Pre-release evaluation of the flea beetle *Heikertingerella* sp. (Coleoptera: Galerucinae: Alticini), a potential biological control agent for the invasive weed *Tecoma stans* L. (Bignoniaceae) in South Africa

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Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy

in the Discipline of Entomology

School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal, Pietermaritzburg

2024

UNIVERSITY OF KWAZULU-NATAL ™

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CONTENTS

DECLARATION

The research described in this thesis was carried out at the Weeds Research Division of the Agricultural Research Council- Plant Health and Protection (ARC-PHP), from 2015 to July 2022 under the supervision of Prof T. Olckers (University of KwaZulu-Natal) and Dr D.O. Simelane (ARC-PHP).

The work presented in this thesis represents the original work of the author and has not been otherwise submitted, in any other form for any degree or diploma, to any other University. Where use has been made of the work of others, this is duly acknowledged in the text.

Signed

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ACKNOWLEDGEMENTS

LIST OF PUBLICATIONS

Chapters from this thesis are published in the international weed biological control literature as follows:

Madire, L., Simelane, D., & Olckers, T. (2021a). Pre-release evaluation of *Heikertingerella* sp. as a potential biocontrol agent for *Tecoma stans* in South Africa. *Journal of Applied Entomology*, 145(1-2), 65–72. (**Chapter 2**)

Madire, L.G., Simelane, D.O., & Olckers, T. (2021b). Competitive interactions between the root-feeding *Heikertingerella* sp. and foliage-feeding *Mada polluta* on the invasive *Tecoma stans*. *Arthropod-Plant Interactions*, 15(2), 265–271. (**Chapter 3**)

Madire, L.G., Olckers, T. Sutton, G.F, & Simelane, D.O. (2024). Predicted impact, establishment and distribution of *Heikertingerella* sp. (Coleoptera, Chrysomelidae), recently released for the biological control of *Tecoma stans* in South Africa. *Agricultural and Forest Entomology*, in press. (**Chapter 4**)

Madire, L.G., Olckers, T. & Simelane, D.O. (2023a). Effect of host-plant age on the performance of the root-feeding flea beetle *Heikertingerella* sp., a biological control agent for *Tecoma stans* in South Africa. *Biocontrol Science and Technology*, 33(3), 258–267. (**Chapter 5**)

Madire, L.G., Simelane, D.O. & Olckers, T. (2023b). Application of nitrogen, phosphorus, potassium + carbon fertilizer improves the growth of *Tecoma stans* and the performance of its root-feeding biological control agent, *Heikertingerella* sp. *Biocontrol Science and Technology*, 33(9), 855–868. (**Chapter 6**)

ABSTRACT

The root-feeding beetle *Heikertingerella* sp. (Coleoptera: Chrysomelidae) was introduced into quarantine in South Africa for evaluation as a biological control agent of the invasive tree *Tecoma stans* L. (Bignoniaceae). Larval feeding damages the roots, potentially reducing the weed's growth and reproduction. Pre-release studies in quarantine included several aspects. Studies on the beetle's biology and host specificity were conducted to confirm its safety for release in South Africa. The impact of varying beetle densities on plant fitness was assessed to determine its likely impact in the field. The effect of local climate on the beetle's potential to establish throughout the weed's range in South Africa was predicted using climate-modelling software. The interaction between *Heikertingerella* sp. and a leaf-feeding agent already established in South Africa was investigated. Finally, the effect of host-plant age and nutrient enhancement on mass-rearing activities was studied, to optimize beetle numbers for releases. The beetle proved host specific resulting in the granting of permission for its release in South Africa. There were significant reductions in plant growth and biomass accumulation in the beetle-exposed plants, relative to the controls, indicating that *Heikertingerella* sp. is sufficiently damaging. Climate matching revealed that *Heikertingerella* sp. is likely to perform best at coastal sites in South Africa, with the colder, more inland, areas within the weed's range proving less suitable. The beetle proved compatible with a defoliating agent established in South Africa, with evidence of positive interactions that are likely to enhance their combined impact on the weed. Trials involving plants of varying age and nutritional enhancement revealed that 3-year-old plants, which were supplemented by either medium or high levels of fertilizer, were best suited for F_1 progeny production and therefore for the mass-rearing of *Heikertingerella* sp. for releases. The results of this study should also benefit other countries in Africa and elsewhere in the world, where the plant is invasive.

CHAPTER 1

General introduction

The impact of invasive plants in South Africa

Invasive alien plant species (hereafter IAPs) are plant species that were brought into an environment outside of their natural range and have spread uncontrollably, leading to the invasion of new habitats in their introduced range (Simberloff et al., 2013). IAPs initially become naturalized, surviving in the new country's landscape without human facilitation. The worst of these species are able to survive, reproduce and spread unaided at alarming rates in their new habitats (Van Wilgen et al., 2001). Globally the number of established alien species, which include plants, are predicted to increase by 36% between 2005 and 2050 (Seebens et al., 2021). IAPs have posed serious threats to the local biodiversity, ecosystem services, environmental quality, food security (IUCN, 2021) and human livelihoods (McLean et al., 2018; Rai & Singh, 2020; Bitani et al., 2022) in several invaded countries including South Africa during the past few decades.

IAPs also have considerable impacts on ecosystem composition and functioning, including potential extinctions of threatened and endangered species. This is because they have a competitive advantage over native flora and are able to alter disturbed regimes, hydrologic cycles, plant productivity and nutrient dynamics, and cause soil erosion (Vitousek & Walker, 1989; Yelenik & D'Antonio, 2013). Alien plant species are the most invasive and damaging group of invasive taxa in South Africa and are reported to cover approximately 7% of the country (Bitani et al., 2022). They reduce available water resources, with significant impact on stream flows and an associated increase in siltation and degradation of water quality. They also increase the impact of fires, floods and soil erosion in several province, notably the Western Cape, KwaZulu-Natal and Eastern Cape (van Wilgen et al., 2022a; Bitani et al., 2022). The

impact of IAPs on ecosystem services, and on biodiversity, are significant and the estimates of their negative effects vary between biomes, regions and countries, but the total costs can amount to tens of billions of US$ each year (McNeely, 2001; Pimentel, 2002; Pimentel et al., 2005; van Wilgen et al., 2008).

In South Africa, substantial amounts are spent annually on the control of IAPs (van Wilgen et al., 2008). According to van Wilgen et al. (2022b), the South African government has spent more than R7.1 billion on the control of IAPs between 1998 and 2020. Many countries worldwide, including South Africa, are committed to global biodiversity goals to prevent or minimise the introduction of high priority invasive species (Moshobane et al., 2019; Bitani et al., 2022). IAP impacts are aggravated by climate change, which facilitates the establishment and spread of both new and existing alien species, and creates new opportunities for naturalized plants to become invasive (IUCN, 2021).

Drivers of invasion

Plant invasions are not linked to one particular driver or mechanism, but various mechanisms, acting either alone or in concert, drive exotic invasions. Theories behind plant invasion include the Empty Niche Hypothesis (ENH), Evolution of Increased Competitive Ability (EICA), Enemy Release Hypothesis (ERH), and Novel Weapons Hypothesis (NWH) (Blossey & Notzold, 1995; Keane & Crawley, 2002; Alpert, 2006; MacDougall et al., 2009; Rai & Singh, 2020).

The ENH was coined by Elton (1958) and refers to the establishment success of exotic populations, which involves either the exploitation of unused resources (empty niches) or enhanced competitive abilities to access a shared resource. The transfer of exotic plant species to a new environment by human activities, changes in temperatures or precipitation, or the

arrival of new possible prey, predators, parasitoids, or vectors (Schmitt, 2020) could create a conducive environment for the ENH.

The EICA was coined by Blossey & Notzold (1995) and predicts that when growing conditions are identical, exotic plants, through increased competitive ability, will produce more biomass than plants from the native range and that herbivores will demonstrate improved performance on native plant species. The NWH was coined by Hierro & Callaway (2003) and argues that allelopathy determines the competitive success of exotic plants in the new environment. Allelochemicals produced by exotic plants comprise novel weapons, which suppress the native plant species while paving the way for colonisation and dominance by IAPs in the new habitat (Batish et al., 2013; Rai & Singh, 2020).

The ERH is a comprehensive and well-known hypothesis in invasion biology, which explains why species are able to establish and spread outside of their native range (Enders et al., 2018; Gozzi et al. 2020). Keane & Crawley (2002) initially formulated this hypothesis for invasive alien plants, which states that plant species, on introduction to an exotic region, experience a decrease in regulation by herbivores and other natural enemies, resulting in a rapid increase in their distribution and abundance. In addition, native natural enemies in the new range are typically unable to regulate the invasive species due to a lack of adaptation to the new species (Jeschke & Heger, 2018). Therefore, the success of classical biological control (see below) is often used as support for the ERH (Keane & Crawley, 2002).

Besides these considerations, other factors such as climate change, increasing global trade and tourism, expansion of human populations, increasing environmental disturbance and biodiversity loss, also facilitate the invasion success of exotic species (Levine & D'Antonio, 2003; Hulme, 2021).

Classical biological control of invasive alien plant species

Classical biological control (hereafter biocontrol) is a sustainable method for the management of invasive alien plants, whereby host-specific, co-evolved natural enemies from the invader's native range are reunited with the invasive plant in the introduced range (Schwarzländer et al., 2018). The aim of classical biocontrol is for the introduced agents to establish in their new environment and increase in abundance to levels capable of inflicting sufficient damage to their target IAPs, in order to reduce their competitiveness, reproductive output and population growth (Fowler et al., 2000; Schwarzländer et al., 2018). Biocontrol is thus a sustainable and cost effective method of controlling the invasiveness of alien plants, once the agents are well established (Fowler et al., 2000; Sheppard et al., 2006). Non-target effects of the potential biocontrol agents are avoided by thoroughly screening these agents and investigating their host-specificity and impact on the target weed before release (Louda et al., 2003; Park et al., 2018; Paynter et al., 2020). In particular, host-specificity testing is an essential part of the pre-release evaluation of the candidate biocontrol agent that minimizes the risks to non-target species (Andres et al., 1976; Wapshere et al., 1989; McFadyen, 1998; Hinz et al., 2019; Paynter et al., 2020).

Pre-release evaluation of candidate agents for the biological control of weeds tended to focus mainly on their safety, to minimise any impact on non-target plants (McClay & Balciunas, 2005). However, pre-release studies have recently included additional aspects, such as the potential impact of the candidate agent on the target weed, its compatibility with the climate of the new range and techniques to optimize mass-rearing and releases of the agent.

The safety of a potential agent is determined by using host-specificity tests to evaluate its host range and hence suitability for release. These studies are sometimes conducted under open-field or laboratory conditions in the native range, but more often in quarantine in the

introduced range (McClay & Balciunas, 2005; Schaffner et al., 2018; Alred et al., 2022; Bowers et al., 2022). Host-range testing includes an assessment of the agent's fundamental host range, which is usually determined by testing all closely related test-plant species under no-choice conditions. However, no-choice tests often reveal a fundamental host range that is broader than the true (realized) host range that is expressed in the field in the native range (Hinz et al., 2014; Schaffner et al., 2018). Choice tests, in which agents are exposed to non-target plants in the presence of their natural host, provide a more realistic assessment of the agent's true host range.

However, testing conducted under laboratory conditions does not include the environmental variability present in the field in the new range and does not test all ecological relationships pertinent to field releases. In particular, insect behaviour in a cage or greenhouse does not reflect the full range of potential field behaviours (Louda et al., 2005; Bowers et al., 2022). Consequently, laboratory host-range testing typically overestimates the risk of non-target attack, which may lead to the rejection of safe and effective agents (Hinz et al., 2014; Bowers et al., 2022). Ultimately, field surveys and open-field testing in the agent's native range provide the most accurate host-range assessments (Schaffner et al., 2018).

Agent impact studies also form part of the pre-release assessment, and assist in predicting the densities that the agent would need to reach in the field to achieve a significant impact on the target weed (McClay & Balciunas, 2005; Gerber et al., 2008; Morin et al., 2009; Alred et al., 2022). Efficacy is an important aspect of pre-release evaluation, although it is affected by many complex, interacting and unforeseen factors in the field. Consequently, not all established agents contribute to the successful control of the target weed (Julien & Griffiths, 1998). Some agents become abundant in the introduced region but do not facilitate effective control of the target weed (McClay & Balciunas, 2005). It is thus important to assess the potential impact of candidate agents prior to release, to maximize agent efficacy in controlling the target weed.

Predictions on the candidate agent's ability to establish and proliferate in the new range have also become important in recent years. These include studies on the agent's thermal tolerances and comparisons of the climate between the native and new countries, using climate-matching tools (Sutherst & Maywald, 1985; Ramanand et al., 2017). Other pre-release studies include interactions between a candidate agent and already established agents, to preclude competitive interactions that could lessen the overall outcomes of the biocontrol programme (Harley & Forno, 1992; Sheppard & Woodburn, 1996). Finally, aspects that promote the mass-rearing of agents (e.g. optimal age and nutritional status of host-plants) are studied prior to release to maximize agent establishment and proliferation in the field (Moran et al., 2014; Hill et al., 2021). The above considerations were all addressed in this study, which targeted the invasive alien plant, *Tecoma stans* L.

Tecoma stans – the target weed

Tecoma stans L. (Bignoniaceae)*,* commonly known as yellow bells, is an evergreen shrub or a small tree that has a wide natural distribution in the tropical and subtropical parts of the Western Hemisphere (Pelton, 1964). This plant is native to Mexico and the southern states of the USA, notably South Florida, and occurs throughout Central America, including the Caribbean and Northern Argentina. *Tecoma stans* is commonly planted in warmer climates throughout the world as an ornamental plant because of its attractive yellow flowers and pinnate foliage (Pelton, 1964; Madire et al., 2011; Madire, 2013; Madire et al., 2021; Madire & Netshiluvhi, 2021; Madire et al., 2023). However, *T. stans* has become invasive in many countries around the world, including Brazil, Australia, and several African countries (Bhat, 2019) that include South Africa (Hussey et al.*,* 1997; Henderson, 2001, 2021). *Tecoma stans* often becomes dominant, forming monospecific stands, thereby altering the invaded habitat's structure, integrity and functioning. Deforestation has also enabled *T. stans* to proliferate and invade many deep soils (Pelton, 1964).

Tecoma stans (Fig. 1) was initially introduced into South Africa as an ornamental plant and was first recorded in 1858 (McGibbon, 1858; Henderson, 2006), but later escaped cultivation and now invades roadsides, urban open spaces, watercourses, and rocky sites in subtropical and tropical savanna (Henderson, 2001, 2021). There has been a rapid increase in populations of the weed in South Africa during the past 25 years. Initially concentrated in Mpumalanga and Limpopo provinces in 1995, the weed has rapidly spread, and has now invaded seven of the nine provinces of South Africa (Fig. 2). It is widely distributed in Gauteng, Mpumalanga, Limpopo, KwaZulu-Natal and the Eastern Cape, as well as in neighbouring countries like Eswatini, Mozambique and Namibia (Henderson, 2001, 2021). The weed continues to extend its range because of seed dispersal by wind and floods. The potential for further spread of *T. stans* is high because some homeowners are not aware of its weed status, and it is still grown (albeit illegally) as an ornamental in some gardens in South Africa, especially in the Western Cape. Despite this, are no possible conflicts of interest in South Africa concerning the control of this invasive weed, although more public awareness on its negative impact is required. *Tecoma stans* is a Category 1b plant species and requires mandatory control according to the Alien Invasive Species Regulations (AIS, 2020 as amended) and National Environmental Management Biodiversity Act (Act No 10, 2004) of South Africa.

Control methods for *T. stans*

Mechanical control of large infestations of *T. stans* has proven to be both ineffective and expensive (Tu et al., 2001). Stem girdling, as a method of killing individual shrubs, is highly labour intensive and largely impractical as *T. stans* plants are multi-stemmed and coppice vigorously when cut back (Tu et al., 2001). The use of fire to destroy *T. stans* infestations is not desirable as the practice is not environmentally friendly, resulting in non-target effects on indigenous plants, and in most cases, induces the germination of other invaders (Tu et al., 2001). Furthermore, the roots of *T. stans* are deep and are difficult to remove via digging or

using a tree-popper. Chemical control options are similarly difficult because costly herbicide applications are only effective with continual follow-up treatments. Besides, no herbicide is currently registered for use against *T. stans* in South Africa (Xact Information, 2005; The Registrar, 2007) and chemical control on a national scale is not possible due to the weed's high density and wide distribution in the country. In light of the above constraints, biological control is considered the most sustainable (Zimmermann et al., 2004; McEvoy, 2018), inexpensive and environmentally friendly method of controlling *T. stans* in South Africa (Madire et al., 2011; Madire, 2013; Madire et al., 2021).
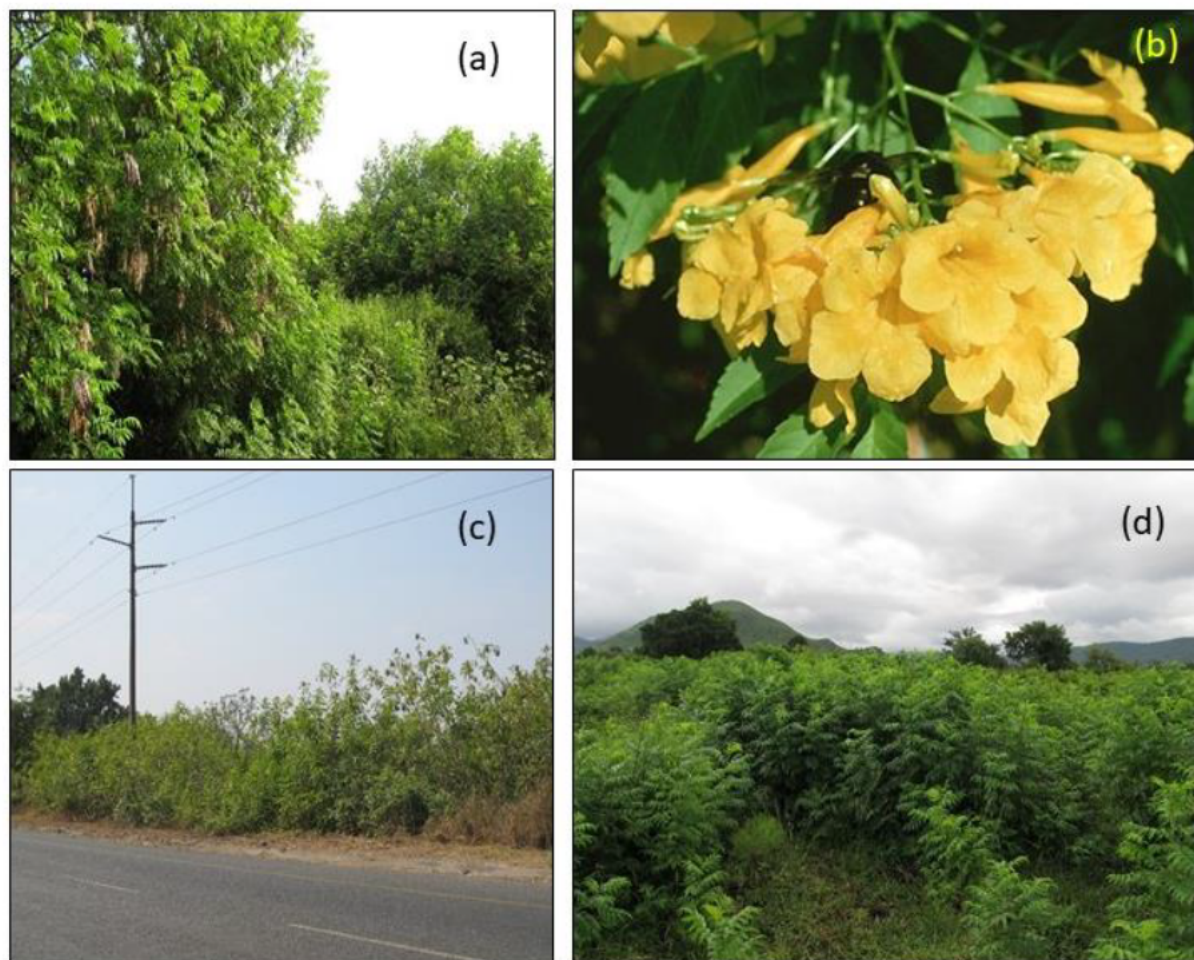
Fig. 1. Images of *Tecoma stans* indicating (a) pod-bearing trees, (b) conspicuous yellow flowers and (c, d) invaded habitats.

Biological control of *T. stans*

Tecoma stans was one of five emerging weed species targeted for biological control since 2003 (Olckers, 2004) by the Agricultural Research Council's then Plant Protection Research institute (now Plant Health and Protection (ARC-PHP). The project was funded by the then Department of Water Affairs and Forestry (now Department of Forestry, Fisheries and the Environment), via the Natural Resource Management Program (NRMP), formerly the Working for Water Programme (WfW). *Tecoma stans* is no longer an emerging weed and is now well distributed across many South African provinces (Fig. 2). Attempts to find suitable biocontrol agents were successful, with three agents sourced from Argentina and Mexico in 2002, 2005 and 2007, respectively. These included a gall-forming rust fungus, *Prospodium transformans* Cummins (Pucciniales: Uropyxidaceae), a leaf-mining fly, *Pseudonapomyza* sp. (Diptera: Agromyzidae), and a defoliating lady beetle, *Mada polluta* Mulsant (Coleoptera: Coccinellidae).

Despite several attempts at inoculation from 2010 onwards, the rust fungus *P. transformans* failed to establish in the field (Wood, 2014). *Mada polluta* was initially released in November 2013, followed by *Pseudonapomyza* sp. in November 2014. Although both insect agents became established, several of the release sites were destroyed by fire or mechanical control, thereby constraining assessments of their establishment and impact in the field. However, *M. polluta* is well established at sites around East London (Eastern Cape Province) with significant damage recorded on *T. stans* populations. Sites where the beetles became established on the KwaZulu-Natal South Coast were recently destroyed by fire and more releases were conducted. These releases are showing signs of initial establishment, with follow-up assessments planned for the future.

Pseudonapomyza sp. has become established in four provinces (Limpopo, Mpumalanga, KwaZulu-Natal and Eastern Cape), but in marginal numbers and with minimal impact. Since

T. stans populations continue to increase and spread in the country, a root-feeding flea beetle, *Heikertingerella* sp. Csiki 1940 (Coleoptera: Galerucinae: Alticini) was imported in 2013 (Import permit: P066301) for screening as an additional agent. *Heikertingerella* sp. targets a different niche compared to the two biocontrol agents already released, and is more resistant to the destruction of trees at release sites due to the underground habitat of its immature stages.
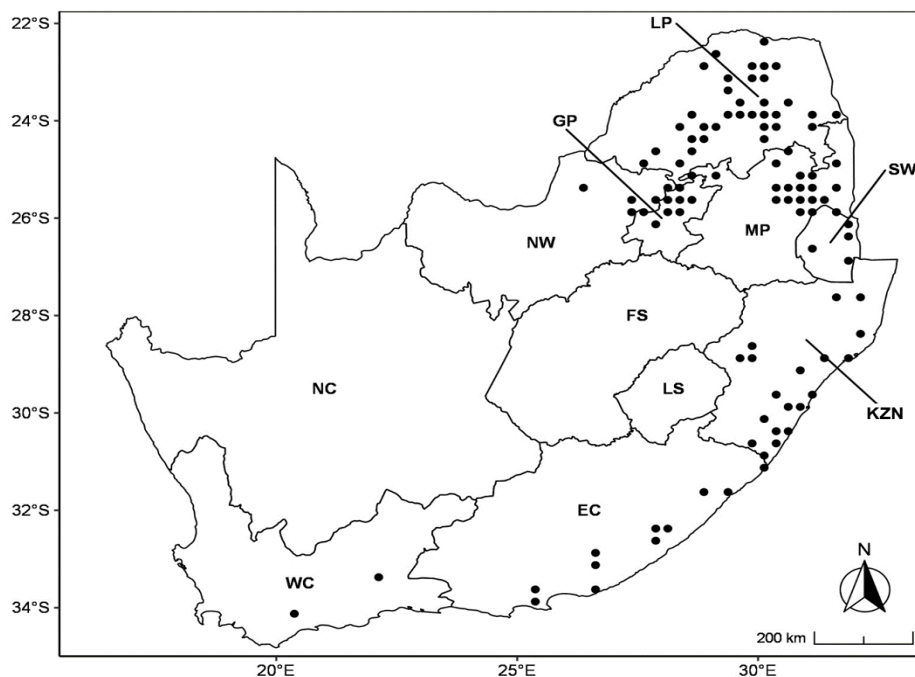
Fig. 2. Distribution of *Tecoma stans* L. in South Africa (drawn by Guy Sutton: SAPIA database, ARC-Plant Health and Protection, Pretoria) (Madire & Netshiluvhi 2021). South African provinces comprise the Eastern Cape (EC), Free State (FS), Gauteng (GP), KwaZulu-Natal (KZN), Limpopo (LP), Mpumalanga (MP), Northern Cape (NC), North West (NW), and Western Cape (WC), while neighbouring countries comprise Lesotho (LS) and Eswatini (SW).

Rationale for the study

Two leaf-feeding biological control agents (*M. polluta* and *Pseudonapomyza* sp.) are established in the field in South Africa, but with limited impact. Because of the severity of *T. stans* invasions in the country, a suite of agents that attack different niches, including the root system and reproductive tissues, is required. Consequently, a root-feeding flea beetle, *Heikertingerella* sp. (Fig. 3) was introduced from Mexico into quarantine in South Africa for assessment as an additional biological control agent. The biocontrol programme against *T. stans* is unique to South Africa and none of the candidate agents have been studied elsewhere in the world. However, since *T. stans* has become invasive in many countries, biological control agents developed in South Africa could also be deployed elsewhere. The current pre-release studies on *Heikertingerella* sp. were intended to demonstrate that the beetle is not only safe for release, but will be able to thrive under differential environmental conditions in South Africa, while inflicting sufficient damage on the target weed that will contribute to its management.

Fig. 3. *Heikertingerella* sp. adult.

Thesis outline

The aim of the study was to determine the safety of the root-feeding flea beetle, *Heikertingerella* sp. (Coleoptera: Galerucinae: Alticini), for release as a biocontrol agent of *T. stans* and to examine various aspects that could potentially influence its establishment, proliferation and effectiveness in South Africa. The structure of the thesis is as follows:

- Chapter 1 provides an introduction to invasive plants, biological control, the target weed and the rationale for the study.

- Chapter 2 evaluates the biology and host range of *Heikertingerella* sp. and determines its potential for release against *T. stans* in South Africa.

- Chapter 3 determines the interactions between *Heikertingerella* sp. and *M. polluta* by examining their survival and reproductive success when confined alone and in combination on potted *T. stans* plants. The individual and combined impact of both beetles on leaf damage, leaf density and plant height was quantified.

- Chapter 4 quantifies the effect of different flea beetle densities on the vegetative growth and biomass accumulation of *T. stans* under quarantine glasshouse conditions. In addition, MaxEnt modelling, together with the beetle's critical thermal limits, were used to predict its potential distribution in South Africa.

- Chapter 5 considered the effect of host-plant age on the performance of *Heikertingerella* sp., since age-based differences can influence the agent's impact on *T. stans* populations of variable age structure. In addition, an optimal host-plant age for mass-rearing of the beetle was determined to boost the numbers available for release.

- Chapter 6 determined the effect of increasing soil nutrient levels (fertilizer treatments) on the growth and biomass of *T. stans* plants and on the subsequent performance of *Heikertingerella* sp. Optimal host-plant nutrient levels were determined to boost the production of healthy beetles in mass-rearing facilities and enhance establishment in the field.

- Chapter 7 summarises the outcomes of the study, with recommendations for the way forward with the biocontrol programme against *T. stans*.

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CHAPTER 2

Pre-release evaluation of *Heikertingerella* sp. as a potential biocontrol agent for *Tecoma stans* in South Africa

Abstract

Native to Central America, *Tecoma stans* (L.) Juss ex Kunth var. *stans*, (Bignoniaceae) is a small tree that is invasive in South Africa, neighbouring countries, and in many countries around the world. The plant was targeted for biological control in South Africa in 2003, with two insect agents released and established so far. The root-feeding flea beetle, *Heikertingerella* sp. (Coleoptera: Galerucinae: Alticini), was imported from Mexico as an additional biocontrol agent and its biology and host-specificity was assessed under quarantine conditions. The beetle displayed a generation time (i.e., from adult to adult) of 49 to 67 days, ensuring four annual generations under laboratory conditions. The beetle's larval and adult stages inflicted high levels of damage on the root system and the leaves of *T. stans*, respectively. No-choice tests with 40 test plant species revealed adult feeding on only two non-target species, *Tecoma* x *alata* and *T. capensis* (Thunb.) Spach, with feeding four times higher on *T. stans*. Larvae developed to adulthood on *T. stans* only. Multi-choice tests involving the three *Tecoma* species confirmed these trends, demonstrating that *Heikertingerella* sp. is host specific. Since, *T.* x *alata* is a hybrid of *T. stans* with invasive tendencies, any unlikely attacks by *Heikertingerella* sp. would be inconsequential in South Africa. The native *T. capensis,* which suffered little leaf damage and produced no F_1 adults, is also at minimal risk of attack. We conclude that *Heikertingerella* sp. is a suitable biocontrol agent for *T. stans* and that permission for its release in South Africa be sought.

Keywords: Biology studies, host-specificity testing, invasive trees, root-attacking agents, yellow bells, weed biocontrol

Introduction

Tecoma stans (L.) Juss ex Kunth var. *stans* (Bignoniaceae), commonly known as yellow bells, is native throughout Central America, including Mexico, the Caribbean Islands and the southern USA (southern Florida) (Pelton, 1964; Madire et al., 2011a). Following its introduction as an ornamental, the plant has invaded roadsides, urban open spaces, watercourses and rocky sites in the sub-tropical regions of South Africa (Madire et al., 2011a). According to the Alien Invasive Species Regulations (AIS) and National Environmental Management Biodiversity Act (NEMBA) (Act No 10. 2004) of South Africa, the plant constitutes a Category 1b species that necessitates compulsory control. *Tecoma stans* has extended its range to seven South African provinces and several neighbouring countries, including Mozambique, Zimbabwe, Namibia and Eswatini (previously known as Swaziland) (Henderson, 2001; Cunningham, 2008; Madire, 2013). The weed is also invasive in many countries around the world, including Argentina, Australia, Brazil and other African countries (Orwa, et al., 2009). It spreads through papery, winged, lightweight seeds, which are easily dispersed by wind and floods. *Tecoma stans* has transformed the vegetation of natural and disturbed habitats and along watercourses where it forms monocultures (Madire et al., 2011a). Due to the inefficiency of mechanical and chemical control, the weed was targeted for biological control in South Africa in 2003 (Madire et al., 2011a),

Two insect agents, the leaf-feeding *Mada polluta* Mulsant (Coleoptera: Coccinellidae) and leaf-mining *Pseudonapomyza* sp. (Diptera: Agromyzidae), were released in South Africa, in 2013 and 2014, respectively. Both defoliating agents have established at various release sites, particularly in the Eastern Cape Province. Because of the severity of *T. stans* invasions in South Africa, a suite of agents is required to attack various parts of the plant, including the root system and the reproductive tissues. The seed-attacking moth *Clydonopteron sacculana* Bosc (Lepidoptera: Pyralidae) was considered but found to be insufficiently host specific for release

(Madire et al., 2011a). During field surveys conducted from 2013 to 2015 in Mexico, the root-feeding flea beetle *Heikertingerella* sp. (Coleoptera : Galerucinae: Alticini) was collected at sites in Chiapas, Guanajuto, Oaxaca and Veracruz provinces and introduced into quarantine in South Africa for assessment as an additional biocontrol agent for *T. stans.*

The genus *Heikertingerella* Csiki includes 125 species and three subspecies, with 106 species recorded from South America and 19 species from Central America (Furth & Savini, 1996; Savini, 1999). *Heikertingerella* is a very diverse genus and has the potential to increase its diversity and distribution in the New World, as more species are discovered and described, (Savini & Furth, 2001). *Heikertingerella*, formerly under the sub-family Alticinae, is now placed under the tribe Alticini of the Galerucinae, since these two subfamilies were combined into Galerucinae (Mohamedsaid & Furth, 2011; Furth et al., 2015). A high proportion of the more effective chrysomelid biological control agents belong to the subfamilies Chrysomelinae and Galerucinae (Syrett et al., 1996). Galerucinae include highly specialized phytophagous insects because of restricted host ranges due to close host-plant associations (Savini & Furth, 2001; Gok et al., 2004). Currently, no species of *Heikertingerella* have been deployed as weed biocontrol agents anywhere in the world (Winston et al., 2014) and the undescribed species involved in this project is the first to be considered as a candidate agent. The identity of this species was confirmed by Dr D.G. Furth from tthe National Museum of Natural History Smithsonian Institution in Washington. The specimens were examined by comparing them with four known *Heikertingerella* species known from Mexico and were further compared to more species from South America and 19 species from Central America (Furth & Savini, 1996) and none matched. Therefore, it was concluded as another undescribed species of *Heikertingerella*. More specimens have been sent to taxonomists to proceed with description.

The study was conducted to evaluate the biology and host range of *Heikertingerella* sp. and determine its potential for release as a biological control agent for *T. stans* in South Africa.

Materials and methods

Glasshouse conditions

The flea beetle cultures were maintained in a glasshouse at the Roodeplaat East quarantine facility of the Agricultural Research Council- Plant Health and Protection (ARC-PHP) in Pretoria, South Africa. The temperature was maintained from 22-29°C at night and 27-29°C during the day, while the relative humidity varied from 47% to 88% throughout the year. The biology studies and host-specificity tests were conducted under natural light conditions during summer and under a 16h L: 8h D photoperiod during winter. The winter photoperiod was maintained using 50W / LED 4000K/ 230 V LED floodlights (Spazio lighting).

Test plants and insect cultures

Tecoma stans host plants and some test plants were grown from seeds that were collected in the field, while other test plants were procured from nurseries. Seeds were germinated on sand media and, once they had grown to 15 cm, were transplanted into 10 litre pots and kept in a nursery shadehouse. A standard soil mixture comprising one part river sand, one part Styrofoam™, one part compost and one part top soil was used to transplant seedlings. Plants were watered regularly with automated overhead sprinklers and were treated with NPK fertilizer (2:3:2 (14%)) once every two weeks prior to their use in the experiments.

The flea beetles were reared in cages (0.55 x 0.55 x 0.95 m) containing potted *T. stans* plants, in which 30-50 adults were confined in a cage to allow oviposition in the soil. After 30 days, the adults were removed and transferred onto fresh plants in another cage. The pots containing the soil-dwelling larvae were monitored until the emergence of the adults (F_1), which normally took around 40 days. The newly emerged adults were used in the biology and host-range studies.

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Biology of Heikertingerella sp.

Biological aspects investigated included pre-oviposition period, developmental period from egg to adult, adult longevity, number of annual generations, and adult body size. Pre-oviposition period was determined by confining a newly-eclosed pair of adults in a perforated container (19 x 14 x 8 cm) with cut leaves placed on moist soil. The containers were inspected daily for the presence of eggs and the period from adult emergence until the commencement of oviposition was recorded as the pre-oviposition period. This trial was replicated six times.

Adult longevity was determined by placing a mating pair of newly emerged beetles in a cage (0.55 x 0.55 x 0.95 m) with a potted *T. stans* plant. The beetles were confined for 20 days and then transferred into another cage containing a fresh plant. This procedure was repeated until both male and female beetles had died. The caged plants exposed to each mating pair were monitored until the new generation of adults emerged. The duration of development of the immature stages from egg to adult was recorded by subtracting the 14-day pre-oviposition period (see below) from the time between the exposures of the P_1 adults to the time of emergence of the F_1 adults. These trials were replicated 20 times.

Body size measurements of the adults of *Heikertingerella* sp. were determined according to the methods of Linzmeier & Ribeiro-Costa (2011). Female and male body size was measured from head to elytral apex, using Vernier callipers under a dissecting microscope.

Host-specificity tests

Adult no-choice and multi-choice tests were conducted under quarantine glasshouse conditions as previously described. Test-plant species were selected according to the centrifugal phylogenetic testing method (Wapshere, 1974), which ensured that a wide range of plant families were represented in the tests. The trials commenced with the taxonomically closest related plants to *T. stans* within the family Bignoniaceae in South Africa and progressed to

more distantly related plants within the order Lamiales (Olmstead et al., 2001). Selected species from plant families that were recorded as hosts of other *Heikertingerella* species in Mexico (Flowers & Janzen, 1997; Konstantinov et al., 2018) were also included in the tests. Several crop species of economic importance in South Africa that belong to unrelated plant orders were also tested. The genus *Tecoma* was represented by the three species found in South Africa (Gentry, 1980, 1992; Wood, 2008; Madire, et al. 2011a), including *T. stans*, a native species (*Tecoma capensis* (Thunb.) Spach) and a hybrid ornamental species (*Tecoma* x *alata*) originally from Peru (Glen, 2002). *Tecoma* x *alata* (orange bells) is regarded as a hybrid between *T. stans* and *T. alata* and is variously referred to as *Tecoma* x 'Burnt Out', *T. alata* 'Orange Jubilee', *T. stans* 'Orange Jubilee' or *Tecoma* x 'Orange Jubilee'.

Adult no-choice trials

Some 40 test-plant species were tested in no-choice situations to determine their suitability for feeding and larval development of *Heikertingerella* sp. Test plants were confined individually in gauze-covered cages (0.55 x 0.55 x 0.95 m) in the quarantine glasshouse. The target weed, *T. stans*, was included as a control. In each cage, five mating pairs of *Heikertingerella* sp. were exposed to the test plant for 30 days to allow feeding and oviposition. The adults were then removed from the cages and the leaves were assessed for adult feeding damage, which was scored on the percentage of leaves damaged per plant. The feeding damage levels included: no leaves damaged; minor damage (1-29% leaves damaged); moderate damage (30-50%) and severe damage (51-100%). After the removal of the adults, the plants were kept in the same cages for a further 30 days, with any emerging F_1 adults recorded daily. Each test-plant species was tested four times, with different plants and beetles used between replicates.

Adult multi-choice trial

An adult multi-choice test was carried out using the three *Tecoma* species found in South Africa (Glen, 2002), since these were the only test-plant species that supported feeding during the adult no-choice trials (see below). Test plants of similar stem height and canopy were selected and sprayed with water, to remove any contaminants prior to testing. Four plants of each of the three *Tecoma* species were arranged in a randomised block design in a large walk-in cage (4 x 4 x 2 m). Forty *Heikertingerella* sp. adults were confined with the plants for 30 days and then removed. After removal of the adults, feeding damage was scored as previously described and individual plants were placed into smaller cages (0.55 x 0.55 x 0.95 m) for a further 30 days to record the emergence of adult F_1 progeny. The trial was terminated after 60 days, by which time it was assumed that all adult progeny had emerged.

Statistical analysis

Analyses were carried out in SPSS 26. Since the data did not conform to normality, we used generalized linear modelling to determine the effect of host plant on the percentage of available leaves that were damaged by adult *Heikertingerella* sp. during the no-choice and multi-choice trials. The models, all corrected for over-dispersion, included a binomial distribution and logit link function, with significance ($P < 0.05$) assessed using Wald chi-square statistics. Where there were significant overall differences between test plant species, post-hoc comparisons (Fisher's Least Significant Difference) were used to separate the means.

Results

Biology of Heikertingerella sp.

Heikertingerella sp. adults are golden brown in colour with black compound eyes and the enlarged hind femora typical of flea beetles. The adult body is oval in shape and ranges in

length from 2.3 to 3.2 mm (mean ± SE = 2.6 ± 0.1 mm; n = 20) as measured from head to elytral apex. There is considerable overlap in body length between the sexes, ranging from 2.3 to 3 mm (mean ± SE = 2.5 ± 0.1; n = 10) in the males and from 2.3 to 3.2 mm (mean ± SE = 2.7 ± 0.1; n = 10) in the females. Adults feed on the leaves of *T. stans* and create small irregular round holes by scraping the leaf epidermis through to the mesophyll and eventually, causing extensive damage.

Feeding by *Heikertingerella* sp. adults commenced shortly after their emergence from the soil and oviposition commenced after a pre-oviposition period of 12 to 15 days (mean ± SE = 13.5 ± 0.5; n = 6). Females confined with potted plants deposited small yellow eggs onto the soil surface. After egg hatch, the first-instar larvae burrowed down into the soil to feed on the secondary roots of *T. stans*. Larvae fed externally on the cortex of the secondary roots and developed on the core of the primary roots, eventually pupating in the soil. Severely damaged plants displayed slow growth after exposure to larval feeding. Some plants suffered mortality after adult emergence. Development from egg to adult emergence ranged from 35 to 53 days (mean ± SE = 47.0 ± 1.10; n = 20).

Due to their subterranean nature, the number of larval instars and the duration of the immature stages were not determined. The adult-to-adult generation time ranged from 51 to 73 days (mean ± SE = 58.9 ± 1.2; n = 25), allowing the beetles to complete four annual generations under the quarantine glasshouse conditions. The male and female adults survived from 41 to 100 days (mean ± SE = 75.7 ± 4.73; n = 20) in the quarantine glasshouse.

Host specificity of Heikertingerella sp.

Adult no-choice trials

Of the 40 plant species tested, adults of *Heikertingerella* sp. fed only on the three *Tecoma* species and were unable to feed on any other species in either the Bignoniaceae or any of the

more distantly related families (Table 1). There were significant differences in the percentage of available leaves that were damaged by the adults ($\chi^2 = 38.741$, *df* = 2, P < 0.0005) between the three test plant species that supported feeding. Feeding was significantly (more than four times) higher on *T. stans* than on *T.* x *alata* and *T. capensis*, while the difference between the latter two species was not significant. Larval development to adult emergence was recorded only on *T. stans*, with an average of 20 beetles reared per trial (Table 1).

Adult multi-choice trials

Adult feeding was recorded on the exotic *T. stans* and *T.* x *alata*, but not on the native *T. capensis* (Table 2). As before, there were significant differences in the percentage of available leaves that were damaged by the adults ($\chi^2 = 122.008$, df = 2, P < 0.0005) with feeding 3.7 times higher on *T. stans* than on *T.* x *alata*. Adult F_1 progeny were recorded only on *T. stans*, with an average of 16 beetles reared per plant (Table 2).

Table 1. Feeding and development to adulthood of *Heikertingerella* sp. during adult no-choice trials on species from several related and unrelated plant families.

Order: Family	Plant species[1]	% Leaves damaged (Mean ± SE)[2]	F_1 adult emergence (Mean ± SE)
Lamiales: Bignoniaceae	*Catophractes alexandri* *	0	0
	Dolichandra unguis-cati **	0	0
	Fernandoa magnifica *	0	0
	Jacaranda mimosifolia **	0	0
	Kigelia africana *	0	0
	Markhamia acuminata *	0	0
	Markhamia zanzibarica *	0	0
	Markhamia obtusifolia *	0	0
	Pyrostegia venusta **	0	0
	Podranea ricasoliana *	0	0
	Tabebuia impetiginosa **	0	0
	Tecoma x *alata* **	22.8 ± 2.3[b]	0
	Tecoma capensis *	20.0 ± 7.8[b]	0
	Tecoma stans (L) **	97.0 ± 1.3[a]	20.8 ± 1.6
	Rhigozum obovatum *	0	0
	Spathodea campanulata **	0	0
Acanthaceae	*Barleria obtusa* *	0	0
	Ruttyruspolia hybrid *	0	0
	Thunbergia natalensis *	0	0
Scrophulariaceae	*Halleria lucida* *	0	0
	Myoporum laetum **	0	0
Verbenaceae	*Lantana camara* **	0	0
	Lippia wilmsii *	0	0

	Lippia rehmannii *	0	0
Lamiaceae	*Mentha spicata* **	0	0
Oleaceae	*Jasminum multipartitum**	0	0
	Jasminum officinale **	0	0
Solanales: Solanaceae	*Capsicum annuum* ***	0	0
	Solanum melongena ***	0	0
	Solanum tuberosum ***	0	0
Chenopodiaceae	*Beta vulgaris* ***	0	0
Fabales: Fabaceae	*Phaseolus vulgaris* ***	0	0
Poales: Poaceae	*Saccharum officinarum* ***	0	0
	Zea mays ***	0	0
Asterales: Asteraceae	*Calendula officinalis* ***	0	0
	Chrysanthemum maximum ***	0	0
	Tagetes erecta ***	0	0
	Lactuca sativa ***	0	0
Apiales: Apiaceae	*Daucus carota* ***	0	0
Cucurbitales: Cucurbitaceae	*Cucumis sativus* ***	0	0

[1] Where * = Native; ** = Exotic; *** = Cultivated.

[2] Means followed by the same letter are not significantly different ($p > 0.05$).

Table 2. Feeding and development to adulthood of *Heikertingerella* sp. during an adult multi-choice trial involving *Tecoma* species.

Test plants[1]	% Leaves damaged per plant (mean ± SE)[2]	F1 adult emergence per plant (mean ± SE)
*Tecoma stans***	58.5 ± 2.2[a]	16.8 ± 1.6
Tecoma x *alata***	15.5 ± 2.8[b]	0
*Tecoma capensis**	0	0

[1] Where * = Native; ** = Exotic

[2] Means followed by different letters are significantly different (p < 0.05)

Discussion

Our study showed that both adults and larvae of *Heikertingerella* sp. caused severe feeding damage to *T. stans*, suggesting that the beetle can reduce the weed's invasiveness in the field. Blossey & Hunt-Joshi (2003) reported that over 50% of root-feeding agents contributed to the suppression of invasive plant populations, with the beetle families Curculionidae and Chrysomelidae comprising the most effective taxa. For example, flea beetles in the genus *Aphthona* Chevrolat are among the more successful biocontrol agents in the management of leafy spurge, *Euphorbia esula* L. (Euphorbiales: Euphorbiaceae), across North America (Hansen, et al., 1997; Kirby, et al., 2000). Blossey & Hunt-Joshi (2003) also reported that the establishment rates of root feeders exceeded those of aboveground herbivores by 54% and were more likely to contribute to control of their target weed species. Species of *Longitarsus* Latreille, which are close relatives of *Heikertingerella* sp. under the same flea beetle clade (Alticini) (Furth, et al., 2015), have achieved success as biocontrol agents against various weed species in Australia, the United States of America (USA), Canada and New Zealand (Simelane, 2005). For example, the root-feeding *Longitarsus jacobaeae* (Waterhouse) brought the

invasive tansy ragwort *Jacobaea vulgaris* Gaertn. (Asteraceae) under control in the USA (McEvoy, et al., 1991). According to Crawley (1989), Chrysomelidae was the third most successful insect family with 23% of popularity as a source of biological control agents (Julien, 1992). However, not all the chrysomelid candidate biological control agents had become successful in controlling their target weeds. For example, *Disonycha argentinensis* Jacoby failed to control alligator weed *Alternanthera philoxeroides* (Mart.) Griseb in Australia (Sainty et al., 1997). Although the flea beetle species *Agasicles hygrophila* Selman & Vogt (Coleoptera: Chrysomelidae) was effective in some regions of North America and New Zealand, it was not effective on the terrestrial form of alligator weed in Florida, USA (Julien & Chan, 1992). On condition that there are no ecological and climatic barriers to the establishment and persistence of *Heikertingerella* sp. in South Africa, we expect the beetle to perform like other successful root feeders.

Unlike aboveground herbivores, the belowground life stages of *Heikertingerella* sp. may protect populations against wild fires and natural enemies (Hawkins, et al., 1993; Blossey & Hunt-Joshi, 2003; Kim & Holt, 2012). The root-feeding *Longitarsus bethae* Savini & Escalona, released against *Lantana camara* L. (Verbenaceae) in South Africa, survived several wild fires in the field due to its subterranean immature stages (Simelane & Mawela, 2018). In contrast, the establishment and dispersal of the leaf-feeding lady beetle *M. polluta*, released against *T. stans* in South Africa, is negatively affected by winter wild fires at various release sites. Furthermore, subterranean niches often provide a refuge that limits the recruitment of native natural enemies by the immature stages of biocontrol agents in their introduced range (Hawkins, 1988; Hawkins, 1990; Blossey & Hunt-Joshi, 2003).

Host-specificity testing confirmed that *Heikertingerella* sp. is highly host specific to *T. stans* and is unlikely to pose any threat to non-target plant species in South Africa. During adult no-choice and multi-choice trials, *Heikertingerella* sp. caused severe damage to the target

weed, but only minor leaf damage to the non-target *T.* x *alata* and *T. capensis.* Inspection of

the roots of *T.* x *alata* and *T. capensis* at the end of the trials revealed no signs of larval feeding

damage, which corresponded with the absence of any F_1 adult progeny reared from them.

Consequently, neither *T.* x *alata* nor *T. capensis* will be able to sustain populations of

Heikertingerella sp. in the field. The low levels of adult leaf feeding on these two non-target

species are typical of conservative laboratory trials, notably no-choice trials. Indeed, several

studies have described such cage artefacts whereby confined cage conditions force insects to

feed on non-target plant species that would otherwise be avoided in the field (e.g. Balciunas,

et al., 1996; Simelane, 2005; Madire, et al., 2011b; Madire, 2013). Many herbivorous insect

species have displayed extended host ranges under laboratory conditions, which have seldom

been realised under field conditions (e.g. Harris, 1984; Hill & Hulley, 1995; Olckers, et al.,

1995; Kaufman & Landis, 2000; Simelane, 2005; Madire, et al., 2011b). Despite feeding on

some non-target plant species during laboratory no-choice tests, both *Pseudonapomyza* sp. and

M. polluta, have only been recorded on *T. stans*, six years after their release in South Africa

(Madire, et al. 2011b; Madire 2013). Similar trends were observed with the leaf-mining fly

*Ophiomyia camara*e Spencer (Agromyzidae) and root-feeding *L. bethae*, which fed on a few

non-target species of *Lippia* L. in the laboratory (Simelane, 2002, 2005) but have only been

recovered on the target *L. camara*, since their release in South Africa over 11 years ago

(Simelane & Mawela, 2018).

Because of the high abundance of *Heikertingerella* sp. observed in its native range in

Mexico, we suspect that the beetle's reproductive output during the host-specificity tests is an

underestimation of its performance. In particular, the number of F_1 adults reared from

individual *T. stans* plants ranged from 14 to 25. Because *Heikertingerella* sp. spends its pre-

adult life cycle under the soil surface, the factors affecting its biology are poorly understood,

causing difficulties in determining an optimum mass-rearing procedure. We have also observed

that glasshouse cultures of *Heikertingerella* sp. display differential performance in F_1 adult production on *T. stans* plants of variable age. Studies to determine the effect of plant age on *Heikertingerella* sp. performance were subsequently completed (Chapter 5). In addition, results from the pre-release impact study in quarantine showed a high potential of efficacy of *Heikertingerella* sp. on individual *T. stans* plants (Chapter 4); therefore we presume that it will be effective once released.

This study has demonstrated that *Heikertingerella* sp. is safe for release as a biocontrol agent against *T. stans* in South Africa and possibly elsewhere in the world. As a root-feeding agent, we believe that *Heikertingerella* sp. will complement the two established leaf-feeding agents. An application for its release in South Africa was thus submitted to the regulatory authorities.

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CHAPTER 3

Competitive interactions between the root-feeding *Heikertingerella* sp. and foliage-feeding *Mada polluta* on the invasive *Tecoma stans*

Abstract

Despite potential negative interactions between biological control agents, the release of multiple agents against invasive alien weeds is often justified. The leaf-feeding beetle *Mada polluta* Mulsant (Coleoptera: Coccinellidae), released against *Tecoma stans* (L.) Juss ex Kunth var. *stans* in South Africa, has so far been unable to contain the weed. Consequently, the root-feeding flea beetle *Heikertingerella* sp. (Coleoptera: Galerucinae) was introduced to complement *M. polluta*. The effects of the interaction between the two beetles on their performance and on the target weed were studied on potted *T. stans* plants in a quarantine glasshouse to assess whether they were additive, synergistic or negative. There was no significant difference in the percentage survival of the P_1 adults of either beetle when tested alone or in combination. *Mada polluta* produced significantly more F_1 adult progeny than *Heikertingerella* sp. when tested alone, while both beetles produced significantly fewer offspring when tested in combination. Leaf damage by *M. polluta* alone was higher than that caused by *Heikertingerella* sp. alone, but in combination was not significantly higher than damage by *M. polluta* alone. Although both beetles on their own caused a significant reduction in leaf density relative to the control, leaf density was significantly lower when in combination. Despite large reductions in plant height relative to the control, the differences between the three beetle treatments were not significant. Although competitive interactions caused a trade-off between agent proliferation and their impact on the growth of *T. stans*, these data need to be confirmed in the field.

Introduction

The release of multiple agents to control invasive plant species has been applied in many biological control programmes, with success often increasing with the number of agents released (Denoth et al., 2002). While there has been support for the release of multiple agents in both concept and practice (e.g. Hoffmann & Moran, 1998; Jimenez & Balandra, 2007), there has also been criticism of this approach (e.g. Myers, 1985; Myers et al., 1989; McEvoy & Coombs, 2000; Denoth et al., 2002; Crowe, 2003). Critics have described this as a "lottery approach" (McEvoy & Coombs, 2000), arguing that control arising from the release of multiple agents is due to the increased probability of releasing an effective agent, rather than the combined impact of the suite of agents. Although competition among phytophagous insects appears rare in their native ranges (Rathke, 1976; Strong et al., 1984), introduced herbivores typically experience a decrease in regulation by their natural enemies and thus higher population densities (Harley & Forno, 1992; Keane & Crawley, 2002), which could have profound negative consequences for established biocontrol agents. In particular, greater competition for shared food resources or the same niches can cause antagonistic effects on agent populations (Denno et al., 1995; Paynter & Hennecke, 2001; April et al., 2011).

The invasive Central American tree, *Tecoma stans* (L.) Juss ex Kunth var. *stans* (Bignoniaceae), commonly known as yellow bells, was targeted for biological control in South Africa in 2005 (Madire et al., 2011a; 2011b). *Tecoma stans* has invaded much of South Africa, as well as neighbouring countries in southern Africa and other countries in the world. Increasing infestations of *T. stans* during the past 20 years has been a concern in South Africa, leading to the initiation of the biological control programme. The programme has resulted in

the release of two insect agents, the leaf-feeding beetle *Mada polluta* Mulsant (Coleoptera: Coccinellidae) and a leaf-mining fly *Pseudonapomyza* sp. (Diptera: Agromyzidae) in 2013 and 2014, respectively. *Mada polluta* has since established and become abundant at a few sites in KwaZulu-Natal (KZN) and the Eastern Cape (EC) provinces, while very small populations of *Pseudonapomyza* sp. have been recorded in KZN, EC, Limpopo and Mpumalanga provinces. Due to the severity of *T. stans* invasions in South Africa, it has been argued that a suite of agents is required to attack various parts of the plant, including the root system and the reproductive organs (Madire et al., 2011a).

The Mexican root-feeding flea beetle *Heikertingerella* sp. (Chrysomelidae: Galerucinae: Alticini) was introduced into quarantine in South Africa for assessment as an additional biocontrol agent for *T. stans*. While the adult flea beetles can cause extensive leaf damage, the larvae feed on the roots of the plant, often reducing growth (Madire et al., 2021). Host specificity testing has demonstrated that *Heikertingerella* sp. is suitable for release in South Africa (Madire et al., 2021). Research on plant-mediated interactions should form part of pre-release evaluation protocols to assist in decision-making about which agents to introduce in classical biological control programs, in order to achieve the greatest impact on invasive weeds (Milbrath & Nichols, 2014).

Assuming that *Heikertingerella* sp. will be cleared for release, this study examined the consequences of the simultaneous release of *Heikertingerella* sp. and *M. polluta* on the performance of each agent species on their shared host and on the growth of the target plant. When released from their natural enemies in the introduced range, biocontrol agents may compete with other agents that share the same host (Harley & Forno, 1992; Sheppard & Woodburn, 1996). Such competitive interactions for the same resources may be mediated by their host plant through changes in food quality or induced defences in response to herbivore attack (Denno et al., 1995; Gerber et al., 2007), with negative consequences for one or all

agents. Below-ground herbivores can be effective agents by causing substantial damage to roots, which may have a more severe impact on plant fitness than above-ground damage (Gerber et al., 2007; Johnson & Cushman, 2007). However, interactions between below-ground herbivores and their host plant could have a profound influence on above-ground herbivores (e.g. Simelane, 2006), and this effect could be positive or negative for *Heikertingerella* sp. and *M. polluta*.

In this study, we assessed the interactions between *M. polluta* and *Heikertingerella* sp. by examining their survival and reproductive success when confined alone and in combination on potted *T. stans* plants in cages. We also assessed the individual and combined impact of both herbivores on leaf damage, leaf density and plant height.

Materials and methods

Laboratory conditions

This study was conducted in a quarantine glasshouse at the Agricultural Research Council-Plant Health and Protection, Roodeplaat facility in Pretoria, South Africa (25°36'8780"S; 28°21'9230"E). The temperature and relative humidity during the trial was set at 28-33°C and 47-60%, respectively. This study was conducted under natural light conditions during summer and under a 16:8 L: D photoperiod during winter. The winter photoperiod was maintained using 50W / LED 4000K/ 230 V LED floodlights (Spazio lighting). *Tecoma stans* plants were propagated from seeds collected in the field, using river sand only as the growth medium. After the seeds had germinated, the seedlings were transplanted into 2-litre pots containing a standard growing mixture of one part each of top soil, river sand, compost and vermiculite. These plants were watered twice a day and Wonder Nitrogen, Phosphorus and Potassium fertilizer (2:3:2 [14%]) was applied every three weeks to promote plant growth. Plants were maintained until

they were one year old and then used in the trial. *Heikertingeralla* sp. and *M. polluta* cultures that provided individuals for this study were reared on *T. stans* under the same conditions in the quarantine glasshouse, in gauze-covered cages (0.55 x 0.55 x 0.95 m).

Life history of the study organisms

Adults of the root-feeding flea beetle *Heikertingerella* sp. feed on the leaves of *T. stans* and create small, irregular round holes by scraping the leaf epidermis through to the mesophyll, eventually causing extensive damage. The females deposit eggs onto the soil surface of potted plants. *Heikertingerella* sp. larvae feed on the secondary roots and develop on the core of the primary roots, eventually pupating in the soil until adult emergence. The flea beetle has a generation time from adult to adult of 49 to 67 days (Madire et al., 2021)

Both adults and larvae of the lady beetle *M. polluta* feed on the leaves of *T. stans*. Adults feed on the upper surface of the leaves, whereas the larvae feed on the under surface. Female *M. polluta* deposit their eggs in clusters on the under surface of the leaves and all larval instars develop on the leaves until pupation. This lady beetle has a generation time of ca. 36 days (Madire, 2013).

Experimental design

Sixteen 1-year old *T. stans* plants of similar stem height, ranging from 18-20 cm tall (Mean ± SE = 19.56 ± 0.16; *n* = 16) and leaf density, ranging from 16-29 leaves (Mean ± SE = 19.94 ± 0.80; *n* = 16) were selected from the nursery for the experiment. Plants were sprayed with water and cleaned to remove any unwanted insects or contaminants before they were moved to the quarantine glasshouse. Individual plants were placed in separate gauze-covered cages (0.55 m x 0.55 m x 0.95 m) prior to their exposure to the insects. The four treatments included controls (with no insects), Heiker only (five mating pairs of *Heikertingerella* sp. only), Mada only (five mating pairs of *M. polluta* only) and a combination of *Heikertingerell*a sp. and *M. pollut*a

(Heiker combined and Mada combined) that included three mating pairs of each beetle species. Insect densities were chosen based on the results of preliminary assays conducted to determine the range of insect densities in which considerable damage was observed on the growth and development of the plant. Newly emerged adults (P_1) were used in this study and each treatment was replicated four times. After 20 days, the surviving P_1 adults of *Heikertingerella* sp. and *M. polluta* were counted and removed from the plants in all treatments, while their immature stages (i.e., eggs, larvae and pupae) were allowed to develop to adulthood over 60 days and then recorded. To determine the effect of the treatments on the two agents, we compared the percentage survival of P_1 adults over the 20-day period and the number of emerging F_1 progeny over the 60-day period, between the individual and combined exposures. To determine the response of the host plants to each treatment, we compared adult foliar damage, leaf density and plant height between the controls and the three beetle treatments after the 60-day period.

Data analysis

The statistical analyses were conducted using IBM SPSS version 26.0. Since the datasets did not meet the assumptions of normality, generalized linear modelling was used to determine the effect of treatment on the numbers of surviving P_1 adults, F_1 progeny, leaves damaged, leaves produced and the size of the plants. The models that analysed count data incorporated a Poisson distribution (corrected for over-dispersion) with a log link function. The model that analysed plant size data incorporated a Tweedie distribution (corrected for over-dispersion) with a log link function. Significance ($P < 0.05$) was assessed using Likelihood ratio chi-square statistics because of the small sample sizes. When treatment had a significant influence, post-hoc paired comparisons (Fisher's Least Significant Difference) were performed on the means.

Results

Survival of P₁ adults of Heikertingerella sp. and M. polluta

The percentage survival of the P$_1$ adults of *M. polluta* and *Heikertingerella* sp. did not differ between the single and combined treatments during the 20-day period (χ^2 = 1.100; df = 3; p = 0.777). The percentage survival of *M. polluta* in both single and combined treatments was slightly higher than that of *Heikertingerella* sp. in the same treatments, with 73% and 71% of *M. polluta* adults surviving in single and combined treatments, respectively, compared to 67% and 68% of *Heikertingerella* sp. adults surviving in the same treatments (Fig 1).
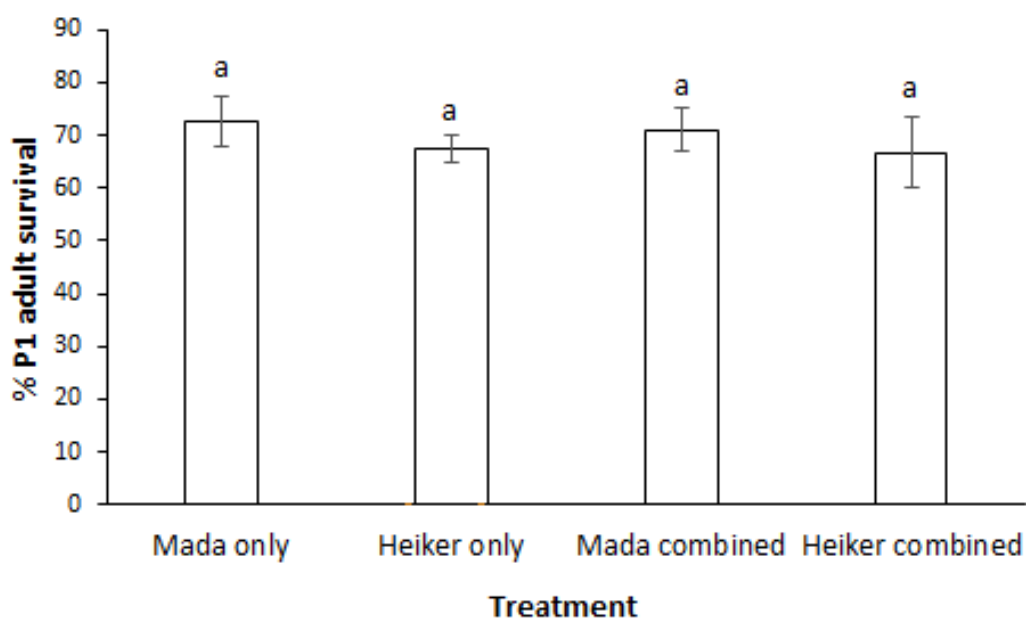
Fig. 1. Mean (± S.E.) percentage of P$_1$ adults of *Heikertingerella* sp. and *M. polluta* that survived in single and combined treatments during a 20-day period. Heiker only = *Heikertingerella* sp. alone; Mada only = *M. polluta* alone; Heiker combined and Mada combined = both beetles in combination. Bars with the same letter are not significantly different (Fisher's Least Significant Difference).

Emergence of F₁ adult progeny of Heikertingerella sp. and M. polluta

There were significant differences in the number of F_1 adult progeny emerging from single and combined treatments of *Heikertingerella* sp. and *M. polluta* ($\chi^2 = 63.547$; df =3; $p < 0.001$). The numbers of adult progeny produced by both beetle species in the single treatments were significantly higher than those produced in the combined treatments (Fig 2). When confined alone on *T. stans*, *M. polluta* produced 46% more adult progeny than when confined with *Heikertingerella* sp. Similarly, *Heikertingerella* sp. produced 65% more adult progeny when confined alone than when confined with *M. polluta* (Fig 2).
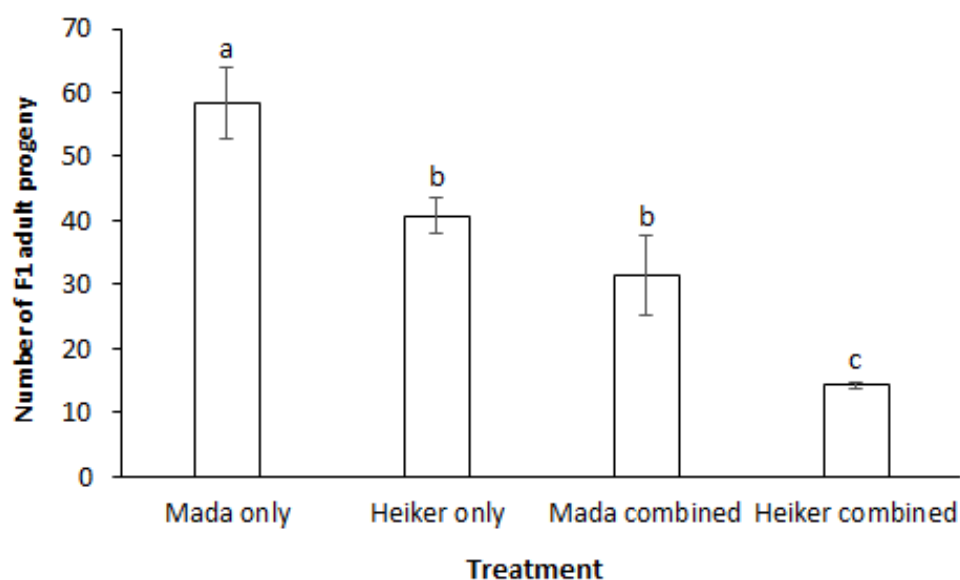
Fig. 2. Mean (± S.E.) number of *Heikertingerella* sp. and *M. polluta* F₁ adult progeny that emerged from single and combined treatments. Heiker only = *Heikertingerella* sp. alone; Mada only = *M. polluta* alone; Heiker combined and Mada combined = both beetles in combination. Bars with different letters are significantly different (Fisher's Least Significant Difference).

Leaf feeding damage and leaf density

There were significant differences in leaf damage between single and combined exposures of *T. stans* to *Heikertingerella* sp. and *M. polluta* ($\chi^2 = 101.099$; df = 2; p < 0.001). Exposure to *M. polluta* only and a combination of both species caused significantly more leaf damage than exposure to *Heikertingerella* sp. only, with no significant difference between the *M. polluta* only and combined treatments (Fig 3).

Fig. 3. Mean (± S.E.) number of damaged leaves on *Tecoma stans* plants exposed to *Heikertingerella* sp. alone (Heiker only), *Mada polluta* alone (Mada only) and both beetles in combination (Heiker + Mada). Bars with different letters are significantly different (Fisher's Least Significant Difference).

Exposure to *Heikertingerella* sp. alone, *M. polluta* alone and a combination of the two beetle species significantly reduced leaf density on *T. stans* relative to the control ($\chi^2 = 44.964$; df = 3; p < 0.001). Exposure to each of *Heikertingerella* sp. and *M. polluta* alone reduced leaf density by 20% and 29%, respectively, while exposure to a combination of both species reduced leaf density by 43% (Fig 4).
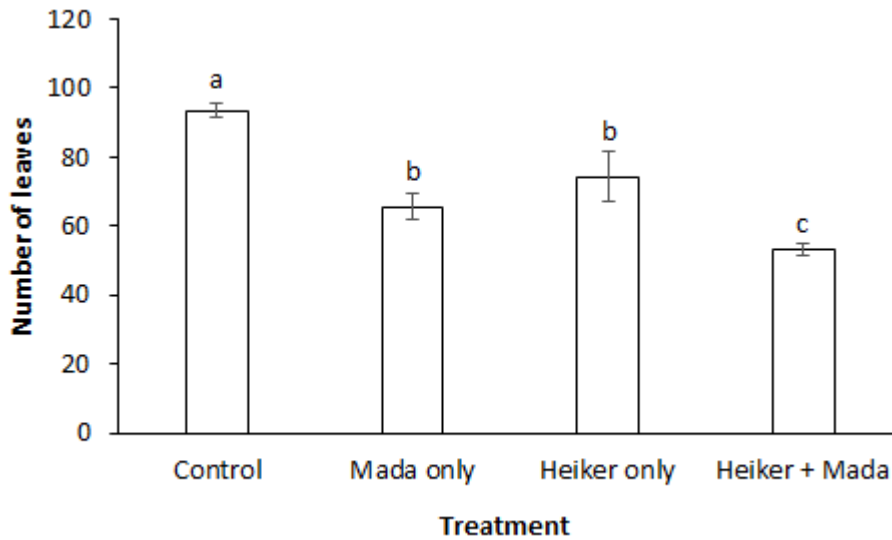
Fig. 4. Mean (± S.E.) number of leaves produced by *Tecoma stans* in response to exposure to *Heikertingerella* sp. alone (Heiker only), *Mada polluta* alone (Mada only) and both beetles in combination (Heiker + Mada). Bars with different letters are significantly different (Fisher's Least Significant Difference).

Plant height

Single and combined exposures to *Heikertingerella* sp. and *M. polluta* caused significant reductions in plant height ($\chi^2 = 62.890$; df = 3; $p < 0.001$) in relation to the control. However, there were no significant differences in plant height between the three beetle exposure treatments. Plant height in the *Heikertingerella* sp. only, *M. polluta* only and combined treatments were reduced by 20.3%, 20.8% and 21.3%, respectively, relative to the control (Fig. 5).
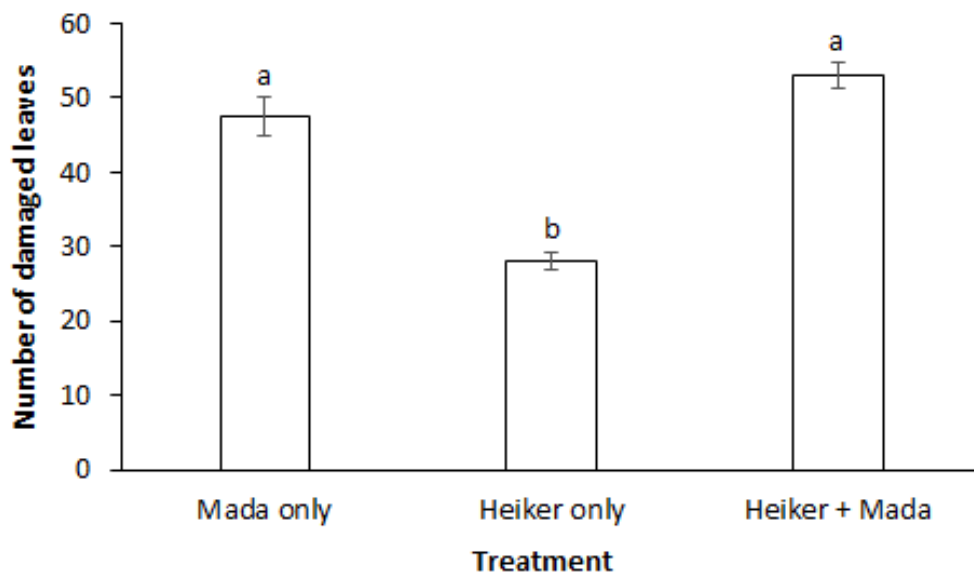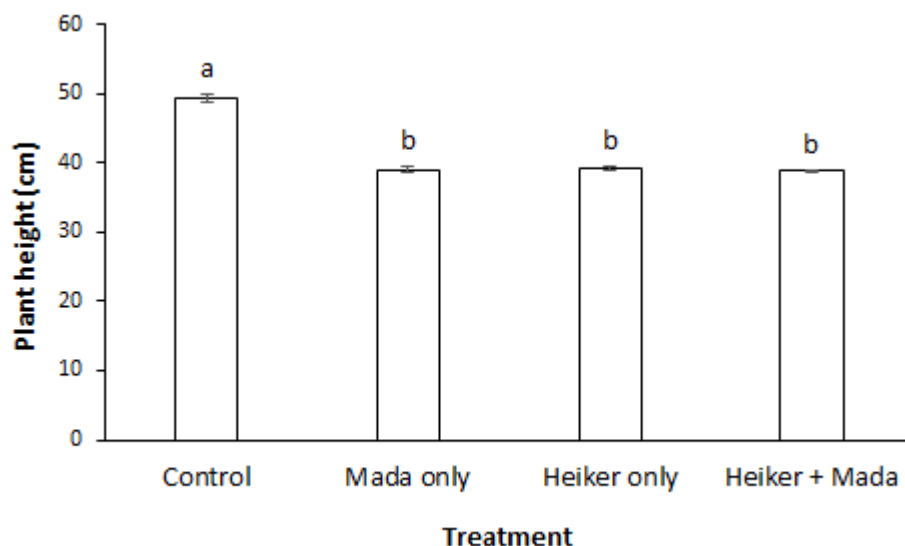
Fig. 5. Mean (± S.E.) height of *Tecoma stans* plants exposed to *Heikertingerella* sp. alone (Heiker only), *Mada polluta* alone (Mada only) and both beetles in combination (Heiker + Mada). Bars with the same letter are not significantly different (Fisher's Least Significant Difference).

Discussion

Although there seems to be a trade-off between the additive effect of *Heikertingerella* sp. and *M. polluta* on *T. stans* and a reduction in their reproductive success, the two beetle species had a greater impact when combined than when confined individually. On their own, *Heikertingerella* sp. and *M. polluta* reduced leaf density by 20% and 29%, respectively, but together caused a 43% reduction because of higher levels of leaf damage. Reductions in plant height through insect attack were similar for the single and combined treatments relative to the control. Although it is uncertain how *Heikertingerella* sp. and *M. polluta* will affect weed density, our study suggests that they could complement each other in the field, particularly since the larvae of *Heikertingerella* sp. are root feeders.

The additive impact of *Heikertingerella* sp. and *M. polluta* implies that releases of both species as biocontrol agents could be more effective than the release of a single species. This

supports the notion that weed biocontrol success rates improve with releases of multiple agents (Denoth et al., 2002; Seastedt et al., 2007). For example, the release of an undescribed leafhopper (Hemiptera: Cicadellidae), formerly referred to as *Zygina* sp., and the rust fungus *Puccinia myrsiphylli* (Thuem.) Winter (Pucciniaceae) against *Asparagus asparagoides* (L.) Druce (Asteraceae) in Australia had an additive impact on various plant growth parameters (Turner et al., 2010). Furthermore, the combined impact of the leaf- and stem-mining *Neurostrota gunniella* Busck (Lepidoptera: Gracillariidae) and the fungus *Phloeospora mimosa-pigrae* H.C. Evans & Carrion (Ascomycotina) in reducing the leaf density of *Mimosa pigra* L. (Mimosaceae) in Australia was higher than that caused by each species on its own (Paynter & Hennecke, 2001).

Stiling and Cornelissen (2005) concluded that multiple releases of biocontrol agents against insect and plant pests decreased pest abundance by 27.2% more, when compared to single-species releases. While our study revealed an antagonistic interaction between *Heikertingerella* sp. and *M. polluta*, with fewer F_1 progeny produced in combination than in isolation, this is unlikely to diminish their combined negative effect on the target weed. For example, despite decreased populations of the thistle-head weevil *Rhinocyllus conicus* Froelich (Coleoptera: Curculionidae) in the presence of the rosette weevil *Trichosirocalus horridus* Panzer (Coleoptera: Curculionidae), their combined attack reduced viable seed production in *Carduus nutans* L. (Asteraceae) by 59%, while the presence of *R. conicus* alone caused a 45% reduction (Milbrath & Nechols, 2004). Several other studies (Crawley, 1983; Kinsmann & Platt, 1984; Marquis, 1984; Strauss, 1991; Karban & Strauss, 1993; Poveda et al., 2003) have also demonstrated that releases of multiple biocontrol agents may be needed to inflict sufficient damage on target weed populations.

The interaction between *Heikertingerella* sp. and *M. polluta* deserves further investigation under field conditions, since our laboratory trials may have exacerbated interference between

the two species, more than would otherwise occur under unconfined conditions. Although F_1 progeny production by both beetle species was reduced by competition, *Heikertingerella* sp. was more affected. The shorter developmental period of *M. polluta* (36 days) (Madire 2013) and its potential for rapid population increase might have reduced food quality for *Heikertingerella* sp., thereby negatively affecting its performance due to a longer developmental period (49-67 days) (Madire et al., 2021). However, unlimited food resources under field conditions are likely to mitigate any niche overlap (i.e. leaf feeding) between the two beetle species, particularly since biocontrol agents often avoid plant tissues infested by competitors in the field (e.g. Rayamajhi et al., 2006), thereby reducing competition. Furthermore, the size of both the potted plants and the cage might have limited the development of the root system and the foliage of *T. stans*, thereby affecting both species; particularly *Heikertingerella* sp. which displays longer larval development and feeding activity (Brown & Gange, 1990; Masters et al., 1993). Buccellato et al. (2019) also found that the results of glasshouse trials on agent interactions were not predictive of the field results, and attributed this to variation in biotic and abiotic environmental factors, which are excluded in the controlled conditions of a glasshouse trial. Thus uncertainty makes it difficult to select the best possible agent for a target weed (i.e. "silver bullet") and biocontrol programmes thus often tend towards the "cumulative stress" approach (e.g. Dauer et al., 2012).

Although the two beetle species have been found in similar habitats in their native range in Central America, populations of *M. polluta* appear to peak earlier in the season than those of *Heikertingerella* sp. This could allow resource partitioning over time (Denno et al., 1995) and promote co-existence between the two agents in the field. However, an increase in the intensity of herbivory by early-season *M. polluta* could cause rapid deterioration of the host plants, with adverse effects on the performance of late-season *Heikertingerella* sp. (e.g. Hunter, 1990; Denno et al., 1995; Kaplan & Denno, 2007). Nonetheless, we speculate that unlimited

food resources under field conditions in South Africa are likely to dampen the effects of competition between early- and late-season feeding agents (Rayamajhi et al., 2006).

Blossey & Hunt-Joshi (2003) also argued that the performance of root-feeding herbivores could be compromised if food quality and quantity is reduced by aboveground herbivores, which is likely to be exacerbated on potted plants under confined conditions. However, such events are likely to be rare under field conditions (Hunt-Joshi & Blossey 2005), emphasizing the need to confirm these results with field trials, once *Heikertingerella* sp. is released from quarantine. Indeed, entire defoliation of purple loosestrife *Lythrum salicaria* L. (Lythraceae) shoots by the leaf-feeding beetle *Galerucella calmariensis* L. (Coleoptera: Chrysomelidae) in field cages had no negative impact on leaf herbivory by adults of the root-feeding weevil *Hylobius transversovittatus* Goeze (Coleoptera: Curculionidae) (Hunt-Joshi & Blossey 2005). While additional long-term data under field conditions are needed, our data suggest that the simultaneous release of *Heikertingerella* sp. and *M. polluta* appears likely to complement the biocontrol programme against *T. stans.*

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CHAPTER 4

Predicted impact, establishment and distribution of *Heikertingerella* sp. (Coleoptera, Chrysomelidae), recently released for the biological control of *Tecoma stans* in South Africa

Abstract

Biological control practitioners are required to demonstrate that candidate agents are not only safe for release, but will establish widely in their new range with significant impact on the target weed. We conducted this study in quarantine to provide insight into the impact and distribution of the root-feeding flea beetle *Heikertingerella* sp. (Chrysomelidae), released in 2023 against the invasive tree *Tecoma stans* (L.) (Bignoniaceae) in South Africa. We studied the effects of low and high beetle densities on plant growth and biomass, and F_1 progeny production by the beetle. There were significant reductions in plant growth and biomass accumulation in the beetle-exposed plants, relative to the controls. P_1 adults damaged significantly more leaflets, with significantly higher F_1 progeny production, at high beetle densities. The MaxEnt model predictions suggested that most of South Africa is climatically highly suitable for *Heikertingerella* sp., particularly along the southern and eastern coast where the weed is most prevalent. Only the inland regions of the Northern Cape Province, depicting the driest and hottest part of the country where the weed does not occur, was predicted to be unsuitable for *Heikertingerella* sp. The beetle's thermal physiology, as determined by its critical thermal minimum (CT_{min}) and maximum (CT_{max}) temperatures and lower and upper lethal temperatures (LT_{50}), suggests tolerance to temperature extremes in at least four of the eight provinces that fall within the range of *T. stans* in South Africa. Our results further justify the release of *Heikertingerella* sp. in areas predicted as suitable in South Africa.

Keywords: Agent efficacy, MaxEnt modelling, pre-release impact, thermal physiology, weed biocontrol.

Introduction

Pre-release evaluations of candidate agents for the biological control of weeds typically confirm their safety and suitability for release. However, these also include studies to demonstrate that agents are likely to establish and control the target weed in their new range (Holt & Hochberg, 2001; Pearson & Callaway, 2003; McClay & Balciunas, 2005; van Klinken & Raghu, 2006; Morin et al., 2009). Consequently, pre-release efficacy assessments facilitate agent selection based on their likelihood of establishment and quantified impact, thereby increasing the chances of releasing effective agents (Sheppard, 2003; Balciunas, 2004; Gerber et al., 2008). However, factors that can contribute to the non-establishment and inefficiency of agents, once released, include poor climate matching (e.g., Byrne et al., 2004; Harms et al., 2021), recruited natural enemies (e.g., Tipping et al., 2013; Paynter et al., 2019) and mismatching of agent and plant genotypes (e.g., Smith et al., 2018; Gaskin et al., 2023).

Pre-release efficacy assessments involve exposure of the target plant to known population densities or levels of attack by a candidate agent, to measure its effects on plant performance parameters such as growth rate, biomass accumulation, seed production, or competitive ability. Such trials are conducted either in the field in the target weed's native range (Brun et al., 1995; Briese, 1996; Goolsby et al., 2004; Gerber et al., 2007; Morin et al., 2009) or under quarantine laboratory conditions (Shishkoff & Bruckart, 1996; Klöppel et al., 2003; Balciunas & Smith, 2006; Baars et al., 2007; Morin et al., 2009). For example, Goolsby et al., (2004) used an acaricide-based exclusion treatment to demonstrate the impact of the mite *Floracarus perrepae* Knihinicki and Boczek (Eriophyidae) on *Lygodium microphyllum* (Cav.) R. Br. (Lygodiaceae) in its native range in Australia. Under quarantine conditions, Balciunas & Smith (2006)

demonstrated the impact of two densities of a gall-forming fly from South Africa, considered for release against *Delairea odorata* Lem. (Asteraceae) in the USA.

Whilst complex, interacting, and unforeseeable factors often make it difficult for pre-release studies to predict agent performance and efficacy in the field (Buccellato et al., 2019), they are an important aspect in the evaluation of candidate agents (Gerber et al., 2008) and can preclude the release of insufficiently damaging agents (McClay & Balciunas, 2005). For example, pre-release efficacy studies justified the release of an unidentified sap-sucking leafhopper, previously referred to as *Zygina* sp. (Hemiptera: Cicadellidae), against *Asparagus asparagoides* L. Druce (Asparagaceae) in Australia (Kleinjan et al., 2004). These results were subsequently validated by the agent's performance in the field, despite annual fluctuations caused by recruited parasitoids (Morin & Scott, 2012). Besides efficacy assessments, species distribution modelling has become an increasingly popular tool in recent years for predicting a biocontrol agents' potential establishment and distribution (Elith *et al.,* 2010).

The Maximum Entropy species distribution model (MaxEnt) is popular tool for climate modelling studies and has performed well relative to alternative modelling techniques (Wisz et al., 2008; Phillips et al., 2017). It uses maximum entropy to distinguish between environmental conditions at sites where the focal taxon is present from those at sites where its presence is unconfirmed (Elith et al., 2011). MaxEnt is used extensively in studies to predict the geographic distribution of species and their potential habitats (Elith et.al., 2011; Merow et al., 2013; Park et al., 2018). It creates a model of the focal species' range using a set of georeferenced occurrence locations and a set of layers or environmental variables such as elevation, precipitation and temperature (Park et al., 2018).

MaxEnt can thus determine the likelihood of establishment and distribution of weed biocontrol agents in their new range and hence their potential impact on their targets

(Mukherjee et al., 2021). Higher MaxEnt climatic suitability scores typically correlate with higher biocontrol agent establishment rates (e.g. Sutton & Martin, 2022). In support of climate modelling, pre-release thermal physiology studies can also determine an insect agent's response to variations in temperature, as responses to constant temperatures in the laboratory are indicative of its optimal temperature range in the field (e.g., Sutherst & Maywald, 1985; Ramanand et al., 2017). Pre-release assessments of an agent's tolerance to thermal extremes can predict its climatic adaptability and avoid the release of agents that cannot cope with climatic extremes in the field (Byrne et al., 2004).

Native to Mexico, *Tecoma stans* (L.) (Bignoniaceae) is an invasive alien tree that continues to extend its range in South Africa and its neighbouring countries (Cunningham, 2008; Madire et al., 2011; Henderson, 2021; Madire et al., 2021a, b; Madire et al., 2023), but also other countries on the African continent (Orwa et al., 2009) and around the world (Pelton, 1964; Madire, 2013). The plant grows aggressively and occurs widely in several provinces in South Africa. The distribution of *T. stans* in southern Africa encompasses a wide range of climatic conditions, from high rainfall tropical areas to semi-arid areas, and includes various soil types (Pelton, 1964; Madire et al., 2011; Madire & Netshiluvhi, 2021). The plant's ability to tolerate a wide range of abiotic conditions, rapid growth and abundant seed production, contributes to its high invasive potential. Two biocontrol agents have been deployed against *T. stans* in South Africa, namely the leaf-feeding lady beetle *Mada polluta* Mulsant (Coleoptera: Coccinelidae) and leaf-mining fly *Pseudonapomyza* sp. (Diptera: Agromyzidae). Due to the severity of *T. stans* invasions in South Africa, a suite of agents is required to attack additional parts of the plant, notably the root system (Madire et al., 2011).

The Mexican root-feeding flea beetle *Heikertingerella* sp. (Coleoptera: Chrysomelidae) was thus introduced to exert additional pressure on *T. stans* infestations. *Heikertingerella* sp. adults feed on the leaves of *T. stans* and oviposit in the soil at the base of the plant. The

subterranean larvae feed on the cortex of the secondary roots and develop on the internal parts of the primary roots. Developmental time from adult-to-adult ranges from 51 to 73 days, with adult longevity ranging from 41 to 100 days, under quarantine glasshouse conditions (Madire et al., 2021a). Host-specificity studies confirmed that the flea beetle is safe for release in South Africa (Madire et al., 2021a), with the first release undertaken in early 2023 and confirmation of establishment still pending.

Despite difficulties in working with root feeders and generally lower numbers available for release (Hight et al., 1995), root-feeding agents are more likely to contribute to the suppression of invasive plant populations than other herbivorous guilds (Blossey & Hunt-Joshi, 2003). In later successional habitats, root feeders become more abundant and appear to be a strong force in driving plant performance and plant community composition (Blossey & Hunt-Joshi, 2003). For example, the root-feeding flea beetles *Aphthona* spp. (Chrysomelidae) effectively reduced the density of the invasive *Euphorbia esula* L. (Euphorbiaceae) across a variety of locations and environmental conditions in the USA (Lym & Nelson, 2000; Butler et al., 2006). The ragwort flea beetle, *Longitarsus flavicornis* (Waterhouse) (Chrysomelidae), first released in Tasmania (Australia) in 1979, established at over 90% of release sites and reduced the density of *Jacobaea vulgaris* Gaert. (Asteraceae) by over 90%, at many sites in the State (Ireson et al., 2000).

In this study, which was concluded prior to the agent's release, we examined the effect of different densities of *Heikertingerella* sp. on the vegetative growth and biomass accumulation of *T. stans* under quarantine glasshouse conditions. In addition, we used MaxEnt modelling together with the beetle's critical thermal limits to predict its potential distribution in South Africa and determine where releases are best carried out. We envisage that the results will direct releases of *Heikertingerella* sp. across the weed's invaded range in South Africa.

Materials and Methods

Plant material and insect culture

The study took place in a quarantine glasshouse at the ARC-Plant Health and Protection Roodeplaat facility in Pretoria, South Africa (25°37'00" S 28°22'00"E), where the temperature and humidity were maintained at 22-32°C and 47-60%, respectively. *Tecoma stans* plants were propagated from field-collected seeds, with individual seedlings transplanted into 10 litre pots containing a standard soil mixture of top soil, river sand, compost and vermiculite in equal amounts. The plants were maintained in a nursery, watered twice a day and a granular fertilizer (N: P: K- 2:3:2) of 14% SR was applied to promote growth. The study commenced under natural light conditions during summer and under a light/dark regime of 16:8 during winter. The winter photoperiod was maintained using 50 W/LED 4000 K/230 V LED floodlights (Spazio lighting). Trials commenced once the plants reached a height of around 40 cm.

The flea beetle culture was maintained under quarantine glasshouse conditions described previously (Madire et al., 2021a). Adults for the trials were obtained by confining 30-50 beetles with a single plant in a cage (0.55 x 0.55 x 0.95 m) to allow oviposition in the soil. The adults were removed after 30 days to avoid overexploitation and after around 50 days, newly emerged adults were collected from the cages and used in the trials.

Impact assessment

Thirty-six *T. stans* plants (1-year old) of similar height and canopy size (mean ± SE = 43.8 ± 0.82 cm, n = 36) were selected from the propagated plants. Prior to the trials, unwanted insects or pests were removed from the plants and their number of leaflets, height and basal stem diameter (above the soil surface) were recorded using a measuring tape and a Vernier calliper, respectively. The plants were placed individually into gauze-covered cages (0.55m x 0.55m x 0.95m) in the quarantine glasshouse. These were grouped into three treatments of 12 plants,

namely the control (without beetles), low-density treatment (five pairs of beetles) and high-density treatment (10 pairs of beetles). These densities were determined through observations during culturing in quarantine and field observations in Mexico. Newly emerged adults were confined with the plants for 30 days, to allow feeding and oviposition. The number of surviving adults were recorded before their removal from the cages.

From 50 days onwards, all emerging F_1 adults were counted and removed in each cage. The experiment was terminated after 70 days, by which time all immature stages had developed to adulthood, and plant parameters that included plant height, stem diameter, numbers of leaflets (including those damaged by adult feeding) were recorded. The plants were harvested and separated into leaves, stems and roots. The soil was completely removed from the roots by rinsing with water, with broken pieces collected. The plant material was oven-dried at 70°C for 72 hours and the above- and below-ground dry biomass was recorded for each individual plant.

Prediction of geographic distribution

MaxEnt modelling

To determine the potential distribution of *Heikertingerella* sp. in South Africa, species distribution modelling was utilized using known distribution records from its native distribution and climatic data. Sixteen native-range occurrence records, recorded by the authors during the field collection trips in the Veracruz and Chiapas provinces of Mexico, were available for *Heikertingerella* sp. Since spatial autocorrelation is an important factor that may affect species distribution model outputs (Veloz, 2009), only one occurrence record per 2.5 min grid cell was used for model calibration. Species occurrence datasets were thinned using the '*spThin*' package (Aiello-Lammens et al., 2015), and spatial autocorrelation analyses were performed using the '*ecospat*' package (Di Cola et al., 2017). Spatial autocorrelation was

present for GPS records that were up to 1 km apart and, as such, spatial thinning was performed to retain only GPS records >1 km apart. In total, 15 occurrence records were retained for model calibration.

Climate data were obtained by downloading the standard set of 19 bioclimatic variables from the WorldClim ver. 2.1 database (Fick & Hijmans, 2017) (data available at: www.worldclim.org/download.html). This dataset is representative of annual and seasonal averages and variation in temperature and precipitation metrics averaged over the 1950–2000 period (current climate) at a 2.5-minute resolution. We constructed a set of uncorrelated climate variables to fit as covariates during model calibration (Dormann et al., 2013). Pearson's correlation coefficients were computed for all pairs of predictors, whereby predictors that were highly correlated ($|r| > 0.70$) were excluded from the final predictor set. The reduced set of environmental predictors consisted of six climatic variables, including: bio2 – mean diurnal temperature range [mean of monthly (max temp – min temp)], bio4 – temperature seasonality (standard deviation x 100), bio6 – minimum temperature of the coldest month, bio9 – mean temperature of the driest quarter, bio16 – precipitation of the wettest quarter, and bio17 – precipitation of the driest quarter (see Fick & Hijmans (2017) for further details).

We modelled the climatic suitability for *Heikertingerella* sp. in southern Africa using MaxEnt (ver. 3.4.3), implemented in the '*dismo*' R package (Hijmans et al., 2023). Given that MaxEnt is a presence/pseudo-absence modelling algorithm, model building requires a user-defined geographic background to sample the climate of representative grid cells where the focal species is assumed to be absent (i.e., background points or pseudo-absences). The background should ideally represent the geographic areas available to the focal species, omitting areas where species absence is due to historical factors, dispersal constraints and/or biotic interactions (Sanín & Anderson, 2018). We defined the model background using the Köppen-Geiger climate classification system (available at: http://koeppen-geiger.vu-

wien.ac.at). Only Köppen-Geiger climate zones that contained at least one native-range occurrence record for *Heikertingerella* sp. were used as the background area from which background points were drawn for model calibration. We randomly sampled 1000 background points from within this background definition (Martin et al., 2020). All raster manipulation and spatial analyses were performed using the '*terr*a' R package (Hijmans, 2023).

Model tuning was applied to guide the selection of optimal MaxEnt parameter configurations (feature classes and regularization multipliers) (Merow et al., 2013; Sutton & Martin, 2022). Model tuning was performed by building MaxEnt models with varying (1) feature class combinations (H = Hinge only, L = Linear only, and LQH = Linear, Quadratic and Hinge features) and (2) regularization multipliers (1, 2, 4, 6). In total, 12 MaxEnt models were specified. Model performance and optimal parameter configurations were assessed using 4-fold spatial block cross validation in the '*ENMeval*' R package (Kass et al., 2021). Optimal parameter configurations were determined by selecting model configurations which produced the lowest value for the Akaike Information Criterion corrected for small sample sizes (AICc) (i.e., AICc = 0; following Kass et al. (2021)). The optimal parameter configuration for the MaxEnt model included: (1) linear, hinge and quadratic feature classes, and (2) a regularization multiplier value of 2. Otherwise, MaxEnt models were parameterized with default settings for multiple parameters, including: convergence = 10^5, maximum number of iterations = 500 and prevalence = 0.5. The 'fade by clamping' option was selected to prevent extrapolation well outside the range of climatic values in the model training area (Phillips et al., 2017). Model predictions were obtained using the 'logistic output' to create continuous climatic suitability raster layers scaled between 0 (climatically unsuitable) and 1 (climatically suitable). All species distribution modelling was conducted in *R* ver. 4.3.0 (R Core Team 2023).

Critical thermal limits and lethal temperatures

We adopted the methods of Terblanche et al. (2007) and Griffith et al. (2019) to determine the critical thermal minimum (CT$_{min}$) temperature (i.e., chill coma) of *Heikertingerella* sp. adults. These trials were conducted in the glasshouse using a programmable water bath (Model Julabo F12) filled with distilled water. Ten adults (1-day old) were placed individually into transparent glass vials sealed with plastic lids. The lids were filled with expanded polystyrene foam to prevent evaporative cooling by the beetles and were sealed with plastic film to prevent water leakage into the vials. The beetles were submerged in the water bath and left to equilibrate for 15 minutes at room temperature (25°C). The temperatures of the beetles in the vials were monitored using an Oregon scientific thermo sensor (Model THG 312) that was inserted into a vial placed in the water bath. The water temperature was cooled at the rate of 0.25°C min^{-1} and, every minute, the vials were turned and assessed for adult mobility. The number of beetles that became immobile and impaired were recorded at 10°C, 8°C, 5°C, and 2°C; however, the observations were conducted at every temperature degree interval. This range of minimum temperatures is likely to occur at potential release sites during cold months. The beetle's critical thermal limit was determined as the temperature at which its locomotory function ceased. Thereafter, the beetles were left to recover for 24 hours in a Petri dish at room temperature. Four sets of trials were conducted with 10 newly emerged beetles used in each trial (n = 40).

The beetle's critical thermal maximum (CT$_{max}$) temperature (i.e., heat stupor) was determined in a similar manner, after initially keeping the beetles at 25°C for 15 minutes to equilibrate their body temperatures. The vials were submerged as before and the temperature was gradually increased at the rate of 0.25°C min^{-1} until 50°C was reached. Every minute the vials were turned and assessed for adult mobility. The loss of mobility was recorded at 40°C, 42°C, 45°C, 47°C and 50°C; however, the observations were conducted at every temperature

degree interval. As before, four sets of trials were conducted, with 10 newly emerged beetles used in each trial.

To determine the beetle's lower lethal temperature (LT_{50}), the temperature was decreased gradually from 25°C to each experimental temperature that included 2°C, 0°C, -2°C, -5°C, -7°C and -12°C. Holts® liquid anti-freezing medium at 96% concentration was used to prevent freezing. At each temperature, different sets of 10 adult beetles were placed individually into glass vials and submerged in the water bath, where the temperature was lowered gradually until the desired experimental temperature. The beetles were exposed to each experimental temperature for two hours and then removed from the water bath. The adults were placed in a Petri dish containing *T. stans* leaves and left to recuperate for 24 hours at 25°C, with the numbers that fully recovered recorded. The upper lethal temperature (UT_{50}) of the beetle was determined in the similar manner, in which the temperatures were increased from 25°C to each experimental temperature that included 40°C, 42°C, 45°C, 47°C and 50°C. Both lethal temperature trials were replicated four times, with 10 different individuals used for each experimental temperature during each trial.

Minimum temperatures in localities invaded by Tecoma stans

Weather stations of the South African Weather Services supplied minimum temperature data for 2020 (SAWS 2021), pertaining to localities invaded by *T. stans* in South Africa. Average minimum temperatures from localities in eight provinces (North West, Gauteng, Mpumalanga, Limpopo, KwaZulu-Natal, Eastern Cape, Western Cape and Free State) were compared to the beetle's laboratory-determined minimum threshold (CT_{min}) to predict where it is likely to survive and establish. The minimum temperature data, generated by averaging the monthly data for 2020, were plotted graphically against the beetle's CT_{min} to determine the months when temperatures fell below the CT_{min} threshold (see Cowie et al., 2016, 2023). We used CT_{min} as

it is typically a stronger predictor of realised insect distributions across a range of taxa (Anderson et al., 2015).

Data analysis

Data from the impact assessment trial were analysed using IBM SPSS Statistics 28. The data did not meet the assumptions of normality and equality of variances and were analysed with Generalized Linear Models. Models comparing the increases in plant height and stem diameter, as well as final plant biomass, over the experimental period included a Tweedie distribution and a log link function. Models comparing the increase in leaf production and F_1 beetle progeny production between the treatments included a Poisson distribution and a log link function, while the model assessing percentage leaf damage included a binomial distribution and a logit link function. Following corrections for over-dispersion, Wald chi-square statistics determined significance ($P < 0.05$). Pairwise post-hoc comparisons involved Fisher's Least Significant Difference test. Data from the thermal tolerance trials were analysed using Tibco Statistica 13.3 (StatSoft Inc., 2020), with the critical thermal limits determined by means and standard deviations and lethal temperatures (LC_{50}) of *Heikertingerella* sp. determined by probit regression analysis. Mean minimum temperature data for sites in provinces across the invaded range of *T. stans* in South Africa were compared graphically to the beetle's laboratory-determined minimum threshold (CT_{min}).

Results

Effect of Heikertingerella sp. adult density on T. stans

Feeding by adults of *Heikertingerella* sp. caused a significant reduction in plant height increment relative to the control plants ($\chi^2 = 23.475$, df = 2, $P < 0.001$), but only at the low beetle density. The differences between the low (29% reduction) and high (9%) population

densities were significant (Fig. 1A). There were no significant differences in stem diameter increment between the control and beetle-exposed plants (χ^2 = 1.184, df = 2, P = 0.553). The increase in leaflet production differed significantly between the control and beetle-exposed plants (χ^2 = 33.057, df = 2, P < 0.001), with significant differences between the high- and low-density treatments. *Tecoma stans* plants subjected to the high-density treatment produced 15% fewer leaflets relative to the control, compared to 29% in the low-density treatment (Fig. 1B).

There were significant differences in the percentage of leaflets damaged by the P_1 adults between the two density treatments (χ^2 = 39.762, df = 1, P < 0.001), with leaf damage 21% higher in the high-density treatment (Fig. 1C). Survival of the P_1 adults over the 30-day period was the same in the low and high-density treatments (29%). However, significantly more F_1 adult progeny were produced in the high-density treatment (χ^2 = 85.301, df = 1, P < 0.0005) over the 60-day period, with progeny production 68% higher than in the low-density treatment (Fig. 1D).

There were also significant differences in the mean biomass of the above-ground (χ^2 = 1580.311, df = 2, P < 0.0005) and below-ground (χ^2 = 822.520, df = 2, P < 0.0005) components of *T. stans* plants between the treatments. There were significant reductions in above-ground biomass at both low (37%) and high (45%) beetle densities relative to the controls (Fig. 1E), with significant differences between the low and high-density treatments. Similarly, there were significant reductions in below-ground biomass at both low (31%) and high (57%) beetle densities relative to the controls (Fig. 1F), with significant differences between the low and high-density treatments.
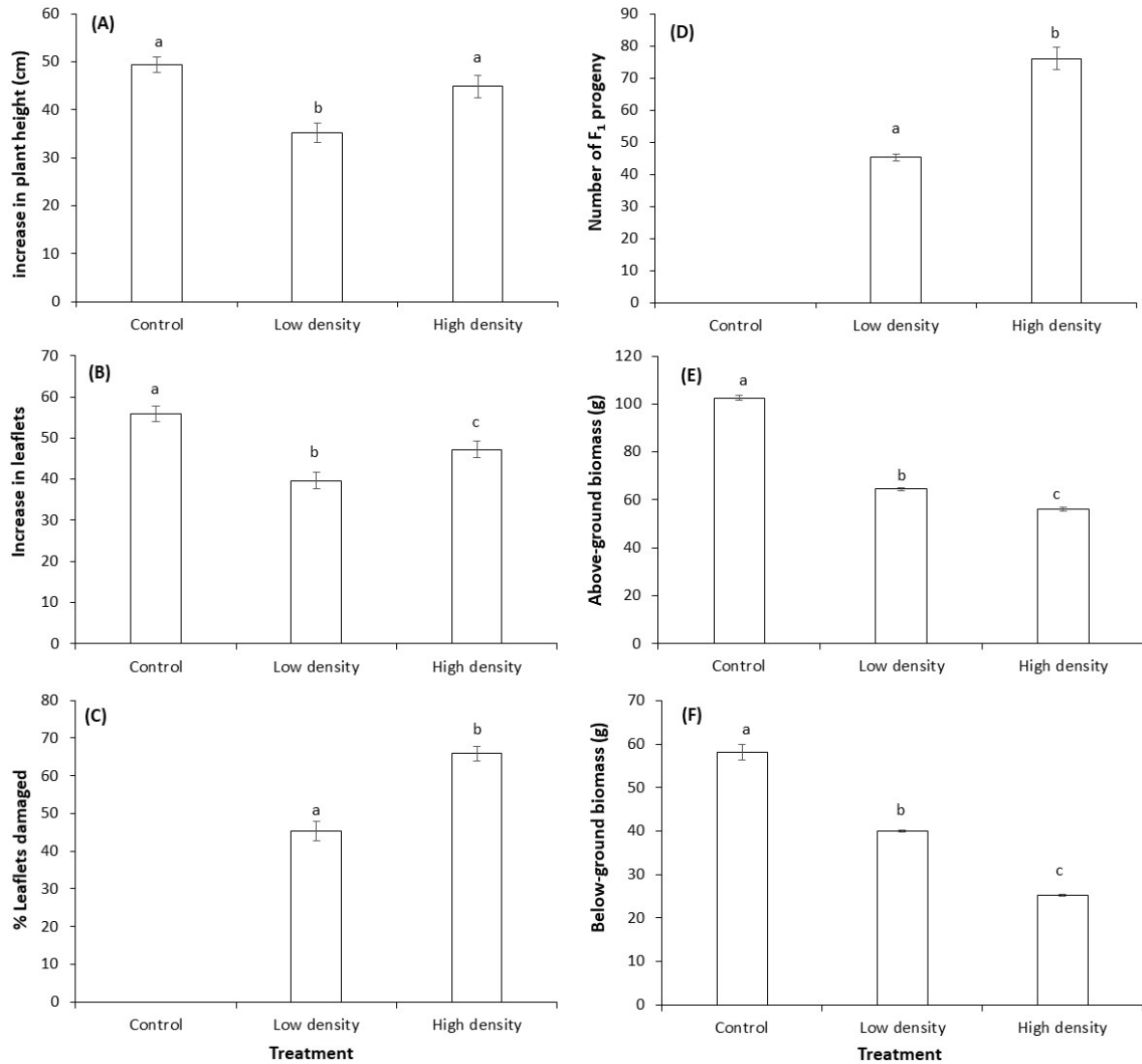
Fig. 1. Mean (± SE) increases in height (A) and leaflet production (B) of *Tecoma stans* plants, percentage leaflet damage inflicted by P_1 adults (C) and number of adult F_1 progeny (D) of *Heikertingerella* sp., and above-ground (E) and below-ground (F) biomass of plants, following their exposure to low- and high-density treatments of *Heikertingerella* sp. relative to the untreated controls. Means with different letters differed significantly.

Predicted geographical distribution of Heikertingerella sp. in South Africa

The MaxEnt model displayed high discriminatory ability and predictability, as indicated by the high area under curve (AUC) value (mean ± SE = 0.89 ± 0.16). The model predicted that most

of South Africa is climatically highly suitable for *Heikertingerella* sp., particularly along the southern and east coast where the weed is most prevalent (Fig. 2). Only the inland regions of the Northern Cape Province, which is the driest and hottest part of the country, and where the weed is currently absent, was predicted to be unsuitable for *Heikertingerella* sp. The highest climatic suitability (suitability scores > 0.9) occurred within approximately 50 km of the coast from Cape Point (Western Cape Province) to the most northerly point in KwaZulu-Natal Province, in addition to a few small regions in the Mpumalanga and Limpopo provinces (Fig. 2). Outside of South Africa, high climatic suitability is predicted across Lesotho, eSwatini and Mozambique, with a slightly less climatically suitable region present in south-eastern Zimbabwe (Fig. 2).
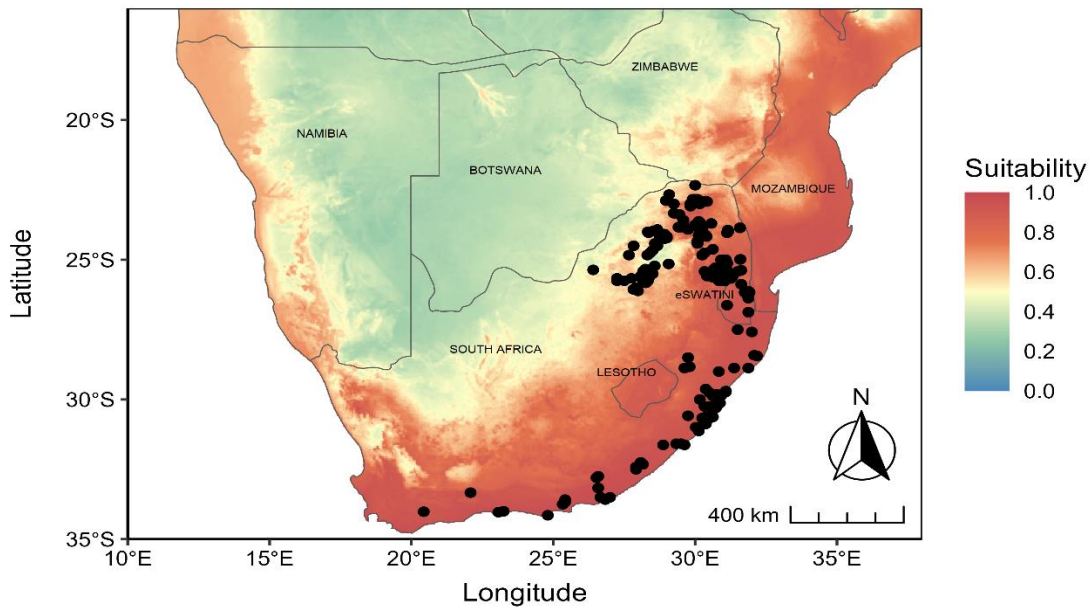
Fig 2. Potential geographic distribution of *Heikertingerella* sp. on *Tecoma stans* in southern Africa as predicted by MaxEnt modelling. Suitability scale: 0.0–0.1 = unsuitable; 0.2–0.3 = marginally suitable; 0.4–0.5 = suitable; and 0.6–1.0 = highly suitable areas. Closed circles

represent the weed's current distribution in South Africa (SAPIA database, ARC–Plant Health & Protection, Pretoria).

Critical thermal limits and lethal temperatures

Based on their mobility, the mean ($\pm$ SD) critical thermal minimum (CT_{min}) of *Heikertingerella* sp. adults was determined as $5.45 \pm 1.69^{\circ}C$ (n = 40) and the mean critical thermal maximum (CT_{max}) as $47.3 \pm 1.31^{\circ}C$ (n = 40). The calculated lower lethal temperature was $-4.0 \pm 0.5^{\circ}C$. After 2-hr exposures to low temperatures, there were no adult mortalities at $2^{\circ}C$ but there were gradual increases in mortality as temperatures declined from $0^{\circ}C$ to $-5^{\circ}C$, with no survival beyond $-5^{\circ}C$ (Fig. 3A). After 2-hr exposures to high temperatures, there were no mortalities at $40^{\circ}C$ but there were gradual increases in mortality as temperatures increased from $43^{\circ}C$ to $47^{\circ}C$, with no survival at $50^{\circ}C$ (Fig. 3B). The calculated upper lethal temperature of *Heikertingerella* sp. was thus $45.0 \pm 0.5^{\circ}C$.
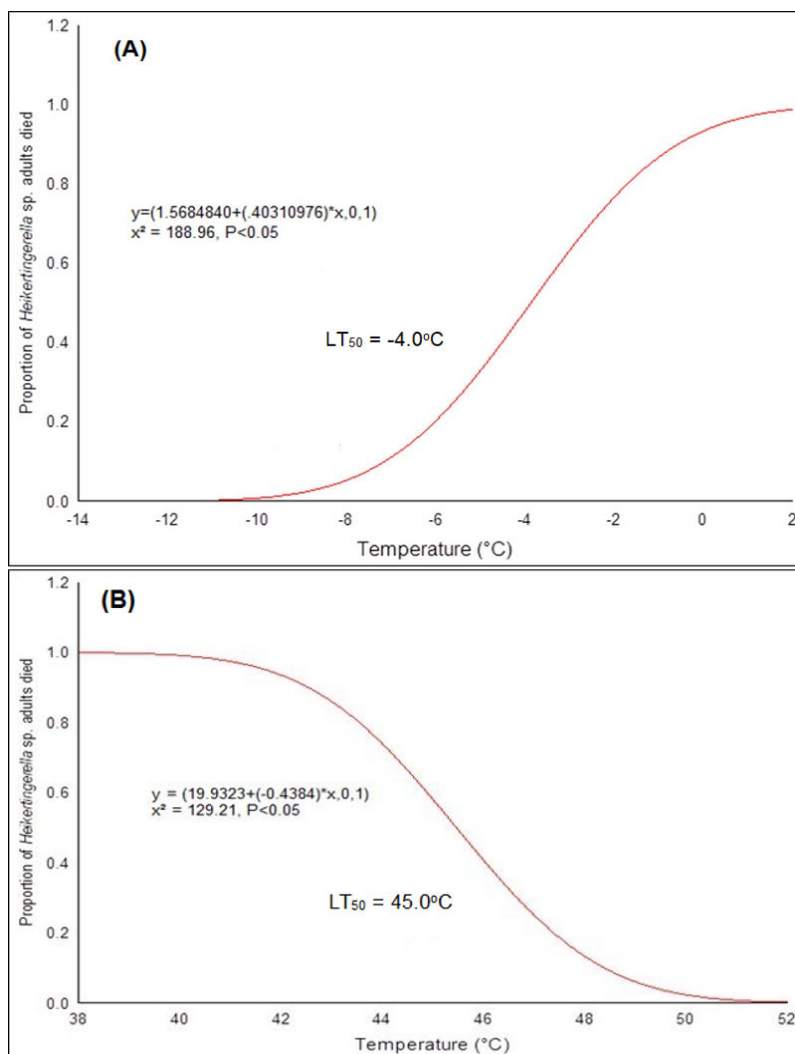
Fig. 3. Probit regression analysis indicating the lower (A) and upper (B) lethal temperatures of adults of *Heikertingerella* sp.

Minimum temperatures in localities invaded by Tecoma stans

The average minimum temperatures in the Eastern Cape, KwaZulu-Natal, Western Cape and Limpopo provinces of South Africa are typically above the critical thermal minimum (CT_{min} = 5.45°C) of *Heikertingerella* sp. (Fig. 4), suggesting likely establishment. In contrast, the average minimum temperatures in the Mpumalanga and North West provinces fall below the beetle's CT_{min} during the winter months, suggesting less likelihood of establishment. Since the

winter temperatures in Gauteng, and particularly the Free State, are clearly below the beetle's CT_{min} (Fig. 4), establishment in these provinces seems unlikely.
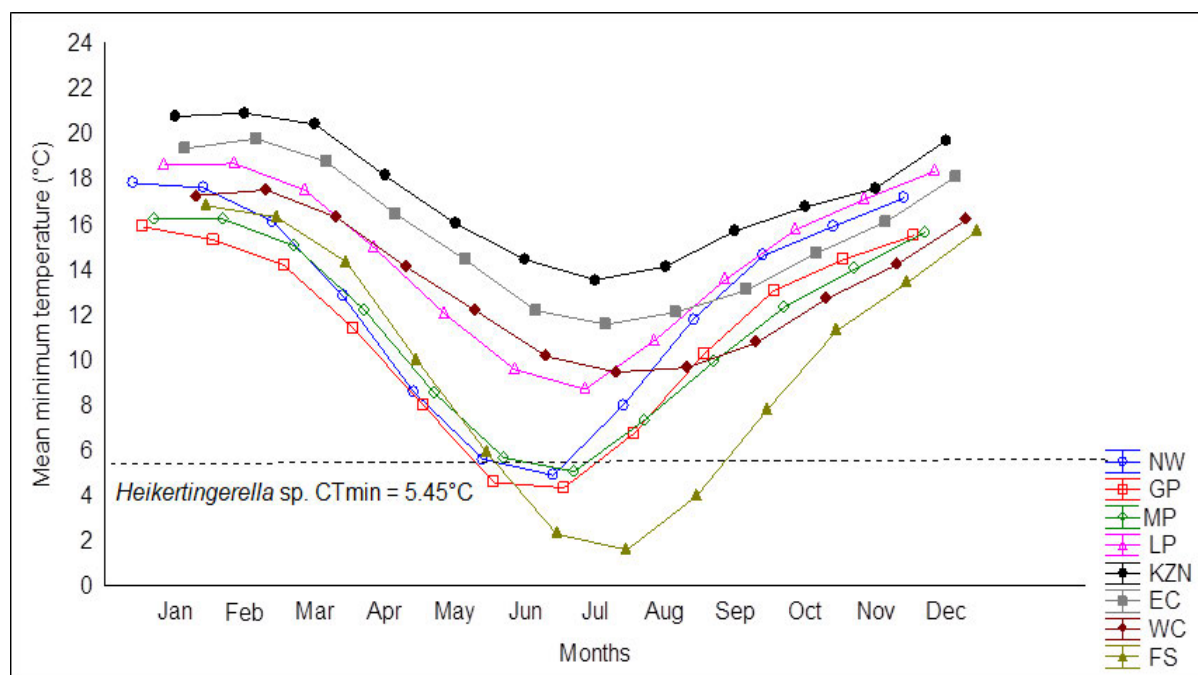
Fig. 4. Mean (± SE) minimum monthly temperatures for South African provinces invaded by *T. stans* in relation to the laboratory-determined minimum temperature threshold (CT_{min} = 5.45°C) of *Heikertingerella* sp. Provinces are: North West (NW), Gauteng (GP), Mpumalanga (MP), Limpopo (LP), KwaZulu-Natal (KZN), Eastern Cape (EC), Western Cape (WC) and Free State (FS).

Discussion

This study demonstrated that the growth and biomass accumulation of *T. stans* was significantly reduced by exposure to the root-feeding *Heikertingerella* sp. Reduced plant height, leaf production and both above- and below-ground biomass was generally evident at both low (10 adults/plant) and high beetle densities (20 adults/plant). Significantly higher increases in plant height and leaflet production in the high-density than in the low-density treatment were unexpected, particularly since above-ground biomass was significantly lower

in the high-density treatment. Although these results are contradictory, the loss of biomass by plants in the high-density treatment may be the result of substantially higher leaflet damage (i.e. reduction of leaf area), despite the increases in plant height and leaflets. It is possible that the higher levels of damage suffered by plants in the high-density treatment stimulated compensatory growth (see Gerber et al., 2008) in the above-ground tissues, albeit not measurable in biomass but clearly at the expense of resources extracted from the roots (i.e. below-ground biomass).

Our results are consistent with those of Conrad & Dhileepan (2007) where a single generation of the leaf-sucking lace bug *Carvalhotingis visenda* (Drake and Hambleton) (Hemiptera: Tingidae) significantly reduced the leaf chlorophyll content, plant height and leaf biomass of the confamilial *Dolichandra unguis-cati* (L.) Gentry (Bignoniaceae) under laboratory conditions. Based on precedents from other studies, our laboratory results suggest that *Heikertingerella* sp. could suppress the growth of *T. stans* under field conditions. For example, during field-cage studies on invasive *Onopordum* species (Asteraceae) in the native range, the weevil *Lixus cardui* Olivier (Coleoptera: Curculionidae) reduced plant growth and biomass by up to 50%, and seed viability by 80%, (Briese, 1996). Post-release evaluations in the introduced range in Australia confirmed these results, where *L. cardui* reduced plant growth and seed production by 33% and 65%, respectively (Swirepik et al., 2008).

During our exploration for potential biocontrol agents in Chiapas Province (Mexico), we observed *Heikertingerella* sp. causing considerable damage and defoliation to *T. stans* populations. The effects of *Heikertingerella* sp. on plant height, leaf density and biomass were thus consistent with these observations. In addition, as demonstrated in an earlier laboratory study (Madire et al. 2021b), the impact of *Heikertingerella* sp. and the leaf-feeding beetle *M. polluta* are likely to complement each other and thereby enhance the biocontrol programme

against *T. stans*, particularly in the eastern coastal region of South Africa where *M. polluta* is already well established (Madire, 2019).

Furthermore, MaxEnt modelling predicted that most of South Africa is climatically highly suitable for *Heikertingerella* sp., particularly the areas where the weed is most prevalent (Henderson, 2021). High climatic suitability was similarly predicted for neighbouring southern African countries. Since *T. stans* is also prevalent in a few East African countries (Orwa et al., 2009), the likely spread of *Heikertingerella* sp. further north (see Pratt & Center, 2012) has benefits for invaded sub-Saharan countries, where, given the beetle's monophagous nature (Madire et al. 2021a), there are no non-target species at risk of attack.

The thermal physiology of *Heikertingerella* sp. adults suggests a degree of adaptability to South African field conditions. The CT_{min} (5.45°C) and lower lethal temperature (-4°C) suggest that the beetle displays a degree of cold tolerance that could support survival during the coldest winter months, in at least four of the eight provinces invaded by *T. stans*. Temperatures below -4°C fall below the beetle's lethal threshold and will cause substantial mortality. Based on the 2020 temperatures recorded in South African provinces (SAWS, 2021), the minimum daily winter temperatures in Limpopo (-1.7°C to -4.2°C), KwaZulu-Natal inland (-3.8°C to -6.6°C), Western Cape coast (-2.1°C to -6.5°C) and Eastern Cape coast (1.6°C to -4.8°C), suggest that *Heikertingerella* sp. will display moderate winter compatibility with these areas. In contrast, the high CT_{max} (47.3°C) and upper lethal temperature (45.0°C) indicated high heat tolerance. Since the 2020 temperatures in South Africa rarely exceeded 40°C (SAWS, 2021), *Heikertingerella* sp. seems highly unlikely to succumb to high summer temperatures. However, these thermal tolerance predictions require verification by monitoring of the persistence of the beetle's populations across seasons. In addition, these predications are based on adult physiology and may underestimate the resilience of the subterranean immature stages, which may be buffered by warmer

belowground temperatures. Also, colder temperatures in certain regions may induce earlier diapause in the immature stages.

Although we acknowledge that the MaxEnt predictions depend on broad climatic comparisons of temperature, elevation and precipitation (Park et al., 2018), the combination of this model and the beetle's critical thermal limits enhances our predictions of its establishment and distribution in southern Africa. However, microclimates may play an important role within the broader climatic predictions (e.g. allow establishment in areas predicted to be unsuitable). In addition, the influence of local biotic factors such as regional topography, genetic traits of the host plant, and recruited natural enemies, on the beetle's establishment and distribution require consideration (Samways et al., 1999). These considerations warrant the need for post-release evaluations to verify the predictions of this study.

In conclusion, *Heikertingerella* sp. is predicted to have a significant impact on the growth and biomass accumulation of *T. stans* populations in areas that are most suitable for its establishment. In particular, the eastern coastal regions of South Africa that are severely invaded by *T. stans* are best suited for the beetle, while the colder more inland regions may constrain its efficacy. The results of this study, together with the beetle's very high level of host specificity (Madire et al., 2021a), strongly supported the first release of *Heikertingerella* sp. in South Africa in early 2023.

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CHAPTER 5

Effect of host-plant age on the performance of the root-feeding flea beetle *Heikertingerella* sp., a biological control agent for *Tecoma stans* in South Africa

 Abstract

Age influences host-plant quality, which is a key determinant of the fitness of herbivorous insects and hence biological control agents of invasive plants. We assessed the effect of host-plant age on the performance of the root-feeding flea beetle *Heikertingerella* sp. (Coleoptera: Chrysomelidae: Galerucinae), which was recently approved for release against the invasive tree *Tecoma stans* (L.) Juss ex Kunth var. *stans* (Bignoniaceae) in South Africa. Using similar-sized plants that were aged from one to three years, our aim was to determine an optimal host-plant age to facilitate mass-rearing of the beetle for impending releases. While significantly more F_1 progeny were produced with increasing plant age, presumably due to larger root masses in progressively older plants, plant age did not influence the size of the F_1 adults. There was no significant effect of plant age on the survival and percentage of leaves damaged by the P_1 adults. *Tecoma stans* plants of all ages produced significantly fewer leaves when exposed to feeding by *Heikertingerella* sp. adults and their larval progeny, relative to the unexposed control plants. Irrespective of plant age, adults displayed higher feeding intensity on the younger leaves at the top sections of the plants than on the older leaves on the middle and bottom sections. Although plants of all three ages were suitable for adult feeding and survival in quarantine cultures, progressively older plants are best suited for F_1 progeny production and therefore for the mass-rearing of *Heikertingerella* sp. for releases.

Keywords: Agent performance, host-plant age, mass-rearing, root-feeding beetles, weed biocontrol

Introduction

Tecoma stans (L.) Juss. ex Kunth var. *stans* (Bignoniaceae), commonly known as yellow bells, is an invasive shrub or small tree in South Africa and neighbouring countries that grows aggressively and typically forms multi-stemmed shrubs after clearing (Madire et al., 2011). The plant has a wide natural distribution in tropical and subtropical parts of the Western Hemisphere (Pelton, 1964). In its native range in Central America, *T. stans* is associated with a wide range of herbivores, many of which have been considered as biological control agents (Madire et al., 2011; Madire & Netshiluvhi, 2021). The flea beetle *Heikertingerella* sp. (Coleoptera: Chrysomelidae: Galerucinae: Alticini) has recently been approved for release in South Africa, following confirmation of its host specificity (Madire et al., 2021a) and compatibility with other agents (Madire et al., 2021b). The adult beetles feed on the leaves of *T. stans* while the subterranean larval stages feed externally on the cortex of secondary roots (Madire et al., 2021a).

During the establishment of *Heikertingerella* sp. cultures in the quarantine glasshouse, variable performance was observed on *T. stans* plants of different ages, suggesting that host-plant age may be an important factor in optimising beetle numbers during mass-rearing. Mass-rearing for field releases involves the production of very high numbers of healthy insects (Parra & Coelho, 2022), using minimum labour and resources (Moran et al., 2014) and is a well-established protocol in South Africa (Hill et al., 2021). Effective mass-rearing can enhance the establishment of certain biocontrol agents, such as the slow-dispersing *Mada polluta* (Mulsant) (Coleoptera: Coccinellidae), an established agent on *T. stans* in South Africa. For a successful mass-rearing programme, it is imperative to ensure high-quality plants, a suitable rearing environment and culturing practices that promote genetic integrity of the agent's populations (Moran et al., 2014; Hill et al., 2021). This study on the effects of host-plant age was thus

undertaken to contribute towards an understanding of the host-plant quality required for optimal mass-rearing of *Heikertingerella* sp.

Several studies (e.g., Price, 1991; Bowers & Stamp, 1993; Wait et al., 2002) have shown that host-plant age plays a vital role in the development and fitness of insect herbivores. Herbivorous insects typically perform better on younger plants that are more vigorous, photosynthetically more active, and higher in limiting resources (e.g. nitrogen) than older plants (e.g., Krischik & Denno, 1983; Harper, 1989; Bowers & Stamp, 1993). In such instances, the impact of insect damage is likely to decrease with increasing host-plant age. Consequently, age influences plant quality (often expressed as leaf nitrogen content), which directly affects food consumption and the development and survival of insect larvae (Awmack & Leather, 2002). Plants with high leaf nitrogen content typically display faster growth, greater leaf area and higher leaf production than plants with low nitrogen content (Wait et al., 2002), which directly affect insect herbivore performance. Physiological ageing of plants also reduces insect fitness traits such as size, longevity and reproductive output (e.g., Leather, 1990; Albert & Bauce, 1994; Dodds et al., 1996; Tammaru, 1998; Campos et al., 2003). In contrast, other studies have demonstrated improved performance (e.g., population growth) of insect herbivores on older plants than on younger ones (e.g., Hard, 1985; Bauce et al., 1994).

Although several studies have reported the effect of host-plant age on above-ground herbivores (e.g., Hard, 1985; Bauce et al., 1994), few studies have involved below-ground herbivores. However, Smith & Story (2003) recorded higher numbers of root-feeding larvae of *Agapeta zoegana* L. (Lepidoptera: Tortricidae) with increased root diameter in spotted knapweed *Centaurea stoebe* L. subsp. *micranthos* (Gugler) Hayek (Asteraceae), suggesting that larger (older) plants are preferred. Withington et al. (2006) attributed the preference of root herbivores for longer-lived roots in older plants to lower levels of defensive chemicals. Hunter (2008) also reported that the growth, survival and reproduction of many root-feeding

herbivores are limited by nitrogen availability in the roots, and hence high concentrations of nitrogen in the soil and roots are likely to favour root herbivores. Root herbivores can significantly change the concentration of secondary plant compounds and reduce root biomass, resulting in decreased nutrient uptake by plants (Wang et al., 2018).

The aim of this study was to determine the effect of host-plant age on the performance of the root-feeding *Heikertingerella* sp., since age-based differences can influence the agent's impact on *T. stans* populations of variable age structure. Since the beetle is currently being mass-reared for initial releases, determining an optimal host-plant age can boost the numbers available for release and improve the monitoring of beetle populations in the field.

Materials and methods

Laboratory conditions, test plants and insect cultures

The study was conducted in a quarantine glasshouse at the ARC- Plant Health and Protection Roodeplaat facility in Pretoria, South Africa (25°36'8780" S; 28°21'9230" E), with the temperature and relative humidity set at 28-33°C and 47-60%, respectively. Natural light conditions were utilized during summer, while a 16h L: 8h D photoperiod was maintained during winter using 50W / LED 4000K/ 230 V LED floodlights (Spazio lighting). Test plants were initially propagated from seeds collected from mature *T. stans* plants in the field. After germination, the seedlings were transferred to 10-litre pots to facilitate root and vegetative growth. The growth medium comprised equal amounts of river sand, top soil, compost and vermiculite to facilitate drainage of the soil. Plants were maintained in a shade house and watered twice a day with overhead irrigation sprinklers. A 2:3:2 (14%) mixture of N: P: K fertilizer was applied every month to sustain growth. The plants were tagged according to their age (1-3 years old) and above-ground biomass was pruned during the spring of each year to

encourage both root growth (i.e., by becoming deeper and more fibrous) and branch development and to maintain a standard plant size that fitted inside the experimental cages (see below). Individuals of *Heikertingerella* sp. were provided by the quarantine culture and were reared on potted *T. stans* plants under the abovementioned glasshouse conditions.

Performance of Heikertingerella sp. on different aged plants

Sixty *T. stans* plants, comprising 20 plants from each of three different age classes (1-3 years old), were selected from the shade house. These included 10 beetle-exposed and 10 control plants for each of the three age classes. Prior to testing in the quarantine glasshouse, the plants were sprayed with water to remove any insect pests or contaminants. Individual plants were placed in gauze-covered cages (55 x 55 m x 95 cm) prior to their exposure to the flea beetles and the number of leaves were recorded. Ten mating pairs of newly-emerged beetles were released onto each caged experimental plant to allow feeding and oviposition in the soil. After 30 days, all surviving P_1 adults were counted and removed from the plants in all age treatments and the leaves were counted and assessed for adult feeding damage. After removal of the adults, the plants were kept in the same cages for a further 30 days to enable the development of immature stages to adulthood (47 days on average from egg to adult; Madire et al., 2021a) and the numbers of emerging adult progeny (F_1) were recorded. In each age treatment group, 10 F_1 adults were collected at random (i.e., one per experimental plant and 30 across treatments), and their body length (head to end of the elytra) was determined for comparison between the treatments. The trials were terminated 60 days after their initiation, by which time all F_1 adults had emerged. The numbers of leaves produced during the 60-day trial period were recorded in the beetle-exposed and control plants.

Positional feeding preferences of Heikertingerella sp. adults

Twelve *T. stans* plants, comprising four plants from each of the three age classes (1-3 years old), were selected as in the previous experiment and transferred into the quarantine glasshouse. Individual plants were confined with 15 mating pairs of beetles in a gauze-covered cage (55 x 55 m x 95 cm) for 30 days. After 30 days, the intensity of feeding was assessed by recording the number of feeding scars in the top-, middle- and bottom-third sections of each plant.

Statistical analysis

All analyses were conducted using SPSS version 28 (IBM Inc.). The datasets did not meet the assumptions of normality and generalised linear modelling was thus used, with the models corrected for over-dispersion and significance ($P < 0.05$) determined by Wald chi-square statistics. The models testing the influence of host-plant age on the percentage survival of P_1 adults, and percentage of leaves damaged, incorporated a binomial distribution and a logit-link function. Models testing the effect of plant age on F_1 progeny production and F_1 adult size incorporated a Poisson distribution with a log-link function and a Tweedie distribution with a log-link function, respectively. The increases in leaf production by the different aged plants were compared between the beetle-exposed and control plants using a model that incorporated a Poisson distribution and a log-link function. The proportion of adult feeding scars on leaves in different positions (i.e. bottom, middle and top sections) on the plants were compared using a model that incorporated a binomial distribution and a logit-link function. Where significant differences were demonstrated, Least Significant Difference (LSD) tests were used for pairwise comparisons of the means.

Results

Performance of Heikertingerella sp. on different aged plants

There was no significant effect of host-plant age on the percentage of P_1 adults that survived the 30-day period of exposure to the *T. stans* plants ($\chi^2 = 2.912$; d.f. = 2; P = 0.233). Mean ($\pm$ SE) percentage P_1 adult survival varied only slightly between the 1-year old ($56.0 \pm 7.7\%$), 2-year old ($65.5 \pm 5.6\%$) and 3-year ($61.0 \pm 1.6\%$) old-plants (Fig. 1a). In contrast, host-plant age had a significant effect on the numbers of F_1 progeny produced ($\chi^2 = 765.447$; d.f. = 2; P < 0.001). Mean ($\pm$ SE) F_1 adult numbers increased significantly between the 1-year old (32.0 ± 0.6), 2-year old (35.5 ± 0.7) and 3-year old (56.2 ± 0.6) plants (Fig. 1b). Despite higher progeny production with increasing host-plant age, there was no significant effect on F_1 adult size ($\chi^2 = 0.226$; d.f. = 2; P = 0.893). Mean ($\pm$ SE) F_1 adult lengths were very similar between the 1-year old (2.51 ± 0.13 mm), 2-year old (2.58 ± 0.16 mm) and 3-year old (2.60 ± 0.15 mm) plants (Fig. 1c). There was also no significant effect of host-plant age on the percentage of available leaves that were damaged by P_1 adult feeding ($\chi^2 = 4.858$; d.f. = 2; P = 0.088). The mean ($\pm$ SE) percentage of leaves damaged increased marginally between the 1-year old ($50.7 \pm 2.9\%$), 2-year old ($57.2 \pm 3.4\%$) and 3-year old ($63.8 \pm 5.7\%$) plants (Fig. 1d).
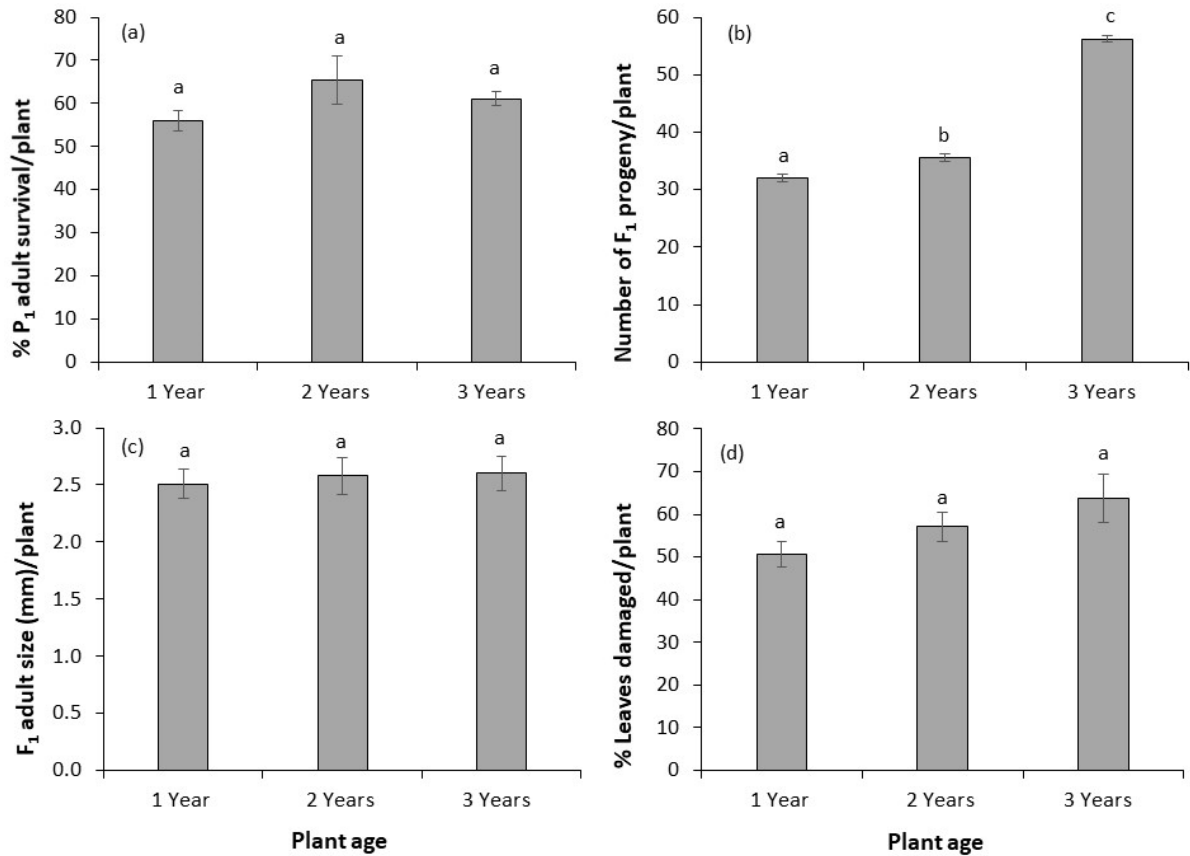
Fig. 1. Response of *Heikertingerella* sp. following exposure to *Tecoma stans* plants of varying age (1-3 years), as represented by the mean ($\pm$ SE) (a) percentage survival of P_1 adults, (b) number of F_1 progeny produced, (c) body size (length) of F_1 progeny and (d) percentage of leaves damaged by P_1 adults. Bars with different letters are significantly different ($P < 0.05$).

Impact of Heikertingerella sp. feeding on leaf production

There were significant reductions in leaf production by *T. stans* plants when exposed to *Heikertingerella* sp., relative to the unexposed controls ($\chi^2 = 530.540$; d.f. = 5; $P < 0.001$) and this trend was consistent across all age classes. On average, leaf production was reduced by 34.4% in the 1-year old, 23.7% in the 2-year old and 26.9% in the 3-year old plants (Fig. 2). Marginally, but significantly, more leaves were produced by the 2-year old and 3-year old exposed plants relative to the 1-year old exposed plants (Fig. 2).

Fig. 2. Mean (± SE) increase in leaf production by *Tecoma stans* plants of varying age (1-3 years) when exposed to feeding by *Heikertingerella* sp. adults and their larval progeny. Control plants were not exposed to the beetles. Bars with different letters are significantly different (P < 0.05).

Positional feeding preferences of Heikertingerella sp. adults

Heikertingerella sp. adults displayed positional feeding preferences, with significantly higher proportions of leaves with feeding scars ($\chi^2 = 81.373$; d.f. = 2; P < 0.001) on the top sections of the plants (50.0 ± 2.7%), than on the middle (34.8 ± 2.2%) and bottom (15.2 ± 2.6%) sections (Fig. 3). This trend was not influenced by plant age ($\chi^2 = 0.186$; d.f. = 2; P = 0.911) and there was no significant interaction between age and feeding position ($\chi^2 = 5.498$; d.f. = 2; P = 0.240).
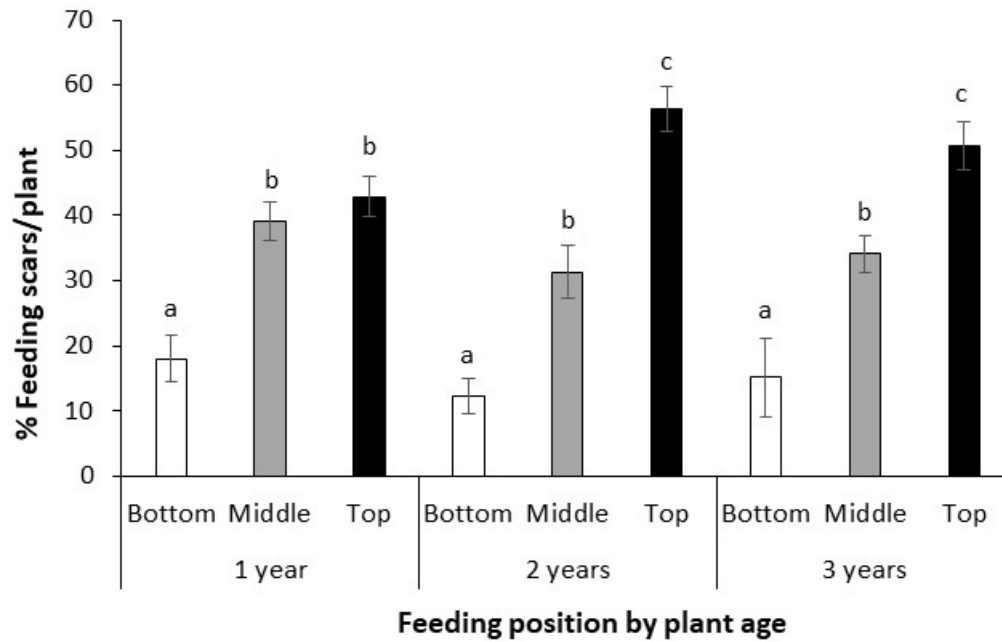
Fig. 3. Mean (± SE) percentage of feeding scars of *Heikertingerella* sp. adults recorded on the leaves in the bottom, middle and top sections of *Tecoma stans* plants of varying age (1-3 years). Within the same age class, bars with different letters are significantly different (P < 0.05).

Discussion

Our study revealed that while the feeding and survival of P_1 *Heikertingerella* sp. adults was not affected by the age of *T. stans* plants, progressively older plants supported substantially higher levels of F_1 progeny production. The numbers of F_1 adults emerging from 3-year old plants were substantially higher than those from 2-year old and 1-year old plants, with no effect on adult size, suggesting that older roots improved larval development and survival. During the study, we observed that some of the younger plants that were used to culture the beetles were unable to recover after the emergence of the F_1 adult progeny, suggesting that inadequate food resources (i.e., resulting from reduced root volume) may also have increased the mortality of the subterranean immature stages. Since younger plants are generally less tolerant of root-feeding herbivores than older plants because of smaller root systems (Stout et al., 2002), it

seems likely that older *T. stans* plants produced more F_1 adult progeny of *Heikertingerella* sp. because of larger root size or volume. Also, because younger plant stages are often more susceptible to herbivory, with proportionally more undifferentiated and actively-growing tissues than older plant stages, their ability to induce chemical defences is greater than in mature plants (Karban & Baldwin, 1997). Consequently, the higher production of *Heikertingerella* sp. adults on older *T. stans* plants could be due to higher tolerance to herbivory, resulting from a combination of higher root reserves and lower levels of chemical defences (Strauss & Agrawal, 1999; Haukioja & Koricheva, 2000).

Despite the above considerations, the size of the F_1 *Heikertingerella* sp. adults was not affected by host-plant age and was contrary to the widespread phenomenon that lower resource availability or dietary constraints during larval development cause a reduction in adult body size (e.g., Boggs & Ross, 1993; O'Brien et al., 2004). However, larval survival does not necessarily correlate with adult body size. For example, Thiery et al. (2014) demonstrated that while high larval densities significantly increased larval mortality in *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae), adult body size was not affected by larval crowding.

There is substantial evidence that leaf-feeding insect species often prefer younger plants or leaves to more mature ones for feeding (e.g., Stowe et al., 2000; Wait et al., 2002). Our study confirmed that, irrespective of plant age, younger leaves in the top sections of *T. stans* plants were preferred for feeding by adult *Heikertingerella* sp., relative to leaves in the lower sections. The absence of an effect of plant age was expected, since leaf age was consistent across treatments, due to the pruning of the test plants prior to exposure. The shoot tips of all *T. stans* plants exposed to the beetles comprised young vigorously growing leaves, which supported around 50% of adult feeding scars. This trend is common in both chewing and sucking insects (Kursar & Coley, 1991) because of lower toughness and higher digestibility of younger leaves

(Coley, 1983), but also higher nutritional value with up to four times more nitrogen content than mature leaves (Coley & Aide, 1991). However, there are notable deviations from these trends, where older plants were more attractive and suffered higher levels of insect herbivory than younger plants (e.g., Blais, 1958; Schmidt & Frye, 1977; Hard, 1985; Kearsley & Witham; 1989; Karban, 1990; Bauce et al., 1994).

Our study has provided important insights into the effect of host-plant age on the performance of *Heikertingerella* sp. We conclude that *T. stans* plants of all ages tested (i.e. 1-3 years) are suitable for sustaining laboratory cultures of *Heikertingerell*a sp., but that progressively older plants are best suited for the purposes of mass-rearing to facilitate field releases. It seems plausible that even older and larger *T. stans* plants (e.g. 4-5 years) with higher root volumes will be able to boost the numbers of *Heikertingerella* sp. adults for field releases. These results may also guide the selection of release sites. In particular, old stands of *T. stans* (i.e., with large and well-developed root systems), in which some plants are pruned pre-release to stimulate new leaf growth, could provide optimal release sites. However, there is no real need to prune old plants because new leaves are produced every spring, after flowering. Furthermore, most *T. stans* populations support plants of variable age, due to recurring infestations. However, this study was unable to conclude whether the variations in F_1 adult emergence across different aged plants were due to intraspecific competition for limited root resources, variations in chemical defences, better nutritional value, or a combination of these factors. Additional experiments (e.g., controlling root biomass or size through pruning) could be conducted to address these uncertainties.

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CHAPTER 6

Application of Nitrogen, Phosphorus, Potassium + Carbon fertilizer improves the growth of *Tecoma stans* and the performance of its root-feeding biological control agent, *Heikertingerella* sp.

Abstract

The invasive tree *Tecoma stans* (L.) Juss. ex Kunth var *stans* (Bignoniaceae) has been targeted for biological control in South Africa since 2003. Plant nutrients contained in fertilizers typically improve host-plant quality, thereby enhancing the fitness of insect herbivores deployed as weed biocontrol agents. We investigated the effect of increasing nutrient levels (fertilizer treatments) on the growth of potted *T. stans* plants and on the subsequent performance of the root-feeding beetle *Heikertingerella* sp. (Chrysomelidae). Enhanced performance of *Heikertingerella* sp. in culture will improve mass-rearing initiatives for releases in South Africa. Ten newly emerged mating pairs of *Heikertingerella* sp. were exposed under glasshouse conditions to caged plants that were treated with four regimes of nitrogen: phosphorus: potassium 2:3:2 (14%) + carbon (8%) fertilizer, namely zero (control), low (5.6 g/m^2), medium (9.4 g/m^2) and high (13.2 g/m^2). Increasing nutrient levels significantly increased leaf production and above- and below-ground biomass accumulation in *T. stans* plants. Similarly, the performance of *Heikertingerella* sp. was improved substantially, with significantly and progressively higher levels of P_1 adult feeding and F_1 progeny production, and significantly and progressively reduced F_1 developmental times, under increasing nutrient levels. While F_1 adult size was significantly increased by fertilizer application relative to the controls, there were no significant differences between the three nutrient levels. Although the high fertilizer application rates were the most suitable for the culturing and mass-rearing of *Heikertingerella* sp. adults, medium fertilizer applications may also be suitable to improve host

plant quality as there were no significant differences between high and medium fertilizer application.

Keywords: Biocontrol agent biology, fertilizer application rates, host-plant quality, mass-rearing, weed biocontrol

Introduction

Plant nutrients have a significant effect on plant-herbivore interactions and ecosystem dynamics (Vitousek et al., 2002). According to the "plant vigour" hypothesis, plants grown in nutrient-rich soils produce biomass of greater nutrient quality, which is more attractive and beneficial to insect herbivores (Price et al., 1980; Price, 1991; Leather, 1994). Consequently, the application of fertilizers is expected to enhance several biological features of insect herbivores including food consumption, developmental rate and survival of the immature stages, and body size, reproductive ability and longevity of the adults (Price et al., 1980; Albert & Bauce, 1994; Leather, 1994; Tammaru, 1998; Campos et al., 2003; Hancock et al., 2013).

Several studies have indicated that herbivorous insects can respond to very small levels of nutritional and non-nutritional compounds in plants, which can influence their acceptance as hosts (Dale, 1988; Bernays & Chapman, 1994; Facknath & Lalljee, 2005). Also, several studies support the contention that increased nutrient levels decrease plant resistance to insect herbivory (e.g., Herms, 2002) and thus favour insect herbivores at individual and population levels (e.g., Kyto et al., 1996). However, increased nutrient availability does not always enhance insect performance and may have a negative or neutral effect (see McCullough & Kulman, 1991; Casey & Raupp, 1999; Herms, 2002).

Tecoma stans (L.) Juss. ex Kunth var. *stans* (Bignoniaceae), also known as yellow bells, is a fast-growing small tree or shrub that produces thousands of viable light-weight papery

seeds (Madire et al., 2011). It is native to Mexico, the southern states of the USA and throughout Central America, including the Caribbean region (Pelton, 1964; Madire, 2013). *Tecoma stans* is invasive in several countries including South Africa, where it has been targeted for biological control since 2003 (Madire et al., 2011). A root-feeding flea beetle, *Heikertingerella* sp. Csiki 1940 (Coleoptera: Galerucinae: Alticini), was imported from Mexico for screening as a potential biocontrol agent (Madire et al., 2021a, b; 2023) and subsequently cleared for release in South Africa in 2022. While adults feed on the leaves, the subterranean larvae feed and develop externally on the roots of *T. stans* (Madire et al., 2021a). Under quarantine glasshouse conditions, the adult-to-adult developmental time ranged from 51 to 73 days, while the longevity of both male and female adults ranged from 41 to 100 days (Madire et al., 2021a).

Following the protocols of quarantine studies, potted plants of *T. stans* were used for maintaining the cultures of *Heikertingerella* sp. and conducting the pre-release studies. The confinement of plants in pots often obstructs the development of the primary root, while promoting an increase in the number of lateral roots (Peterson et al., 1991; NeSmith & Duval, 1998). The roots of potted plants, particularly trees, thus display restricted growth, but also suffer from a limited supply of oxygen and essential nutrients. Suboptimal root development in potted *T. stans* plants is thus likely to reduce host-plant quality for the root-feeding larvae of *Heikertingerella* sp. This is particularly true with small pots, as pot size is positively related to the growth of below- and above-ground plant components (Latimer, 1991; NeSmith & Duval, 1998). The application of fertilizer can counteract this problem, by improving the nutritional quality of potted plants, and thereby increase the performance of *Heikertingerella* sp., which is currently being mass-reared for releases in the field. A successful mass-rearing programme requires high-quality host plants, optimal rearing conditions and culturing practices that

promote the agent's genetic fitness (i.e. its abillity to produce many generations without loss of fitness) (Moran et al., 2014; Hill et al., 2021; Madire et al., 2023).

This study was conducted to determine the effect of increasing nutrient levels (fertilizer treatments) on the growth and biomass of *T. stans* plants and on the subsequent performance of the root-feeding *Heikertingerella* sp. We expected that increasing soil nutrient levels (i.e., concentrations of nitrogen (N), phosphorus (P) and potassium (K) and Carbon (C)) would increase host-plant quality (i.e., higher biomass), resulting in increased insect performance (i.e., higher feeding rates, increased progeny production, quicker development and increased body size). These outcomes could substantially boost the production of healthy beetles in mass-rearing facilities and enhance establishment in the field.

Materials and Methods

Plant material and laboratory conditions

Mature seeds of *T. stans* were collected from several trees at three locations around Pretoria, Gauteng Province, South Africa, in February 2019, namely Roodeplaat (25°36'36"S; 28°21'42"E), Pretoria West (25°45'38"S; 28°1'43"E), and Kameeldrift (25°39'00"S 28°19'00"E). Seeds were dried in brown paper bags at room temperature for four months. In July 2019, seeds were sown in seed trays containing river sand at temperatures ranging from 15 to 36°C. Following germination, a standard soil mixture comprising one part river sand, one part Styrofoam™, one part compost and one part top soil was used to transplant the seedlings. Seedlings were initially transplanted into 2-litre pots for two months and then transferred into 10-litre pots for 30 days to promote root development. After 30 days, forty similar-sized potted plants were selected and cleaned to remove any arthropod contaminants, and then transferred to a quarantine glasshouse for the fertilizer trials. The glasshouse was located at the

Agricultural Research Council-Plant Health and Protection (ARC-PHP) Roodeplaat quarantine facility in Pretoria (25°37'00" S; 28°22'00" E), where the temperature and relative humidity were maintained at 28-33°C and 47-60%, respectively, for the duration of the trials. The trials were conducted under natural light conditions for a period of four months.

Experimental design

Nitrogen (N), Phosphorus (P), Potassium (K) and Carbon (C) were applied as a granular basal fertilizer to a depth of 5 cm when the seedlings were 15 cm in height and when transplanted into the 10-litre pots. This was applied in the form of N:P:K 2:3:2 (14%) + C (8%), which is traded as "Wonder Plant Starter All Purpose 2:3:2 (14) + C (8) SR®". The experiment comprised four fertilizer application rates, namely: zero (control), low (5.6 g/m^2), medium (9.4 g/m^2) and high (13.2 g/m^2). The application rates did not exceed the recommendations of the fertilizer manufacturers, and were in accordance with the methods developed by Uyi et al. (2016). Both control and treatment plants were provided with equal amounts of water every morning using a watering can. The quantities of each nutrient element in each of the fertilizer treatments are presented in Table 1.

In each fertilizer treatment, 10 *T. stans* plants were placed separately in individual gauze-covered cages (0.55 m x 0.55 m x 0.95 m) which were placed randomly on benches in the glasshouse, with the number of leaves recorded for each. Three weeks after the fertilizer applications, 10 mating pairs of newly emerged adults of *Heikertingerella* sp. were added to each cage and randomly assigned as P_1 adults for each plant. After 30 days, the surviving P_1 adults were removed from the treatment and control cages and their numbers recorded. The numbers of leaves on each plant, and those damaged by the P_1 adults, were also recorded after the 30-day exposure period. The numbers of F_1 adult progeny emerging from the treatment and control cages were recorded over the following 40-60 days, since it takes 47 days on average

for the beetle to develop from egg to adult (Madire et al. 2021a). The F_1 developmental period from egg to adult was determined by recording the time between the exposure of the P_1 adults to the time of emergence of the first F_1 adult in each cage, and then subtracting the 14-day pre-oviposition period (Madire et al., 2021a). Ten F_1 adult *Heikertingerell*a sp. from each treatment (i.e., one from each cage) were randomly selected and their body length (head to end of the elytra) measured. The trials were terminated 60 days after removal of the P_1 adults from the plants. The soil was removed from the roots by washing with water, and each plant was separated into leaves, stems and root crown with its associated roots, and placed in brown paper bags. The plant material was oven-dried at 70°C for 72 hours and the above-ground (leaves and stems) and below-ground (roots) biomass was recorded for each individual plant.

Table 1. Nutrient composition (g/m^2) of the three fertilizer treatments used to enhance the host-plant quality of *Tecoma stans*.

Nutrients	Low	Medium	High
Nitrogen	0.224	0.376	0.528
Phosphorus	0.336	0.564	0.792
Potassium	0.224	0.376	0.528
Carbon	0.448	0.752	1.056
All	1.232	2.068	2.904

Data analysis

Statistical analyses were conducted using SPSS version 28 (IBM Inc.). Since the datasets did not conform to normality and/or equality of variances, the effects of the fertilizer treatments on the recorded plant and insect variables were analysed using generalized linear modelling. All models were corrected for under-dispersion and Wald chi-square statistics were used to

determine significance (P < 0.05). Models testing the effect of the treatments on new leaf production and biomass accumulation by *T. stans* plants included a Poisson distribution with a log-link function, and a Tweedie distribution with a log-link function, respectively. Models assessing the percentage of leaves damaged by the P_1 adults, and their percentage survival, included a binomial distribution and a logit-link function. Models assessing F_1 progeny production included a Poisson distribution with a log-link function, while those assessing F_1 developmental periods and adult size included a Tweedie distribution with a log-link function.

Results

Effect of fertilizer on P_1 adults

Fertilizer application had a significant and positive effect on the production of new leaves by *T. stans* plants during their 30-day exposure to the P_1 adults (χ^2 = 195.603; df = 3; P < 0.0005). All fertilizer treatments were significantly different to the control, and to each other, with mean new leaf production increasing by 34%, 77% and 135% relative to the control, in the low, medium and high treatments, respectively (Fig. 1a). The same trend occurred in the percentage of leaves damaged by the P_1 adults during their exposure period (χ^2 = 416.845; df = 3; P < 0.0005) (Fig. 1b). Relative to the control, the mean percentage of leaves damaged increased by 36%, 58% and 135% in the low, medium and high treatments, respectively. However, this trend was not consistent with the survival of the P_1 adults, despite significant differences between the treatments (χ^2 = 172.964; df = 3; P < 0.0005). While there was no significant difference in P_1 adult survival between the control (67%) and low fertilizer treatment (72%), survival was significantly higher at the medium treatment (84%) but significantly lower at the high treatment (43%) (Fig 2a).
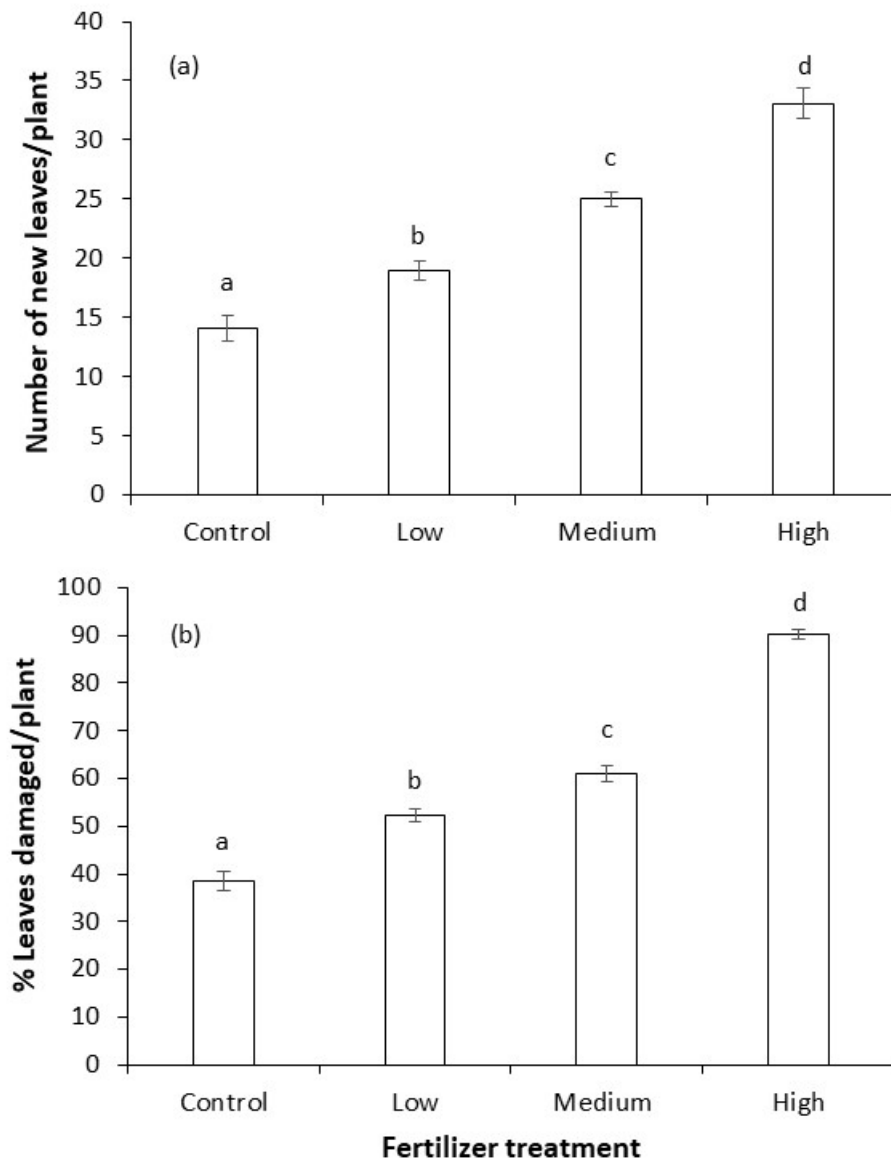
Fig. 1. Mean (± SE) (a) number of new leaves produced by *Tecoma stans* plants subjected to different fertilizer treatments (see Table 1) and (b) percentage of leaves damaged by P_1 adults of *Heikertingerella* sp., when exposed to these plants. Bars with different letters are significantly different (P < 0.05).

Effect of fertilizer on F_1 progeny

Fertilizer application also had a significant and positive effect on the production of F_1 progeny by the P_1 adults, during their 30-day exposure to the *T. stans* plants ($\chi^2 = 1536.926$; df = 3; P <

0.0005). All fertilizer treatments were significantly different to the control, and to each other, with mean F_1 progeny production increasing by 65%, 113% and 230% relative to the control, in the low, medium and high treatments, respectively (Fig. 2b).
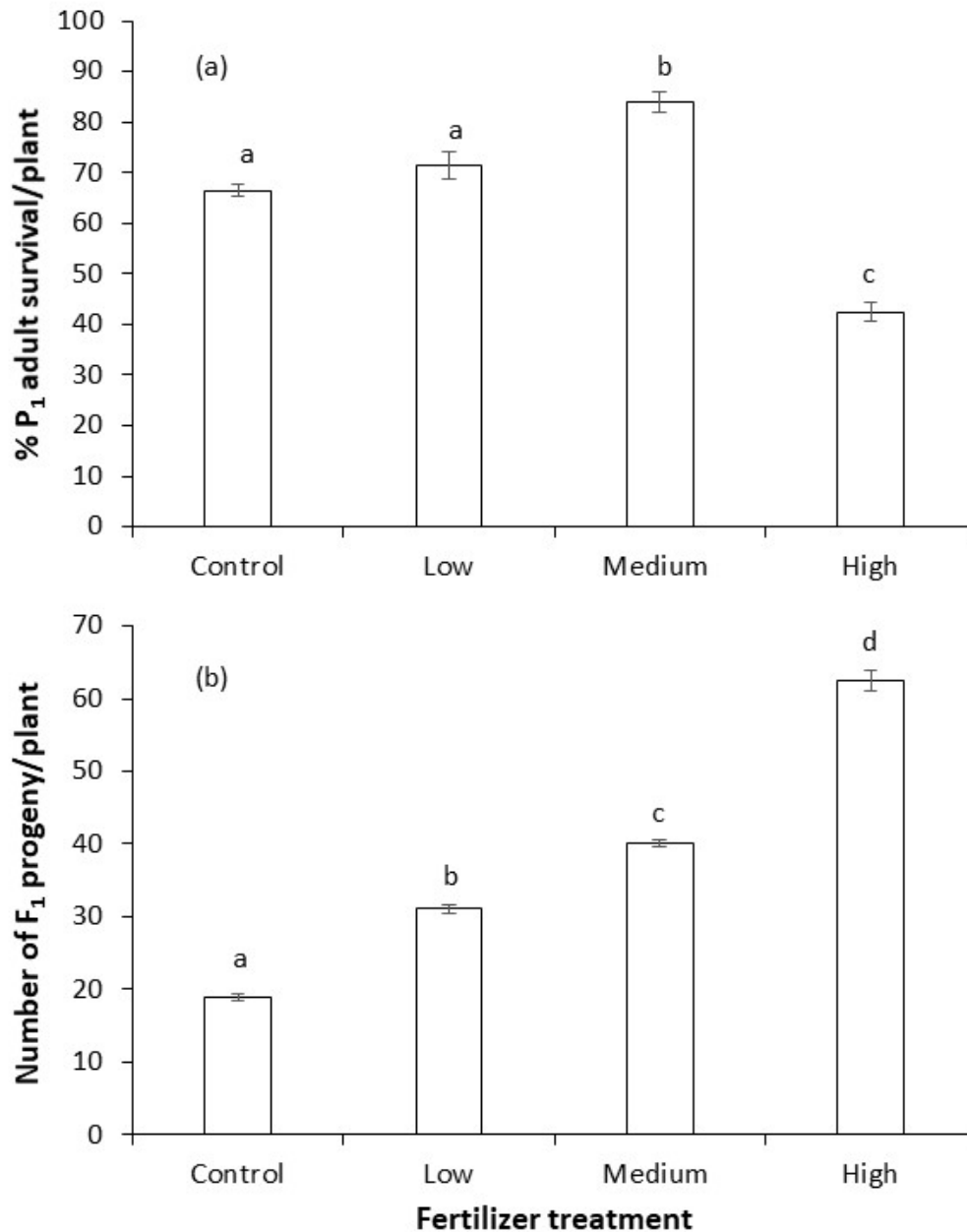

Fig. 2. Mean ($\pm$ SE) (a) percentage survival and (b) number of F_1 progeny produced by P_1 adults of *Heikertingerella* sp., when exposed to *Tecoma stans* plants subjected to different fertilizer treatments. Bars with different letters are significantly different (P < 0.05).

Fertilizer application also had a significant and positive effect on the duration of development to adulthood of the F_1 progeny ($\chi^2 = 487.415$; df = 3; $P < 0.0005$). All fertilizer treatments were significantly different to the control, and to each other, with mean developmental duration decreasing by 8%, 12% and 16% relative to the control, in the low, medium and high treatments, respectively (Fig. 3a). Fertilizer application had a significant and positive effect on the size of the F_1 progeny ($\chi^2 = 39.301$; df = 3; $P < 0.001$). While all fertilizer treatments produced significantly larger F_1 adults than the control, there were no significant size differences between the low, medium and high treatments, in which adults were 20%, 25% and 22% larger, respectively (Fig. 3b).
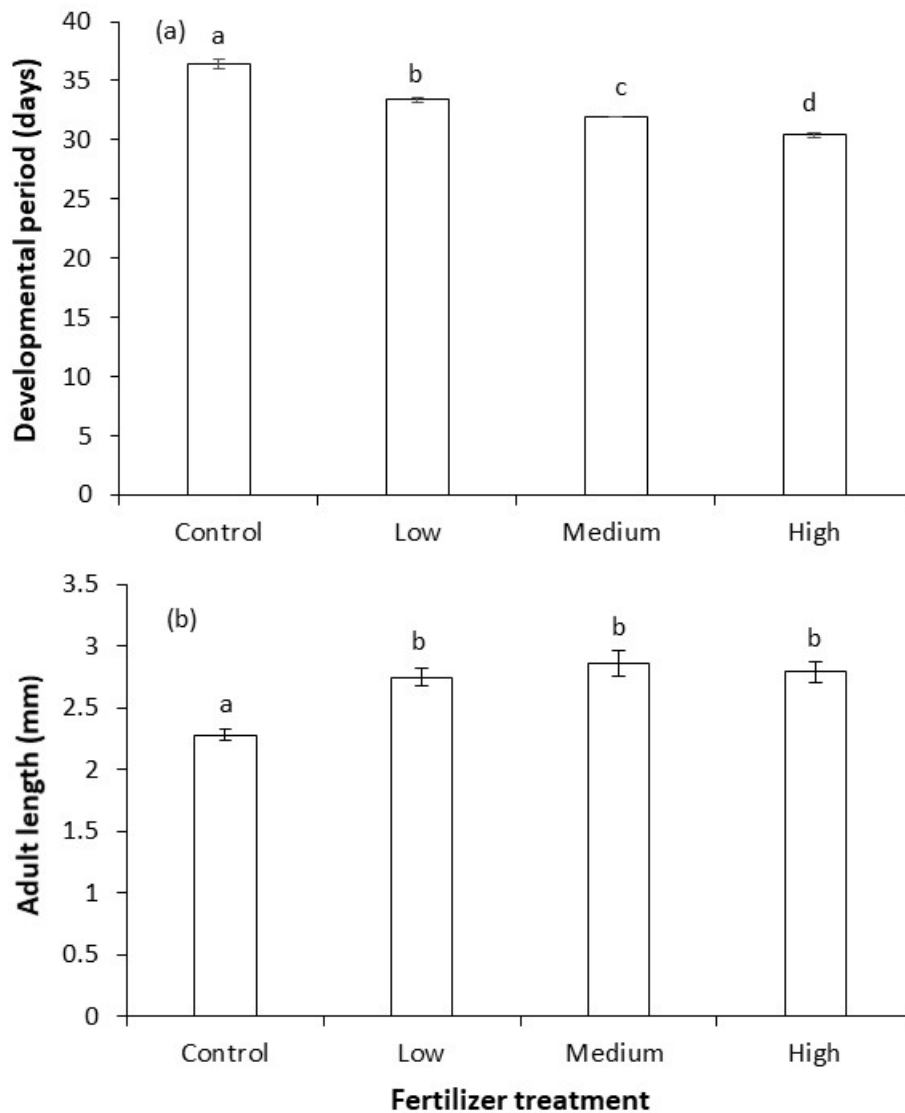
Fig. 3. Mean ($\pm$ SE) (a) developmental period and (b) body length of F_1 adults of *Heikertingerella* sp. reared on *Tecoma stans* plants subjected to different fertilizer treatments. Bars with different letters are significantly different (P < 0.05).

Effect of fertilizer on plant biomass

Fertilizer application had a significant and positive effect on the accumulation of above-ground ($\chi^2 = 572.826$; df = 3; P < 0.0005) and below-ground ($\chi^2 = 263.601$; df = 3; P < 0.0005) biomass of the *T. stans* plants. All fertilizer treatments were significantly different to the control, and to each other, with mean above-ground biomass increasing by 55%, 90% and 127% (Fig. 4a) and

below-ground biomass increasing by 30%, 53% and 125% (Fig. 4b), relative to the control, in

the low, medium and high treatments, respectively.

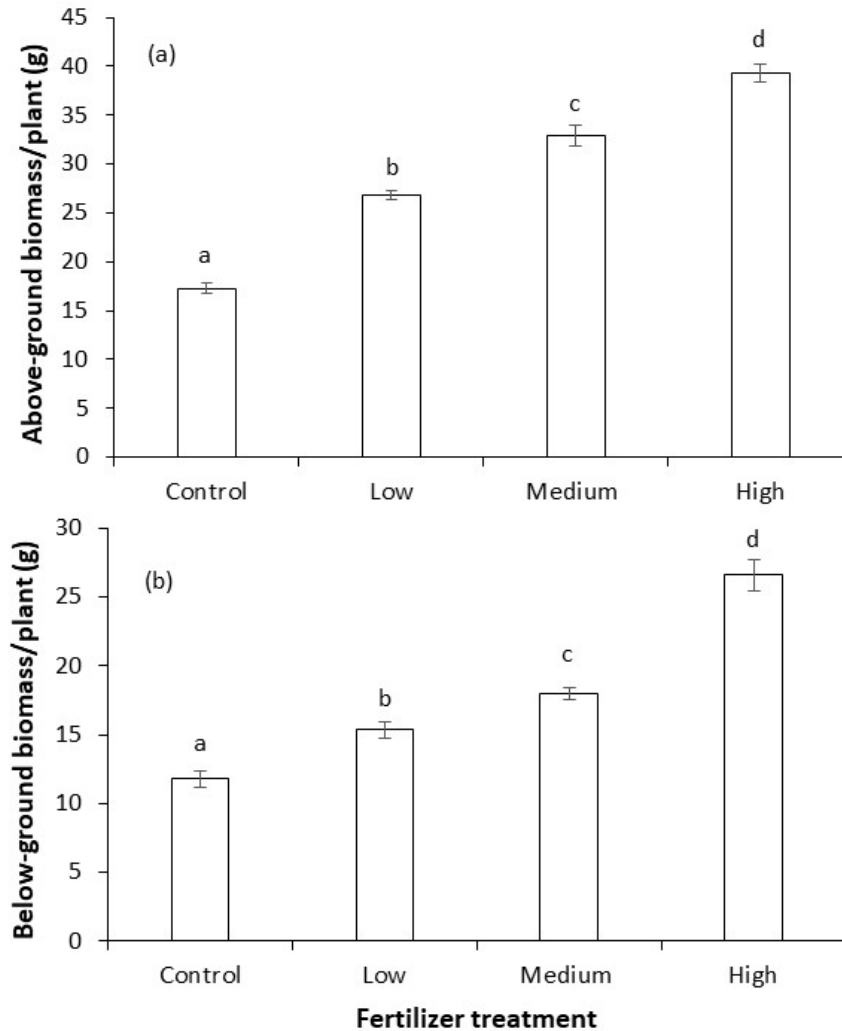**Figure 4.** Mean (± SE) (a) above-ground and (b) below-ground biomass of *Tecoma stans* plants

subjected to different fertilizer treatments. Bars with different letters are significantly different

(P < 0.05).

Discussion

The performance of *Heikertingerella* sp. was improved substantially when reared on fertilizer-

treated *T. stans* plants compared to untreated controls, presumably as a result of enhanced host-

plant quality. Higher plant quality directly increases the feeding, survival and development of herbivorous insect larvae, which extends to adult traits such as increased size, reproductive ability and longevity (e.g., Albert & Bauce, 1994; Leather, 1994; Dodds et al., 1996, Tammaru, 1998; Campos et al., 2003).

Newly emerged P_1 adults displayed variable levels of survival during the 30-day exposure period, in all fertilizer treatments including the controls, which was surprising given their average longevity of 76 days (Madire et al., 2021a). While percentage survival in the medium fertilizer treatment was 26% higher than in the control, survival in the high fertilizer treatment was 36% lower. Although the latter result was unexpected, higher nutrient content may not always benefit herbivorous insects. For example, Hancock et al. (2013) reported a decrease in the abundance of the weevils *Mecinus labilis* Herbst and *M. pascuorum* Gyllenhal (Coleoptera: Curculionidae), on *Plantago lanceolata* L. (Plantaginaceae) after fertilizer application. Also, increased nitrogen levels decreased the size and viability of the locust *Oedaleus decorus asiaticus* Bei Bienko (Orthoptera: Acrididae) (Cease et al., 2012) and caused higher larval and pupal mortality, and a reduction in adult size, of the butterfly *Lycaena tityrus* (Lepidoptera: Lycaenidae) (Fischer & Fiedler, 2000).

It is possible that the high fertilizer treatment may have increased the biosynthesis and accumulation of secondary metabolites (Yang et al., 2018), which reduced the longevity of the P_1 adults of *Heikertingerella* sp. Indeed, phytochemical studies on *T. stans* have reported the presence of several primary and secondary plant metabolites, including alkaloids (e.g. tecomanine), iridoid glycosides, and naphthoquinones (e.g. lapachol) (Havsteen, 2002; Larbie et al., 2019), which serve as defences against phytophagous herbivores and pathogens (Khare et al., 2020; Mrid et al., 2021; Divekar et al. 2022). However, a simpler explanation could be that substantially higher oviposition and progeny production in the high fertilizer treatments

(see below) could have incurred physiological costs in the P_1 adults, leading to reduced longevity. Nevertheless, our study demonstrated that fertilizer application had an increasingly positive effect on all of the remaining insect-related parameters investigated (see below).

The developmental period of the immature stages of *Heikertingerella* sp. was significantly reduced on plants treated with fertilizer compared to the controls. According to the "slow growth - high mortality" hypothesis, quicker development is advantageous as it reduces mortality from natural enemies, unfavourable environmental conditions and other factors, by limiting exposure to these (Benrey & Denno, 1997; Williams, 1999; Fordyce & Shapiro, 2003). Regarding other weed biocontrol agents, high nutrient treatments in *Schinus terebinthifolius* Raddi (Anacardiaceae) caused quicker development of *Episimus unguiculus* Clarke (Lepidoptera: Tortricidae) (Manrique et al., 2009), while *Parthenium hysterophorus* L. (Asteraceae: Heliantheae) plants subjected to high and medium nutrient treatments facilitated faster larval development of *Zygogramma bicolorata* Pallister (Coleoptera: Chrysomelidae) (Cowie et al., 2019).

The production of F_1 progeny increased significantly at all fertilizer application rates, particularly at the high rate where 3.3 times more progeny were produced than in the control. This occurred despite the decrease in P_1 adult survival at the high fertilizer rate. The substantial improvement in root development (i.e. increased biomass) with increased fertilizer application was presumably responsible for increased larval survival and higher F_1 progeny production. Our results are consistent with other studies on coleopteran biocontrol agents, where nutritional enhancement of *Cynoglossum officinale* L. (Boraginaceae) plants significantly increased progeny production in *Mogulones cruciger* Herbst (Curculionidae) (van Hezewijk et al., 2008), with the same trend recorded with Z. *bicolorata* on *P. hysterophorus* (Cowie et al., 2019).

Our study also revealed a significant increase in the size (body length) of adult F_1 progeny in the three fertilizer treatments relative to the control. These results are consistent with those of Cowie et al. (2019) who reported an increase in the body size of female *Z. bicolorata* fed on vigorously growing and high quality *P. hysterophorus* plants. Similarly, Uyi et al. (2016) reported that caterpillars of *Pareuchaetes insulata* (Lepidoptera: Erebidae), a biocontrol agent of *Chromolaena odorata* L. (Asteraceae), increased in size when fed on leaves produced under medium and high fertilizer treatments. Furthermore, Rashid et al. (2017) reported an increase in the size of the brown planthopper, *Nilaparvata lugens* Stal (Hemiptera: Delphacidae) adults when higher nitrogen doses were applied onto potted rice plants.

P_1 adults of *Heikertingerella* sp. displayed progressively higher levels of feeding damage on the leaves of plants treated with increasing levels of fertilizer, presumably because of increased nutrient levels but also because of increased new leaf production or an interaction between these factors. Rashid et al. (2017) also recorded that nitrogen-rich rice plants stimulated higher levels of feeding by *N. lugens*, with higher levels of feeding damage. Phytophagous insects typically respond rapidly to different fertilizer treatments by either increasing or decreasing their feeding (White, 1993; Awmack & Leather, 2002), depending on plant resource allocation and insect nutritional requirements (Maschinski & Whitham, 1989).

The progressive increase in above- and below-ground biomass in fertilizer-treated *T. stans* plants improved the performance of *Heikertingerella* sp. in terms of higher P_1 adult feeding, shorter larval developmental periods and an increased production of larger F_1 adult progeny. The fertilizer treatments improved root growth, which subsequently increased food resource availability for the developing larvae (Shah, 2017; Bala et al., 2018). Pierce et al. (2001) reported that high levels of nitrogen increased the height of Bt cotton plants, while Uyi et al. (2016) reported that *C. odorata* plants receiving medium and high fertilizer treatments had

longer shoots, increased basal stem diameter and higher plant biomass compared to low fertilizer treatments. Increased nitrogen levels are generally associated with improved plant quality (Hinz & Muller-Scharer, 2000; Moran & Goolsby, 2014; Uyi et al., 2016), which are likely to increase the survival of *Heikertingerella* sp. larvae while compensating for increased leaf damage by the P_1 adults (Teetes, 1980; Listinger, 1993).

In conclusion, our results support the contention that fertilizer applications substantially improve the performance of weed biocontrol agents, by increasing the nutritional status of their host plants (e.g., Heard & Winterton, 2000; Manrique et al., 2009; Bownes et al., 2013; Uyi et al., 2016; Cowie et al., 2019). In particular, applications of high and medium rates of NPK + C fertilizer on potted plants can increase the production of *Heikertingerella* sp. adults in mass-rearing facilities and provide high numbers for field releases. This can be enhanced by using plants of optimal age (i.e., three or more years) that have high root volumes (Madire et al., 2023). Albeit speculative, there is also the possibility that the addition of fertilizer to small populations of *T. stans* at selected field sites or biocontrol reserves could facilitate the beetle's establishment and population proliferation.

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CHAPTER 7

General discussion and conclusions

Biological attributes and suitability of *Heikertingerella* sp. as a biocontrol agent

Biocontrol practitioners are acutely aware of the dual expectations placed on them to achieve successful control of alien invasive plants while avoiding damage to non-target plants. The current pre-release studies on *Heikertingerella* sp. were carried out to demonstrate that the beetle is not only safe for release, but will be able to thrive under differential environmental conditions in South Africa, while inflicting sufficient damage on the target weed that will contribute to its management. The results of the biology and host range studies of *Heikertingerella* sp. (Chapter 2) showed that the flea beetle is safe, has a short lifecycle and is capable of producing four generations per year under laboratory conditions. Both larval and adult stages of the beetle cause severe feeding damage to the plant, suggesting that it can potentially reduce the weed's invasiveness in the field. Host specificity testing has also clearly demonstrated that *Heikertingerella* sp. is suitably host specific to *T. stans* and is unlikely to pose any threat to non-target plant species in South Africa. Indeed, *Heikertingerella* sp. was approved for release in South Africa in March 2022, following rigorous scrutiny of the release application by the Biological Control Review Committee (BCRC) of the Department of Agriculture, Land Reform and Rural Development (DALRRD). The BCRC has developed and implemented national guidelines for assessing the potential risks associated with the release of biocontrol agents in South Africa (Ivey et al., 2021).

The agent competition study (Chapter 3) also showed that simultaneous release of *Heikertingerella* sp. and the established lady beetle *M. polluta* should complement the biocontrol of *T. stans*. Despite the antagonistic interaction between *Heikertingerell*a sp. and *M. polluta*, which was manifested in the reduction of their reproductive successes, the combined

effect of these agents on *T. stans* was additive. For example, the two beetle species had a greater impact on leaf density reduction when combined than when confined to the plants individually, and yet there was a reduction in their F_1 progeny production. The additive impact of *Heikertingerella* sp. and *M. polluta* implies that releases of both species as biocontrol agents could be more effective than the release of a single species. As argued by April et al. (2021), spatial and temporal partitioning of food resources under field conditions is likely to further mitigate competitive interactions, while enhancing additive and synergistic interactions between the two beetle species. The outcome of the competition study supports the notion that weed biocontrol success rates improve with releases of multiple agents (Denoth et al., 2002; Seastedt et al., 2007; Schwarzlander et al., 2018). These outcomes, together with the host-range results, further demonstrate that *Heikertingerella* sp. is suitable for release as a biocontrol agent against *T. stans* in South Africa and possibly elsewhere in the world. Like other subterranean species, *Heikertingerella* sp. could be particularly effective in controlling *T. stans* given that the majority of woody plant biomass is allocated belowground (DiTommaso et al., 2005; Milbrath & Nechols, 2014). Also, the direct effect of *Heikertingerella* sp. larvae on water and nutrient uptake (Blossey & Hunt-Joshi, 2003), and the suppression of plant growth by both *M. polluta* and *Heikertingerell*a sp. (Chapter 3) render the two beetle species potentially effective in controlling *T. stans*.

Prediction of efficacy, establishment and distribution of *Heikertingerella* sp.

Pre-release efficacy assessments are often conducted in laboratories and glasshouses, or in the field in the weed's native range, to predict the impact of candidate agents on individual plants or populations (Holt & Hochberg, 2001; Pearson & Callaway, 2003; McClay & Balciunas, 2005; van Klinken & Raghu, 2006; Raghu et al. 2006; Conrad & Dhileepan 2007; Morin et al., 2009). Furthermore, these studies should facilitate agent selection based on their quantified

impact, thereby increasing the chances of releasing effective agents (Sheppard, 2003; Balciunas, 2004; Gerber et al., 2008). The efficacy study predicted that *Heikertingerella* sp. is likely to establish and inflict substantial damage on the target weed in South Africa (Chapter 4). Both low and high population levels of *Heikertingerella* sp. were able to reduce the growth and biomass accumulation of *T. stans* plants. Therefore, this suggests that even lower numbers of the beetle could be able to exert considerable pressure on *T. stans* populations in the field.

In addition, the Maximum Entropy (MaxEnt) species distribution model, together with the beetle's critical thermal limits, were used to predict the potential distribution of *Heikertingerella* sp. in South Africa (Chapter 4). MaxEnt modelling predicted that the southern and east coast of South Africa, where the weed is most prevalent (Henderson, 2021), is climatically suitable for *Heikertingerella* sp. Therefore, the selection of initial release sites should focus along the coastal regions of South Africa. These results are consistent with thermal physiology studies, which demonstrated that the beetle should overwinter in the eastern region of the country, but is likely to be poorly adapted to extreme cold and hot conditions, which are prevalent in the Free State and Northern Cape provinces, respectively. However, neither of the Free State or Northern Cape provinces are presently invaded by *T. stans*.

Overall, *Heikertingerella* sp. is predicted to have a significant impact on the growth and biomass accumulation of *T. stans* populations in areas that are most suitable for its establishment. The results of this study, together with the beetle's very high level of host specificity (Chapter 2), strongly supported the first release of *Heikertingerella* sp. at East London in the Eastern Cape Province of South Africa in early 2023.

Importance of plant quality on the performance of *Heikertingerella* sp.

Variations in the quality and quantity of host plants can influence many aspects of a phytophagous insect's life history at both individual and population scales (Awmack & Leather,

2002). For example, weed biocontrol agents are strongly constrained by the physiological suitability and nutritional value of their host plants (Price, 2000). Since *Heikertingerella* sp. is currently being mass-reared for initial releases, determining an optimal host-plant quality is essential for improving the chances of its establishment in the field. In separate studies, the effects of host-plant age on the performance of *Heikertingerella* sp. and the effects of increasing nutrient levels (fertilizer treatments) on the growth and biomass of *T. stans* plants, and on the subsequent performance of *Heikertingerella* sp., were determined.

The host-plant age study revealed that while the feeding and survival of *Heikertingerella* sp. adults was not affected by the age of *T. stans* plants, progressively older plants supported substantially higher levels of progeny production (Chapter 5). The study also confirmed that, irrespective of plant age, younger leaves in the top sections of *T. stans* plants were preferred for feeding by adult *Heikertingerella* sp., relative to leaves in the lower sections. According to Wang et al. (2018), older plants have greater ability to tolerate herbivore damage because of developmental changes to plant architecture, storage capacity and resource allocation, compared to younger plants that have lesser capacity to compensate for herbivore damage. Price (2000) argued that food of high quality is a necessity for every individual insect herbivore species because of its overwhelming impact on the distribution, abundance and demography of insect populations in regulating plant populations.

The effects of increasing nutrient levels (fertilizer treatments) on the growth and biomass of *T. stans* plants and on the subsequent performance of *Heikertingerella* sp. were further assessed (Chapter 6). Increasing soil nutrient levels (i.e. concentrations of nitrogen (N), phosphorus (P), potassium (K) and Carbon (C)) substantially improved the performance of *Heikertingerella* sp., presumably because of enhanced host-plant quality. This suggests that plants in the mass-rearing facilities need to be manipulated using fertilizer to produce higher

adult numbers for releases, in order to increase the chances of establishment of *Heikertingerella* sp. at release sites.

Higher plant quality directly increases the feeding, survival and development of herbivorous insect larvae, which extends to adult traits such as increased body size, reproductive ability and longevity (Albert & Bauce, 1994; Leather, 1994; Dodds et al., 1996; Tammaru, 1998; Campos et al., 2003). Similarly, host-plant quality can affect the establishment, survival and population growth rates of biocontrol agents at field release sites (Bownes et al., 2013; Cowie et al., 2019). Although food of high quality is likely to be limiting in the field, because rapid growth is usually seasonally brief, optimal sites for plant growth are often scattered over a landscape, and physiological aging in plants typically lowers plant vigour and quality through time (Price, 2000), massive infestations of *T. stans* in South Africa should ensure a continuous supply of food resources within growing seasons and across seasons (Carpenter & Cappuccino, 2005).

Conclusions and recommendations

1. The biology and host specificity studies have demonstrated that *Heikertingerella* sp. is safe for release as a biocontrol agent against *T. stans* in South Africa and possibly elsewhere in the world, subject to the completion of similar studies as conducted in South Africa. *Heikertingerella* sp. was therefore approved for release in South Africa in March 2022, following rigorous scrutiny of the release application by the BCRC of the DALRRD.

2. Despite the antagonistic interaction between *Heikertingerell*a sp. and the established beetle *M. polluta*, the combined effect of these agents on *T. stans* was additive. The additive impact of *Heikertingerella* sp. and *M. polluta* implies that simultaneous releases of both species will complement the biocontrol programme against *T. stans.*

3. *Heikertingerella* sp. is predicted to have a significant impact on the growth and biomass accumulation of *T. stans* populations in areas that are most suitable for its establishment. In particular, the coastal regions of South Africa that are severely invaded by *T. stans* are best suited for the beetle, while the dry hotter and more inland regions are likely to constrain its efficacy. The results of this study, together with the beetle's very high level of host specificity, supported the release of *Heikertingerella* sp. in South Africa.

4. This study has also provided important insights into the effect of host-plant age on the performance of *Heikertingerella* sp. *Tecoma stans* plants of all ages tested (i.e., 1-3 years) are suitable for sustaining laboratory cultures of *Heikertingerell*a sp., but progressively older plants are best suited for the purposes of mass-rearing to facilitate field releases. It seems plausible that even older and larger *T. stans* plants (e.g., 4-5 years) with higher root volumes will be able to boost the numbers of *Heikertingerella* sp. adults for field releases. These results may also guide the selection of release sites. In particular, old stands of *T. stans* (i.e., with large and well-developed root systems), in which some plants are pruned to stimulate new leaf growth, could provide optimal release sites.

5. This study also supports the contention that fertilizer applications substantially improve the performance of weed biocontrol agents, by increasing the nutritional status of their host plants. In particular, applications of high and medium rates of NPK + C fertilizer on potted plants can increase the production of *Heikertingerella* sp. adults in mass-rearing facilities and provide high numbers for field releases. This can be enhanced by using plants of optimal age (i.e., three or more years) that have high root volumes.

6. The outcomes of these pre-release studies have demonstrated that *Heikertingerella* sp. is suitable for release as a biocontrol agent in South Africa. However, post-release evaluations should be conducted in the field to verify the predictions of these laboratory

trials, notably those regarding establishment success, distribution, interactions between different agents and impact on weed populations.

7. Since *T. stans* populations continue to increase yearly in South Africa, a suite of biocontrol agents is required to control the weed. To increase the chances of suppressing *T. stans* invasions, additional surveys in the native region of *T. stans* should be conducted to identify natural enemies that target other niches such as flowers, seeds and stems. In addition, surveys in the colder regions of the native range can identify candidate agent species that are more tolerant of colder conditions and thus better suited for the inland areas of South Africa. The need for additional agents will be considered following post-release evaluations on agents already established.

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