB *C. odorata* that could reduce generalist herbivores and enhance *C. odorata*, also the presence of PAs could reduce *P. insulata* predators and therefore improve the establishment and spread of the moth in southern Africa. To achieve full representation of *C. odorata* in South Africa, plants were collected from the release and

establishment point of *P. insulata*, i.e. Umkomaas, where the moth has been released in 2001 and been persisting since 2004; locations where *P. insulata* was never recorded, *viz*. Thohoyandou in Limpopo province and Pietermaritzburg in the KZN Midlands, and in Komatipoort, Mpumalanga, where *P. insulata* was recently discovered.

8.3 Materials and methods

8.3.1 Study locations and plant collection

Plant materials were collected from two habitats (shaded and full-sun) from each of four sites (Umkomaas, Thohoyandou, Komatipoort and Pietermaritzburg) in three provinces (KZN, Limpopo and Mpumalanga). Generally, the selected plants were dug up using a standard spade, thereafter, cut at the base of the stem and below ground materials (roots) were placed in brown paper bags and taken back to the Warren laboratory, Chemistry Department, University of KwaZulu-Natal. Further information on the sites and how plant materials were collected are detailed in Chapter Six.

8.3.2 Plant processing

Roots collected from Umkomaas, Thohoyandou, Komatipoort and Pietermaritzburg were washed with cold water, dried in a potting shed on a sunny day with a maximum temperature of 30 °C, put in paper bags and stored at room temperature (25 °C). Plant material of *C. odorata* was prepared by grinding the dried roots with a mill into small pieces of about 0.01-1.0 mm.

8.3.3 Alkaloid extraction

Pyrrolizidine alkaloids were obtained using the method previously reported in Thoden et al. (2007) by soaking 10 g of milled roots in 50 mL methanol and stirred vigorously for 24 hr with Corning stirrer bars on a Spectrum magnetic stirrer, at 350 rpm in 125 mL conical flasks at room temperature.

8.3.4 Thin-layer chromatography

The extract was then passed through a filter paper (size 90 mm, CHMLAB, Barcelona, Spain) into a 100 mL round-bottomed flask (Schott, Duran, Germany) and methanol was

removed by evaporation in a rotary evaporator at 45-50 °C, yielding extracts as indicated in Table 8.1. To confirm the presence of alkaloids, each extract was dissolved in 3 mL methanol and subjected to TLC analyses on aluminium-backed TLC plates covered with Silica gel 60 F254, (Merck, Darmstadt, Germany). Ten to 20 µL of each sample was spotted on the plates and the plates were developed in a mixture of methanol-dichloromethane-ammonia (1.5:8.2:0.3, v/v). To detect the PA *N*-oxides, the plates were air-dried and then sprayed with acetic anhydride, heated for 10 min at 70 °C and finally resprayed with Ehrlich reagent (10g 4-dimethylaminobenzaldehyde in 90 ml hydrochloric acid).

Table 8.1. Mass of crude extracts of *Chromolaena odorata* roots.

Name of an extract	Mass of extract (g)
NED-FSC-4R	0.339
NED-FSM-5R	0.384
NED-FSL-5R	0.403
NED-FSP-5R	0.345
NED-ShC-2R	0.458
NED-ShP-5R	0.509
NED-ShM-5R	0.410
NED-ShL-1R	0.039

8.3.5 Preparation of samples for Gas Chromatography-Mass Spectrometry (GC-MS)

To obtain the PAs from the *N*-oxides, the extracts prepared as above were dissolved in 35 mL aqueous sulfuric acid (2 M) to which 2 g of zinc dust was added to reduce the *N*-oxides into their free bases. The solutions (31 mL) were stirred for 4 h at room temperature, again passed through filter papers into 125 mL conical flasks, washed 3 times with 31 mL diethyl ether in a 250 mL separating funnel (in acidic medium, the protonated PAs are soluble in water and will be in the lower aqueous layer and not in the upper diethyl ether layer) and basified (pH 10-12) with 25% ammonia solution resulting in exothermic reaction. In a basic medium, the alkaloids are no longer protonated and are now soluble in organic solvents. The aqueous solution was extracted 3 times with 62 mL dichloromethane (DCM) (bottom layer), collected and dried under nitrogen. Samples from Umkomaas, Thohoyandou, Komatipoort and Pietermaritzburg from shaded and full-sun habitats were then dissolved

in 10 mL DCM, transferred to size 8 polytop vials, concentrated under nitrogen gas in a fumehood and the resulting DCM extracts were sent for GC-MS analysis (Table 8.2).

Table 8.2: Masses of pyrrolizidine alkaloid extracts from *Chromolaena odorata* roots prepared for GC-MS analysis.

Name of an extract	Mass of Extract (g)
NED-FSC-1R	0.009
NED-FSC-2R	0.015
NED-FSC-4R	0.02
NED-FSC-5R	0.01
NED-ShC-1R	0.05
NED-ShC-2R	0.023
NED-FSL-1R	0.025
NED-FSL-2R	0.028
NED-FSL-3R	0.019
NED-FSL-4R	0.03
NED-FSL-5R	0.036
NED-ShL-1R	0.039
NED-ShL-2R	0.027
NED-ShL-3R	0.034
NED-ShL-4R	0.014
NED-ShL-5R	0.031
NED-FSM-1R	0.021
NED-FSM-2R	0.021
NED-FSM-3R	0.012
NED-FSM-4R	0.008
NED-FSM-5R	0.068
NED-ShM-1R	0.042
NED-ShM-2R	0.020
NED-ShM-3R	0.041
NED-ShM-4R	0.045
NED-ShM-5R	0.059
NED-FSP-1R	0.022
NED-FSP-2R	0.022
NED-FSP-3R	0.026
NED-FSP-4R	0.0179
NED-FSP-5R	0.016
NED-ShP-1R	0.042
NED-ShP-2R	0.026
NED-ShP-4R	0.051
NED-ShP-5R	0.086

Extracts were re-dissolved in 0.1% formic acid and were transferred into a GC vial for analysis. Samples were analysed using a Shimadzu QP2010-SE Gas Chromatograph-Mass Spectrometer, fitted with a Zebron ZB-5MSplus column (30 m x 0.25 mm x 0.25 μm). Two microlitres of extract was injected in split mode with a ratio of 5.0, at a temperature of 280 °C. Helium was used as the carrier gas with a column flow rate of 1.13 mL/min. The oven temperature profile was as follows: 100 °C for 1 min, ramping at 20 °C/min to 200 °C, then 10 °C/min to a 5-minute hold. The MS transfer line was set at 280 °C. Mass spectra were obtained at a scan range of 10-500 m/z.

In an attempt to separate rinderine and intermidine, pure PAs were obtained from 400 g of pulverised roots, collected at Peter Brown Drive, Pietermaritzburg (from both the full sun and shaded habitats) by soaking the pulverised roots in 1950 mL methanol (just covering plant material) in a 4000 mL conical flask, and shaking for 24 hrs on a shaker at room temperature. The extract was then passed through a filter paper (size 90 mm, CHMLAB, Barcelona, Spain) portion-wise into a 500 mL round-bottomed flask (Schott, Duran, Germany) and the methanol was removed by evaporation in a rotary evaporator at 45-50 $^{\circ}$C. To obtain the PAs, the 19.81g extract was dissolved in 350 mL aqueous sulfuric acid (2 M) to which the 20 g of zinc dust was added to reduce the *N*-oxides into the free bases. The solution was stirred for 4 h at room temperature, passed through filter paper into a separating funnel, washed 3 times with 320 mL diethyl ether (between two separate layers diethyl ether on top) and basified (pH 10-11) with 25% ammonia solution resulting in exothermic reaction. The solution was extracted 3 times with 590 mL dichloromethane (DCM) (in the bottom) and the organic solvent removed under vacuum on a rotavapor, resulting in 1.187 g DCM extract.

8.3.6 Isolation of PAs by column chromatography and analysis of fractions by nuclear magnetic resonance (NMR)

500 mg of the 1.187 g DCM extract was dissolved in DCM:MeOH, mixed with 2 g of silica and dried in a fumehood to absorb the extract on the silica gel. 40g of silica was packed in a column using MeOH-DCM-NH_4OH (10:87:30). The sample was then added on top of the column. The column was initially eluted with MeOH-DCM-NH_4OH (10:87:3)

(fractions 1-15, 20 mL per fraction), and then with MeOH-DCM-NH_4OH (15:82:3) (fractions 16-29). The fractions were left in a fume hood to dry. Based on a TLC evaluation, fractions 13-18 were combined and dried. The material (0.0572 mg) was dissolved in $CDCl_3$ and analysed by ^1H NMR.

^1H NMR spectra were recorded on either a Bruker Avance III 500 or Bruker Avance III 400 spectrometer at frequencies of 500 MHz/400 MHz (^1H) and 125 MHz/100 MHz (^{13}C) using one of a 5 mm BBOZ probe $^{19}F/^{31}P$-^{109}Ag-{1H}, a 5 mm BBIZ probe ^1H-{^{31}P-^{109}Ag}, or a 5 mm TBIZ probe ^1H-{^{31}P}-{^{31}P-^{103}Rh}. All proton and carbon chemical shifts are quoted relative to the relevant residual protonated solvent signal (for $CHCl_3$: ^1H, 7.26 ppm, ^{13}C, 77.0 ppm). Coupling constants (*J*) are reported in Hertz. All experiments were conducted at 30 °C unless specified otherwise.

8.4 Results

8.4.1 Thin-Layer Chromatography (TLC)

A purple/magenta colour (Fig 8.2) after spraying TLC plates with Ehrlich reagent indicated the presence of alkaloids in the methanol crude extracts of roots of the SAB *C. odorata* collected at Umkomaas, Thohoyandou, Komatipoort and Pietermaritzburg. Alkaloids were found in both full sun and shaded habitats from all 4 locations.

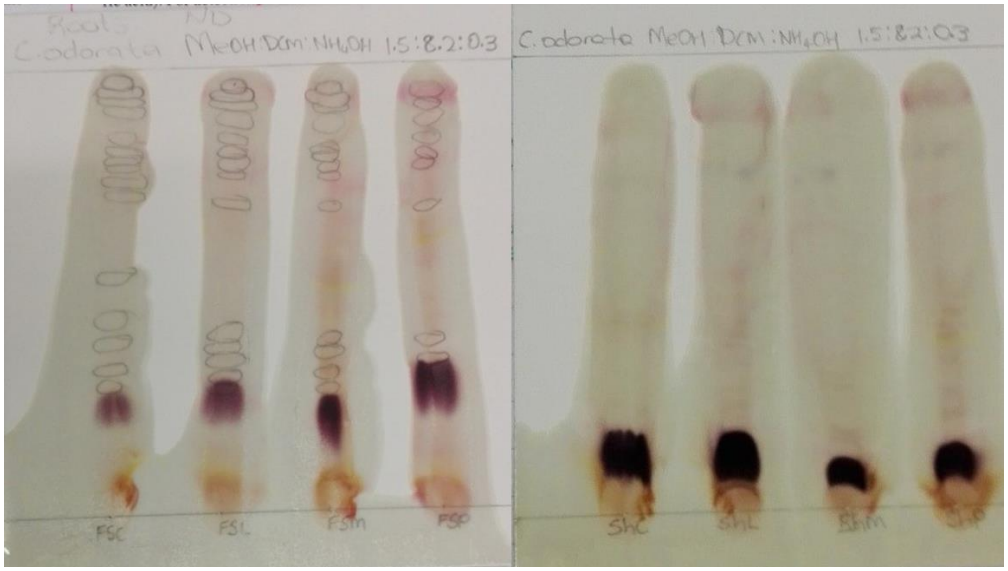

Figure 8.2: TLC tracks of a methanol crude extract showing PAs from *Chromolaena odorata* roots collected from full- sun and shaded sites Umkomaas (FSC and ShC), Thohoyandou (FSL and ShL), Komatipoort (FSM and ShM) and Pietermaritzburg (FSP and ShP).

8.4.2 Gas Chromatography-Mass Spectrometry and ^{1}H, ^{13}C Nuclear Magnetic Resonance

A GC-MS chromatogram of the DCM extract of FSC 2R (full sun Umkomaas quadrat 2 roots) showed two major peaks at retention times 11.444 and 11.612 minutes (Fig. 8.3). The mass spectra of the two compounds (Fig. 8.4) are virtually identical and display a molecular ion at low intensity at *m/z* 299, which is in agreement with a molecular formula of $C_{15}H_{25}NO_5$, the molecular formula of both rinderine and intermidine. Other major fragments observed in the mass spectra of both compounds are at *m/z* 138 (base peak) and *m/z* 93. A library search identified both compounds as the acetate of lycopsamine. Rinderine, intermidine, and lycopsamine are three stereoisomeric PAs. Rinderine and intermidine differ in an opposite configuration at C-7 only. The configuration at C-13 is the only difference between the structures of intermidine and lycopsamine. Mass spectrometry cannot differentiate between stereoisomers; therefore, rinderine, intermidine and lycopsamine will give the same mass spectrum. The library search indicated the closest hit for the two PAs as lycopsamine acetate. However, the compounds at R_t 11.444 and

11.612 minutes showed a molecular ion at *m/z* 299, consistent with a PA that is not acetylated. Furthermore, for an acetate, a fragment with *m/z* 43 would be expected. This fragment was not observed in the two mass spectra. The information obtained from the GC-MS identified the two compounds with R_t 11.444 and 11.612 as either rinderine, intermidine or lycopsamine. According to Biller et al. (1994), rinderine (45-49%) and intermedine (26-33%) are the two major PA N-oxides in the roots of *C. odorata*. An attempt was made to isolate the two pure PAs by column chromatography. Although the two PAs could be separated from other minor compounds, separation of the two major PAs was not achieved because these two compounds are stereoisomers with similar chromatographic properties. The ^{1}H NMR spectrum of the mixture of the two PAs (Fig. 8.5) was compared to the NMR spectra of PAs reported by Colegate et al. (2014). Based on this comparison, the PAs were identified as rinderine (major compound) and intermidine.

Rinderine and intermidine occurred in all other analysed samples (as per Table 8.3) from full sun and shade Umkomaas, Thohoyandou, Komatipoort and Pietermaritzburg, ranging in time between 11.391-11.570 minutes for peak number 4 and between 11.557-11.737 minutes for peak number 5 (Fig. 8.3).

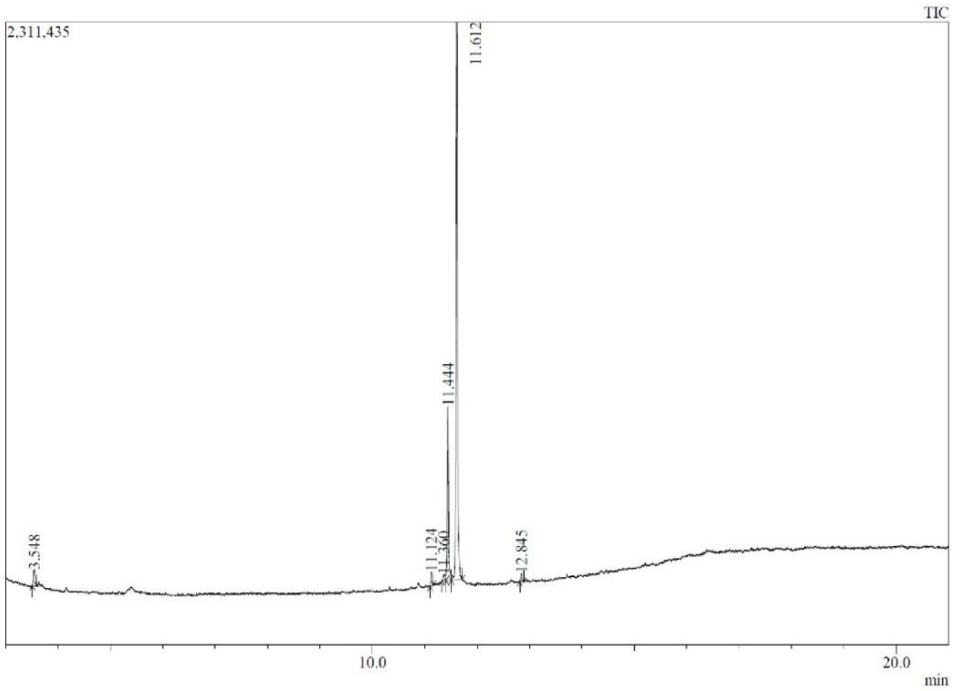

Figure 8.3: Chromatogram of at pyrrolizidine alkaloids rinderine at 11.44 and intermidine at 11.612 minutes extracted from *C. odorata* root samples from full sun and shade, Thohoyandou, Komatipoort, Pietermaritzburg and Umkomaas.

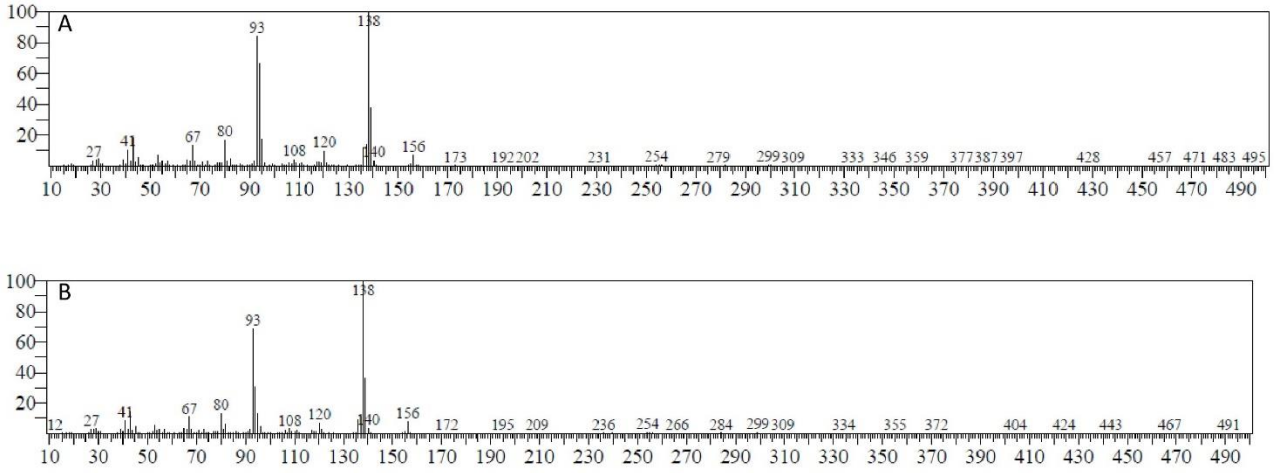

Figure 8.4: Mass Spectrometer of pyrrolizidine alkaloids (A) rinderine and (B) intermidine extracted from *C. odorata* roots samples from full sun and shade Thohoyandou, Komatipoort, Pietermaritzburg and Umkomaas.

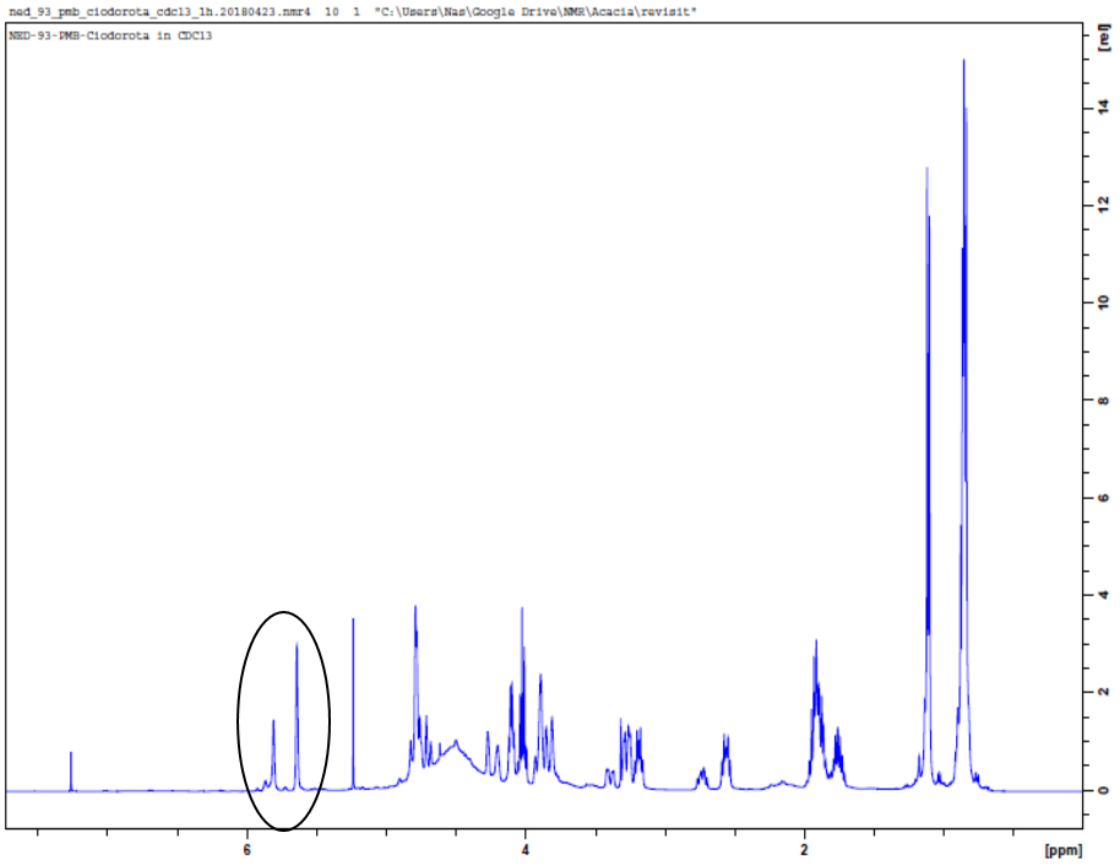

Figure 8.5: ^{1}H-NMR spectrum of a mixture of rinderine and intermidine between 5.5-6 ppm.

8.5 Discussion

In contrast to an earlier study on the AWAB *C. odorata* that reported the isolation of 5 major PAs (Biller et al. 1995), the present GC-MS and ^{1}H NMR analysis of the DCM extract of *C. odorata* root powder, showed the major presence of the N- oxides and free base forms of the monoesters rinderine and intermidine. Rinderine and intermidine are major stereoisomers and are difficult to separate.

Insects are known to have specific biochemical activities to handle and maintain PAs acquired from plants (Pasteels et al. 2003). For example, *Platyphora eucosma* (Stål) (Coleoptera: Chrysomelidae) larvae could largely epimerize O^{7}- (oxygen at position 7 in a structure) and to some degree O^{3}- of rinderine to intermidine and lycopsamine, and are able

to transfer the stored PAs as insect alkaloids from their blood through the pupal stage into the defensive secretions of adults (Pasteels et al. 2003).

Although PAs are known for their role as a plant defence mechanism against generalist herbivores, these compounds also serve as phagostimulants in other generalists such as the PA-adapted arctiine moth *Estigmene acraea* Drury (Lepidoptera: Erebidae) at lower concentrations (Bernays et al. 2002). Additionally, PA-containing butterflies are well protected against predation by tropical spiders, and arctiine moths protect their eggs by endowing them with PAs against predators (Pasteels et al. 2003). In arctiine moths, PAs in their host plants are known to produce sex pheromones such as hydroaxydanaidal (Boppré 1990; also see review in Conner 2009). The function of such male pheromones in arctiine behaviour is known to be the induction of sexual acceptance by the female (Schneider et al. 1992) and PAs stored in spermatophores are essential for the protection of females and eggs (Bezzerides et al. 2004; Conner 2009).

Generally, PA contents of the plants vary significantly and can be influenced by the condition of the plant such as withering or drying, the stability of the PAs, plant parts (flowers, leaves and roots) or the plant age (seedlings or matured plants) (Boppré, 1990). A previous study (Omokhua et al. 2017) could not detect alkaloids from the leaves, hence the current study only examined PAs in the roots of SAB *C. odorata* (as per the methods of Thoden et al., 2007). The Asian/West African *C. odorata* biotype, with 5 major PAs, is a strong competitive biotype (Yu et al. 2014), invasive in South, East and South-East Asia, parts of Oceania and in West, Central and East Africa, whereas the SAB *C. odorata* with only 2 major PAs (in this study) is so far only invasive in southern Africa (Zachariades et al. 2009). However, it is not known how the number of PAs present in a plant influences its invasiveness. Biller et al. (1994) found high concentrations of rinderine and intermidine in flower heads of *C. odorata* compared to the roots, and found the lowest concentrations in the leaves. This study could not quantify the amount of rinderine and intermidine in locations with *C. odorata* infested by its specialist herbivore *P. insulata* and those without *P. insulata*, and thus its ultimate objective was not met. However, the confirmation of rinderine and intermidine in SAB add to the factors that substantiate the establishment and

spread of *P. insulata* in southern Africa, as it should result in easily found host cues, enhanced mating of the adults and reduced predation of the moth population. Studies to elucidate re-association of *C. odorata* with *P. insulata* and its implications for PA contents and biological control of *C. odorata* (possibly including plant material from the native range of the SAB *C. odorata*) are recommended.

8.6 References

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CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS

Ongoing globalisation and universal trade increase the introduction and naturalisation of plant species outside their native range. Species that become invasive impose negative effects on key parameters of many indigenous plants, species richness and diversity, agriculture and forest production, nutrient, water and fire cycles, recreation and tourism, incur high control costs, and pose negative effects to people's source of income and health (Hinz et al. 2019). Chemical and mechanical control methods provide only short-term solutions, require follow up and are often expensive. Therefore, biological control, which entails the introduction of natural enemies (mostly insects) from the native range of the invasive alien plant is regarded as a more cost-effective, environmentally friendly and self-perpetuating control measure (Seibert 1989; Mack 1995; Zimmermann et al. 2004; Culliney 2005). Henderson and Wilson (2017) indicated that South Africa has an increasing number of invasive alien plants, with records of more than 770 species, including grasses and reeds, climbers, terrestrial herbs, aquatics, trees and shrubs.

Despite initiation of biological control of a scrambling shrub, *Chromolaena odorata* (L.) King & Robinson (Asteraceae), in the late 1980s in South Africa, its negative impacts in most parts of the country are still unacceptably high. It threatens biodiversity and agriculture by displacing indigenous plants, inducing allelopathy, altering soil properties, increasing shading, reducing grazing potential for wildlife and livestock, reducing both abundance and diversity of herbivorous insects, and increasing the intensity and frequency of fires in natural forested areas (McFadyen 1989; Mangla et al. 2008; te Beest et al. 2009; Qin et al. 2013; te Beest et al. 2013; Schirmel et al. 2016). In many other parts of its invasive range (i.e. outside of southern Africa) it is often seen more as a threat to agriculture than biodiversity (Zachariades et al. 2009), probably because there is more small-scale cropping, crops grown are more susceptible to *C. odorata*, and biodiversity conservation may be a lower priority in these countries. Genetic studies have confirmed two biotypes in the invaded range i.e. the Asian/West African biotype (AWAB), possibly from Trinidad and Tobago (the most widespread form, invading all areas except southern Africa), and the

southern African biotype (SAB) *C. odorata* from Jamaica and Cuba (the form invading only southern Africa) (Paterson and Zachariades 2013; Yu et al. 2014; Shao et al. 2018). Because of genetic, morphological and/or chemical differences between these two invasive biotypes, biological control agents successful in other countries failed in South Africa. For example, in Sri Lanka *Pareuchaetes pseudoinsulata* Rego Barros (Lepidoptera: Erebidae) larvae caused extensive defoliation and provided partial control of *C. odorata* (Kluge 1990; Waterhouse 1994), and the moth established and is persisting in West Africa (Uyi et al. 2017). However, *P. pseudoinsulata* failed to establish in South Africa even after over 350,000 (larvae and adults) were released in Limpopo province in the late 1990s (Zachariades et al. 2011), possibly due to climatic incompatibility and/or predation (Kluge and Caldwell 1993; Kluge 1994; Robertson et al. 2008; Zachariades et al. 2011). More recent efforts to source insect biocontrol agents for South Africa have largely been focused in Jamaica, where the genotype is identical to the SAB *C. odorata*.

The current study's overall aims were (1) to evaluate the life-history traits of two insects from Jamaica which use *C. odorata* as a host plant there: *Dichrorampha odorata* Brown and Zachariades (Lepidoptera: Tortricidae), a moth with shoot tip-boring larvae and *Polymorphomyia basilica* Snow (Diptera: Tephritidae), a stem-galling fly; and (2) to identify if they will not cause damage to non-target plant species and put them at unnecessary risk, through the use of host-specificity testing on indigenous and economically important plant species. Furthermore, (3) to determine the efficacy of *D. odorata* in the laboratory as a biocontrol agent of *C. odorata*. Finally, (4) to determine the role of the reassociation of *Pareuchaetes insulata* Walker (Lepidoptera: Erebidae: Arctiinae), one of the established agents that is believed to have contributed to the reduction of *C. odorata* in coastal areas of KwaZulu-Natal, on plant growth and defence parameters of *C. odorata* in South Africa, and on growth parameters of *P. insulata*.

Insecta as a group feed upon a highly diverse range of organic constituents, so it is remarkable that most species exhibit a high level of host specificity in their food selection. This is hypothesised to be driven by competition and natural selection, enabling each species to utilise a defined set of resources more efficiently than any of its competitors

(Waldbauer 1968). The chemical defensive mechanisms produced by plants along with the apparency or availability and predictability of the food resources to herbivores arise as vital for any analysis of plant-herbivore relationships (Cates 1980). Insects with narrow host ranges ('specialists') have developed mechanisms to overcome specific secondary chemicals; this enables them to feed and develop on a single plant species (monophagy), or a group of closely related (and thus chemically similar) plant species (oligophagy). Some are even known to be attracted to secondary compounds such as pyrrolizidine alkaloids, which they sequester as defence chemicals or sex pheromones (Biller et al. 1994; Hartmann et al. 1997). In contrast, 'generalist' insects feed on a wide range of plants (polyphagy) but cannot tolerate the secondary chemicals produced by a single plant species, in large doses.

9.1 History of Lepidoptera and Diptera in biological control

Lepidoptera have been used successfully in a number of biocontrol programmes, including those against Asteraceae (Crawley 1989; Winston et al. 2014; Mehelis et al. 2015). For example, the families Pyralidae, Pterophoridae and Tortricidae are all known to feed on *Senecio madagascariensis* Poir. (fireweed; Asteraceae) (Egli and Olckers 2017). The release of Pyralidae against Asteraceae has not been as successful as on other plant families such as *Cactoblastis cactorum* Berg on Cactaceae, and negligible impacts (also valuable in biological control) of Pyralidae on Asteraceae have been recorded (Crawley 1989; Winston et al. 2014). In contrast, moths of the Pterophoridae family released against Asteraceae have caused extensive and significant impacts on their target plants (Winston et al. 2014; Egli and Olckers 2017). Similar to Pterophoridae, Tortricidae used against invasive Asteraceae have had some success in controlling their target weeds. For example, in the biological control of *Jacobaea vulgaris* Gaertn. in Australia, the stem-boring torticid, *Cochylis atricapitana* Stephens, had a significant impact on plant populations (Ireson and McLaren 2012). Consequently, the moth was also released in Canada, where it had a considerable impact on *J. vulgaris* populations in some parts of the country (Winston et al. 2014). The stem-galling tortricid, *Epiblema strenuana* (Walker), similarly had a significant impact on *Parthenium hysterophorus* (L.) in Australia (Dhileepan 2001, 2003) and is currently being considered for release in South Africa (ARC-PHP, unpublished report).

Diptera have also been used extensively in weed biocontrol with varying degrees of success (Crawley 1989; Winston et al. 2014). Most tephritids have narrow host ranges and form galls, which act as nutrient sinks, on plants of the family Asteraceae (e.g. Dodson and George 1986; Fernandes et al. 1996; Balciunas and Mehelis 2010; Buccellato et al. 2012), on roots, leaves or flower heads and most widely spread and commonly on stems (Freidberg 1984; Headrick and Goeden 1998). For this reason, several tephritids have been considered for and are known for their significant success in biological control of invasive alien plants in South Africa and globally (e.g. Harris and Shorthouse 1996; Balciunas and Mehelis 2010; Buccellato et al. 2012; Winston et al. 2014). For example, *Cecidochares connexa* Macquart, a stem-galling tephritid, had a heavy impact on its target plant, *C. odorata* in the Federated states of Micronesia and the northern Mariana Islands, and a medium impact on the weed in Guam (Winston et al. 2014). A number of other stem- and boring tephritid species have had medium impacts on their target weeds (Winston et al. 2014).

9.2 Laboratory studies on life history traits, host range and impact of two candidate biocontrol agents for *Chromolaena odorata*

Knowledge of life-history traits, genetics, and behaviours, among other biological factors, of both the agent and target plant species, all contribute to better predictions of the ecological host range and efficacy of the biological control candidate (Schaffner 2001) and could assist in making a decision about which life stage(s) will be most appropriate for release (personal observations). No-choice trials, which determine the fundamental or physiological host range of an insect species, were employed, because they are the most conservative test. The principle of phylogenetic centrifugal testing was used when selecting which non-target plant species to include in these trials. This posits that test plants more related to the weed in question are more likely to be attacked than more distantly related test plants since they share traits important for the host selection and acceptance behaviour of specialized phytophagous insects (Hinz et al. 2019). Fortunately, *C. odorata* does not have many close relatives native to Africa, nor does it have closely related crop plant species, and this allowed a shorter list of test plants to be used.

9.2.1 Life-history traits of *D. odorata*

The combination of an insect's survivorship, developmental rate and female fecundity that are evident in life-history-trait trials is a key component in determining the effectiveness of a weed biological control agent. Under laboratory conditions, one *D. odorata* female can lay up to 39 eggs in at most 7 days of its lifespan; of these 96% can hatch and survive to adulthood which could facilitate population increases in the field (Chapter 3). This is coupled with a short life-cycle, with several generations a year, all key to enhancing rapid population increases in the field and for success in any biological control programme (Grassmann 1996) regardless of the possibility of attack by native parasitoids and predators (Hill and Hulley 1995). Eggs of *D. odorata* are flimsy, scale-like and laid singly on the leaves, with newly hatched larvae soon moving to the nearest shoot tip and boring into it to complete development to pupation. This could facilitate protection of the larva from environmental factors such as sun and rain. However, eggs of *D. odorata* desiccate immediately after the leaves they are laid on are removed from the plant, hence release of eggs was not advisable. Similarly, adults only eclosed in low numbers per day and have a short lifespan, whilst larvae required extensive labour for harvesting and would result in the destruction of the stock plants containing eggs that may have not yet hatched. Pupae are easy to harvest and can be obtained in large numbers and therefore were recommendable as the most appropriate stage to release. Assuming good climatic compatibility and a low recruitment of natural enemies in South Africa, the high reproductive output and survival of the immature stages of *D. odorata* were deemed likely to sustain high population densities in the field.

9.2.2 Laboratory host range of *D. odorata*

Dichrorampha odorata was only described about a decade ago (Brown and Zachariades 2007) and since its description, no host records have been found to indicate that it feeds on other species of plants in its native range. In no-choice trials in quarantine, first-instar *D. odorata* larvae initially bored into 14 test species other than the control, but intense damage was observed only on *C. odorata*, as was subsequent development to pupation and

adulthood (Chapter 3). One shoot tip of one replicate of *Stomatanthes africanus* (Oliv. & Hiern) R.M. King & H. Rob. (Asteraceae), indigenous to South Africa and closely related to *C. odorata*, experienced initial intense damage but could not support full larval development of *D. odorata*. This minimal acceptance of non-target plants was expected under no-choice trials where the agent is deprived of its host plant.

Sometimes, each life stage of the candidate insect may possess a different host range (van Klinken 2000). For example, adults of a candidate leaf beetle, *Chrysolina aurichalcea asclepiadis* Villa (Coleoptera: Chrysomelidae) demonstrated the ability to sustain feeding on 13 host species in laboratory tests, whereas the larvae were only able to complete development on 6 of the tested species (Weed and Casagrande 2011). For *D. odorata*, limited adult no-choice trials were also undertaken because of concerns that the use of non-naïve larvae in the larval no-choice trials may have biased the results of these trials. However, this does not appear to be the case: of the 7 test plants chosen for adult-no choice trials, from the 14 attacked during larval no-choice trials, oviposition by *D. odorata* was induced on only 4 non-target plants, all within the tribe Eupatorieae, although a strong oviposition preference for *C. odorata* was recorded. The high level of host specificity of *D. odorata* was even more evident in these adult no-choice trials when, even though a high percentage of eggs hatched on most test plants, the newly hatched larvae only accepted *C. odorata* for feeding and development.

The minimal damage and oviposition recorded on some non-target species are most often attributed to cage artefacts and they infrequently happen under field conditions (McFadyen et al. 2002; Simelane 2005; Madire 2013). Failure of *D. odorata* to complete development on test plants, with minimal damage and oviposition, suggested that a population of the moth could not be sustained on species of plants other than the target, *C. odorata*, in the field and nibbling on some target plants was consistent with the centrifugal phylogenetic principle. Furthermore, the inability of any test plants to sustain any life stage of *D. odorata* demonstrated a high level of monophagy in this tortricid. Permission to release the insect as a biocontrol agent against *C. odorata* was issued by the national competent authority in 2013.

Almost 25,000 insects have been released at 17 sites in three provinces since 2013, in a variety of habitats and climates, including almost 7,000 at one site over 13 months. However, although there was some persistence, the moth has not established yet. A thermal biology study (Nqayi 2019) showed that it could complete up to 6.5 generations in parts of South Africa, but the eggs seem susceptible to desiccation and night-time temperatures in winter are much lower than those in Jamaica. Tortricidae, mainly pest species, appear to be susceptible to parasitism in Europe, Australia and Turkey (Aydogdu and Beyarslan 2007; Brockerhoff and Kenis 1996; Paull and Austin 2006). Torgersen and Beckwith (1974) reported that 24 species of parasitoids were found associated with the large aspen tortrix in Alaska, USA. Nor are all leaf-rolling tortricids protected from parasitoids by their behaviour (Berndt et al. 2002). Post-release evaluations found high predation rates of *D. odorata* larvae and pupae in some cases, but this has not been quantified. Likewise, the effects of native parasitoids in South Africa have not been studied.

9.2.3 Laboratory impact of *D. odorata*

This study provided an insight into the effect of *D. odorata* herbivory on growth parameters of *C. odorata*, and demonstrated that continuous shoot-boring activities of larvae of *D. odorata* over a 9-month period significantly reduced plant height, number of flowers and leaf biomass in *C. odorata* plants (Chapter 4). Although herbivory by *D. odorata* increased parameters such as stem- and root-biomass and the number of shoot tips, this does not necessarily translate into *D. odorata* being ineffective as a biological control agent of *C. odorata*. For example, roots are known for their vital role in plant responses to above-ground herbivory by storing photoassimilates and synthesising secondary metabolites involved in leaf defences (Erb et al. 2009) to enable future regrowth; and increase of root biomass in response to herbivory is well documented (Nalam et al. 2013; Paige and Whitham 1987). Several studies have demonstrated increased exportation of carbon from the damage site into the storage organs (stems and roots) after herbivory (Gomez et al. 2012). Similar to other studies (e.g. Schat and Blossey 2005), the increased stem and root biomass in *C. odorata* could be attributed to the excessive production of carbon (unused during photosynthesis) that is stored in the stems and roots, consequent upon attack by *D.*

odorata on the stem tips of the plant. Several years of damage may be necessary to observe the depletion of root- and stem-biomass in long-lived perennial species such as *C. odorata* (e.g. Ringselle et al. 2015). Overall, this study and others demonstrate that plant herbivory results in a decrease in reproductive output such as leaves and flowers rather than in root and stem biomasses (Maschinski and Whitham 1989; Strauss and Agrawal 1999).

Furthermore, the damage to apical meristems in *C. odorata* that resulted from herbivory by *D. odorata*, shortened the apical stem and tended to increase the production of axillary branches, which was the reason for the increased number of shoot tips recorded. The positive effects on lateral growth (increased branching) and negative effect on leader growth (plant height) resulted in a change in *C. odorata* plant architecture. Other studies have also observed a similar pattern. For example, the destruction of the lead shoot of Pinyon pine by the moth, *Diorytria albovitella* Hust (Lepidoptera: Pyralidae), stimulates the lateral buds and the plant changes from a tree to a dense shrub (Whitham and Mopper 1985). Increased branching is not only a vital mechanism involved in increased tolerance of herbivory, but a key mechanism of plant compensation to damage that is commonly observed (Schat and Blossey 2005; Strauss and Agrawal 1999; Trumbule et al. 1993). According to Trumbule et al. (1993), increased branching due to herbivory can reduce plant height thus negatively affecting competition for light and seed dispersal. This was one of the reasons for the introduction of *D. odorata*, because *C. odorata* gains a competitive advantage over other plants in the field in South Africa partly by rapidly outgrowing them in height, thereby shading and smothering them.

Therefore, this study showed that larval feeding damage by the shoot-boring moth *D. odorata* has the capacity to reduce flower production, leaf biomass and plant height in *C. odorata* in a laboratory experiment. Whether such individual-level damage has the potential to impose negative effects on the population dynamics of *C. odorata*, should *D. odorata* establish, depends partly on population levels and seasonal population dynamics of the moth. Impact trials indicated that infestation of 50% of the shoot tips caused as much damage as 100%. During field work in Jamaica, we (N. Dube and C. Zachariades) could not estimate the impact of *D. odorata* on *C. odorata* as it often co-existed with other stem-

damaging insect herbivores such as *Phestinia costella* Hampson (Lepidoptera: Pyralidae: Phycitinae), *Melanagromyza eupatoriella* Spencer (Diptera: Agromyzidae) and/or *P. basilica*. However, the negative effects of *D. odorata* on several fitness parameters of potted *C. odorata* plants suggests that it plays a role in the abundance and population dynamics of *C. odorata* in its native range, in combination with damage caused by the other species. The co-existence of this moth with other insect herbivores in its native range suggests that its impact would probably be complementary to that of other biocontrol agents in South Africa. Because it is the only currently released species using shoot-tips as a resource, it could probably utilise *C. odorata* as a host plant without competing with established biocontrol agents such as *P. insulata* and *C. eupatorivora*, which only utilize the leaves, although the latter two species could be detrimental to *D. odorata* eggs when their populations are high. However, this does not rule out the case that sometimes there is competition between natural enemies in the region of origin or of introduction, such that one affects the other negatively (Impson et al. 2008).

This study suggests that, if it becomes established, *D. odorata* may contribute modestly to reduce the menace caused by *C. odorata* in South Africa (Chapter 4), but recommends more biocontrol agents as a complement for areas in which the moth does not establish.

9.2.4 Life history traits of *Polymorphomyia basilica*

The gall-forming fly *P. basilica* demonstrated positive biological attributes that could facilitate increases in the field and influence its success as a biological control agent (Chapter 5). The fly is multivoltine, with long-lived adults (females and males up to 109 and 126 days, respectively), a single female can lay up to 159 fertile eggs, with high offspring survival (up to 130 adults eclosed per plant. On plants where galled stems died and galls shrivelled as a consequence, adults were still able to eclose from galls with a pupation window) throughout the year in the laboratory. Some of the life history traits of *P. basilica* suggest that it may be able to thrive in relatively dry areas, but it is not known how it will be affected by low or high temperatures. Although climate modelling (Robertson et al. 2008) indicated that Jamaica was not climatically similar to areas invaded by *C. odorata* in southern Africa, the island is mountainous and *P. basilica* was present

over a range of altitudes wherever *C. odorata* grew (Robinson 2012). If *P. basilica* has wide thermal tolerances, establishment would be attainable over a wide area in South Africa including areas like Limpopo which up to now have not sustained any biological control agent. Because *P. basilica* adults are diurnal, they should not be negatively affected by low night-time temperatures to the same extent as nocturnal species such as *P. insulata* (Uyi et al. 2017) or *D. odorata* (Nqayi 2019). The persistence of the stem-galling fly *Procecidochares utilis* Stone (Diptera: Tephritidae), a biocontrol agent of *Ageratina adenophora* (Spreng.) R.M. King & H. Rob. (Asteraceae), in the KZN Midlands, where because of low winter temperatures it had been difficult to establish *C. odorata* biocontrol agents, is a positive sign for establishment of *P. basilica*, although *P. utilis* could have a different thermal biology to that of *P. basilica*.

As the fly is multivoltine, establishment should be achieved in a short time and damage would occur throughout much of the year (*C. odorata* flowers in June-July in South Africa, with little in the way of growing vegetative shoots being produced during this period). In the laboratory, both older plants with developing shoots and very young plants were accepted. Other tephritids, and Diptera more widely, that have been used as biocontrol agents in South Africa have proven easy to establish and disperse quickly over large distances, which, in the case of *P. basilica*, would increase the speed at which they colonise *C. odorata* in climatically suitable areas.

The final level of control given by *P. basilica* to *C. odorata* in South Africa depends, inevitably, on the population density which can be achieved by the fly in the field. This depends not only on climatic factors, but also levels of predation and parasitism, which is difficult to predict. Parasitism rates of *C. connexa* galls in Asia have been surprisingly low, although ants sometimes enter the gall through the window and prey on the pupa inside (McFadyen et al. 2003). Because *P. basilica* galls are smaller and less woody than those of *C. connexa* (C. Zachariades, pers. comm.) they may be more vulnerable to parasitism and predation. *Procecidochares utilis* on *A. adenophora* in South Africa suffers quite high parasitism levels (Kluge, 1991) but is still widespread around Pietermaritzburg, as it was recently found in the areas of KwaNyamazane, Prestbury and Athlone (personal

observations). During a survey conducted on the natural enemies of several indigenous Asteraceae in South Africa, a number of stem-galling tephritids were recorded on several plant species, and parasitoids were obtained from these (Grobbelaar, 2000). This, and the high parasitism rates on *P. utilis*, indicates that *P. basilica* may be subject to significant levels of parasitism in the field in South Africa. Because *P. basilica* was collected from *C. odorata* in Jamaica, which is the origin of the biotype invading South Africa, no biotype incompatibility issues between the biocontrol agent and its host plant are expected, should *P. basilica* be released in South Africa.

The eggs are of *P. basilica* are delicate and small (only visible under a microscope) and physical disruption of the plant tissue is required to access them. Removal of larvae from the galls also requires physical disruption and untimely removal from the galls is not ideal for the suitable growth of the fly (Friedberg 1984). Contrarily, the long-lived adult flies are easy to harvest in large numbers, and are therefore an appropriate stage for releases (personal observations).

Studies of life history traits were conducted using single pairs of adults (n=17). In insect behaviour, it is assumed that females that mate multiple times and allow sperm competition to determine offspring paternity will have more viable offspring than females that mate with a single male (Gershman 2012). Although this could not be tested in this study because of the use of single pairs, it was noteworthy that newly eclosed females which spent their initial 4-11 days with a male did not produce galls (n = 4) (which was expected during their preoviposition period), but those that spent between 18-97 days paired with males continued laying viable eggs even in the absence of the male (where the male had died or escaped) (n = 6). This could mean that *P. basilica* females have multiple matings (although in this case with single male). However, this result rules out a possible motivation for females to mate a large number of times but suggests that they mate enough times. Additionally, this result does not underestimate the role of diet on female fecundity (in *P. basilica*, the possible importance of feeding them with enzymatic yeast hydrolase). This result further recommends the release of pairs of adults that have been confined in a cage (with *C. odorata* as per culturing method of *P. basilica*) for 2 weeks. Success in using

single pairs of adults to determine life history traits motivated the initiation and execution of timely and successful host-range testing even with low numbers of *P. basilica* in the culture, and indicated that a biological control programme does not need to be always delayed by a small insect culture.

9.2.5 Host range of *P. basilica*

Many species among the non-fruit-eating Tephritidae are monophagous or narrowly oligophagous (Headrick and Goeden 1998). *Polymorphomyia* species and a number of other tephritids such as *Urophora solstitialis* (L.) (Diptera: Tephritidae) are known to be gallers of asteraceous plants (Korytkowski 1971; Friedberg 1984; Woodburn 1993), and plants in this family usually possess multiple secondary compounds which are used in the defence of the plant from natural enemies. For example, *Lactuca serriola* L. (Asteraceae) and *C. odorata* contain flavonoids, terpenoids and other secondary chemical compounds (Elsharkawy et al. 2014; Omokhua et al. 2017). These chemical compounds often differ in their absolute and relative concentration and composition between plant species, as in *L. serriola* compared to *Achillea fragrantissima* (Forssk). Sch. Bip. (Asteraceae) (Elsharkawy et al. 2014). Although *P. basilica* has generally manifested a high degree of host specificity, evident in the lack of oviposition and/or high larval mortality recorded from most test plants (Chapter 5), it is not surprising that limited oviposition was recorded in some asteraceous plants other than *C. odorata*. This was inescapable especially in the eat-or-die conditions of no-choice trials, and very low survival of the progeny on a few selected non-target plants further attests to the specificity of this tephritid. Although adults of *P. basilica* do not feed, females have a vital role of choosing whether or not to lay eggs on non-host plants in an "oviposit or leave no progeny" scenario (Jaenike 1990; Gripenberg et al. 2007; Rigsby et al. 2014). During larval no-choice trials of *D. odorata*, *S. africanus*, *A. riparia* and *A. conyzoides* were also nibbled but could not sustain survival of the moth (Dube et al. 2017).

The suitability of a plant species as a host is affected not only by the presence or absence of defensive chemicals but also of those which act to stimulate the insect into eating it. Waldbauer (1968) illustrated that poor growth in insects is attributed to a low rate of intake

due to the absence of a non-nutrient phagostimulant; this might be the case in the plants that were occasionally selected by the female for oviposition but could not sustain significant development of *P. basilica* larvae. The fly completely avoided species in the tribe Senecioneae; this is interesting as, along with the Eupatorieae, plants in this tribe contain pyrrolizidine alkaloids (e.g. Hartmann and Dierich, 1998; Hartmann 2009) and several other species tested as potential biocontrol agents against *C. odorata* have displayed slight feeding on Senecioneae. This further illustrates the level of host specificity *P. basilica* possesses.

In overall, unlike *D. odorata* which seemed to be strictly monophagous, *P. basilica* seemed to be oligophagous with very low sustainability on 2 indigenous plants which were due to laboratory artefacts; this suggests that post-release evaluations should include plants growing interspersed with *C. odorata*. This will confirm the high host specificity of this fly, demonstrated in the ability of female to generally withhold eggs in the absence of the host plant but oviposit as soon as she is reunited with the host plants (Chapter 4). Additionally, it was demonstrated that in isolation (alone in the cage) the non-target plant (*Stomatanthes africanus* Oliv. & Hiern R.M. King & H. Rob. (Asteraceae)) cannot sustain a *P. basilica* population, evident in that the eclosed adult died on the same day it emerged. Contrarily, the non-target plant (*Felicia amelloides* (L.) Voss (Asteraceae)) that sustained 1 *P. basilica* to adulthood was soon moved to the walk-in cage after exposure to *P. basilica* (in no-choice trials). The walk-in cage contained other test plants and many control plants for larval development and thus contained many adults. The fly from *F. amelloides* could thus not be monitored for longevity. Although this result seemed to present spill-over or sustained non target attacks, it also suggests that a population of *P. basilica* will not be sustained without continued access to *C. odorata* (Hinz et al. 2019).

To date, no quantified studies have been conducted in the laboratory in South Africa to determine the impact of *P. basilica* on its host plant *C. odorata*. In the field in Jamaica, the majority of *P. basilica* galls were found on side-shoots, leading to reduced lateral growth. Part of the rationale in employing *P. basilica* as a biocontrol agent in South Africa was that it could be used in conjunction with an insect that mainly curtails the growth of the terminal

shoot tip, to reduce overall stem growth in order to reduce the competitiveness of *C. odorata* with surrounding plants. Currently *D. odorata* is being released to fulfil that role, but other species such as *Melanagromyza eupatoriella* Spencer (Diptera: Agromyzidae), *Carmenta chromolaenae* Eichlin (Lepidoptera: Sesiidae) or *Conotrachelus reticulatus* Champion (Coleoptera: Curculionidae) (Zachariades et al. 2011) could also play the role of a terminal-shoot damager, should *D. odorata* not establish (Nqayi 2019).

Polymorphomyia basilica galls may also have an indirect effect on *C. odorata*. The insect has a similar biology to other tissue-galling tephritids: eggs are laid in meristematic tissue (in this case the shoot tips), larvae hatch, and swell the stem into a gall by causing changes in plant cell growth. *Cecidochares connexa* (Macquart) (Diptera: Tephritidae) galls were demonstrated to act as a nutrient sink by Cruz et al. (2006), and this insect has proved extremely damaging to the Asian/West African biotype of *C. odorata* in South East Asia (e.g. Day et al. 2013) and West Africa (Aigbedion-Atalor et al. 2019). It is not impossible that *P. basilica* galls could also act as a nutrient sink on the SAB of *C. odorata*.

9.3 A potential feedback loop between a biocontrol agent and its host plant: a case study on *Pareuchaetes insulata* and *Chomolaena odorata*

According to the Evolution of Increased Competitive Ability (EICA) hypothesis and its elaborations, the reassociation of a biological control agent with its host plant in the introduction range should decrease the competitive abilities of the invasive species, resulting in a population decline and the creation of more ecologically desirable conditions (Blossey and Notzöld, 1995: Keane and Crawley, 2002; Blossey and Cassagrande, 2016). Given the observed decline of *C. odorata* in parts of KZN, which appeared to be greater than could be explained through direct herbivory by established biocontrol agents alone, this hypothesis was deemed worthwhile to be considered for the moth *Pareuchaetes insulata*. This species was released as a biocontrol agent in 2001, confirmed as established in 2004, and has since spread from its establishment point on the south coast of KZN to northern KZN, Mpumalanga, eSwatini and Mozambique and has contributed to a remarkable reduction of *C. odorata* in some of the areas where it is present (Zachariades et al. 2016).

This hypothesis was tested by assessing defensive chemicals in plants from the field that had been reassociated with *P. insulata* for over 15 years, not exposed at all, or exposed for only a few years. Plant material was collected from full sun and shade to have full representation of habitats occupied by *C. odorata* and *P insulata* in the field in South Africa, and the results from these two habitats differed. Furthermore, fitness parameters of these plants were assessed, as was the performance of larvae of *P. insulata* on these plants. If plant data support EICA, this suggests that the insect would perform better in terms of development, survival and reproduction.

Phytochemical analysis of the leaves of *C. odorata* revealed higher concentrations of phenolic and tannin contents in Thohoyandou (Limpopo) [no *P. insulata*] and Komatipoort (Mpumalanga) [recent *P. insulata*], both of which are at lower latitude compared to Umkomaas [15 years *P. insulata*] and Pietermaritzburg [no *P. insulata*] which are both in KZN. These sites also varied markedly in altitude, but no correlation between altitude and levels of these chemicals was evident. Contrarily, the concentration of flavonoid contents was higher in Komatipoort and Pietermaritzburg than in Thohoyandou and Umkomaas. These findings were inconsistent with the assumption that defence levels (through increased production of quantitative secondary chemicals) of *C. odorata* could be recuperated rapidly after the native specialist herbivore becomes present in the introduced range (Joshi and Vrieling 2005). Instead they could be due to the influence of other abiotic and biotic factors such as light, latitude, altitude, plant structural traits, generalist herbivores, time and allelopathy, which have all been demonstrated to play a vital role in the concentrations of plant chemicals (Bennett and Wallsgrove 1987; Roberts and Paul, 2006; Hanley et al. 2007; Moles et al. 2011; Harvey et al. 2013; Harvey et al. 2015; Dai et al. 2016). Additionally, during a study on *Fallopia japonica* [Houtt.] Ronse Decraene (Polygonaceae), native to Japan and invasive in France, Rouifed et al. (2018) demonstrated that enhanced plant defences can sometimes be manifested through structural rather than chemical means. On the other hand, contrary to the expectations of EICA, Zangerl at al. (2008) showed that uninfested New Zealand populations of *Pastinaca sativa* L. (Apiaceae) native in Europe, contained higher amounts of octyl acetate, a floral volatile used by

webworms *Depressaria pastinacella* Duponchel (Lepidoptera: Oecophoridae) for orientation, than did infested populations. This could be explained by directional selection once the herbivore has become reassociated with its host plant: it would be to the plant's advantage to decrease the chemical so that the herbivore cannot find it as easily

Despite the few studies that consider impact of a specialist herbivore on its host plant in the introduction range relative to EICA thus far conducted, there is increasing evidence that invasive alien plants perform better in terms of growth rate in locations without specialist herbivores compared to locations that are reassociated with specialist herbivores (e.g. Fukano and Yahara 2012; Jogesh et al. 2014; Rouifed et al. 2018). Contrary to phytochemistry studies (Chapter 6) that did not find evidence for the evolution of chemical defences, results in the common garden experiment manifested more rigid *C. odorata* plants with thicker stem diameter and higher reproductive potential from locations (Thohoyandou and Pietermaritzburg) without the specialist herbivore *P. insulata*, and plants with thinner stems and lower reproductive output from Umkomaas and Komatipoort which are reassociated with *P. insulata* (Chapter 6). These results are consistent with the assumption that invasive alien plants benefit from the direct release from natural enemies (Keane and Crawley 2002), consequent to the absence of specialist herbivores for non-indigenous plants, and that over time this leads to an evolutionary shift in resource allocation from defence to growth and increased competitive ability over native plants (Blossey and Notzöld 1995).

A further assumption of the EICA hypothesis is that specialist herbivores will demonstrate improved performance on individual plants originating from an area where plants have been introduced, compared to those from the area into which they have been introduced (Blossey and Notzöld 1995). If this hypothesis is true, the reverse implication is that the defence against herbivory could be restored if a natural enemy is reassociated with the invasive plant or if it also becomes present in the introduced range. This would manifest in reduced performance on the part of the natural enemy, on plants that have been re-associated with it for some time. The study undertaken in Chapter 7, measuring fitness parameters of *P. insulata* that were fed on material from plants originating from an area in

which the insect had been present for >15 years (Umkomaas), an area which it recently colonised (Komatipoort), and two areas from which it had always been absent (Thohoyandou and Pietermaritzburg), demonstrated mixed results regarding this hypothesis. A few other studies exhibited similar trends in results, and these led to the proposal of a more specific hypothesis stating that the introduced plants are expected to exhibit reduced defence against specialist herbivores but increased defence against generalist herbivores (Joshi and Vrieling 2005), because in nature, invasive alien plants in their range of introduction have escaped from specialist herbivores but mostly are still attacked by generalist herbivores.

Consistent with the prediction that a biological control agent will show improved performance on plant individuals originating from an area where plants have been introduced, pupal and total development times were longer in *P. insulata* that was fed on *C. odorata* leaves from Umkomaas than those from Thohoyandou, Komatipoort and Pietermaritzburg, implying that feeding on plants that had not previously been exposed to specialist herbivores improved *P. insulata* performance. Plant cuttings were collected from full sun and shade to have full representation of habitats occupied by *C. odorata* and *P insulata* in the field in South Africa, and the results from these two habitats differed. Larvae that fed on the leaves from shade from Komatipoort had developmental trends intermediate between larvae feeding on the leaves from the shade from Thohoyandou and Umkomaas. Pupae of the larvae that fed on the leaves from full sun Komatipoort showed intermediate trends of development between pupae of the larvae that fed on leaves from full sun Umkomaas and Thohoyandou. The intermediate *P. insulata* performance results demonstrated in Komatipoort (with unknown length of presence of *P. insulata*, probably 2 years), between Thohoyandou (without) and Umkomaas (with 15 years *P. insulata* infestation), were expected. Similarly, Wan et al. (2019) demonstrated better performance of the specialist *Ophraella communa* LeSage (Coleoptera: Chrysomelidae) on uninfested *Ambrosia artemisiifolia* L. (Asteraceae) (native to North America) populations in China (where it is invasive) compared to the infested populations. Additionally, consistent with our study (unexpected high concentrations of phenolics and tannins in Thohoyandou and Komatipoort instead of higher concentrations in Umkomaas and Komatipoort), Wan et al.'s

(2019) trials looking at chemical defences did not yield the expected results as *A. artemisiifolia* plants from infested populations had lower concentrations relative to the uninfested populations.

Results from Chapters 6-8 supported EICA with respect to the reproductive potential of *C. odorata* and *P. insulata* performance, suggesting that *P. insulata* contributed to the remarkable reduction of *C. odorata* on the south coast of KZN in South Africa through an EICA mechanism and not only an ERH mechanism (Zachariades et al., 2016). This may also provide an explanation of why *P. insulata* as well as *P. pseudoinsulata* have consistently been reported as undergoing their highest population outbreaks when first introduced into a region, or along the front of spread of the *P. insulata* population. Subsequent outbreaks are invariably smaller. This has previously been explained by the decreasing density of *C. odorata* infestations over time. *Chromolaena odorata* was first recorded in KZN, South Africa, 72 years ago, while *P. insulata* was introduced only 18 years ago. The contrasting results found for the chemical defences of *C. odorata* with regards to phenolic, flavonoid and tannin contents suggest that (1) contrary to other findings (e.g. Fukano and Yahara, 2012) plant defensive ability is not always easily altered and (2) the reassociation time may not be enough for evolutionary changes to have already occurred in *C. odorata* defence (other studies showed a shift in defence only after over 100 years e.g. Zangerl et al. 2008). Contrasting results could also (3) explain the mixed results in *P. insulata* performance studies observed in some parameters on infested and unifested populations of *C. odorata*. Lastly, (4) they could suggest that *Zonocerus elegans* (L) (Orthoptera: Pyrgomorphidae) (a generalist grasshopper) has inhibited the plant from decreasing its investment in quantitative chemical defences since its introduction, even before *P. insulata* was released, and this may explain for example why such quantitative defences (e.g. flavonoids) do not differ between Limpopo and Umkomaas.

In addition to flavonoid, phenolic and tannin contents, there is increasing evidence that SAB *C. odorata* contains several other secondary compounds (Omokhua, 2018). This study was able to elucidate pyrrolizidine alkaloids (PAs) which are well known for the fitness and mating benefits, such as sexual acceptance of males by females that they confer

on a number of insect species, including *Pareuchaetes* species (Boppré 1990; Schneider et al. 1992; Witte et al. 1993; Biller et al. 1994; Klitzke and Trigo, 2000). Chapter 8 for the first time recorded the presence, at substantial concentration, of the N-oxides and free base forms of the monoesters rinderine and intermidine, through GC-MS and ^{1}H NMR of DCM extracts of *C. odorata* root powder. In contrast, Biller et al. (1994) demonstrated 5 major PAs in the AWAB *C. odorata*. Asian/West African biotype *C. odorata* has a far larger invasive range (southeast Asia, parts of Oceania, Central, West and East Africa) than the SAB *C. odorata* (invasive only in southern Africa). Yu et al. (2014) described the AWAB *C. odorata* as a "genotype with strong competitive abilities" whilst only 2 PAs are only recorded in southern Africa. Nevertheless, this study cannot confidently conclude that the fewer PAs found on SAB *C. odorata* contribute to its smaller invasive range than that of AWAB *C. odorata*, but it is a possibility. Conner (2009) showed that PAs found in the arctiine moths, sequestered from their host plants, make them unpalatable to predators and form the basis for pheromones necessary for courtship success. Therefore, confirmation of rinderine and intermidine in SAB adds to the factors that substantiate the establishment and spread of *P. insulata* in southern Africa as it means easily found host cues, enhanced mating by the adults and reduced predation of the moth population.

Basically, improved *C. odorata* growth rate and larval performance of *P. insulata* in locations uninfested by the specialist herbivore *P. insulata*, compared to locations infested, partly supported EICA. My results also reinforce the evidence on the positive contribution of the specialist herbivore where it is reassociated with the target weed in the country of introduction. This study demonstrates the positive impact of *P. insulata* in the decline of *C. odorata* populations in areas where the moth has persisted. It further encourages the use of similar approaches in post-release studies in weed biological control as it shows the impact of a biological control agent on a target weed. Overall, the phytochemistry of *C. odorata* alone was a poor indicator of the historic presence of *P. insulata* or other specialist herbivores, highlighting the importance of including data on the growth rate of plants and performance of specialist herbivores in such studies.

9.4 General conclusions and recommendations for further research

The introduction of multiple species as biocontrol agents against a target weed has been a debated issue among biological control practitioners (Denoth et al. 2002; Impson et al. 2008). However, *C. odorata* is clearly among the invasive alien plants that require more than one biological control agent to achieve adequate control; this is evident in the number of insect herbivores attacking it in its native range, and that *P. insulata* and *C. eupatorivora* are ineffective in areas away from perennially wet microhabitats. Based on this study, *D. odorata* is likely to only modestly contribute to control of *C. odorata*. However, so far it has been difficult to establish *D. odorata* due to susceptibility of eggs to dehydration, low night-time temperature and high predation levels observed in the field. Therefore, future research steps in this biological control programme should be to get *Recchia parvula* (Lane) (Coleoptera: Cerambycidae) and *P. basilica* established and determine post-released efficacy, and then consider further agents. These could include *M. eupatoriella*, *C. chromolaenae* and *C. reticulatus*. A comparative laboratory study on the performance of *C. connexa* on SAB and AWAB is planned, to confirm that it cannot be employed as a biocontrol agent on the SAB *C. odorata*. Further exploratory surveys in Cuba or Jamaica could be undertaken for further agents which are likely to survive dry conditions; these could also be conducted in other parts of the native range of *C. odorata*, although the use of such agents would raise the possibility of agent-host plant incompatibility.

Life history traits such as measures of fecundity for both *D. odorata* (because of the difficulty of sexing adults) and *P. basilica* (low number of flies in the beginning of host range tests) were determined using single pairs. In general, the methodology of using single pairs appeared successful and appropriate as they demonstrated reasonable fecundity (eggs laid and hatched). However, results obtained here ruled out influences of polyandry (female acceptance of matings from more than one male) such as sperm competition, sperm selection and offspring viability that could be beneficial for some insect species (Simmons 2005) and for studies in insect behaviour. In the same way, these studies eliminated negative polyandrous influences.

Chromolaena odorata does not overlap with *S. africanus* in South Africa and laboratory artefacts could have contributed to acceptance of the more distantly related *F. amelloides* (which falls under tribe Astereae). Survival on other exotic weeds was recorded, but based on the remarkably high survival on *Campuloclinium macrocephalum* (Less.) DC. (Pompom weed) (Asteraceae), *P. basilica* could be cultured on *M. macrocephalum* for evaluation of its potential as a biological control of pompom. Additionally, quantified impact studies are required for this gall-forming fly which seemed to reduce flowering of *C. odorata* (personal observations on plants used during trials), following efforts to release and establish it. Lastly with the phytochemistry experience accumulated in this study (Chapter 6), it is recommended that future host-range trials of *C. odorata* biocontrol agents include chemical analysis of plants that are partly accepted during no-choice tests to determine what could be attracting the agent in question to those plants. This would add to host-specificity techniques in weed biological control programmes that are already advancing, as suggested by Hinz et al. (2019).

It is recommended that similar research to that conducted on *C. odorata* defence (measured by phenolic, flavonoid and tannin contents), plant growth metrics and *P. insulata* performance includes data from the native region of *C. odorata*, and particularly that part of the native range from which the SAB (or AWAB) originates. Another aspect that requires consideration but this study did not include is the case of *Z. elegans* known to overcome and sequester PAs from *C. odorata* for its defense – although it is a generalist, it acts like a specialist on *C. odorata* in South Africa. It is possible that *Z. elegans* has inhibited the plant from reducing its investment in quantitative chemical defences even before *P. insulata* was released, and this may explain for example why such quantitative defences (e.g. flavonoids) do not differ between Limpopo and Umkomaas.

Lastly, although some of the studies revealed that plant populations exposed to specialist herbivores in the country of introduction have lower levels of PAs than plants that were not exposed (e.g. Rapo et al. 2010), quantification of the identified PAs rinderine and intermidine, which this study did not achieve, could assist with understanding the

behaviour of *P. insulata* in the field and add to understanding of the impact of *P. insulata* on *C. odorata* that the analysis of phytochemicals (Chapter 6) could not reveal.

9.5 References

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