Evaluating different biotypes of *Dactylopius opuntiae* and *Dactylopius confusus* (cochineals) for biological control of the invasive cactus *Opuntia engelmannii* in Kenya and South Africa

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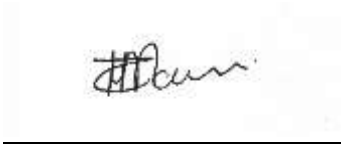
This dissertation is submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa, in fulfillment of the requirements for the degree of Master of Science in the School of Animal, Plant and Environmental Sciences.

23rd April, 2021

DECLARATION

I, Zanele Hariett Machimane, declare that this Dissertation is my own work. It is submitted for the degree Master of Science at the University of the Witwatersrand. It has not been presented before for any degree or examination to any other University.



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DEDICATION

"With your help I can advance against a troop; with my God I can scale a wall."

(II Samuel 22:30, NIV)

This dissertation is dedicated to the wonderful memory of my aunt Mavis Khosa who lost her battle to cancer on the 27th of July 2019. We walked a myriad of journeys together that made me to be a resilient person today. May your soul continue to rest in eternal peace. It is also a heartfelt tribute to all the families who lost their loved ones to cancer and all other chronic illness around the world.

I further dedicate this work to my mother (Maria Machimane), father (Patrick Machimane), brothers (Rodney, Christopher, Guy and Nkateko Machimane), sisters (Fikile, Ingrid, Lorraine and Zandile) and aunt Mamayila Mongwe.

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"Fear not, for I am with you; be not dismayed, for I am your God; I will strengthen you, I will help you, I will uphold you with my righteous right hand" (Isaiah 41: 10, ESV)

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"If I have seen further than others, it is by standing upon the shoulders of giants."

(Isaac Newton)

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LIST OF TERMS and ABBREVIATIONS

ANOVA- Analysis of Variance

Areole - Small spherical or elongated woolly cushion area on the surface of a cactus plant from which spines emerge.

Best suited biocontrol agent - An agent that is host-specific and can bring about complete control of the host plant.

Biotype - A population within a species of arthropods that differ in their ability to cause damage to their host or differ in their capacity to use specific traits within a plant genotype or phenotype when identifying a host.

Cladode - Modified stems or vegetative part of the cactus plant which replaces the leaves in their photosynthetic performance.

Complete control - No other control measures are required to reduce the weed density to an acceptable level.

Crawlers - Refers to a mobile nymph of a cochineal insect.

DEA– Department of Environmental Affairs

EC - Eastern Cape

Effective biocontrol agent - One which via its effects on a host plant causes rapid mortality.

FI- Fitness index

Enemy release hypothesis (ERH) - Suggests that the success of invading alien species in the introduced range is attributed to the fact that they are completely released from their specialised predators in their native range, enabling them to invest resources into reproduction and growth, thus allowing them to rapidly increase in abundance and distribution.

Establishment -The ability of the cochineal insect to be able to settle and reproduce on its host plant.

Enemy tolerance - Suggests that failure of invasive plants to escape all their enemies forces the plants to maintain fitness by becoming tolerant to the damage caused by their enemies.

Glochids - Small, removable pointed bristles.

Gravid female - Females carrying eggs or young.

Induced responses - Changes in plant defences subsequent to stress or damage.

IAPs- Invasive alien plant species

ISSRs- Inter Simple Sequence Repeats

Lineage - A population or species that shares a common ancestor with another lineage but may have evolved into a separate gene pool overtime. In this study, the term will be used to address cochineal insect population.

LM- Limpopo

mg-milligram

Morphology - The physical form or structure of an organism.

NC- Northern Cape

NEM:BA- National Environmental Management: Biodiversity Act

New association - The introduction of natural enemies against alien invasive species in which they have not co-evolved together and therefore the invasive alien species do not have defense mechanisms against these enemies.

Old Association - The introduction of natural enemies against alien invasive species which have co-evolved together.

Resistance - The capacity of plants to reduce the performance of their enemies (Karban and Baldwin, 1997; Van der Putten, 2003).

Settling - The ability of female cochineals to feed and produce a white waxy covering on their host plants.

Species - A group of living organisms that are closely related constituting similar individuals that are capable of interbreeding.

Suitability - Refers to an appropriate cactus (in this study) that the biocontrol agent is able to settle on, complete its development stages, survive and produce offspring and eventually cause damage to its host plant.

Substantial damage - A measure of success in terms of the damage or impact caused by the biocontrol agent, whereby other methods of controlling the weed density are needed, but with less effort required since the infestation or density of the targeted weed would have been reduced.

Tolerance - The ability of plants to regrow and reproduce following herbivory damage and may also allow the insect herbivores to develop and reproduce (Strauss and Agrawal, 1999; Van der Putten, 2003).

USA-United States of America

ABSTRACT

Opuntia engelmannii Salm-Dyck (Cactaceae) is an invasive cactus in Kenya and South Africa. Furthermore, it has become a priority for biological control in South Africa as it is NEM:BA category 1B invader, therefore it must be controlled or removed. *Opuntia engelmannii* varieties are found in different geographic locations in South Africa and Kenya and they vary in their morphology and genetic make-up. Therefore, the term 'lineage' was used in this study based on where they are currently found in Africa. Three of these lineages are found in the Eastern Cape (EC), Northern Cape (NC) and Limpopo (LM) province of South Africa. A fourth *O. engelmannii* lineage is found in Kenya. Biocontrol of *Opuntias* using cochineal insects has an excellent track record. However, success against *O. engelmannii* in Kenya and South Africa has not been achieved because of lack of knowledge on the exact origin of the plant. Therefore, this study aimed at finding a suitable biocontrol agent for the Kenyan and South African (EC) *O. engelmannii* lineages. Classical biocontrol is a preferred method compared to other control measures (e.g., chemical and mechanical control) because it is cost-effective, efficient and sustainable. This study investigated which cochineal population (biotype) of six *Dactylopius opuntiae* (Cockerell) (Hemiptera; Dactylopiidae) and four *Dactylopius confusus* (Cockerell) (Hemiptera; Dactylopiidae) collected from geographically separate lineages of *O. engelmannii* in the United States of America (USA) would perform best on lineages of *O. engelmannii* collected from Kenya and EC Province of South Africa. Each locality where the *Dactylopius* biotypes were collected in the USA was described as a 'site'. Host plant acceptability and suitability of the Kenyan and EC *O. engelmannii* lineages to the 10 *Dactylopius* biotypes were investigated. This was done by measuring the proportion of settling female cochineals on both Kenyan and EC detached cladodes of *O. engelmannii*. Then development and fecundity of the insects was assessed by measuring fitness indices." Results from this study revealed that five (sites 5, 13, 20, 21 and 37) out of the six *D. opuntiae* biotypes settled and developed into gravid females (caring eggs) on detached cladodes of Kenyan *O. engelmannii* lineage and only one (site 23P1) out of the four *D. confusus* biotypes were able to settle and develop into gravid females on the Kenyan *O. engelmannii* lineage. While all populations of both *D. opuntiae* and *D. confusus* settled and developed into gravid females on the EC lineage of *O. engelmannii*. These results described the host plant acceptability of the Kenyan and EC *O. engelmannii* lineage to the 10 *Dactylopius* biotypes. The host plant suitability was assessed by the fitness index. The fitness index (FI), calculated as the proportion of adult females multiplied by the

average number of offspring per female over mean developmental time, show that the EC *O. engelmannii* lineage was more susceptible to both species of *Dactylopius* compared to the Kenyan *O. engelmannii* lineage, where only four (sites 5, 13, 20 and 21) of the six *D. opuntiae* had high fitness index (FI>1) and thus showing a greatest potential to control the Kenyan *O. engelmannii* lineage. No *D. confusus* biotypes had particularly high FI on the Kenyan *O. engelmannii* lineage and thus performed poorly on the Kenyan lineage of *O. engelmannii*. The four *Dactylopius* biotypes with the highest fitness indices were selected for a whole, potted-plant experiment, to assess their impact on plant health. Chlorophyll content, cladode thickness, percentage cover of cochineals and biomass were recorded. All four *D. opuntiae* biotypes (sites 5, 13, 20 and 21) eventually killed the whole plants. However, *D. opuntiae* biotype from site 21 was the most effective biocontrol agent, due to its rapid reduction of plant chlorophyll content of the Kenyan *O. engelmannii* lineage. The biotypes that performed very well on the Kenyan lineage of *O. engelmannii* were biotypes coming from *O. engelmannii* lineages that were morphologically similar to the Kenyan *O. engelmannii* lineage. Geographically, these biotypes were from the same region, south of Texas. This could indicate an old association. The best performing biotypes on the Eastern Cape lineage of *O. engelmannii* are from *O. engelmannii* lineages without the morphological characteristics of the Eastern Cape *O. engelmannii* lineage. This could have been a new association because the Eastern Cape *O. engelmannii* lineage appeared to be a different *Opuntia* species. The Eastern Cape *O. engelmannii* was accepted by all the *D. confusus* biotypes, However, *D. confusus* biotypes were not as damaging as the *D. opuntiae* biotypes on the Eastern Cape *O. engelmannii* lineage. The Kenyan *O. engelmannii* lineage was not a suitable host for any *D. confusus* biotype. Failures and successes of *Dactylopius* biotypes to thrive on their hosts were explained by several suggested hypotheses, including plant defence mechanisms. Findings from this study have important implications for the biological control of *Opuntia*s in Africa.

Keywords: Biocontrol, cacti invasion, lineages, new association, old association, species.

1.1. Introduction

The intentional and accidental introduction of species far from their native ranges has been facilitated by the increased movement of people around the world (Wilson *et al*., 2009; Novoa *et al*., 2015; Richardson *et al*., 2020). A significant number of these organism have notable benefits to humans, yet some have undesirable effects that can bring about significant economic losses as well as changes to the entire ecosystems and social frameworks (McNeely, 2006; Kumschick *et al*., 2012; Novoa *et al*., 2016; Reynolds *et al*., 2020). The family Cactaceae consist of about 1,600 species which are mainly native to North, South and Central Americas (Zimmermann *et al*., 2009; Novoa *et al*., 2015). Many of these species, including *Opuntia engelmannii*, have become invasive in Africa and the world (Volchansky *et al*., 1999; Witt *et al*., 2020). Cacti have sharp spines and numerous tiny glochids which can injure and irritate people, livestock, and wild animals, particularly herbivores (Figure 1.1). Large infestations of the plants also limit the movement of grazing animals (Klein *et al*., 2014) (Figure 1.2). Cactus species are succulent plants that are adapted to survive over a wide range of environments (Cota-Sánchez, 2002). Because of their unique morphological attributes, many of these species are now cultivated mostly as curiosities and ornamentals (Novoa *et al*., 2015; Witt *et al*., 2020). Some cactus species produce edible fruits and are used as fodder, and for commercial purposes (Inglese, 2010; Shackleton *et al*., 2017; Mayer and Cushman, 2019). For example, *Opuntia ficus-indica* (L.) Miller is cultivated in many countries for its fruit and as fodder (Barbera *et al*., 1995; Nobel *et al*., 2003; Mayer and Cushman, 2019), but also used as the main host plant for production of the carmine cochineal insect (*Dactylopius coccus* Costa) for the dye industries in certain countries such as Peru, Chile and Bolivia (Flores-Flores and Tekelenburg, 1995; Müller-Maatsch and Gras, 2016). However, these uses cannot compensate for the weed's overall negative impacts. Amongst several invasive cactus plants in Africa, *O. engelmannii* is one of the major terrestrial invaders in the eastern and southern part of Africa, particularly in Kenya and South Africa (Paterson *et al*., 2011; Wilson *et al*., 2017; Githae, 2018; Witt *et al*., 2018, 2020). The main impact of an *O. engelmannii* invasion on the environment is the displacement of native species (Githae, 2018; Witt *et al*., 2020), therefore in South Africa it is listed as a category 1B invader as per the National Environmental Management Biodiversity Act (NEM:BA) regulations of South Africa (DEA, 2014). According to NEM:BA, plants in

this category must be removed and/or destroyed, any form of trade is strictly prohibited (Van Wilgen, *et al*., 2016). *Opuntia engelmannii* has also been listed as a harmful weed to livestock in other parts of Africa (Githae, 2018; Witt *et al*., 2020). There are several control methods that are being sought, of which classical biocontrol is one. Biocontrol involves the introduction and release of host-specific natural enemies to reduce and control the population density of the targeted weeds in the invaded regions (McFadyen, 1998; van Wilgen *et al*., 2020).

Figure 1.1: The glochids on the prickly-pear cause ulceration and bacterial infection in the lips, tongue and palate (disorder known as "pearmouth") (Cole, 2018).

Figure 1.2: A heavy infestation of the *Opuntia engelmannii* lineage in Kenya; injuring people and affecting grazing animals (Source: A Witt; W Cole).

1.2. Motivation

Biocontrol of Cactaceae involves matching cochineal biotypes to specific populations of Cactaceae (McFadyen, 1998). South Africa has a good track record in this regard but there are some considerations which are important. No suitable match has yet been found for *O. engelmannii* in either Kenya or EC. Therefore, a survey of the native distribution of *O. engelmannii* was conducted in the Southwest United States of America during November 2017 to try find a suitable biocontrol agent for each of the two *O. engelmannii* lineages (i.e., Kenya and EC lineages). Ten *Dactylopius* biotypes were collected from *O. engelmannii* at different sites across the Southwest USA (Figure 1.3) and imported into quarantine at Wits University (26°11'29.4"S 28°01'54.6"E), to test and determine which of the ten biotypes are the most effective agents for each of the two target *O. engelmannii* lineages. These ten biotypes were sourced from a geographic distribution across the native range. In addition, biotypes were collected from morphologically diverse lineages of *O. engelmannii* (Figure 1.4). Moreover, both cochineal insects and *O. engelmannii* samples were collected for genetic analysis in order to identify or confirm species names (van Steenderen, 2020). Of the ten population of *Dactylopius*, six (from sites 5, 13, 20, 21, 37 and 66) were found to be *D. opuntiae,* while the remaining four biotypes (from sites 23 P1, 23 P3, 44 and 51) were *D. confusus* (Figure 1.3) (van Steenderen, 2020). In addition, molecular and morphological data from Mbonani, 2019, indicate that the Kenyan and EC *O. engelmannii* are different lineages. These findings have important implications for selection of biocontrol agents for *O. engelmannii* lineages in Africa.

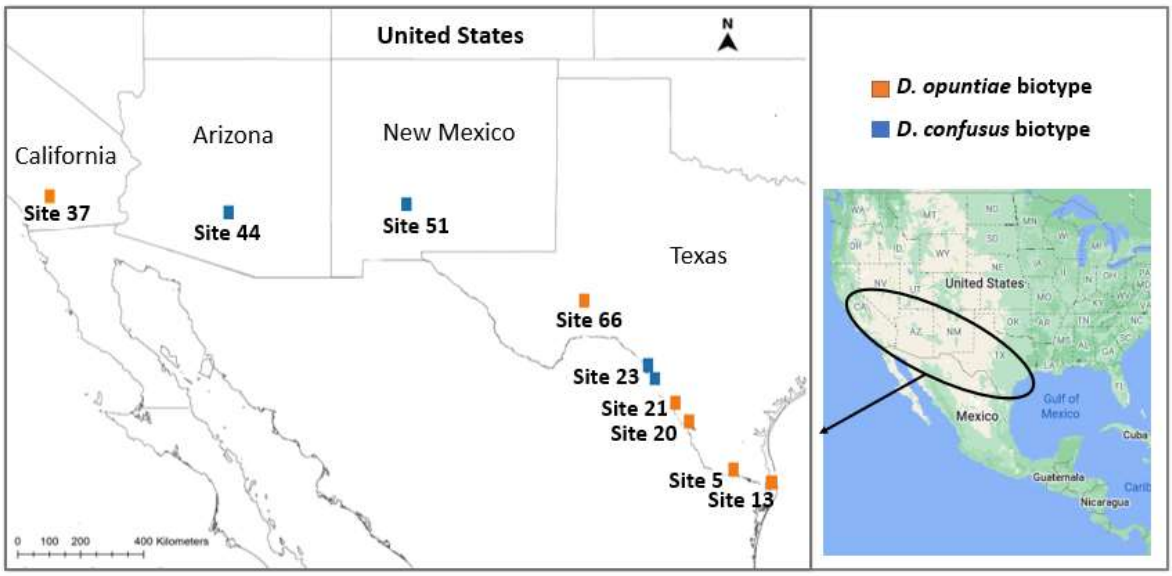

Figure 1.3: Sites at which 10 biotypes of *Dactylopius opuntiae* and *Dactylopius confusus* were sampled during a collection field trip across the Southwest region of United States of America in 2017. Cochineals from sites 5, 13, 20,21, 37 and 66 are *Dactylopius opuntiae* biotypes, while cochineals from sites 23 P1,23 P3, 44 and 51 are *Dactylopius confusus* biotypes (Source: N Venter; C van Steenderen).

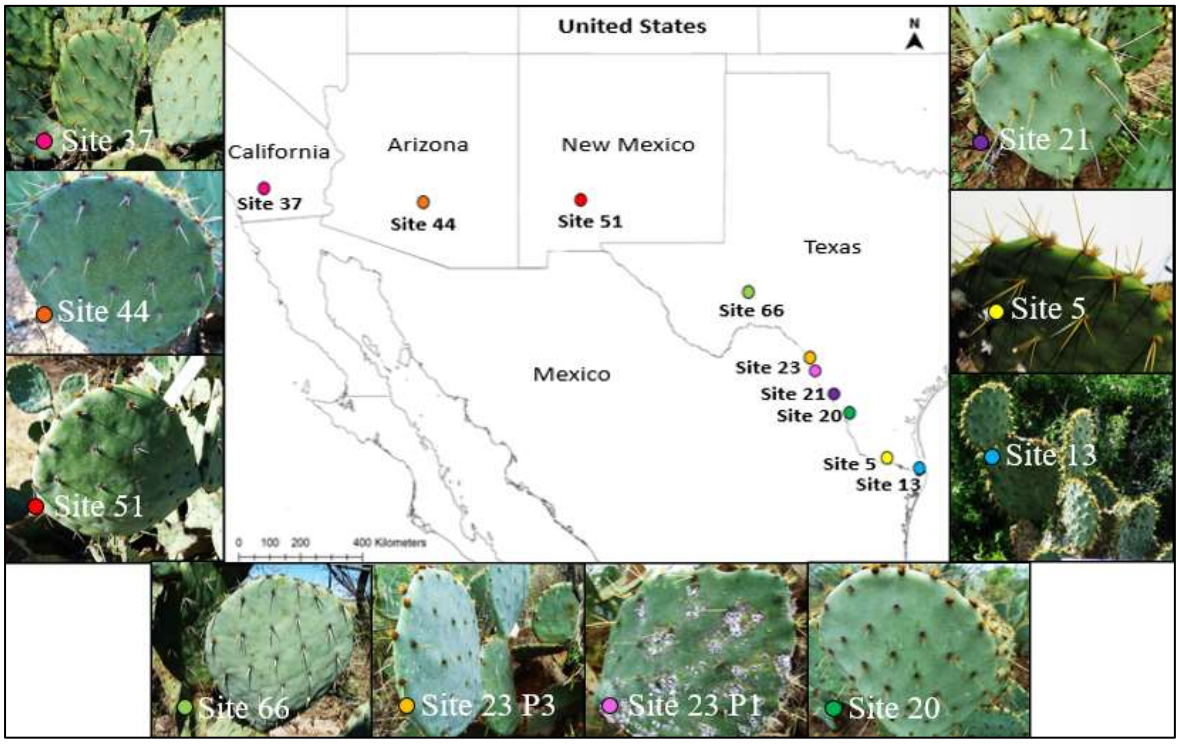

Figure 1.4: Lineages of *Opuntia engelmannii* that were sampled across the Southwest of United States of America in 2017. The 10 lineages shown differ from each other based on their location and morphology. Sites 37, 44, 51 and 66 have white spines, whereas site 21, 23 P3, 23 P1, 13, 20 and 5 have yellow spines with or without brown bases (Source: N venter; ID Paterson).

1.3. LITERATURE REVIEW

1.3.1. Invasive alien plants

Invasive alien plant species (IAPs) are non-native plants that have successfully spread outside their native range (Williamson, 1999; Richardson *et al*., 2000), and have progressively caused significant environmental problems in terrestrial or freshwater environments (Richardson *et al*., 1997; Lindgren *et al*., 2008). Invasive alien plant species alter the ecosystem's structure and/or function in their introduced range, including the composition of species that naturally occur in that environment (Simberloff, 1981; Kourtev *et al*., 2002). The dense infestations of invasive alien plants tend to outcompete native plants (Potter and Rutherford, 2013), as well as introducing other animals and microbes that are not native to the environment (Lovich, 2000). Invasive alien plants can alter the gene pool through hybridization with indigenous species (Riley *et al*., 2003; Kovach *et al*., 2015). Biodiversity is significantly decreased because of reduced rangelands which would otherwise support domestic and wild animals (Richardson and Van Wilgen, 2004; Vavra *et al*., 2007). According to Kotzé *et al*., 2020, IAPs occupy about 5% of the total land surface area on a global scale. In South Africa, an estimated 4% of surface water runoff is consumed by IAPs (Le Maitre *et al*., 2000; Turpie *et al*., 2008; Le Maitre *et al*., 2020), particularly in the Fynbos and Grassland biomes (Turpie *et al*., 2008). The introduction of IAPs is happening at a faster rate as a result of human globalization activities, with international trading being the most predominant facilitator of IAPs (Pimentel, 2011; Faulkner *et al*., 2020; Wilson *et al*., 2020). Invasive alien plant species consistently show harmful effects and pose serious ecological, agricultural and socio-economic challenges (Jeschke *et al.,* 2014; Shackleton *et al*., 2015; Reynolds *et al*., 2020).

Although the invasion of alien plants has been relatively well studied and is increasingly being documented (Richardson *et al*., 1997, Faulkner *et al*., 2020; van Wilgen *et al*., 2020), an important question as to what makes a species invasive and how can it be effectively controlled continues to be a major challenge (Kolar and Lodge, 2001; Hurley *et al*., 2016). There are many hypotheses that have been presented as to what makes an alien species invasive. The Enemy Release Hypothesis (ERH) is one of the most generally accepted theories (Keane and Crawley, 2002; Prior and Hellmann, 2014; Schulz *et al*., 2019). According to the ERH, the invasive alien plant species do not have co-evolved natural enemies to control them in their introduced range, allowing the invasive plant species to invest more resources in reproduction and growth (Keane and Crawley, 2002). Consequently, the invasive alien plants will have an improved competitive ability over the native species, and this might be the reason behind the rapid recruitment and

establishment of the alien species (Maron and Vilà, 2001; Colautii *et al*., 2004). The ERH is based on three principal conditions in the introduced range: i) the lack of specialist enemies in the introduced range, ii) generalist predators are less damaging to the invaders than they are to the native species and iii) specialist predators found on closely related native species are unlikely to 'switch-over' to the invasive alien plant species (Keane and Crawley, 2002; Liu and Stiling, 2006; Cowie, 2016). Although some of these conditions are disputed, the ERH is still accepted and is connected to many other ecological practices and theories (Maron and Vilà, 2001; Schulz *et al*., 2019).

1.3.2. Biological control of invasive alien plants

Biological control, commonly known as biocontrol, is a technique which aims to mitigate and control the population density of invasive species, often weeds, using natural enemies that are host-specific, such as predators, pathogens and parasitoids (McFadyen, 1998). The weeds are called targets, while the natural enemies used in their control are referred to as agents (McFadyen, 1998). Arthropods, mainly insects, are often used as biocontrol agents, given their host-specific nature. Other biocontrol agents include fungi, bacteria, mites and nematodes (Goldson *et al*., 2014). There are three principle techniques of weed biocontrol namely; augmentative, conservation and classical biocontrol (Bellows and Fisher, 1999). Augmentation is a technique which includes all the actions included to artificially increase the numbers or the effectiveness of a biocontrol agent (McFadyen, 1998). Augmentation technique uses mycoherbicides, some insects or grazing animals to control the invasive alien plants (Popay, 1996; McFadyen, 1998). The conservation technique involves the protection or the maintenance of current populations of native biocontrol agents if any (McFadyen, 1998). Classical biocontrol is usually the preferred technique. Classical biocontrol involves the importation of co-evolved monophagous natural enemies from the weed's native range (McFadyen, 1998; Schwarzländer *et al*., 2018). These natural enemies are then tested in quarantine, and if host-specific, mass-reared and released into infested regions (McFadyen, 1998; Bellows and Fisher, 1999). The released agents often establish, spread, attack and suppress the weed's population densities as the agents increase in number under ideal conditions (McFadyen, 1998). Over time, these agent-weed populations can self-regulate, therefore reducing and maintaining the weed's population to an acceptable level (i.e., at a predetermined economic or ecological threshold) (Muniappan *et al*., 2009). For most biological control programmes, it has been assumed that agents collected from their native region could cause damage to plants targeted for control and will therefore be the most appropriate

biological control agents (Gaskin *et al*., 2011). This is often referred to as the use of an 'old association' because the insect and target weed have a long evolutionary history together in the native range (Hokkanen and Pimental, 1984; Stiling, 1990). Classical biocontrol programmes thus use the old association approach for agent selection (Dennill and Hokkanen, 1990; Harris, 1991). However, another approach of selecting agents to control alien invasive plants was advocated by Hokkanen and Pimentel (1984), who suggest that the biocontrol agents should be selected from a close relative of the invasive plant instead of the targeted species. The release of such biocontrol agents on the targeted host plants will establish a new relationship between the agent and the targeted host plant since they would not have co-evolved (Dennill and Moran, 1989; Eilenberg *et al*., 2001; Catton *et al*., 2015; Schulz *et al*., 2019). This is known as new associations (Pimentel, 1991; Eilenberg *et al*., 2001; Schulz *et al*., 2019). An example of new association includes the successful control of *Opuntia stricta* (L.) Mill by *Cactoblastis cactorum* (Pimentel, 1963; Schulz *et al*., 2019). The framework of a classical biocontrol programme consists of six stages namely: (a) Ecology of the targeted weed, (b) the exploration for potential biocontrol agents, (c) evaluation of the biocontrol potential, (d) host-specificity testing, (e) agent release and redistribution and (f) agent post-release evaluation (Figure 1.5).

(a). Ecology of the targeted weed

A comprehensive study of the biology and the ecology of the weed in its introduced range is conducted and if possible, compared with that in its native range (Briese, 2004; Goolsby *et al*., 2006; Poudel *et al*., 2019). This can be very useful in choosing the correct control method and a suitable agent that will reduce the population density of the targeted weed (Müller-Schärer, 2002). Understanding the life history of the weeds can be critical (Denslow and Johnson, 2006; Amarasekare *et al*., 2014). For instance, knowing whether the plant species are perennials or annuals and how they reproduce (e.g., by seeds or vegetatively or both) can assist in selecting the correct type of biocontrol agent (Briese, 2000). Other important information to include is a survey of possible natural enemies, like predators and parasitoids that might attack the weed in its native range (Schärer and Schaffner, 2008; Kenis *et al*., 2019).

(b). The exploration for potential biocontrol agents

The most important aspect in this step is to search for a potential biocontrol agent in the native range of the targeted weed with similar climatic conditions to where the targeted weeds have become invasive (Zachariades *et al*., 2017; Sutton, 2019). This is important because similar climatic conditions will increase the probability of the biocontrol agents establishing, spreading

and eventually persisting over time, thus improving the success of a biocontrol program (Byrne *et al*., 2002; 2004; Cowie *et al*., 2018). Matching of climatic conditions can also be useful in narrowing the search for a biocontrol agent, especially when the geographic distribution of the targeted weed in its native range is broad (Robertson *et al*., 2008). Other important information to consider when searching for a biocontrol agent includes its host range, symptoms or nature of damage to its host, its dispersal capability and associated natural enemies (Schaffner, 2001, Kenis *et al*., 2019).

(c). Evaluation of the biocontrol potential

There are many ways to assess the likely impact of a candidate biocontrol agent. One includes conducting an open-field study of the agents in their native ranges or a closed-field study (e.g., glasshouse or laboratory) in their introduced range (McClay and Balciunas, 2005; Mdlangu, 2010). However, with open-field studies it may be difficult to separate the impact of the agents from biotic and abiotic factors, hence such studies are often conducted over several seasons at many sites across different climatic regions and habitats (Morin *et al*., 2009). The impact of the agents can also be assessed through an estimation of the extent and degree of suppression of the target weed at population level by measuring changes in biomass, number of viable seeds or other propagules produced, reduction in weed density, age structure and their longevity (Hoffmann *et al*., 2019; Hill *et al*., 2020).

(d). Host-specificity testing

The main purpose of host-specificity testing in weed biological control is to determine whether candidate biocontrol agents will attack non-target plants once released in their introduced range (Van Klinken, and Edwards, 2002; Schaffner *et al*., 2018). This involves identifying an agent's physiological host range and its ecological (realized) host range (Marohasy, 1998; Schaffner, 2001; Müller-Schärer and Schaffner, 2008; Hinz *et al*., 2014). The physiological host range of an agent refers to all the host plants on which the agent can successfully complete its life cycle on under artificial (laboratory) conditions in the absence of choice (Marohasy,1998; Briese, 2005; Hinz *et al*., 2014), while an agent's ecological host range includes hosts that are chosen by the agent under natural environmental conditions (Müller-Schärer and Schaffner, 2008; Hinz *et al*., 2014; Musengi, 2018). Before a potential biocontrol agent is selected, researchers would normally have an idea of the host-range of the agent through literature reviews (if available) or through their own observations in the field (Briese, 2000; Kenis *et al*., 2019). Host specificity trial and impact studies are usually conducted in approved quarantine facilities in

the introduced range or may be carried out in the native range (Zilahi-Balogh *et al*., 2002; DeLoach *et al*., 2003). The testing technique usually follows a model called the centrifugal phylogenetic testing procedure (Wapshere, 1974; McEvoy, 1996; Briese, 2000). This procedure involves testing plants that are within the same genus, then within subfamily or tribe, followed by those within same family and lastly those that are gradually less related to the target weed (Briese, 2005). Quarantine testing normally involves placing the biocontrol agents in cages and subjecting them to either choice or no-choice tests (Harley and Forno 1992; Briese, 2000; Murray *et al*., 2010). The no-choice test is the most conservative in that the candidate biocontrol agents are placed in separate cages on individual plant species where they either oviposit, feed or die (Olckers, 2000; Withers *et al*., 2002; Briese and Walker, 2002). However, the results from a no choice test enables us to predict the agent's realized host range in the absence of the target weed (Van Klinken and Edwards, 2002; Hinz *et al*., 2014). The choice test provides the biocontrol agents with more than one potential host species, preventing the induction of hunger stress, which tends to be a common occurrence in no-choice tests (Zwölfer and Harris, 1971; Olckers, 2003). Quantitative measures are often used to identify the agent's responses and thus host specificity (Zwölfer and Harris, 1971; Olckers, 2000). Some of these measures include adult resting or feeding position on the host plant; the choice of oviposition site; adult and/or larval weight gain; number of eggs produced; as well as egg viability (Zwölfer and Harris, 1971; Olckers, 2000, 2004; Cowie *et al*., 2018).

(e). Agent release and redistribution

The initial release and redistribution of biocontrol agents in the field is one of the most critical phases of classical weed biocontrol programmes (Swirepik and Briese, 2000). The numbers of the biocontrol agent are often inadequate for release after rearing in quarantine; hence measures are often needed to make sure that the agent's population is self-sustaining in the field during critical periods (Briese, 2000). Moreover, many highly effective biocontrol agents have relatively slow rates of population increases and/or poor dispersal capabilities (Briese *et al*., 1996; Heimpel and Asplen, 2011). It often requires time for agents to spread through the range of the targeted weeds, but delays add to the costs. Hence, strong emphasis is made on developing effective release and redistribution strategies (Müller-Schärer and Schaffner, 2008). Release strategies of biocontrol agents often differ depending on the agent's life cycle (Briese, 2000; Schärer and Schaffner, 2008). Insect release strategies may involve direct release of adults either in gauze covers on plant parts or in an open field where releases are confined to plants situated in large cages (Harley and Forno 1992). Other strategies can include the

utilization of community group networks (e.g., landholders/landcare, local governments etc.) to ensure rapid distribution of agents (Briese, 2000).

(f). Agent evaluation

The establishment of biocontrol agents in the field is one of the most critical phases of classical weed biocontrol programme (Briese, 2000; Hill *et al*., 2020). Once the agents establish, it is important to precisely evaluate and monitor the population of the agents and its impact on the targeted weeds (McEvoy *et al*., 1991; McFadyen, 1998; Schaffner *et al*., 2020). Ideally, this monitoring should be done at different scales (van Wilgen *et al*., 2012; Hoffmann *et al*., 2019; Schaffner *et al*., 2020). Small-scale monitoring gives a more detailed information about the population dynamics of the agent and its host in the new environment (Dhileepan, 2003) This enables researchers to measure the impact of the agents more accurately (Carson *et al*., 2008; Zachariades *et al*., 2017). However, large-scale monitoring indicates how successful the release programme has been and provides useful information on conditions of the sites, climate and release strategies on establishment of the agents (Blossey and Skinner, 2000; Schärer and Schaffner, 2008; Schaffner *et al*., 2020). In situations where the control levels achieved by biocontrol agents are unsatisfactory or incomplete, evaluations are equally important because the knowledge about reasons for 'failure' of agents to perform, will further assists researchers to develop an understanding of biocontrol and improve on their control strategies (Carson *et al*., 2008; Morin *et al*., 2009; Pliego *et al*., 2011; Hinz *et al*., 2020).

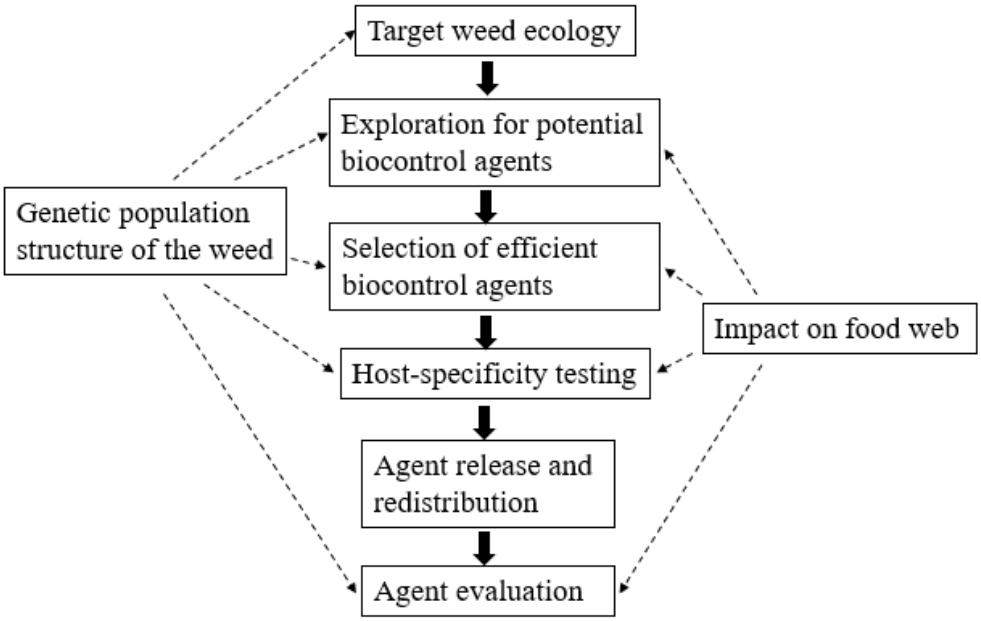

Figure 1.5: The six steps of classical biocontrol (Müller-Schärer and Schaffner, 2008).

1.3.3. Success of biological control of invasive alien plants

The term 'success' is often used to describe the outcomes of a biocontrol programme against IAPS (Syrett *et al*., 2000; Morin *et al*. 2009; Hill *et al*., 2020). However, the term 'success' is subjective and differs between sources (McFadyen, 1998; Carson *et al*., 2008; Schwarzländer *et al*., 2018). Hoffmann (1995) defined success of biocontrol in three categories namely: complete, substantial and negligible. i) Complete control means that no other management techniques are needed, at least in areas where the biocontrol agent is established, ii) substantial control means that other forms of management are still needed to add-on to biocontrol control, but less frequent than before (e.g., less herbicide or less frequent application) and iii) negligible, where despite the damage caused by the biocontrol agents, the control of the weeds is still dependent on other forms of management (McFadyen, 2000). Carson *et al*., (2008) defined a successful biocontrol program as one that will cause enough reduction in the population density of the weeds to allow re-establishment of the native vegetation. In general, the rates of success are expressed as the percentage (%) of agents which established and contribute to a successful control (McFadyen, 1998; Klein, 2011; Zachariades *et al*., 2017). Overall, about 60% of agents establish, with 33% of these resulting in control of the targeted weed (Williamson and Fitter, 1996; McFadyen, 2000). A more recent approach of measuring success of biocontrol against IAPs has been proposed by Hoffmann *et al*., (2019), which is an elaboration of the present

system and is based on conceptual framework that categorizes biocontrol outcomes from noticeable to outstanding results (Hill *et al*., 2020). This system has been currently adopted in South Africa (Hoffmann *et al*., 2019; Hill *et al*., 2020). The success of managing invasive alien plants has been achieved both locally and globally using biocontrol with a success rate of 65. 7% (Schwarzländer *et al*., 2018). In the latest world catalogue of biological control of invasive alien plant species, 65.7% of successful biocontrol have been achieved worldwide (including Africa), with most of the successfully targeted weeds being *Opuntia* (Cactaceae) species (Schwarzländer *et al*., 2018).

1.3.4. Biocontrol of *Opuntia* using *Dactylopius* species

Biocontrol programmes using *Dactylopius* species (cochineal) have been successful against many *Opuntia* species (Hoffmann, 1995; McFadyen, 2000). *Dactylopius opuntiae* and *D. confusus* are amongst the nine species making up the monogeneric, Homoptera family, Dactylopiidae (Volchansky *et al*., 1999; Chávez-Moreno *et al*., 2009). All members feed and reproduce only on cactus plants and almost exclusively on the genus *Opuntia* (De Lotto, 1974; Volchansky *et al*., 1999; Githure *et al*., 1999). These sap-sucking insects are native to North, Central and South America and were introduced into several countries, mainly to Australia, India and African counties to control *Opuntia* species (Foxcroft and Hoffmann 2000; Hoffmann *et al*., 2002; Paterson *et al*., 2011; Spodek *et al*., 2014).

1.3.4.1. *Dactylopius opuntiae*

Amongst the most successful and widely used species of cochineal is *Dactylopius opuntiae*, a sap-sucking scale insect that originates from North, Central and South America (Hoffmann *et al*., 2002; Paterson *et al*., 2011). Female cochineal insects (Figure 1.6) attach to the cladode with their mouthparts and will remain in that position throughout their lives (Mathenge *et al*., 2009a; Melillo, 2014; Dongi *et al*., 2018). They produce a waxy thread covering that provides protection and will continue to develop until they reach maturity at about 50 days (Figure 1.6). After mating with the mobile males, approximately three weeks later they produce large numbers of eggs underneath the white wax which hatch and develop into red coloured nymphs called crawlers (Volchansky *et al*., 1999; Mathenge *et al*., 2009a). Most of the crawlers congregate on the highest point of the cladode where they are dispersed by wind onto new plants (Melillo, 2014). The nymphs undergo two stages of development namely first and second instar, after which the females remain attached to the plant, while the males pupate into short-lived adults with wings (Figure 1.6) (Volchansky *et al*., 1999; Mathenge *et al*., 2009a).

The complete life cycle takes about two to three months. *Dactylopius opuntiae* have successfully contributed to the control of *Opuntia stricta* in Australia and *Opuntia ficus-indica* in South Africa (Volchansky *et al*., 1999; Hoffmann *et al*., 2002; Mathenge *et al*., 2009a), as well as other invasive species of *Opuntia* in other countries (Hoffmann *et al*., 2002; Witt *et al*., 2020). In 1937, *D. opuntiae* was released in South Africa to control *O. ficus-indica* and the biocontrol control program was successful in reducing the density of the weed (Volchansky *et al*., 1999; Hoffmann *et al*., 2002). Given its success in controlling these species, *D. opuntiae* is now being investigated for the control of two lineages of *O. engelmannii*, one occurring in northern Kenya and the other in the EC Province, South Africa.

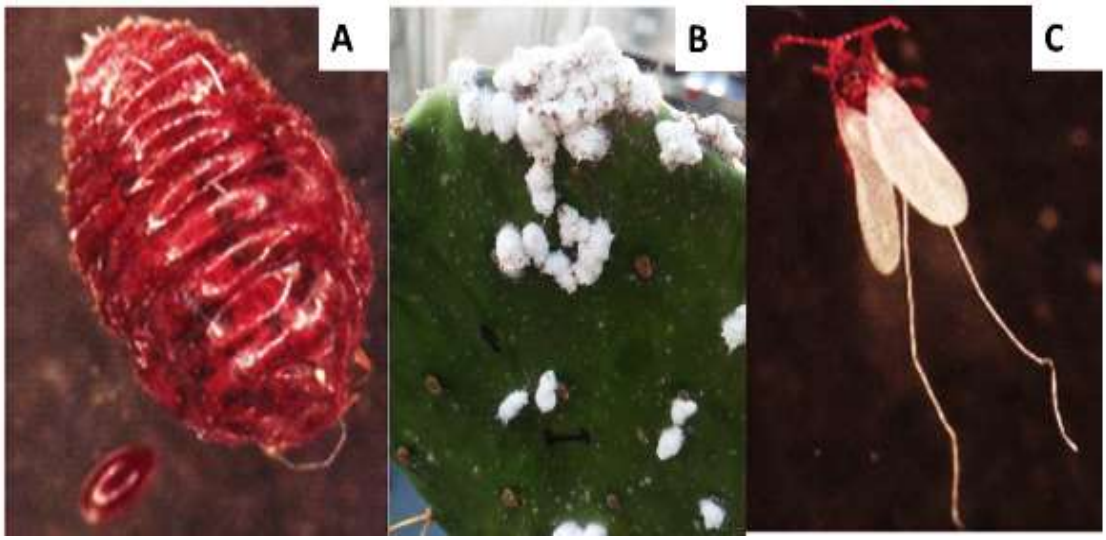

Figure 1.6:(A). Female cochineal insect with wax covering removed. (B) Adult female cochineal congregating on the highest part of the cladode for wind dispersal of their crawlers. (C) Male cochineal insect with wings (Source: CW Mathenge; ZH Machimane).

1.3.4.2. *Dactylopius confusus*

Dactylopius confusus originates from North and South America (Chávez-Moreno *et al*., 2009). Like *D. opuntiae*, female cochineals of *D. confusus* produces great number of eggs which are released into a large and soft mass of waxy thread covering (Cranshaw, 2016). The newly hatched crawlers feed and move actively around the plant for several days before they settle (Cranshaw, 2016). The females take about 42-50 days to reach maturity, then become sedentary (Gilreath and Smith, 1988; Pérez Guerra, 1991; Cranshaw, 2016). *Dactylopius confusus* has never been used as a biological control agent against *Opuntia* species according to my knowledge. If it becomes one of the successful biotypes in this study, it could be a good agent,

because unlike *D. opuntiae* biotypes which are capable of interbreeding (Hoffmann *et al*., 2002), *D. confusus* does not hybridise with other *Dactylopius* species or biotypes (I. Paterson, pers. comm). Hybridisation of cochineal insects can have considerable implications for the efficacy of the biological control agents (Hoffmann *et al*., 2002).

1.3.4.3. *Dactylopius* biotypes

The genus *Dactylopius* consist of several species which are further divided into biotypes. A biotype is a population within a species of arthropods that differ in their ability to cause damage on their host or differ in their capacity to use specific traits within a plant genotype or phenotype when identifying a potential host (Smith, 2005 Michel *et al*., 2011). This has resulted in cochineal biotypes being highly host-specific to a particular lineage of *Opuntia* cactus (Githure *et al*.,1999; Smith, 2005). For example, the 'ficus' biotype of *D. opuntiae* that was initially introduced in South Africa from Australia to control *O. ficus-indica* in 1937, until recently, it was thought to be the same stock that was damaging on *O. stricta* in Australia. However, host-specificity tests confirmed that the newly imported *D. opuntiae* was a different biotype to the one that already existed in south Africa as it barely survived and did not persist on *O. stricta* in South Africa (Volchansky *et al*., 1999). This proved that different biotypes exist within *D. opuntiae* species (Brown *et al*., 1995; Volchansky *et al*., 1999; Musengi, 2018). Biotypes can also be described based on the geographic variation of the cochineal insects or the host plants which the insects are collected from (Diehl and Bush, 1984). *Dactylopius opuntiae* and *Dactylopius confusus* consist of several biotypes, which are being tested to determine the most effective agent in killing the Kenyan and EC *O. engelmannii* hosts.

1.3.5. Cacti invasions in Africa

Cacti (Cactaceae) are one of the most dominant and widespread groups of invasive alien plants in Africa, particularly in South Africa (Nel *et al*., 2004, Novoa *et al*., 2019), with about 35 species listed as invasive under the National Environmental Management: Biodiversity Act 10 of 2004 (NEM:BA) (Zimmermann *et al*., 2009; Paterson *et al*., 2011; Novoa *et al*., 2019). Many of these widespread cacti in Africa include *Opuntia stricta* (Haw.) Haw, *O. engelmannii*, *Opuntia ficus-indica* (L.) Mill., *Opuntia monacantha* Haw and *Opuntia humifusa* (Raf) Raf (Novoa *et al*. 2019; Witt *et al*., 2020). Of these invasive species, *O. stricta* and *O. engelmannii* are considered to be the most problematic in Kenya and South Africa (Witt *et al*. 2018; Musengi *et al*., 2021), having a negative impact on biodiversity and human livelihood (Witt *et al*. 2020). For instance, in Kenya, *O. stricta* formed dense infestations, preventing access to homes, water

resources and pasture and contributed to the ill-health (even death) of livestock (Shackleton et al. 2017; Witt et al., 2020; Musengi *et al*., 2021). To control *O. stricta,* the biocontrol agent *D. opuntiae* '*stricta*' biotype was released in 2014. A preliminary result on the impact of the *D. opuntiae* biotype revealed that it had contributed to a significant reduction in the number of cladodes, fruits and flowers of *O. stricta* in Kenya (Witt *et al*., 2020). Although these results were preliminary, an analysis of the costs of executing this biocontrol programme indicates that it is the most cost-effective management strategy compared to other forms of management (e.g., physical and chemical control) (Witt *et al*., 2020).

There are no known native cacti species in Africa. However, cacti are known for being more invasive in Africa than on any other continent outside their native range (Novoa *et al*., 2015). This is because of the continent's arid interior providing favourable conditions for the establishment and proliferation of drought tolerant species, such as cacti (Novoa *et al*., 2015; Kaplan *et al*., 2017). Moreover, cacti have high water-use efficiency, increasing their ability to survive in wide ecological ranges that may have extremes of heat and cold (Musengi, 2018). As a result, South Africa is considered a global hotspot of cacti invasion, having the highest diversities of cacti species that have naturalised outside their family's native range (Novoa *et al*., 2015; Novoa *et al*., 2019). The Cactaceae family is native to North America and South America (Edwards *et al*. 2005). The widespread horticultural trading of cacti is the main cause of many cacti species spreading rapidly in many countries around the world, including African countries (Walters *et al*. 2011). The Cactaceae family consist of three recognized subfamilies: Pereskioideae, Opuntioideae and Cactoideae (Novoa *et al*., 2014). Of the 1922 cactus species, 57 have been classified as invasive (Novoa *et al*., 2015).

1.4. Aim:

To determine which *Dactylopius* biotypes are best suited as biocontrol agents for the Kenyan and Eastern Cape (South Africa) lineages of *Opuntia engelmannii*.

1.5. Objectives:

(a). Evaluate the acceptability and suitability of the Kenyan and the Eastern Cape lineage of *Opuntia engelmannii* to ten cochineal biotypes collected from the USA, using detached cladodes.

(b). Assess the impact of the four best cochineal biotypes on whole Kenyan and Eastern Cape *O. engelmannii* potted plants.

1.6. Key Questions:

(a). Which biotype of *Dactylopius opuntiae* is the most suitable biocontrol agent for the Kenyan and EC *O. engelmannii* lineages?

(b). Are the cochineal biotypes collected from USA *O. engelmannii* lineages morphologically similar to the targeted lineages be the most suitable biocontrol agents?

1.8. Dissertation outline

This dissertation is broken down into the following chapters:

Chapter 1: This chapter discusses the motivation, aim, objectives, key questions and literature review.

Chapter 2: Title: Screening *Dactylopius opuntiae* and *Dactylopius confusus* biotypes as biocontrol agents for invasive *Opuntiae engelmannii*, using detached cladodes: This chapter gives an insight into the acceptability and suitability of the Kenyan and Eastern Cape *Opuntia engelmannii* lineages to 10 *Dactylopius* biotypes.

Chapter 3: Title: Assessing herbivory damage caused by four *Dactylopius* biotypes on whole potted plants of Kenyan and Eastern Cape *O. engelmannii* lineages. This chapter determines which cochineal herbivory will result in the most rapid plant mortality.

Chapter 4: A Synthesis of the findings of this research, implications for biocontrol, conclusion and recommendations.

CHAPTER TWO: Screening *Dactylopius opuntiae* biotypes as biocontrol agents for invasive *Opuntiae engelmannii*, using detached cladodes.

Abstract

Opuntia engelmannii or small round-leaved prickly pear is a drought tolerant perennial shrub native to North and Central USA. *Opuntia engelmannii* has invaded many countries around the world, including African countries. South Africa in particular, has achieved an outstanding biocontrol success of selected alien invasive weeds since its beginning in 1913. Nonetheless, success against *O. engelmannii* has not been attained since there have not been any effective biocontrol agents to control it. Therefore, *O. engelmannii* is spreading and increasing in density at rates that are concerning. This study uses proportions of female cochineals settling on detached cladodes of *O. engelmannii* lineage to determine host plant acceptability. Fitness indices (FI), developmental times, female masses and number of crawlers produced by each female cochineal were used to measure host suitability. High fitness indices (FI >1) were the best indicators of the host plant suitability. The Kenyan *O. engelmannii* lineage was highly acceptable and suitable for the *D. opuntiae* biotype from site 21. While the Eastern Cape *O. engelmannii* lineage was highly acceptable to all 10 *Dactylopius* biotypes. However, the Eastern Cape *O. engelmannii* was highly suitable for the *D. opuntiae* from sites 66 and *D. confusus* 23 P3.

Keywords: fitness index, host acceptability, host suitability, host tolerance, settling.

2.1.1. Biocontrol of *Opuntias*

2.1.1.1. History of successful biocontrol in Africa

South Africa has achieved exceptional biocontrol success of alien invasive weeds since its commencement of weed biocontrol in 1913 (Zimmermann, 2010; Hill *et al*., 2020). This success of biocontrol began in the late 18th century, when a cochineal insect *Dactylopius ceylonicus* Green (Hemiptera: Dactylopiidae) from South America was mistakenly introduced to India while trying to obtain a commercially valuable dye-producing cochineal, *Dactylopius coccus* Costa (Hemiptera: Dactyliiopidae) on *O. ficus-indica* (Paterson *et al*., 2011; Moran *et al*., 2013). As a result, there was a large die-back of a different, but very problematic cactus plant called *O. monacantha* between 1796 and 1809. Subsequently, *D. ceylonicus* was introduced into Ceylon (Sri Lanka) where it also successfully controlled the invasive *O. monacantha*. News of this so called 'Deadly Indian Cochineal' reached South Africa and Kenya only in 1913 and 1958 respectively, and consequently *D. ceylonicus* was imported and released as a biological control agent against *O. monacantha* (Paterson *et al*., 2011; Moran *et al*., 2013; Witt *et al*., 2020). Since then, biocontrol agent entities (species or biotypes) have been released against many *Opuntia* species (e.g., *Opuntia aurantiaca* Lindl., *O. ficus-indica*, and *O. stricta* in Africa, particularly South Africa (Hill *et al*., 2020). This lead to notable successes and biocontrol of invasive alien cacti which in turn led to an effective method for reducing population densities of many other important invasive plants (Hill *et al*., 2020). South Africa has achieved a remarkable success rate of 72% from the worst invasive weeds, of these, 39% are under complete or substantial control (Zachariades *et al*., 2017). In many biocontrol programmes of Cactaceae, successful agents are selected either from an old or new association. An old association involves agents that have coevolve with the targeted host (Dennill and Hokkanen, 1990). While new association agents are those that did not coevolve with the targeted host and thus the host lacks resistance towards the agents (Harris, 1991; Eilenberg, 2001). Nonetheless, it is not known whether the past successful biocontrol agents on *Opuntia* were collected from new or old associations.

2.1.1.2. *Opuntia engelmannii*: Invasive traits and invasion in Africa

The genus *Opuntia* is an extensive group within the Cactaceae family, with more than 300 species and *O. engelmannii* is one of them (Novoa *et al*., 2014). *Opuntia engelmannii*, commonly known as small round-leaved prickly pear, is a drought tolerant perennial shrub that can grow up to 1.5 m in height. It is a succulent plant with large fleshy leaf-like pads called cladodes (Zimmermann *et al*., 2009; Klein *et al*., 2014; Novoa *et al*., 2015). The cladodes are

flattened, round to egg shaped with a narrow end at the base and grey-green in colour (Figure 2.1). *Opuntia engelmannii* cladodes have sharp, white to yellow spines and smaller clusters containing small sharp glochids that emerge from the areoles (rounded or elongated structures on the cladode surface) (Figure 2.1). The plant produces yellow to orange flowers which develop into fleshy reddish-purple fruits (Feugang *et al*., 2006; Zimmermann *et al*., 2009) (Figure 2.1). *Opuntia engelmannii* is native to northern Mexico, south-central and southwestern USA (Mondragón-Jacobo and Pérez-González, 2001).

Over the last century, *O. engelmannii* has emerged as one of the most problematic invasive alien cactus species in Africa (Henderson and Wilson, 2017) because of its negative impact on rural livelihoods and grazing animals (Wilson *et al*., 2017; Githae, 2018; Witt *et al*., 2020). *Opuntia engelmannii* has invaded many countries located in the eastern and southern regions of the African continent, where conditions favour the growth and spread of *O. engelmannii* (; Kaplan *et al*., 2017; Wilson *et al*., 2017). However, like many other *Opuntia* species, *O. engelmannii* has been mainly introduced into Africa as an ornament or a source of fodder (Shackleton *et al*., 2017; Richardson *et al*., 2020; Hill *et al*., 2020; Witt *et al*., 2020). In South Africa, there are three morphologically distinct lineages i.e., one lineage each within the NC, LM and EC provinces respectively (Figure 2.2). A fourth lineage has become problematic in Kenya (Figure 2.2) (Klein, 2015b). These four African lineages of *O. engelmannii* differ from each other in morphological traits and in their ability to be utilized by cochineal biotypes as hosts (Musengi, 2018; Mbonani, 2019). The differences in appearance and location where these *O. engelmannii* are found in South Africa led to the use of the term 'lineage' in this dissertation, to describe the different varieties of *O. engelmannii.*

The number of different lineages of *O. engelmannii* in Africa (and around the world) complicate the efforts towards biocontrol because the potential agent, *Dactylopius opuntiae* Cockerell (Hemiptera: Dactyliiopidae) is very host specific and is only effective when the correct plant species or lineage is matched with the correct insect biotype (i.e., a population within the species of arthropods). Given that the intraspecific taxonomy of *O. engelmannii* is a highly diverse and extremely complex (Weniger, 1978), causing confusion amongst authors. Some authors have combined all the lineages and closely related species in one group, while others have divided all plants with any morphological differences from known taxa into new species. For this study, the taxonomy of Weniger (1978) will be used, because in my opinion, it is the most inclusive and thorough review of *O. engelmannii* and its close relatives. Weniger (1978), recognises 11 variants of *O. engelmannii* in Texas and New Mexico alone, and there

are almost certainly other lineages present in Arizona, California and Mexico. Resolving this taxonomy is not one of the aims of this project, but it is important that we understand which of these lineages is present in Kenya and South Africa and where these lineages may have originated from in order to control them.

A recent MSc study used morphological and molecular data to determine the variation present within and amongst these four African lineages compared to *O. stricta* (Mbonani, 2019). Results from this study were useful as they will further assist in finding the correct biotypes for the targeted *O. engelmannii* host plants and help researchers to identify the different *O. engelmannii* lineages in the field (Mbonani, 2019). According to morphological data, spine morphology was similar between LM and Kenyan lineages, while EC was more analogous to a sister taxon, *O. stricta*. The NC lineage was however different in its morphology to all the other lineages of O. *engelmannii* and different to *O. stricta*. Molecular data shows that the NC lineage was genetically different from all the other *O. engelmannii* lineages and different to *O. stricta* (Mbonani, 2019). The EC lineage was found to be more genetically similar to *O. stricta* than the other *O. engelmannii* lineages and could have potentially been an agricultural variety of *O. engelmannii*. Apart from the morphological resemblance shown by LM and Kenyan lineages, these two lineages were also found to be genetically similar with a great deal of overlap, which suggests that these could potentially be the same lineage that were most likely recently separated by humans. Both morphological and molecular studies provide useful information when it comes to finding appropriate biocontrol agents for *O. engelmannii* lineages in South Africa and Kenya. If an old association will result in the most effective biocontrol control agent for Kenyan and EC *O. engelmannii* lineages, then the most appropriate lineages of the plants to collect from would be morphologically similar (or identical) lineages in their native range.

Figure 2.1: Different characteristic morphological traits *of Opuntia engelmannii*. (A) Oval-shaped, grey-green cladode of *Opuntia engelmannii* with sharp spines and small sharp glochids. (B) *Opuntia engelmannii* with orange and yellow flowers. (C) Fleshy fruits of *Opuntia engelmannii* (Source: N Venter).

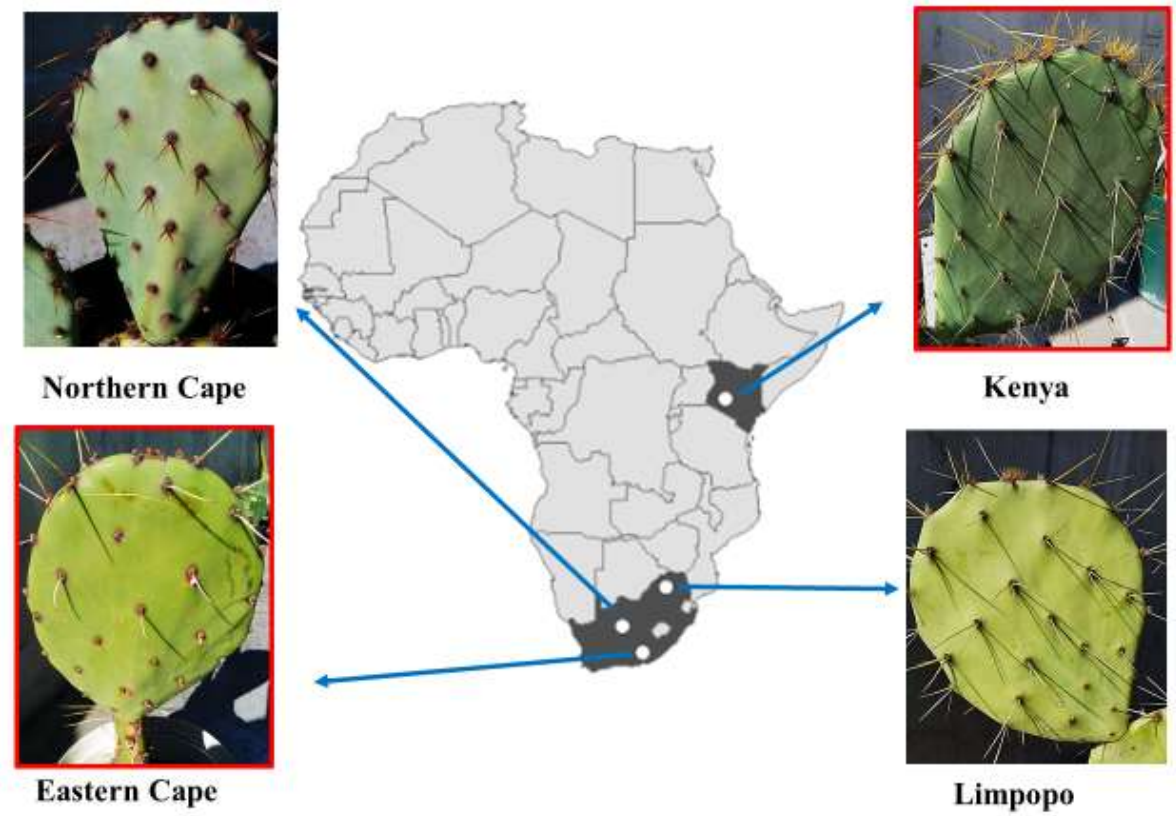

Figure 2.2: The distribution of the four *Opuntia engelmannii* lineage in Africa. Three lineages of *Opuntia engelmannii* have been found in South Africa, one in Limpopo (Mokopane), one in the Northern Cape (Douglas) and one in the Eastern Cape (Bedford). A fourth lineage of *Opuntia engelmannii* occurs in the northern part of Kenya (Loisaba). The blue arrows indicate where the lineages are found on the African map and the red colour highlights the species of interest. (Source: N Venter; S Mbonani).

2.1.3. Response of candidate agents to cactus host plant cladodes

It is imperative to assess how a candidate biocontrol agent responds when introduced to a host (Musengi, 2018). There are two aspects to the question of whether a plant can be host for a biocontrol agent (Browne and Withers, 2002; Musengi, 2018). The first one is called host acceptability and the second is host suitability (Browne and Withers, 2002; Musengi, 2018). These two terms are further discussed below.

(a). Host acceptability

Acceptability refers to the ability of the biocontrol agent to feed and or oviposit on the host plant (Browne and Withers, 2002). The degree of acceptability is addressed by the proportion of insects settling until they reach maturity (Browne and Withers, 2002). The proportion of

cochineal insects that settles is expressed as the number of crawlers that begin to develop a white waxy covering over the total number of crawlers that were initially inoculated on the targeted host plant.

(b). Host suitability

For biocontrol agents to be regarded as successful, they must be able to utilize resources, avoid being killed before contributing viable offspring to the next generation and form self-sustaining populations on its target plant (Coombs *et al*. 2004). Host suitability is critical for successful biocontrol agents. Suitability is defined as the ability of the host plant to support development of the biocontrol agent from neonate to a reproducing adult (Browne and Withers, 2002). In cochineals, the level of suitability can be addressed by fitness index. Fitness index is used as an estimate to measure how many female cochineals that survives, reach maturity and produce offspring over time (Mathenge *et al*., 2009a).

2.1.4. Settling

Settling in cochineal insects occurs when cochineal insects start feeding and producing a white waxy covering on their host plant (Browne and Withers, 2002; Mow *et al*., 2008; Musengi, 2018). When a cochineal insect finds a host plant it may not always settle on it, potentially due to lack of stimuli to identify it as a potential host (Musengi, 2018). There are many factors that affect settling of insects onto a host plant. These may include the condition and phytochemical aspects of host plant and developmental stage or age of female crawlers (Prager *et al*., 2014; Musengi, 2018).

2.1.5. Developmental time

Developmental time of cochineal insects is described as the time from inoculation of crawlers to maturity of females (Mathenge *et al*., 2009a). The developmental time of cochineals can differ between biotypes of the same species on the source or different plants (Rule and Hoffmann, 2018). The time taken for cochineal insects to develop and reach maturity depends on many factors such as climatic conditions (e.g., temperature and humidity) and physiological traits of the host plant and host suitability (Müller-Maatsch and Gras, 2016). However, for an insect to develop and reach maturity on its host, a ‘proper fit’ between the traits of the plant and insect is needed (Musengi, 2018). The physiological and physical traits of both the insect and its host plant are subjected to temporal changes (Musengi, 2018). Hence, even when a host plant has been utilized by a cochineal insect to some level, there are chances that the host plant

will prove at some level to be unsuitable and the cochineal insect will not be able to develop further (Musengi, 2018).

2.1.6. Fitness index (FI)

Fitness index is a measure of host suitability, expressed as combination of the proportion of individual cochineals surviving to maturity, the female fecundity (the mean number of crawlers produced per female) and the mean developmental time (Volchansky *et al*., 1999; Mathenge *et al*., 2009a). Fitness index is used to determine how the insects will perform on the target host plant (Roitberg and Boivin, 2001). There are three subjective categories that best describe the host plant suitability using fitness indices (FI) and behaviour of the *Dactylopius* species or biotypes (Mathenge *et al*., 2009a). The first category is for *Dactylopius* species or biotypes that thrive on their host plant (FI $\geq$ 1), this includes female cochineals that are highly fecund and crawlers that settle quickly, begin to feed and freely develop into mature females through subsequent stages (Mathenge *et al*., 2009). The second category is for those that survive on their host plant (FI < 1 but > 0), female insects are not highly fecund, and the crawlers take some time to settle and develop slowly in their life stages (Mathenge *et al*., 2009a). The last category is for those dying on their host plant (FI = 0), most of the cochineal insects fail to settle and feed, and none of them complete their life cycle (Mathenge *et al*., 2009a). These three categories will be used in this study to describe the host suitability of Kenyan and EC *O. engelmannii* lineages. Another important parameter in determining host suitability is the body mass of female cochineals (Volchansky *et al*., 1999; Rule and Hoffmann, 2018). Female body mass has direct effect on the number of crawlers produced by each female (fecundity). A study by Zanuncio *et al*., 2002, has shown a positive linear relationship between number of nymphs and female mass of *Podisus rostralis* (Stål) (Heteroptera: Pentatomidae).

2.2. Methods and Materials

Cladodes of *O. engelmannii* plants were collected in the Eastern Cape province of South Africa (32˚41’16.37” S 26˚06’23.04” E) and in Loisaba, Kenya (0˚22’37.70” N 36˚47’18.40” E). The cladodes were then planted in eight litre pots in the quarantine facility at the University of the Witwatersrand, Johannesburg, South Africa, for further experimental use.

A total of 10 *Dactylopius* biotype cultures from the USA were maintained on Kenyan and EC cladodes of *O. engelmannii* in quarantine at Wits University. Cladode trials were conducted in separate rooms with a near constant temperature between 20℃ and 30℃ and relative humidity at about 50% and a 12 hour to 14 hour daylight phase to offer conditions that are known to be

favourable for development, reproduction and survival of the *Dactylopius* biotypes (Pérez Guerra and Kosztarab, 1992; Volchansky *et al*., 1999).

To determine the host plant suitability of the 10 *Dactylopius* biotypes, a standard method was followed (Githure *et al*., 1999, Mathenge *et al*., 2010; Rule and Hoffmann, 2018) using mature cladodes harvested from stock plants of Kenyan and EC *O. engelmannii* in the Wits quarantine facility. A single cladode was placed in a separate eight litre plastic container with a gauze lid to prevent the insects from escaping (Githure *et al*., 1999). Six cladodes per insect biotype were inoculated with thirty randomly selected crawlers from gravid females that were harvested from the USA cultures and kept on Kenyan and EC *O*. *engelmannii* lineages. This was done for both lineages. The total sample size was n= 30 crawlers per cladode x 6 cladodes per cactus lineage. A fine paint brush was used to transfer the crawlers to the cladodes from the Kenyan and EC *O*. *engelmannii* lineages. The progress of each cochineal biotype on the Kenyan and EC cladodes was observed daily until the newly settled fertile females produced crawlers and the lifecycle had been completed. The parameters recorded include; (a) the number of crawlers that had settled after 14 days, (b) the mass and fecundity of the resultant mature females and (c) their development time from settling to sexual maturity. To determine the female fecundity, gravid females were removed from the cladode together with their wax covering as soon as the first offspring were detected as crawlers near them. If crawlers were produced prior to removal, they were noted. The wax coating was removed, and the female was weighed and placed inside an Eppendorf tube and sealed with cotton wool to allow air passage. The number of offspring produced in the Eppendorf tube was recorded. Although there was some uncertainty about the effect that this technique had on reproduction, since females detached from cladode stop laying eggs earlier than those attached to cladode, it still provided a good measure of relative fecundity (Volchansky *et al*., 1999). Out of the 10 US cochineal biotypes, the four best biotypes with the highest female fitness index (see equation below) were selected for whole plant trials. The number of male insects were not assessed.

Three subjective categories were used to best describe the host plant suitability of the Kenyan and the EC *O*. *engelmannii* lineages (Mathenge *et al*., 2009a) (Appendix I). These categories were based on fitness indices (FI) of the *Dactylopius* species or biotypes (Mathenge *et al*.,2009) (Appendix 1). Four *Dactylopius* biotypes with the highest FI were selected and further tested in whole potted - plant experiments (Chapter 3) to assess their impact on plant mortality rates. Due to the insect taking much longer time to produce crawlers which were needed for the EC whole-potted plant trial, biotypes from site 5, 21, 23 P3 and 66 were selected for the EC whole-

potted plant trial. These biotypes (sites 5, 21, 23 P3 and 66) were selected based on high proportions of settled female cochineals.

Fitness index = Proportion of females surviving until maturity x (Mean number of offspring per female / Mean developmental time)

2.3. Statistical analysis

Proportions of settled female insects, developmental times and fitness indices were compared between treatments using one-way ANOVA with a Tukey HSD post hoc (StatSoft, 2014). Linear regressions were fitted to number of crawlers against female body mass for the 10 different *Dactylopius* biotypes on both Kenyan and EC *O. engelmannii* lineages.

2.4. Results

2.4.1. Proportion of settled females

2.4.1.1. Kenyan lineage

Dactylopius opuntiae biotypes from sites 5, 13, 20 and 21, along with *D. confusus* from site 23 P1, had a high proportion of settled female cochineal insects compared to the other biotypes (Figure 2.3). There was, however, no significant difference in the proportion of settled females between biotypes from sites 5 and 21 ($F_{1,12}$ = 1.2125, P > 0.05). *Dactylopius opuntiae* from site 21 had the highest proportion of settled females, while *D. opuntiae* from site 66, together with *D. confusus* from sites 23 P3, 44 and 51 had no settled females. Overall, proportions of settled females were significantly different between sites of origin ($F_{9,8}$ = 8.6602, P < 0.001).

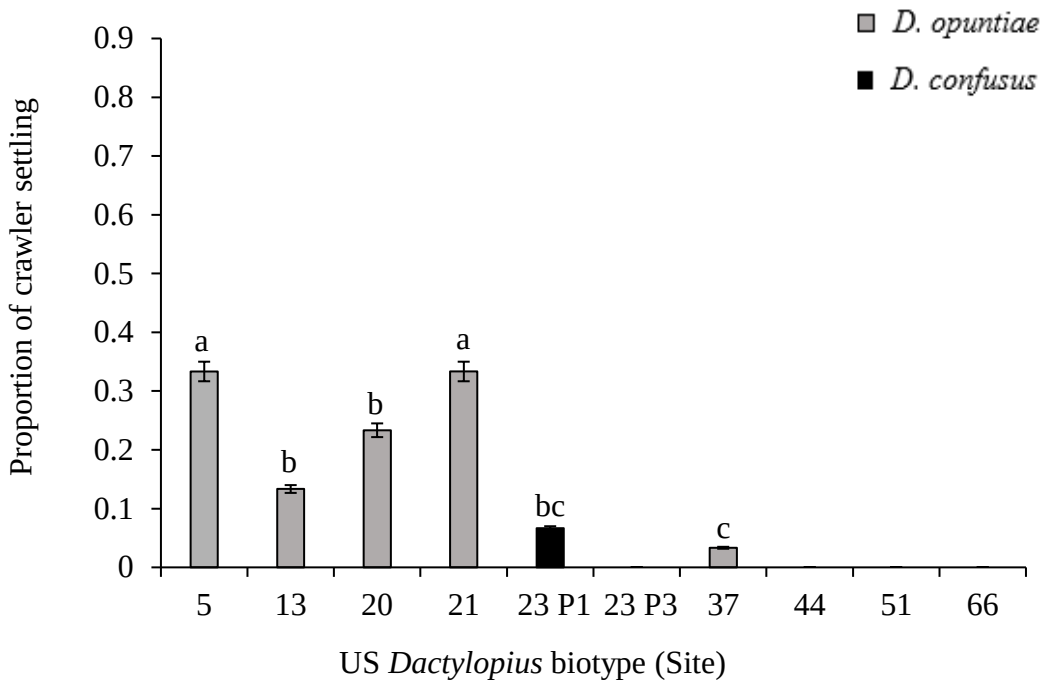

Figure 2.3: The mean proportions of cochineal crawlers that settled and developed into gravid females, calculated as the number of females that began to develop a white waxy covering over the total number of crawlers (n=30) that were initially inoculated onto the cladode (n = 6 ± SE) for each of the 10 *Dactylopius* biotypes on the Kenyan lineage of *Opuntia engelmannii*. Biotypes from Sites 23 P1, 37, 44, 51 and 66 did not settle. The overall difference between sites was assessed using a one-way ANOVA. Different lowercase letters indicate significant differences between sites ($P < 0.001$).

2.4.1.2. Eastern Cape lineage

Proportions of settled females of *D. opuntiae* and *D. confusus* biotypes were very high on the EC lineage of *O. engelmannii* compared to the Kenyan lineage *O. engelmannii*, with biotypes from sites 5, 37, 44 and 66 having the highest proportions of settled female cochineals (Figure 2.4). *Dactylopius opuntiae* from site 37 however had the highest proportion of settled female cochineals, whereas *D. opuntiae* from site 21 had the lowest proportion of settled female cochineals. It is worth noting that all the biotypes (Sites: 23 P1, 23 P3, 37, 44, 51 and 66) that did not perform very well on the Kenyan lineage, showed a high acceptance on the EC lineage, producing high proportions of settled cochineal crawlers (Figure 2.4). Overall, proportions of settled females of D. *opuntiae* and *D. confusus* were not statistically different between sites of origin on the EC lineage ($F_{17,43} = 6.2747$, $P > 0.05$).

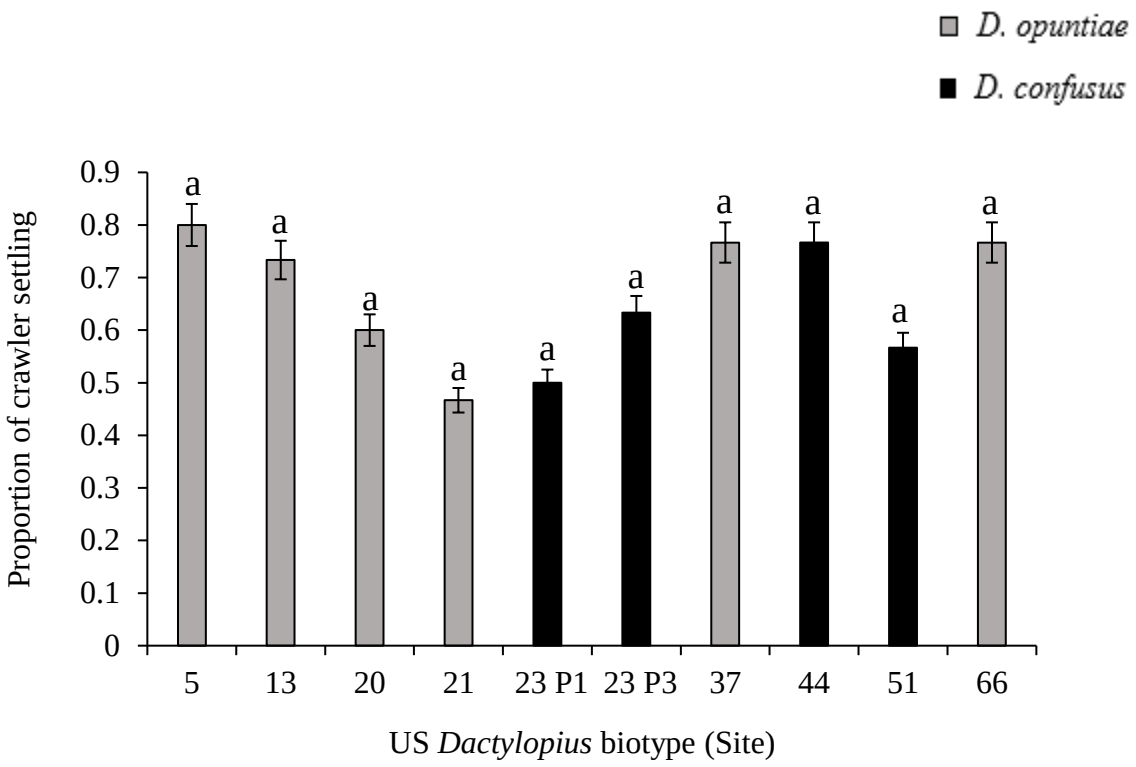

Figure 2.4: The mean proportions of cochineal crawlers that settled and developed into gravid females, calculated as the number of females that began to develop a white waxy covering over the total number of crawlers (n = 30) that were initially inoculated onto the cladodes (n = 6 ± SE) for each of the 10 *Dactylopius* biotypes on the EC lineage of *Opuntia engelmannii*. The overall difference between sites was assessed using a one-way ANOVA.

2.4.2. Female body mass

2.4.2.1. Kenyan lineage

Body mass of mature, fertile females of *D. opuntiae* and *D. confusus* differed greatly between sites of origin, ranging from 1.1 mg (site 23P1) to 24.0 mg (site 20) ($F_{6, 171} = 27.088$, $P < 0.001$) (Figure 2.5). There was a positive, linear relationship between the number of crawlers produced by each female and body mass. However, *D. opuntiae* from sites 5, and 21 showed a very strong relationship ($r > 0.7$, n = 7 biotypes) between the number of crawlers produced by individual females and body mass, while all the other remaining biotypes, including *D. confusus* showed weak ($0.3 < r < 0.5$, n = 7 biotypes) to moderate ($0.5 < r < 0.7$, n = 7 biotypes), positive linear association between the number of crawlers produced by each female and body mass (Figure 2.5). *Dactylopius opuntiae* from site 21 had a much steeper slope than all the other biotypes (sites 5, 13, 20, 23 P1, 37 and 66) (Figure 2.5). For the same mass, the site 21 female cochineals produced more crawlers. For example, a 20 mg female produced above 500

crawlers (Figure 2.5). The average female body mass of *D. opuntiae* from sites 5 and 21 were the same (Figure 2.5). However, *D. opuntiae* females from site 20 were heavier than females of the other biotypes (sites 5, 13, 21, 23P1, 37 and 66). Female cochineal of *D. confusus* from site 23 P1 weighed lighter than all the other biotypes (sites 5, 13, 20, 21, 23 P1, 37 and 66) (Figure 2.5).

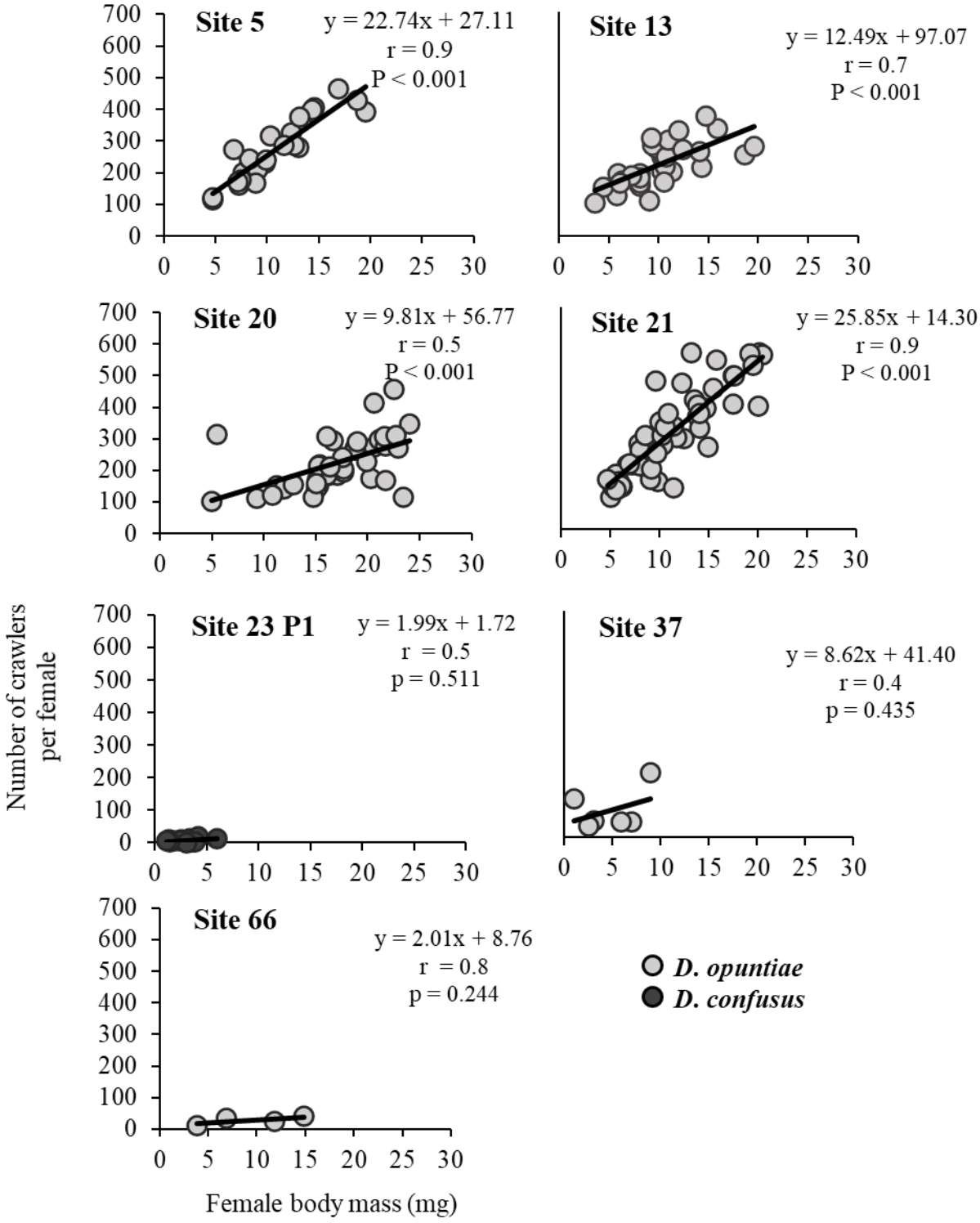

Figure 2.5: The relationship between the number of crawlers produced by gravid females and female body mass from (a) Site 5, (b) site 13 etc. on Kenyan *Opuntia engelmannii* cladodes (n = 6 ± SE). Out of the 10 biotypes assessed, only seven biotypes produced gravid females (biotypes from sites 5, 13, 20, 21, 23 P1, 37 and 66), while biotypes from sites 23 P3, 44 and 66 did not produce any gravid females. P- value indicates significance difference between number of crawlers and female body mass.

2.4.2.2. Eastern Cape lineage

On the EC lineage, the number of crawlers produced by each female cochineal was positively correlated to female body mass. Female body mass varied greatly between insect sites of origin, ranging from 5.4 mg to 32.9 mg ($F_{10, 374} = 16.846$, $P < 0.001$) (Figure 2.6). Contrary to the Kenyan lineage, a moderate ($0.5 < r < 0.7$, $n = 10$) to strong ($r > 0.7$, $n = 10$) relationships between the number of crawlers and female body mass of *D. opuntiae* (collected from sites 37 and 66) and *D. confusus* (collected from site 51) was displayed. However, *D. opuntiae* females from site 13 had a greater mean mass than the females from the other biotypes (sites 5, 20, 21, 23 P1, 23 P3, 37, 44, 51 and 66). The mass of female cochineals of *D. confusus* from site 23 P3 was less than that of the other biotypes (sites 5, 20, 21, 23 P1, 37, 44, 51 and 66. It is worth noting that *D. opuntiae* from site 21 showed a very weak relationship ($r = 0.3$, $n = 10$) between number of crawlers produced by individual females and body mass on the EC lineage of *O. engelmannii* (Figure 2.6).

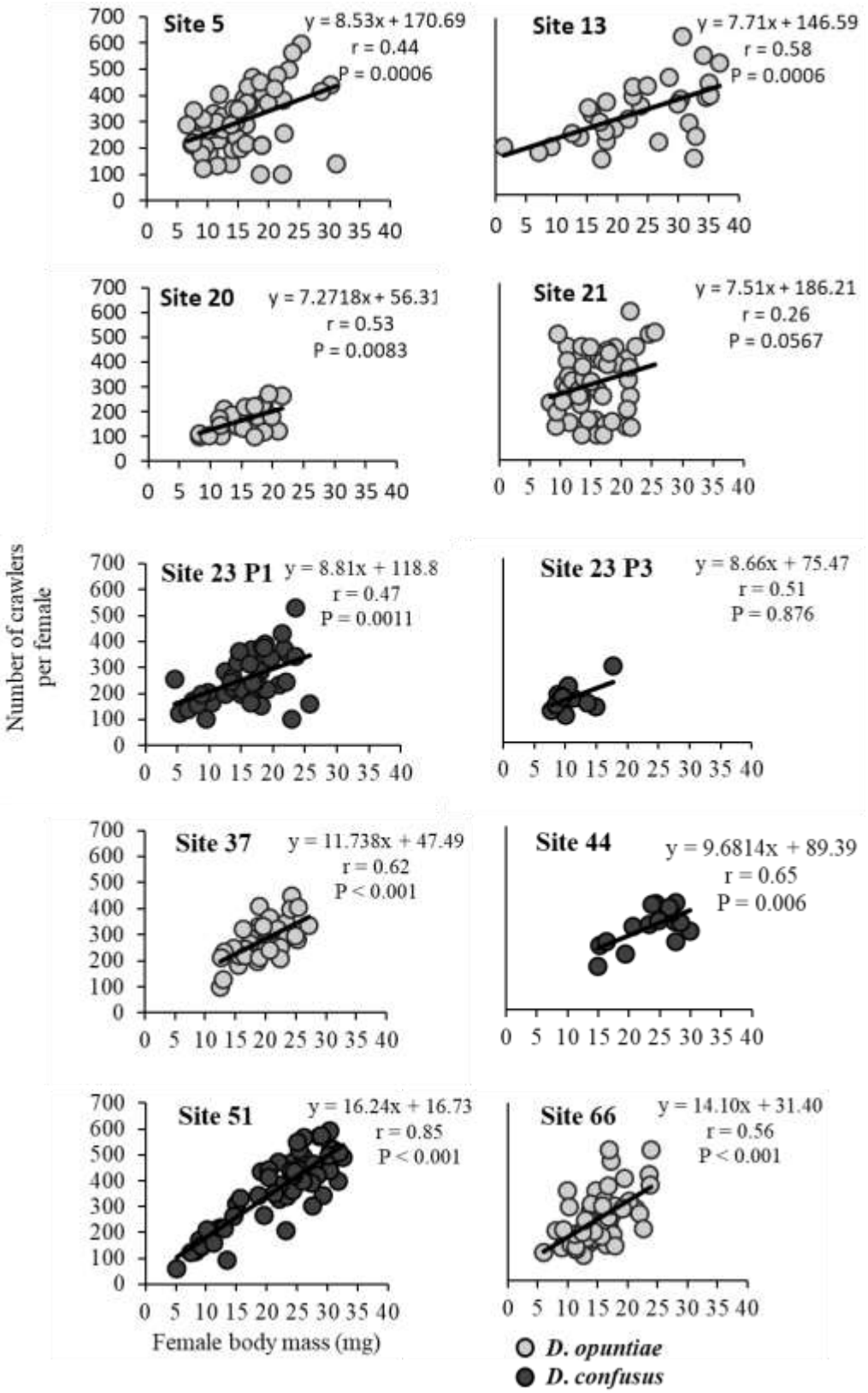

Figure 2.6: The relationship between the number of crawlers produced by gravid females and female body mass for each of the *Dactylopius* biotypes on the EC lineage of *Opuntia engelmannii* (n = 6 ± SE). Only nine biotypes produced gravid females (sites 5, 13, 20,21,23 P1, 23 P3, 37,51 and 66). P- value indicates significance difference between number of crawlers and female body mass.

2.4.3. Developmental time

2.4.3.1. Kenyan lineage

Dactylopius opuntiae from site 5 took significantly longer to reach maturity than the other biotypes (sites 13, 20, 21, 23 P1 and 37) ($F_{9,50} = 32.298$, $P < 0.001$). *Dactylopius opuntiae* from site 13 took less time to reach maturity compared to the other biotypes, but there was no significant difference in developmental time between *D. opuntiae* from sites 13, 37 and *D. confusus* from site 23 P1($F_{2,15} = 1.3312$, $P > 0.05$) (Figure 2.7). Developmental time was also not significantly different between *D. opuntiae* from sites 20 and 21($F_{1,10} = 1.8182$, $P > 0.05$) (Figure 2.7). *Dactylopius opuntiae* from site 66 and *D. confusus* from sites 23 P3, 44 and 51 did not develop.

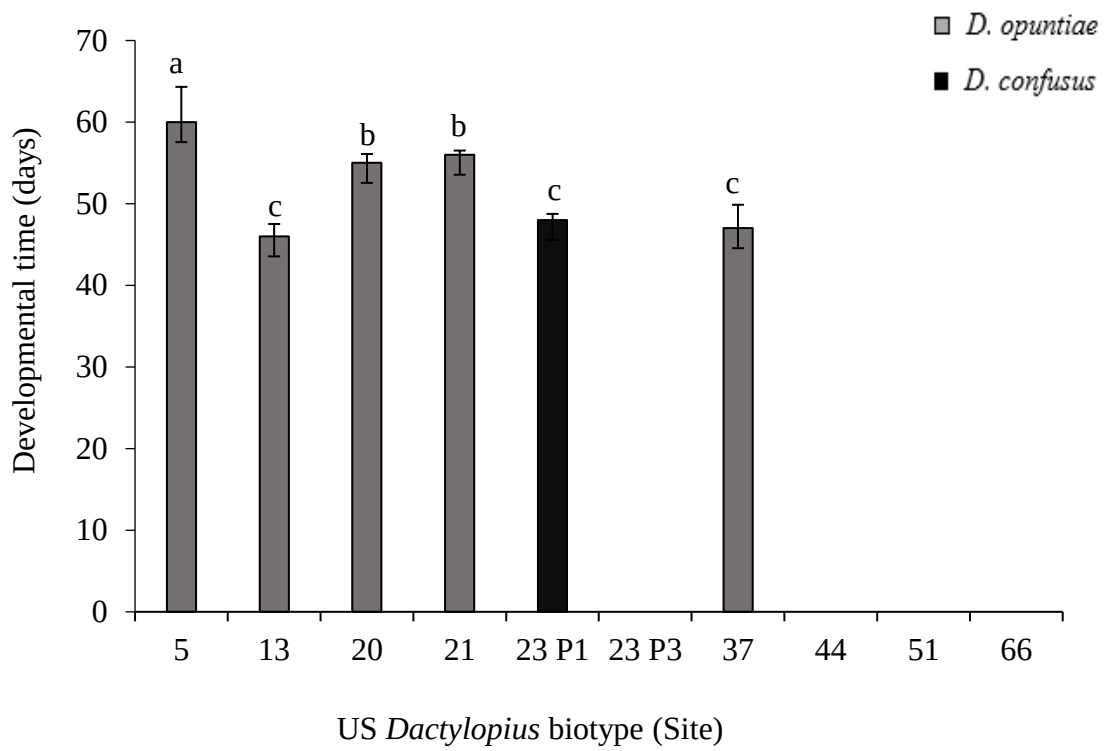

Figure 2.7: The mean developmental time of female cochineal insects to reach maturity on Kenyan *Opuntia engelmannii* cladodes (n = 6 ± SE), using 10 USA cochineal biotypes. Different lowercase alphabets indicate significant differences between sites of origin ($P < 0.001$). The overall difference between sites was assessed using a one-way ANOVA. *Dactylopius opuntiae* from site 66 and *Dactylopius confusus* from sites 23 P3, 44 and 51 did not produce any crawlers (grey - *Dactylopius opuntiae*; black – *Dactylopius confusus*).

2.4.3.2. Eastern Cape lineage

Developmental time on the EC *O. engelmannii* lineage was significantly longer for *D. opuntiae* biotypes from sites 5, 21 and 66. However, *D. opuntiae* from sites 13, 20, 37 and 51, along with *D. confusus* from sites 23 P1 and 23 P3 had shorter developmental times on the EC lineage of *O. engelmannii* (Figure 2.8). *Dactylopius opuntiae* from site 21 took a longer time to develop and reach maturity compared to the other biotypes. Nonetheless there was no significant difference in developmental time between *D. opuntiae* from sites 5 and 21 ($F_{1,10} = 1.4545$, $P > 0.05$). *Dactylopius confusus* from site 23 P3 took significantly less time reach maturity compared to the other biotypes (Figure 2.8). Overall, there was a significant difference in developmental time between the different sites of origin ($F_{9,50} = 1220$, $P < 0.001$).

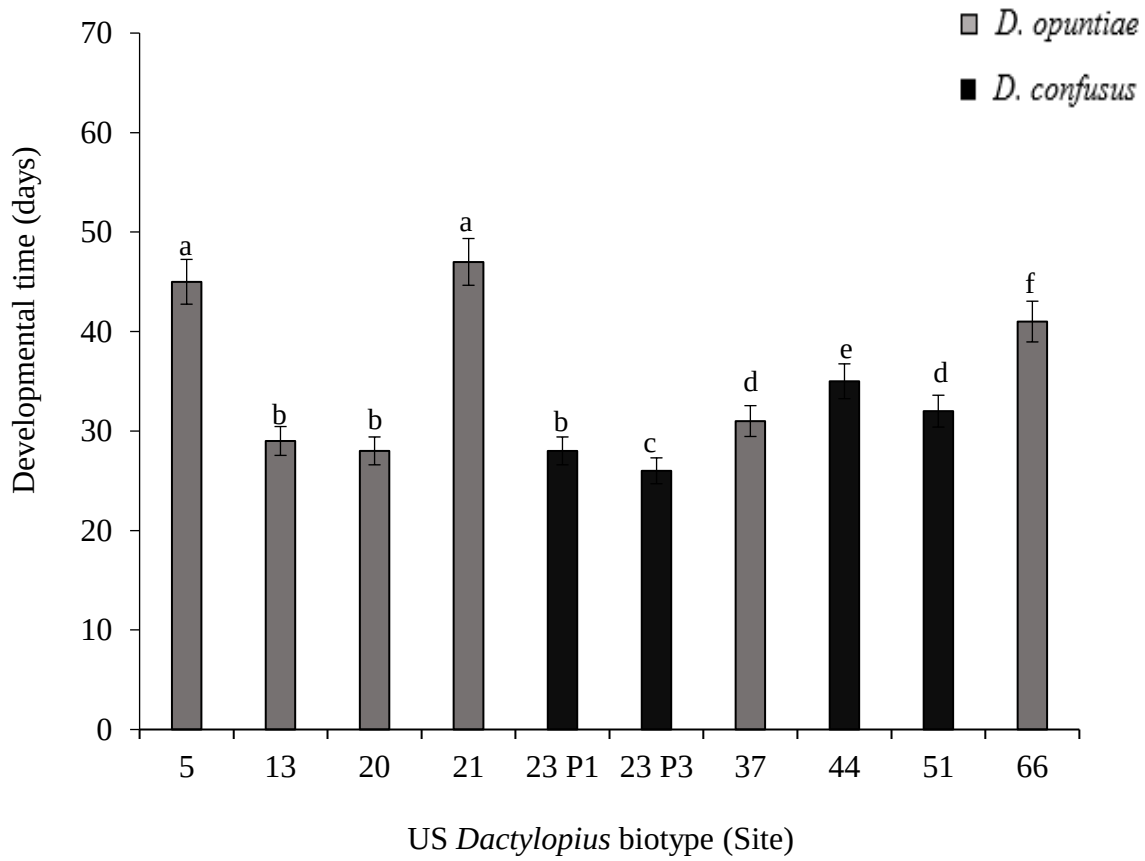

Figure 2.8: The mean developmental time of female cochineals to reach maturity on EC *Opuntia engelmannii* cladodes (n = 6 ± SE), using 10 USA cochineal biotypes. The overall difference between sites was assessed using a one-way ANOVA. Different lowercase letters indicate significant differences between sites of origin ($P < 0.05$).

2.4.4. Fitness index

2.4.4.1. Kenyan lineage

Fitness Index (FI) values were significantly different between *D. opuntiae* biotype sites of origin on the Kenyan lineage of *O. engelmannii*, with *D. opuntiae* from site 21 having a significantly higher FI ($F_{7,28}$ =12.848, P < 0.001) (Figure 2.9). But the FI was not statistically different between *D. opuntiae* from sites 5, 13 and 20 ($F_{2,15}$ = 1.7139, P > 0.05). Furthermore, FI did not differ between *D. opuntiae* from sites 13 and 37 ($F_{1,10}$ = 6.5334, P > 0.05). *Dactylopius confusus* biotypes from sites 23 P3, 44 and 51 did not produce any crawlers from parental generation of insects on the Kenyan *lineage of O. engelmannii*. Hence, they had no fitness index (Figure 2.9).

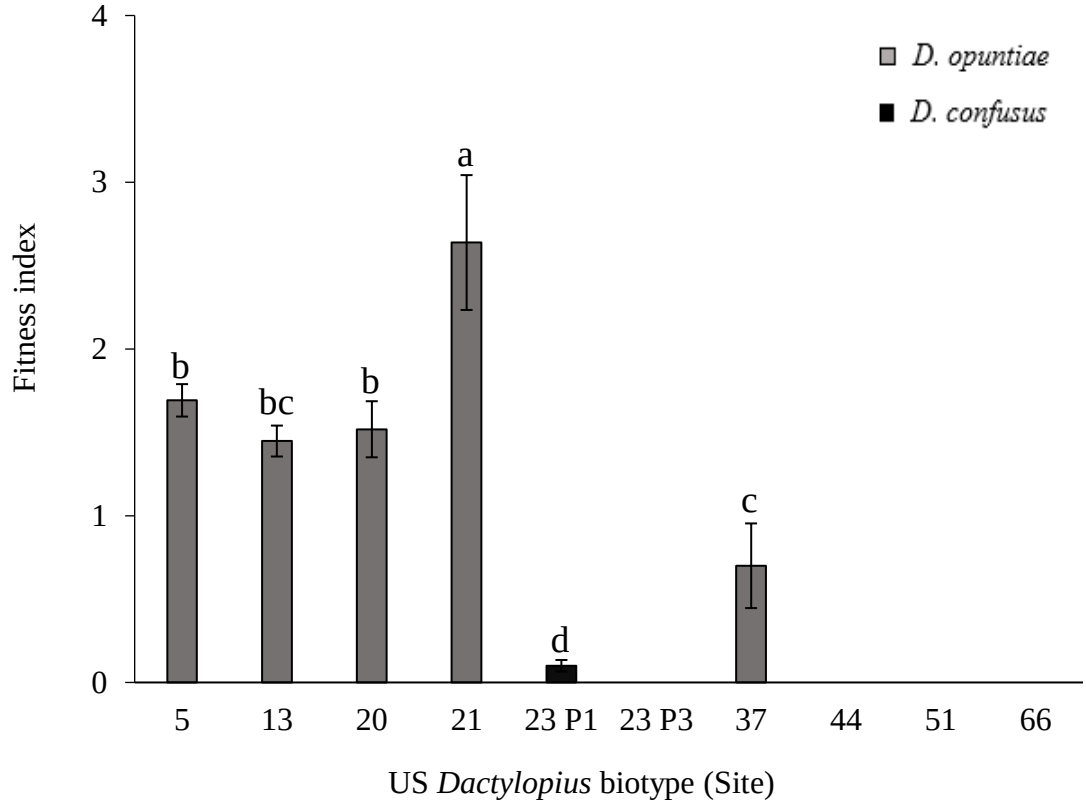

Figure 2.9: The mean fitness index (FI) of 10 *Dactylopius* biotypes from USA on Kenyan *Opuntia engelmannii*, calculated as the proportion of adult females multiplied by the average number of offspring per female over mean developmental time. n = 30 crawlers/cladode (n = 6 ± SE). Different lowercase letters indicate significant differences between sites of origin (P < 0.001).

2.4.4.2. Eastern Cape lineage

All the biotypes of *D. opuntiae* showed a high acceptance for the EC *O. engelmannii* lineage by exhibiting a higher FI compared to the same biotypes on the Kenyan lineage. The EC lineage is a clear suitable host for the three D. *confusus* biotypes (Figure 2.10). The Fitness Index was high for *D. opuntiae* from sites 13, 37, 66 and for *D. confusus* from site 51, with *D. opuntiae* from site 37 showing high acceptance for the EC *O. engelmannii* lineage. The biotype with the lowest FI was *D. opuntiae* from site 20 (Figure 2.10). Overall, there was a significant different in FI between sites of origin on the EC lineage ($F_{8,43} = 4.4248$, $P < 0.001$). However, there was no significance different in FI between *D. opuntiae* from sites 5,13, 37,66 and *D. confusus* from site 51 ($F_{4,25} = 1.5890$, $P > 0.05$).

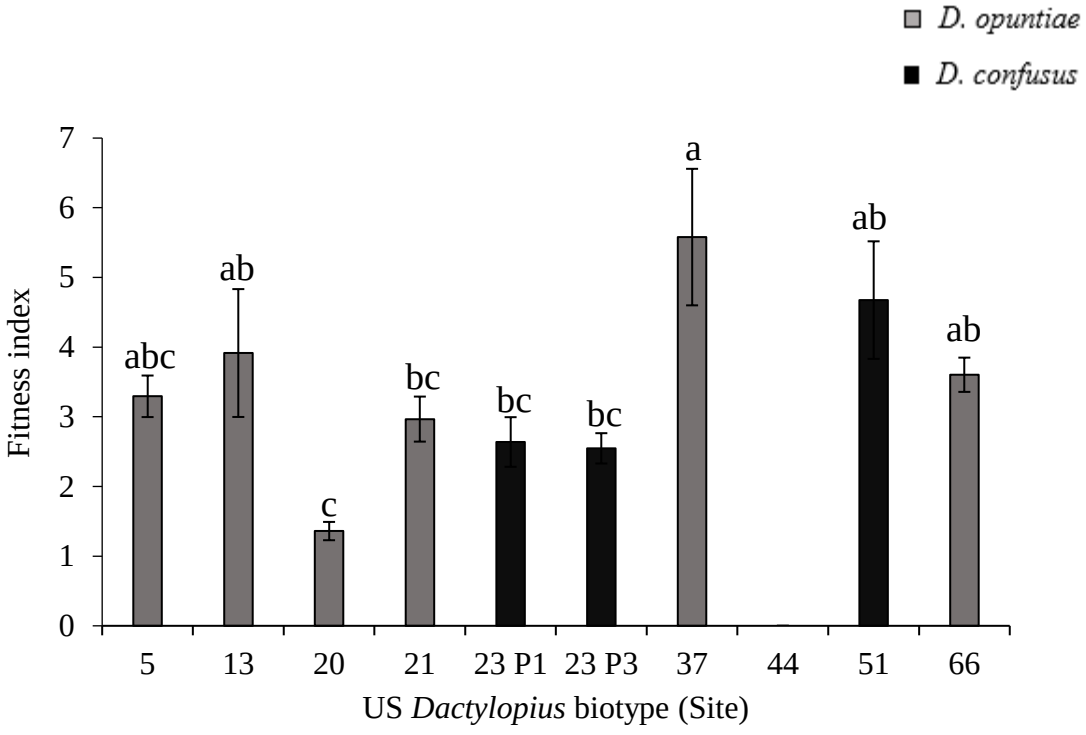

Figure 2.10: The mean fitness index (FI) of 10 *Dactylopius* biotypes from USA on EC *Opuntia engelmannii*, calculated as the proportion of adult females multiplied by the average number of offspring per female over mean developmental time. n = 30 crawlers/cladode (n = 6 ± SE). Different lowercase letters indicate significant differences between sites of origin ($P < 0.001$).

2.5. Discussion

These results show that the biotypes within the *D. opuntiae* and *D. confusus* cochineal species differed in their degree of acceptance and suitedness to both the Kenyan and EC *O. engelmannii* lineages. The Kenyan *O. engelmannii* lineage was the least acceptable and suitable host, while the EC *O. engelmannii* lineage was the most acceptable and suitable host. Differences in the degree of acceptability and suitability shown by the *O. engelmannii* lineages might be due to the cochineals' host specific nature (Githure *et al*., 1999; Volchansky *et al*., 1999). For example, in cross-over tests of the host plant affinities of the 'stricta' and 'ficus' biotypes of *D. opuntiae*, the survival of the *D. opuntiae* biotypes was much greater on their conventional host plants than it was on their alternate host plants (*O. stricta* and *O. ficus-indica* respectively) (Githure *et al*., 1999; Volchansky *et al*., 1999; Rule and Hoffmann, 2018). This suggests that each plant species is an imperfect host for the other biotype of *D. opuntiae* (Rule and Hoffmann, 2018). Differences in the degree of acceptability and suitability of the *O. engelmannii* lineages may also be a result of different plant traits such as the level of amino acids, phytochemical and morphological traits such as spine morphology, tannins, pH and the amount of acid in the cladodes of the host plants (Meraz-Maldonado *et al*., 2012; El-Mostafa *et al*. 2014; Musengi, 2018). For example, Musengi (2018) found that the most acceptable host plants for the 'stricta' biotype of *D. opuntiae* were *O. stricta* and *O. humifusa*, which showed low levels of acidity in their organic acids compared to the *O. engelmannii* lineages that had more alkaline organic acids (Musengi, 2018).

The proportions of settled females of *Dactylopius* biotypes on detached cladodes was much lower on the Kenyan *O. engelmannii* lineage than it was EC *O. engelmannii* lineage. This is similar to a study conducted by Musengi, (2018), where the 'stricta' biotype showed a lower proportion of settled females on the Kenyan O. *engelmannii* lineage than on *O. humifusa, O. stricta*, and LM *O. engelmannii* lineages. Githure *et al*., (1999) and Volchansky *et al*., (1999), also observed a similar trend in the proportion of settled females, where the '*stricta*' and '*ficus*' biotypes of *D. opuntiae* showed lower proportions of settled females on alternate host plants than they did on their conventional host plants (*O. stricta* and *O. ficus-indica* respectively).

The positive linear relationship between the body mass of gravid females and the number of crawlers produced by each female on both Kenyan and EC *O. engelmannii* lineages is a common phenomenon among insects (Moran and Cobby, 1979) and has been observed in other *Dactylopius* species (Githure *et al*., 1999; Mathenge *et al*., 2009a). The average body mass of gravid females and the number of crawlers produced per female cochineal were much greater

on the EC *O. engelmannii* lineage in comparison with that observed on the Kenyan *O. engelmannii* lineage and that of other species of *Dactylopius* (Volchansky *et al*., 1999; Mathenge *et al*., 2009b). The high number of crawlers produced by each gravid female on the EC *engelmannii* lineage suggest that the crawlers have the ability to build high population levels within a short period and thus the impact of the cochineals in high numbers could eventually causes the plants to succumb. Contrary to the findings of the developmental times of *D. tomentosus* (Lamarck) biotypes by Mathenge *et al*., (2009a), prolonged development time was observed in biotypes that produced more heavy and fecund female cochineal insects on both the Kenyan and EC lineages. However, when comparing the developmental times of the *Dactylopius* biotypes on the Kenyan and EC *O. engelmannii* lineages, the developmental time was much shorter on the EC *O. engelmannii* lineage. This is similar to what was observed in *D. tomentosus* (Mathenge *et al*., 2009a) and *D. opuntiae* biotypes (Rule and Hoffmann, 2018), suggesting that all the *Dactylopius* biotypes perform best on the EC lineage.

From the 10 biotypes tested, four *D. opuntiae* biotypes (sites 5, 13, 20 and 21) thrived on the Kenyan *O. engelmannii* lineage (FI $\geq$ 1), therefore they fall under category 1 of fitness indices (Appendix I). The remaining six biotypes ranged between surviving (sites 37 and 23 P1) and dying (sites 23 P3, 44, 51,66) on the Kenyan *O. engelmannii* lineage, falling under category 2 and 3 respectively (Appendix I). The FI of *Dactylopius* biotypes on the EC *O. engelmannii* lineage were very high in comparison with those observed in other species of *Dactylopius* (Mathenge *et al*., 2009a; Mathenge *et al*., 2010). This suggests that the EC *O. engelmannii* lineage is a more suitable host for these biotypes.

In general, the best performing biotype on the Kenyan *O. engelmannii* lineage were biotypes collected from US *O. engelmannii* lineages that have similar morphological traits to the Kenyan *O. engelmannii* lineage, both having yellow-spined cladodes and brown bases (Figure 4.1). Geographically, these biotypes are in the same region (south of Texas) in USA (Figure 4.1). This could perhaps indicate an old association (i.e., the insect biotype and its host have co-evolved over time). Meanwhile the best performing biotypes on the EC lineage of *O. engelmannii* were biotypes coming from white-spined *O. engelmannii* lineages, of which none had the same morphological characteristics as the EC lineage. These *O. engelmannii* lineage are geographically more isolated in the north of the US collection sites (Figure 4.1). This might represent a new association, since it is unlikely that there is a shared evolutionary between the targeted EC lineage and the *Dactylopius* biotypes, nor there is any morphological resemblance between the EC *O. engelmannii* lineage and the US *O. engelmannii* lineage sampled to suggest

that any of the two could be the same lineage or even species. The differences in morphology between the American *O. engelmannii* lineages and the EC *O. engelmannii* lineage suggest that the EC *O. engelmannii* might be a different species of *Opuntia*. The principal component analysis (PCA) of spine morphology and genetic analysis *O. engelmannii* lineages from a study by Mbonani (2019), affirms that the EC *O. engelmannii* is a different species of cactus. van Steenderen, (2020), used DNA barcoding and Inter Simple Sequence Repeats (ISSRs) to successfully delineate species of *Dactylopius* and lineages (van Steenderen *et al*., 2021), and one of his findings was that, of the ten population of *Dactylopius*, 6 (sites 5, 13, 20, 21, 37 and 66) were *D. opuntiae* species, while the remaining 4 biotypes (sites 23 P1, 23 P3, 44 and 51) were *D. confusus* species. This has an important implication for agent selection as these species are host-specific. These findings also help to further explain the different feeding behaviours of the ten *Dactylopius* biotypes on the Kenyan and EC *O. engelmannii* hosts and suggest that the Kenyan and EC *O. engelmannii* might be different species of *Opuntia*.

Even though *D. confusus* biotypes thrived (F>1) on the EC *O. engelmannii*, they appeared not to be causing any substantial damage to their host plant compared to *D. opuntiae* biotypes. This could be influenced by many factors intrinsic to a particular host plant such as the chemical compounds of the plant (Musengi, 2018). However, one of the factors that might have contributed to this is host tolerance. This is best explained by the enemy tolerance hypothesis. The enemy tolerance hypothesis proposes that, when some plants cannot escape their natural enemies, they will maintain their reproductive fitness by becoming tolerant to the damage or negative effects caused by their natural enemies (Ashton and Lerdau 2008; Espinosa and Fornoni, 2006; Schulz *et al*., 2019). Plant inducible defense mechanism can also be a contributing factor. For instance, sap-sucking insects like *D. confusus* primarily feed on the xylem and phloem (vascular tissue of a plant transporting soluble organic substances made during photosynthesis) (Meyer and Whitlow, 1992; Schaeffer *et al*., 2017) of the plant, however, it might be difficult to get to this phloem sap because the plant is defending itself by releasing alkaline organic acids (especially *O. engelmannii* lineages) that makes the plant to be less palatable (Dungan *et al*., 2007; Musengi, 2018; Cowie *et al*., 2018).

The results from this chapter together with the widely separated *Dactylopius* sites on the US map (figure 4.1) suggest that not all ten *Dactylopius* biotypes (including the different species) might be from old associations. We suggest that the isolated *Dactylopius* sites might represent new associations. The reasons as to why would a new association or an old association kill or fail to kill the plants are further explored in Chapter three.

CHAPTER THREE: Assessing herbivory damage caused by four *Dactylopius* biotypes on whole potted plants of Kenyan and Eastern Cape *Opuntia engelmannii* lineages

Abstract

Plant responses to insect herbivory are often poorly understood because they are complicated, dynamic and widespread. The co-evolutionary relationship between plants and insects have existed for Millenia. This evolutionary arms race between plants and insects has resulted in plants evolving intricate and advanced defense mechanisms, which affect insects performance, behaviour or preference. The shared evolutionary history between insects and their hosts in their native range has enabled insects to evolve feeding strategies that successfully counteract some of these plant defense mechanisms and use them as feeding stimulants. This forms an old association between the insects and their hosts. New associations occur when the insects and their hosts have no shared evolutionary history in their introduced range. Effective biocontrol was selected based on which *Dactylopius* biotypes caused rapid mortality on whole, potted plants of Kenyan and EC *O. engelmannii* lineage. Chlorophyll content, cladode thickness, biomass and insect percentage cover on plant surfaces were used as measures of plant-insect responses. Based on the reduction in chlorophyll content, cladode thickness and biomass of the host plants, the *D. opuntiae* biotype from collection site 21 was the most effective biocontrol agent on the Kenyan *O. engelmannii* lineage. This is assumed to be an old association between the insect biotype and its host plant. Meanwhile, *D. opuntiae* biotype from collection site 66 was the most effective biocontrol agent on the EC *O. engelmannii* lineage, and this is suggested to be a new association between the cochineal biotype and its host plant. The Kenyan and EC *O. engelmannii* lineages were less susceptible to herbivory damage inflicted by the *D. confusus* biotypes, suggesting that *D. confusus* biotypes will not be good biocontrol agents for either *O. engelmannii* lineages. These findings and the morphological and genetic differences between the African and American *O. engelmannii* lineages, suggest that the EC *O. engelmannii* lineage might be a South American *Opuntia* species. These successes and failures of biocontrol agents on their host plants have important implications for the biological control of *Opuntia*s in Africa.

Keywords: Chlorophyll content, cladode thickness, effective agent, enemy release, percentage cover, inducible defenses.

3.1. Introduction

Plant responses to insect herbivory are often dynamic and complex, depending on the feeding style and herbivore damage (e.g., sap-feeders versus leaf feeders), and the plant parts or structures being attacked (e.g., feeding on stems versus feeding on whole plants) (Meyer, 1993; Van Zandt and Agrawal, 2004; Zvereva *et al*., 2010). Removing a cactus cladode from a whole plant and subjecting it to insect herbivory will cause the cladode's physiology to respond differently to that of the whole plant which is rooted on the ground. This is due to the plant's defense mechanisms. Plant defense mechanisms play an important role in controlling the host selection and performance of herbivorous insect populations (Zangerl and Berenbaum, 1993; Nomikou *et al*., 2003; Harvey *et al*. 2011). Plants and insects have been co-existing for so long that both have evolved strategies than can either avoid or suppress each other's defense systems (War *et al*., 2012; Bentur *et al*., 2016). This evolutionary arms race between plants and insects has resulted in plants evolving sophisticated and advanced defense mechanisms that enable them to recognise damaged cells and activate their immune responses against herbivorous insects (War *et al*., 2012). These plant defense mechanisms against insect herbivory are also shared across most plant families (Stotz *et al*., 1999; Kant *et al*., 2015). The co-evolutionary theory suggest that some insects have been successful in counteracting some of the plant's defense mechanism (Consales *et al*., 2012; Kumar, 2019) and have used those defense mechanism as feeding stimulants for those insects that specialize on the plant (Chown and Nicolson, 2004). In the case of sap-sucking insects like *D. opuntiae*, the shared evolutionary history between the cochineal insects and their host plants in their native range has enabled the insects to evolve feeding strategies such as specialized mouthparts called stylets that secrete watery and gelling saliva that protect the insects against the plants defense chemicals, enabling the insects to feed and successfully counteract the host immune response (Harris, 1991; War *et al*., 2012; Jiang *et al*., 2019). This is known as an old association (Dennill and Hokkanen, 1990). A new association would be when an insect and its host have no co-evolutionary history (Hokkanen and Pimentel, 1989). The word defense, is often reserved for traits that increases the plant's fitness when it is under attack (Agrawal, 1999; Karban and Baldwin,1997; Kessler and Baldwin, 2001, 2002). Plant response mechanism elicited by insect herbivory can be broadly categorised into two strategies namely: defensive (resistance) and tolerance (Cowie *et al*., 2018). Defensive mechanisms include morphological/physiological traits (e.g., thorns) or secondary chemical compounds that are toxic (e.g., glucosides) or have no nutritional value to the insect herbivores (Dungan *et al*., 2007; Cowie *et al*., 2018). These defense mechanisms are

further subdivided into two groups namely: constitutive and inducible defenses (Agrawal, 1999; Chen, 2008; Kaplan *et al*., 2008). A plant is either constitutively defensive due to pre-formed resistant traits, or it becomes defensive after an attack as a result of herbivore-induced changes that are either localised on certain parts of the plant or expressed on the whole plant. This study will focus on inducible defense mechanism.

3.1.1. Inducible defense

Inducible defenses are those that are activated after an insect has attacked a plant. There are two types of inducible defences: direct and indirect (Chen, 2008; Gols, 2014; Aljbory and Chen, 2018) (Figure 3.1). Direct defenses include all plant traits that assist the plants to defend themselves against their enemies and help reduce the performance or preference of their enemies (Chen, 2008; War *et al*., 2012; Schulz *et al*., 2019). These include reducing nutrient value, cell wall strengthening, and releasing chemicals that physically damage the attacking insect (e.g., protease, secondary metabolites, protein inhibitors of insect digestive enzymes etc.) (Kessler and Baldwin, 2001; Dicke *et al*., 2003; Chen, 2008; War *et al*., 2012). Indirect defenses are plant traits that increase the chances of attracting inherent enemies of the insect herbivore (Burger and Louda, 1994; Ched, 2008; Carmona *et al*., 2011). The time between first insect attack on a plant and the defense activation make inducible defenses inherently inferior to constitutive defenses (Baldwin, 1998). Although inducible defense strategies have some metabolic costs, they are important in reducing stress of immediate concern, as most of these chemical compounds are produced in response to an attack evoked by insect herbivory (Chehab *et al*., 2008; War *et al*., 2012; Zhou *et al*., 2015).

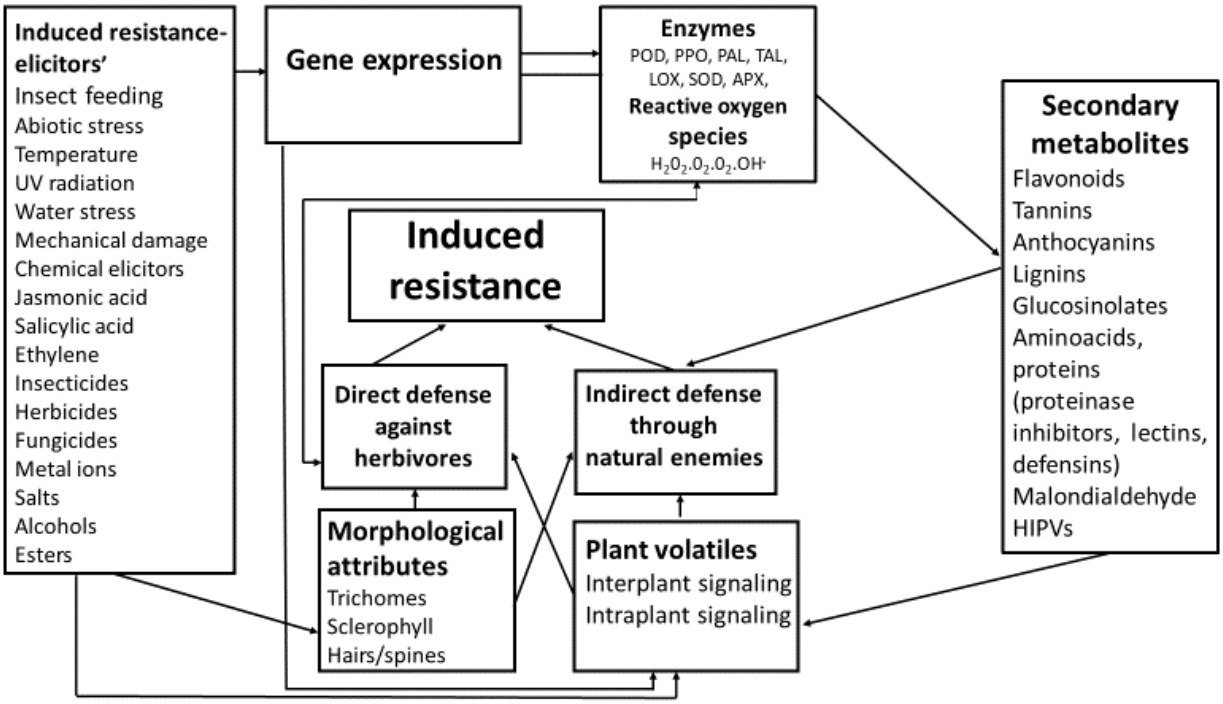

Figure 3.1: Inducible defense (resistance) mechanism in plants against insect herbivory (War *et al*., 2012).

3.1.2. Tolerance

Tolerance mechanisms are defined as those plant traits that reduce detrimental effects caused by herbivory on plant fitness (Leimu and Koricheva, 2006). Tolerance mechanisms are mainly by compensatory growth (Dungan *et al*., 2007), and include: formation of new leaves, increased photosynthetic activity in remaining leaves and increased biomass or seed production (Strauss and Agrawal, 1999; Dungan *et al*., 2007; Cowie *et al*., 2018). These mechanisms are generally described as induced or 'active' responses of tolerance due to herbivore damage that causes physiological changes that do not occur in undamaged plants (Tiffin, 2000). Other responses include changes in phenology and utilization of stored reserves (Suwa and Maherali, 2008).

3.1.2.1. Compensatory growth

The damage caused by insect herbivory can change plant growth trajectories, allowing plants to counteract or offset the losses incurred during and after herbivory (Tiffin, 2000; Cowie *et al*., 2018). Regrowth in plants can take place through the forming new leaves or by replacement of removed tissues (Dungan *et al*., 2007). The formation of new leaves is done either through the activation of apical dormancy or through reallocation of resources to foliage production at the cost of reproductive output (Strauss and Agrawal, 1999). Regrowth of aboveground plant tissue after defoliation is linked to reduction in root growth (Bardgett *et al*., 1998; Hudgeons

et al., 2007). Reduced roots will have smaller volume, limiting the acquisition of resources such as nutrients and water and thus reducing plant fitness (Wise and Abrahamson, 2005; Craine and Dybzinski, 2013). Compensatory regrowth can also incur plant fitness cost because of the activated apical meristems which compete with the developing fruits and flowers for nutrients, sugars and water subsequent to the damage caused by insect herbivory (Strauss and Agrawal, 1999; Avila-Sakar, 2020). Plants may also compensate by increasing photosynthetic rates in the residual leaf tissues, particularly in developing leaves or in neighbouring leaves that are not damaged (Delaney, 2008; Cowie *et al*., 2018). However, this heavily relies on resources available to the plants (i.e., stored reserves) (Suwa and Maherali, 2008; Cowie *et al*., 2018). Moreover, an increase in photosynthetic activities may not be substantial enough to overcome severe defoliation (Kant *et al*., 2015; Cowie *et al*., 2018). Plants may increase their below (root) biomass in response to insect herbivory (Strauss and Agrawal, 1999; Johnson *et al*., 2016). Plants with high root-shoot ratios may be more tolerant to herbivory since they are better able to acquire nutrients for seed production or regrow after losing nutrients stored in tissues that have been consumed by insect herbivores (Strauss and Agrawal, 1999; Tiffin, 2000). Changes in plant phenology is one of the most common, but less studied mechanism of plant tolerance (Hofmann and Todgham, 2010; Vitasse *et al*., 2014). Numerous studies have shown that a delay in plant phenology caused by herbivory may result in genetic alteration for tolerance (Tiffin and Rausher, 1999; Weinig *et al*., 2003; Barton, 2013). For example, plants living in seasonal environments where the end of the growing season may limit reproduction (Tiffin, 2000), a delay in seed maturation caused by herbivory may cause the genotypes that experience the shortest delay after damage to be the most tolerant since only those genotypes will be able to produce seeds before the end of the season (Tiffin, 2000; Mitchell *et al*., 2016). Empirical evidence suggests that the delay in plant phenology is dependent on the type of species and the type of insect herbivory (e.g., partial defoliation versus meristem damage. etc.) (Meyer, 1998b; Tiffin, 2000).

Plant responses to sap-feeding insects like *Dactylopius* species are mostly physiological rather than chemical or structural (Dungan *et al*., 2007; Cowie *et al*., 2016). Hence, the following physiological plant responses were evaluated to assess which cochineal biotype's herbivory resulted in the most rapid plant mortality: chlorophyll content, cladode thickness, biomass and percentage cover of cochineals.

3.1.3. Chlorophyll Content

Chlorophyll is a pigment that gives plants their characteristic green colour and determines the plant's photosynthetic capacity (Palta, 1990; Huang *et al*., 2013). Chlorophyll content in plant tissue is considered as one of the primary measures used in host plant-insect interactions because it is a good indicator of plant photosynthetic performance (Palta, 1990; Goggin *et al*., 2015; Golan *et al*., 2015). Changes in chlorophyll content is influenced by both biotic stress factors (e.g., pathogen infections and insect feeding) and abiotic stress factors (e.g., nutrition deficiency, pigment inhibiting herbicide damage, light etc.) (Strauss and Agrawal, 1999; Zvereva *et al*., 2010). Deficiency in plant nutrients such as phosphorus, nitrogen, magnesium etc., can manifest as a reduction in chlorophyll content in leaf tissue (Curran *et al*., 1990; Kalaji *et al*., 2017). A decrease in an overall plant chlorophyll content affects the plant's photosynthetic ability (Li *et al*., 2018). Photosynthesis plays a vital role in all parts of plants physiology since it provides energy and assimilates for growth and reproduction (Lawlor, 2009; Huang *et al*., 2013).

3.1.4. Cladode thickness

Plant thickness is an important parameter involved in the interaction between sap-sucking insects and their host plants (Khalil *et al*., 2017). Sap feeding insects like *D. opuntiae* remove vital compounds in leaf tissues (e.g., chlorophyll, sugars, water etc.) and reduce the plant's vigor (Khalil *et al*., 2017). In cases where there is serious damage, drooping, wilting and reduction in leaf thickness occur (Abro *et al*., 2004; Khalil *et al*., 2017). Dongi *et al*., (2018) showed that an increase in density of cochineal insects (both nymph and female adults) reduces cladode thickness of *O. ficus-indica* and thus stunt plant growth.

3.1.5. Insects and plants Percentage cover

Percentage cover is one of the measures of species relative abundance of sessile plants and animal (Ewers and Didham, 2006; Damgaard, 2014). There are three common techniques used for measuring percentage cover namely; visual estimates, random-point -quadrats (RPQ) and digital methods (Connel, 2005; Drummond and Connell, 2005). The visual estimate method is very subjective, while the RPQ method is more precise and objective. The digital method is the most preferred and accurate technique for measuring percentage cover (Whorff and Griffing, 1992; Connel, 2005). The digital method uses photographs and newly developed software like imageJ to accurately estimate percentage covers (Rasband, 1997; Cai *et al*., 2011).

3.1.6. Plant Biomass

Biomass is defined as "the weight of living plant material contained above and below a unit of ground surface area at a given point in time" (Roberts *et al*., 1993). Biomass can be measured for individuals or a group of species or expressed as the total weight of both plants and animals (Catchpole and Wheeler, 2006). Biomass is an important indicator of ecological and management process in an ecosystem. As an ecological indicator, biomass measures species dominance within an ecosystem because it reflects the amount of resources such as water, minerals and light energy captured by the plants which are later turned into plant mass (Catchpole and Wheeler, 2006). Biomass estimates also affects the hydrologic properties of an area including runoff, infiltration and erosion. Biomass estimates can be used to assess rangeland conditions, for instance, measures of standing crops reflect the amount of energy stored in the crops, which then indicate the potential productivity of the vegetation (Pieper, 1988; Ramoelo *et al*., 2012). Plant biomass can be divided into two parts, below-ground (roots) and above-ground biomass (stem and leaf) (Drake *et al*., 2003; Niklas, 2005). The relationship between below-ground and above-ground biomass can reflect growth responses of plants to ambient ecological conditions or to experimental manipulation (Hunt and Lloyd, 1987; Niklas, 2005).

3.2. Methods and Materials

The main purpose of this experiment was to determine which cochineal biotype would result in the most rapid mortality of its test plant. To determine the impact of the cochineal on potted plants, the four best performing biotypes of *D. opuntiae* and *D. confusus* (defined as those with the highest female fitness index on either the Kenyan or EC *O. engelmannii* lineage) were selected. Five potted *O. engelmannii* treatments (for each of the four *Dactylopius* biotypes) and five *O. engelmannii* controls (with no insects), making a total of 25 potted plants per lineage were used. These potted *O. engelmannii* plants were of similar sizes and were kept under the same room conditions where they received natural light with temperature ranging between 20℃ and of 30℃ and a relative humidity of about 50% in the Wits quarantine facility. Each of the five potted plants of Kenyan or EC *O. engelmannii* lineage were inoculated with two gravid females of *D. opuntiae* and *D. confusus* biotypes respectively, then placed in separate cages. Control plants were also kept in cages for both Kenyan and EC *O. engelmannii* lineages. The *O. engelmannii* treatment and control plants were given the same amount of water when it was necessary during the experiment. The four *Dactylopius* biotypes used on this experiment for the Kenyan *O. engelmannii* lineage were *D. opuntiae* biotypes collected from sites 5, 13, 20

and 21 in USA (Appendix II). While the biotypes used for the EC *O. engelmannii* lineage were *D. confusus* biotype collected from site 23P3 and *D. opuntiae* biotypes collected from sites 5, 21 and 66 in the USA (Appendix II). The following parameters were used to assess the impact of the cochineal biotypes: (a) chlorophyll content, (b) cladode thickness, (c) insect percentage cover and (d) biomass. Measurements were taken every two weeks over the duration of 8-9 months. Measurements were taken for both treatments and controls. The *O. engelmannii* test plants are considered to be dead when they are completely dry, with the cladodes changing colour from green to brown.

(a). Chlorophyll content

The chlorophyll content of the infested (treatment) and the uninfested (control) cladodes was measured non-destructively on each potted plant using a chlorophyll content meter (CCM-300) (Opti-sciences, Inc. USA). Five measurements were taken non-destructively per cladode on each potted plant at random. Then an average of the total cladode measurements of chlorophyll content were done for each potted plant on both *O. engelmannii* lineages. These measurements were taken every two weeks for a duration of 8-9 months.

(b). Cladode thickness

The cladode thickness (mm) of each potted plant was measured non-destructively using a Vernier Calliper on the lateral side of the cladode. This was done consistently on the same area of the cladode in order to note the damage or changes in cladode thickness of the plant. A single measurement was taken per cladode on each potted plant. Then an average of the total cladode thickness was done for each potted plant on both *O. engelmannii* lineages. Measurements were taken every two weeks over the duration (8-9 months) of the experiment.

(c). Insect percentage cover

The percentage cover of cochineal on the cladode surface was measured by taking images of the infested potted plants using a digital camera. The images were taken (using a standard procedure of taking photographs) twice per week over a period of 244 days. The images were analysed using ImageJ software, which was used to calculate the area of the plant infested by cochineal insects and the total area of the plant (in pixels) separately. Those values were used to calculate the proportions of insect cover on the potted plants using the equation below.

Percentage cover = Area of the plant covered by insects (pixels) / Total Area of the plant (pixels) x 100

(d). Plant biomass

After the plants have been killed by the insects, they were placed in air dry oven at 60 ℃ for a period of seven days, then separated into below ground biomass and above ground biomass. The below ground biomass consisted of roots (> 2mm in diameter) and the above ground biomass included the cladodes and spines. These were then weighed using a balance. The plant biomass was only measured for the Kenyan *O. engelmannii* plants because not all the plants of the EC *O. engelmannii* lineage had died by the end of the trial.

3.3. Data analysis

Data analysis of plant physiology was performed using STATISTICA Version 12.5 (StatSoft 2014). Data were tested for normality. If the data were normal, a Tukey HSD post hoc test was used or alternatively if not normal, a Kruskal-Wallis test was used. Proportions or percentage data that were not normally distributed were Arcsine transformed. Plant chlorophyll content and plant thickness were compared between treatments using one-way analysis of variance (ANOVA) with a Tukey HSD post hoc (StatSoft, 2014). Percentage cover between treatments over time was compared using one-way repeated measures (time) ANOVA with a Tukey HSD post hoc (StatSoft, 2014).

3.4. Results

3.4.1. Chlorophyll content

3.4.1.1. Kenyan lineage

After 16 weeks, there was a significant decline in the chlorophyll content of potted *O. engelmannii* plants in all the treatments compared to the control ($F_{4,313} = 59.246$, $P < 0.05$) (Figure 3.2). Chlorophyll content was significantly lower in the site 21 treatment than in all the other treatments (sites 5,13 and 20). There was, however, no significant difference in chlorophyll content between treatment from sites 5,13 and 20. Based on the greatest reduction in chlorophyll content, *D. opuntiae* from site 21 qualified as the most damaging biotype.

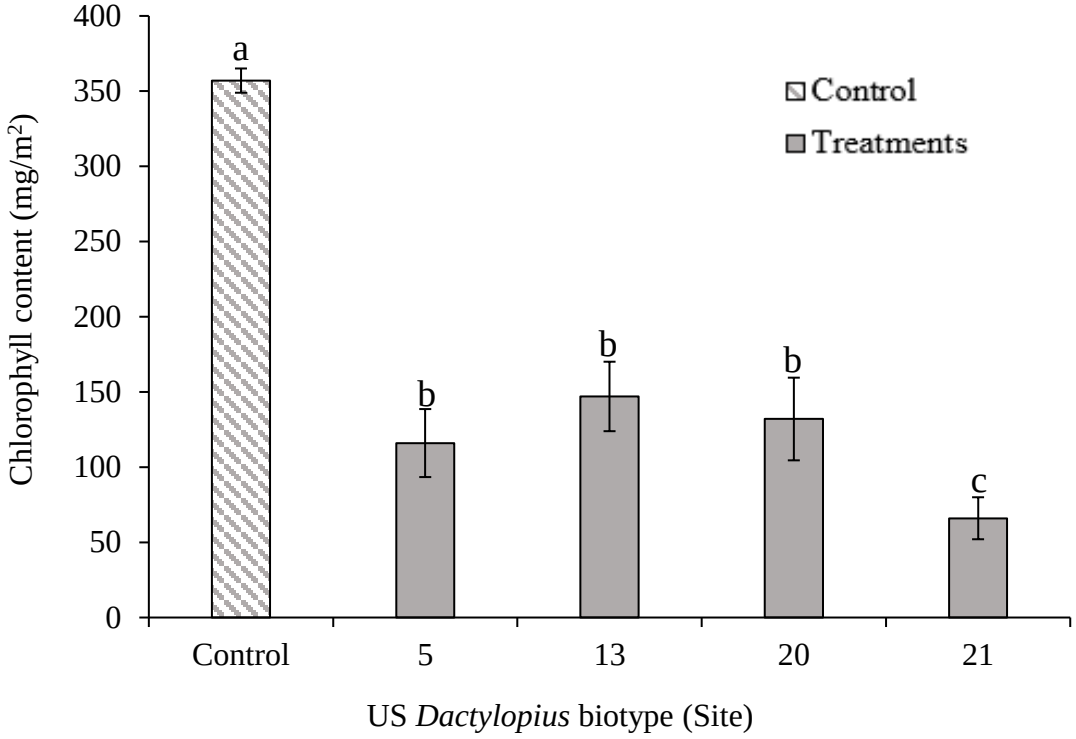

Figure 3.2: The mean chlorophyll content of potted *Opuntia engelmannii* Kenyan lineage in response to herbivory (eight months; 01st of March- 31st of October 2018) by the four United States of America cochineal biotypes that had the highest fitness index from the cladode trials. (n = 5 ± SE). Different lowercase letters indicate significant differences between treatments one-way ANOVA ($P < 0.05$) (grey- *Dactylopius opuntiae*).

3.4.1.2. Eastern Cape lineage

The chlorophyll content of the treatment plants of the EC *O. engelmannii* lineage was significantly reduced in comparison to their control ($F_{4,863} = 62.081$, $P < 0.05$). There was, however, no significant difference in chlorophyll content between plant treatment with *D. opuntiae* from sites 5, 21 and 66. Although the plant treatment with *D. confusus* from site 23 had a significant reduction in chlorophyll relative to the control, it had a significantly higher chlorophyll content compared to the other treatments (sites 5, 21 and 66) (Figure 3.3). Based on the greatest reduction in chlorophyll content, *D. opuntiae* from site 66 qualifies as the most damaging biotype.

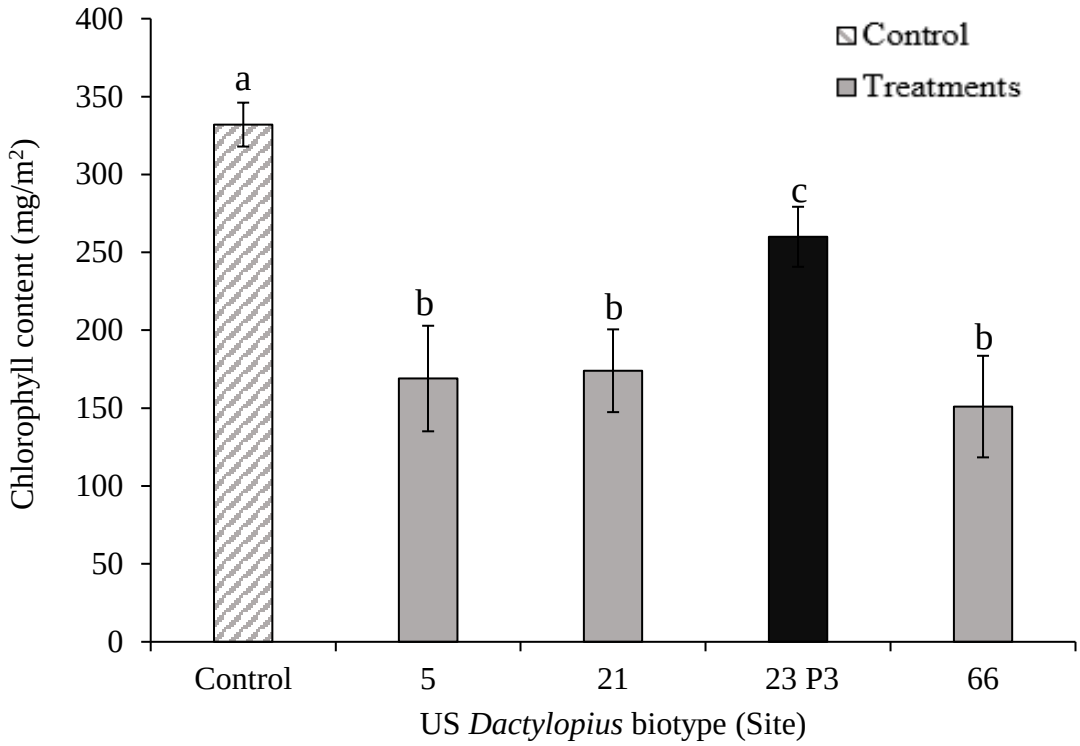

Figure 3.3: The mean reduction in chlorophyll content of potted *Opuntia engelmannii* Eastern Cape lineage in response to herbivory (nine months; 01st of April- 31st of December 2019) by the four United States of America cochineal biotypes that had the highest fitness index from the cladode trials. (n=5 ± SE). Different lowercase alphabets indicate significant differences between treatments and control using one-way ANOVA (P < 0.05) (grey- *Dactylopius opuntiae*; black- *Dactylopius confusus*).

3.4.2. Cladode thickness

3.4.2.1. Kenyan lineage

The thickness of cladodes from potted O. *engelmannii* Kenyan lineage plants with *D. opuntiae* infestation were significantly reduced in all the treatments (sites 5, 13 20 and 21) relative to the control ($F_{4,313} = 59.246$, $P < 0.05$) (Figure 3.4). However, there was no significant difference in cladode thickness between sites 5, 13 and 20 ($F_{2,42} = 0.15471$, $P > 0.05$) (Figure 3.4). *Dactylopius opuntiae* from site 21 was the most damaging biotype since it showed the greatest reduction in cladode thickness compared to the other biotypes (sites 5, 13 and 20).

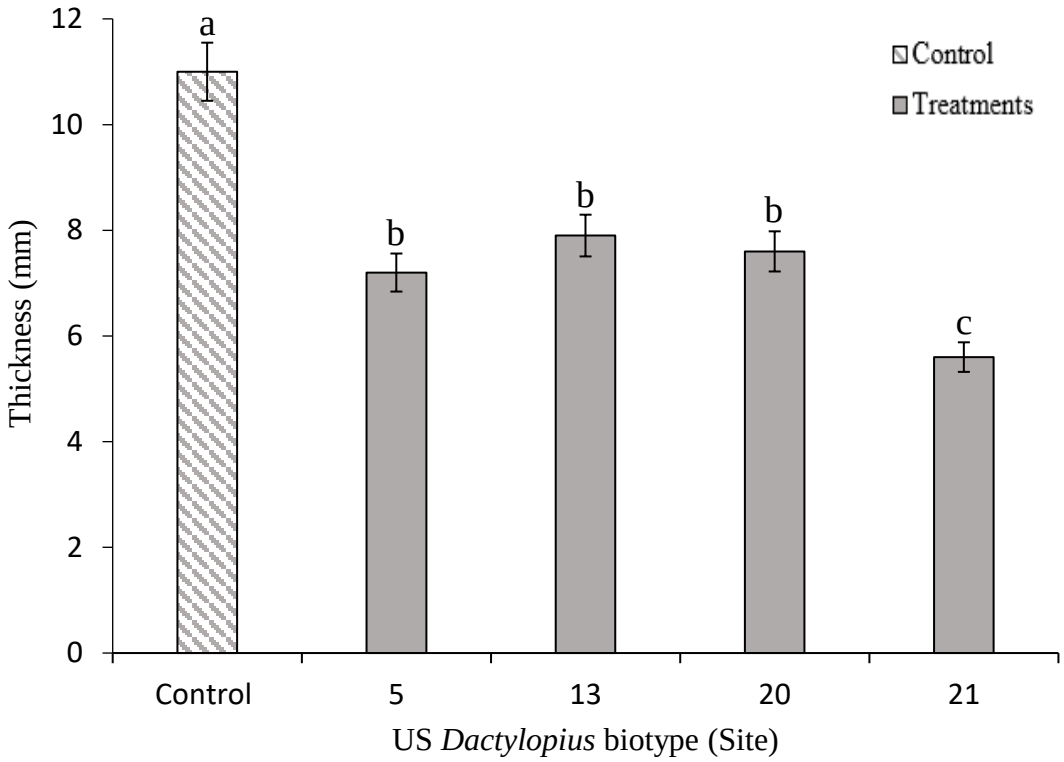

Figure 3.4: The mean thickness of potted *Opuntia engelmannii* Kenyan plants in response to herbivory (eight months; March-October 2018) by the four United States of America cochineal biotypes that had the highest fitness index from the cladode trials. (n=5 ± SE). Different lowercase alphabets indicate significant differences between treatments and control using one-way ANOVA ($P < 0.05$) (grey - *Dactylopius opuntiae*; black- *Dactylopius confusus*).

3.4.2.2. Eastern Cape lineage

The thickness of cladodes from potted *O. engelmannii* EC plants infested with *D. opuntiae* and *D. confusus* biotypes was significantly reduced in all the treatments (sites 5, 21, 23 P3 and 66) compared to their control ($F_{4,220} = 8.3403$, $P < 0.05$) (Figure 3.5). However, there was no significant difference in cladode thickness between treatments (i.e., between sites 5, 21, 23 P3 and 66) ($F_{3,171} = 8.3403$, $P > 0.05$). *Dactylopius opuntiae* from site 66 was the most damaging biotype since it caused greatest reduction in cladode thickness compared to the other biotypes (sites 5, 21, 23 P3 and 66).

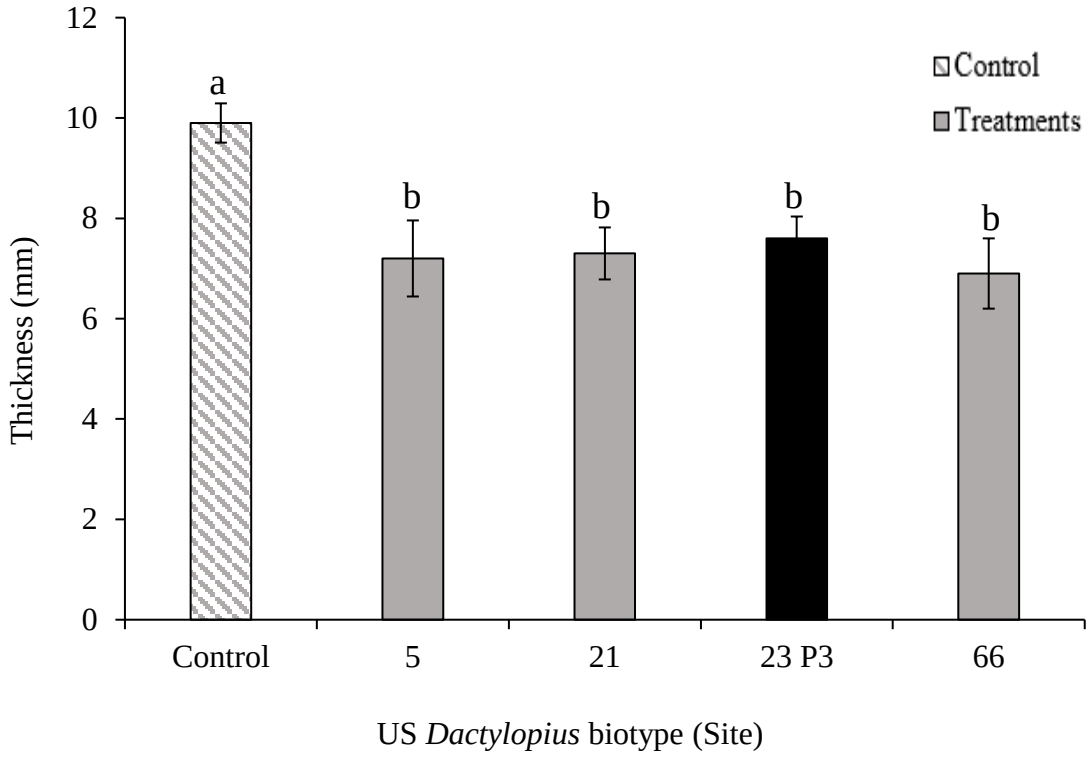

Figure 3.5: The mean thickness of potted *Opuntia engelmannii* Eastern Cape plants (n=5 ± SE) in response to herbivory (nine months; April-December 2019) by the four United States of America cochineal biotypes that had the highest fitness index from the cladode trials. Different lowercase alphabets indicate significant differences between treatments and control using one-way ANOVA ($P < 0.05$) (grey - *Dactylopius opuntiae*; black – *Dactylopius confusus*).

3.4.3. Cochineal percentage cover

3.4.3.1. Kenyan lineage

Dactylopius opuntiae from site 21 had the lowest cochineal percentage cover over time compared to the other biotypes (sites 5, 13 and 20) (Figure 3.6). However, there was no significant difference in cochineal percentage cover between *Dactylopius* biotype collection sites ($F_{3,16} = 0.7043$, $P > 0.05$). Time had a significant effect on percentage cover of the different *Dactylopius* biotypes (sites 5, 13, 20 and 21) ($F_{2,32} = 31.1610$, $P < 0.05$). Nonetheless, there was no significant interaction between time and biotype (site) in percentage cover of the different *Dactylopius* biotypes ($F_{6,32} = 0.4292$, $P > 0.05$). By day 152, all the cochineal biotypes from the different *Dactylopius* sites had killed the *O. engelmannii*, Kenyan lineage plants (as indicated by arrows on Figure 3.6). Both insects and plants had died by day 244.

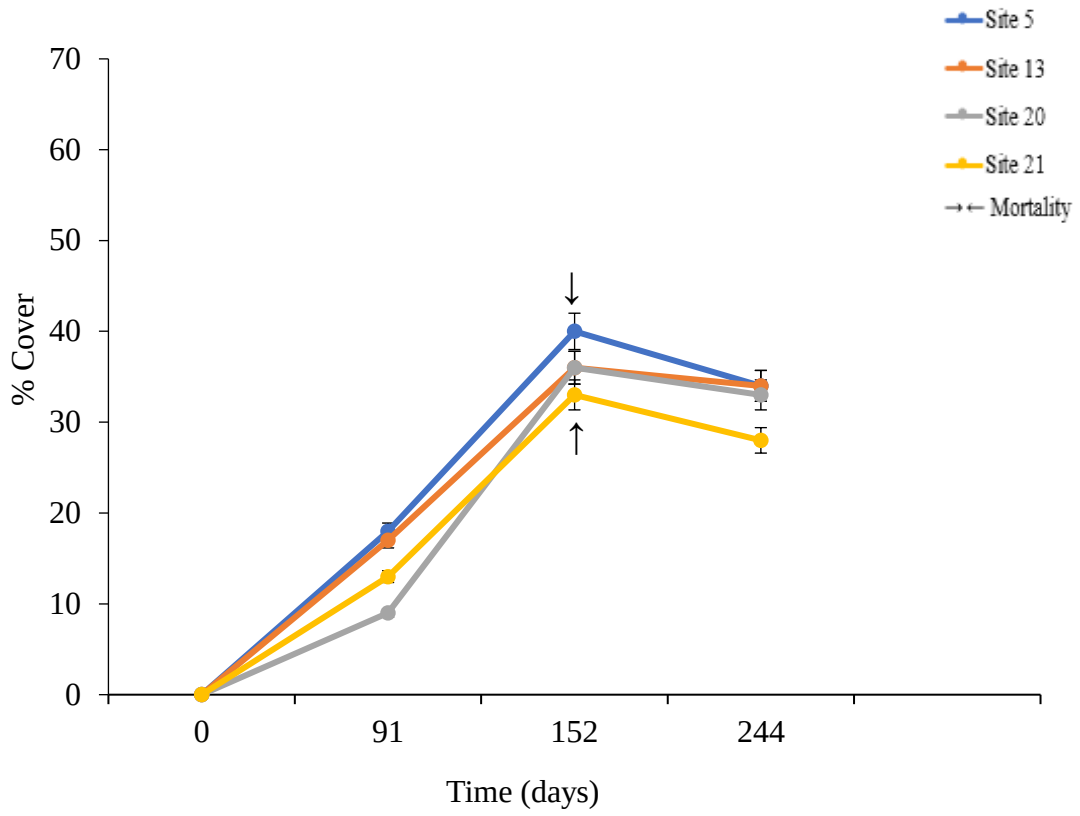

Figure 3.6: Percentage cover of cochineal insects on whole potted Kenyan *Opuntia engelmannii* lineage plants over a period of 244 days (March-October 2018). n = 5 potted plants for each data point (mean ± SE). (Fstat and P-value indicate overall difference between sites using repeated measures ANOVA). Arrows indicate the point at which the insect had killed the plants.

3.4.3.2. Eastern Cape lineage

Percentage cover of cochineals differed significantly between *Dactylopius* biotype collection sites on the EC lineage of *O. engelmannii* ($F_{3,8} = 5.969$, $P < 0.05$) (Figure 3.7). *Dactylopius opuntiae* biotype from site 66 ended up with the highest insect percentage cover. By Day 152 all the insect biotypes had killed the plants (indicated by arrows), except for *D. confusus* from site 23 P3, where the plants are persisting to date (Figure 3.7). Time had a significant effect on insect percentage cover ($F_{2,6} = 36.128$, $P < 0.05$). However, the interaction between time and biotype (site) did not differ on percentage cover of the different *Dactylopius* biotypes ($F_{16,6} = 1.146$, $P < 0.05$).

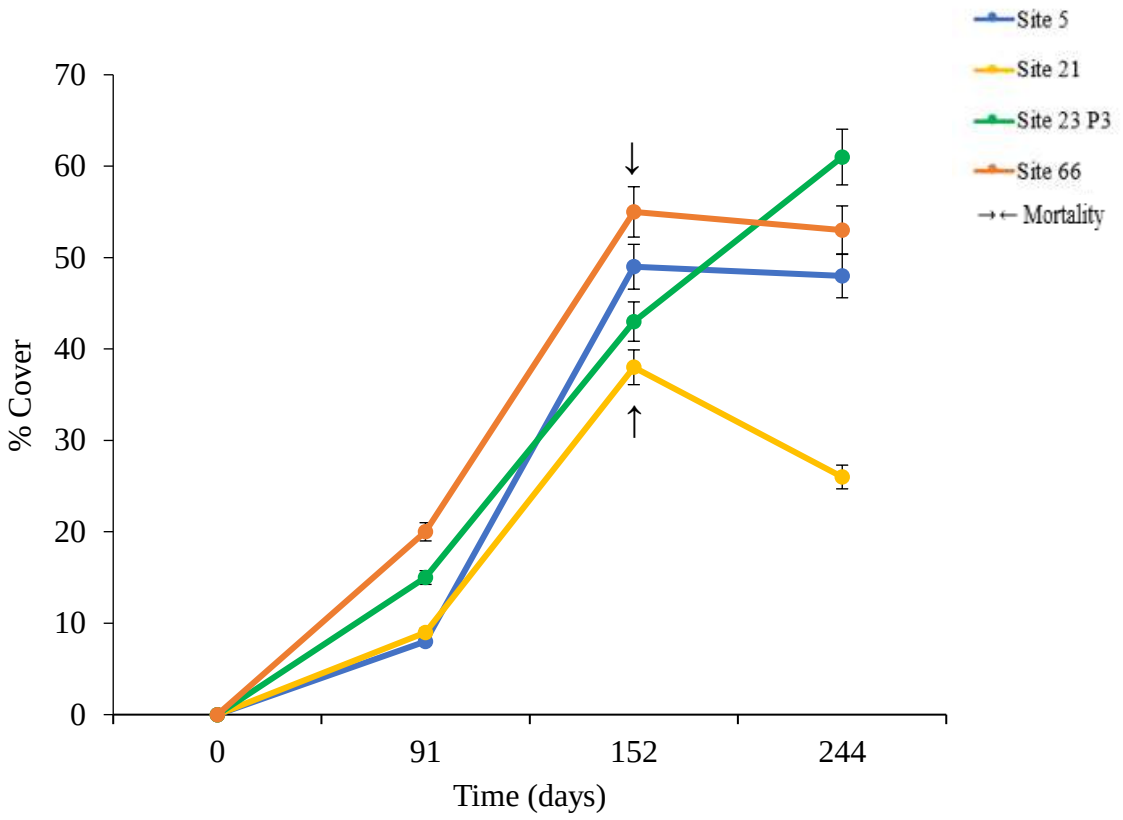

Figure 3.7: Percentage cover of cochineal insect on whole potted Eastern Cape *Opuntia engelmannii* plants over a period of 244 days (April-December 2019). n = 5 potted plants for each site (mean ± SE). (Fstat and P-value indicate overall difference between sites using repeated measures ANOVA). Arrows indicate the period in which the insect had killed the plants.

3.4.4. Plant Biomass

3.4.4.1. Kenyan lineage

Below ground biomass increased in the treatment plants of the Kenyan *O. engelmannii* lineage compared to the control (Figure 3.8), however, this was not significant ($F_{4,20}$ =1.1278, P >0.05). Above ground biomass was reduced in treatment plants relative to the control, however, this was not significant ($F_{4,20}$ = 0.55906, P >0.05) (Figure 3.8). Given that not all the plants of the EC *O. engelmannii* lineage had died (i.e., plants infested with *D. confusus* biotype), no biomass measurements were taken.

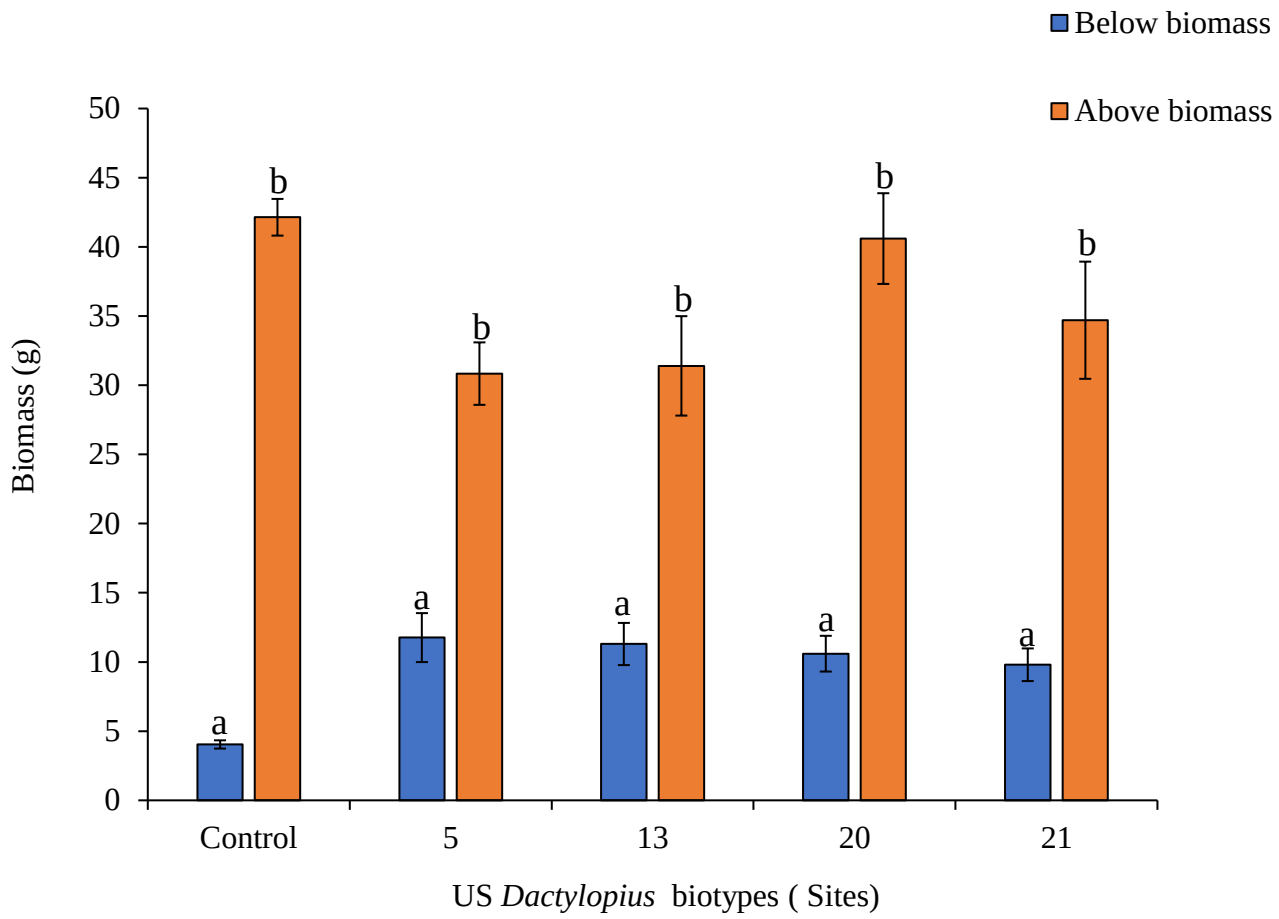

Figure 3.8: The mean plant biomass of potted *Opuntia engelmannii* Kenyan lineage plants (n = 5 ± SE) in response to herbivory by the four United States of America cochineal biotypes, using one-way ANOVA with a Tukey HSD post hoc tests. Different lowercase letters indicate significant differences between sites of origin (P > 0.05).

3.5. Discussion

Herbivory of the *D. opuntiae* biotypes from sites 21 and 66 significantly reduced the overall chlorophyll content on the Kenyan and EC lineages of *O. engelmannii*, respectively. Numerous studies on sap-feeders have associated a reduction in the chlorophyll content of plants with reduced photosynthetic rates (Cabrera *et al*., 1994, Zvereva *et al*., 2010; Cowie *et al*., 2016). The siphoning of water and other vital cladode compounds, particularly chlorophyll and photosynthates, which are essential in plant's physiological pathways affect the photosynthetic rates on damaged cladodes (Nagaraj *et al*., 2002; Cowie *et al*., 2016) and ultimately affects the long-term growth of the plant (Smith and Schowalter 2001). The reduction in chlorophyll content resulted in the *O. engelmannii* lineages suffering from chlorosis, accompanied by premature cladode abscission. Premature cladode abscission is an induced response of the plant

to insect herbivory to counteract the feeding damage (Stiling and Simberloff, 1989; Cowie *et al*., 2018). It allows the plant to enhance photosynthetic capacity in undamaged tissues (Maia *et al*., 2019).

Changes in cladode thickness and cochineal percentage cover were also used as measurable determinants of cochineal effectiveness on Kenyan and EC *O. engelmannii* hosts. Relative to the control, the cladode thickness was significantly reduced by *D. opuntiae* biotypes from sites 21 and 66 on both Kenyan and EC *O. engelmannii* lineages respectively. This could perhaps be attributed to the increased numbers of sap sucking insects on the plants. According to Dongi *et al*., (2018), increases in density of adult and nymph insects on cladodes, or other plant parts slows down the plant's growth rate and consequently results in stunted development, or in this case death. As these cochineal insects suck the plants, they inject toxins that are thought to cause the eventual death of the plant tissue (Moran, 1981; Mathenge *et al*., 2009a; Dongi *et al*., 2018). The observed effects of the *D. opuntiae* biotype on its host plant, resonate with previous studies in South Africa where *Dactylopius tomentosus* (Lamark), the '*cholla*' biotype was used as a biocontrol agent against two varieties of *Cylindropuntia fulgida* (Engelm.) namely; *Cylindropuntia fulgida* var. fulgida Engelm. ('chain-fruit cholla') and *Cylindropuntia fulgida* var. mamillata (Schott ex Engelm.) ('boxing- glove cactus'), where it caused damage on the cladodes of the plants and eventually killed them (Klein *et al*., 2020).

Percentage cover of cochineal insects increased over time on the Kenyan and EC *O. engelmannii* lineages. Percentage cover of *D. opuntiae* from site 21 was the lowest on Kenyan lineage of *O. engelmannii*, but it still killed the plant. This suggests that the biotype was effective in killing its host at a low level of infestation. Nonetheless, the percentage cover of all the *Dactylopius* biotypes sites (sites 5, 13, 20 and 21) was not statistically different, suggesting that all the biotypes were equally effective in killing their host plants. All the *D. opuntiae* biotype (sites 5, 13, 20 and 21) displayed an increasing trend in cochineal percentage cover. The *Dactylopius* biotypes (sites 5, 21, 23 P3 and 66) on the EC *O. engelmannii* lineage showed the same increasing trend of insect percentage cover over time as those on the Kenyan *O. engelmannii* lineage. However, *D. confusus* from site 23 P3 showed a continuing increase in percentage during the trial and showing a significant increase in plant chlorophyll content along with a slight increase in cladode thickness on the EC *O. engelmannii* lineage, suggesting that *D. confusus* biotype is less effective on target host. Therefore. it is considered as not a good biocontrol agent for the EC *O. engelmannii* lineage. Interestingly, the percentage cover of the *D. opuntiae* biotype from site 66 was higher than the other biotypes (sites 5, 21 and 23 P) on

the EC *O. engelmannii* lineage. This could mean that this biotype was able to produce many offspring and kill its host within a short period, making it a potential candidate agent for control of the EC *O. engelmannii* lineage.

In response to the stress caused by cochineal herbivory, the below ground biomass increased slightly in the treatment plants of the Kenyan *O. engelmannii* lineage compared to the control. This phenomenon is common in plants under any form of stress (Pugnaire and Luque, 2001; Geng *et al*., 2007). Relative to the control, above-ground biomass was slightly reduced on Kenyan *O. engelmannii* potted plants. This is expected since the cochineal insects were feeding on the above-ground biomass, which is mainly cladodes. The results also suggest that there was no significant reduction in the overall plant biomass of the Kenyan *O. engelmannii* even though the above-ground biomass was slightly reduced by cochineal herbivory. There are few suggested explanations for this observation: (i). The different feeding style of the insect herbivores is probably one of the factors that might influence how plants respond to the damage inflicted by insect herbivores (Meyer and Whitlow, 1992; Schaeffer *et al*., 2017). For instance, the effects of herbivory damage caused by leaf-feeders (folivore) versus sap-feeders on their host plant are different (Stadler *et al*., 2001). Folivores feed directly on plant tissues, reducing leaf area (Cowie *et al*., 2018). While sap-feeders like cochineal insects remove assimilates from the xylem and phloem, disrupting the flow of fluids and nutrients within the plant (Schowalter, 2013) and thus affecting the plant's photosynthetic capacity (Meyer and Whitlow, 1992; Kehr, 2006; Schaeffer *et al*., 2017). Zvereva *et al*., (2010), showed that the different plant parts, responded similar to the damage inflicted by sap-feeders, indicating that sap-feeders do not cause a change in resource allocation in plants, but they have an impact on plant photosynthesis. (ii). Different plant parts or structures also respond differently to the damage caused by insect herbivores (Meyer, 1993; Trumble *et al*., 1993).

The results in this study reveal that *D. confusus* biotypes are not good biocontrol agents for either the Kenyan or the EC *O. engelmannii* lineages in that they failed to kill their target host plant, while *D. opuntiae* biotypes did kill their hosts and have the potential of being good biocontrol agents for both Kenyan and EC *O. engelmannii* lineages. Failures of *D. confusus* biotypes in killing their hosts could be attributed to the plant's inducible defense mechanisms. The EC *O. engelmannii* lineage was acceptable and suitable for the *D. opuntiae* from sites 5, 21 and 66. While *D. confusus* found the EC *O. engelmannii* lineage to be neither an acceptable nor a suitable host for it. Musengi, (2018), revealed that the less acceptable hosts of *O. engelmannii* lineages to the 'stricta' biotype of *D. opuntiae* had higher levels of tannins than

the acceptable hosts, suggesting that the host plants could be synthesising and releasing a lot of complex blends of volatile organic compounds that acted as repellents to the cochineal insects (Séquin, 2017). The EC *O. engelmannii* lineage might have also exhibited some level of tolerance towards *D. confusus* biotypes (Ashton and Lerdau 2008; Schulz *et al*., 2019). This was evident in the high chlorophyll content displayed by the EC *O. engelmannii* host plants. Also, the EC *O. engelmannii* hosts were starting to form new shoots towards the end of the experiment showing that the plants were less susceptible to *D. confusus* (Appendix III). This is a form of compensatory growth.

The results reveal that *D. opuntiae* biotype from collection site 21 was the most effective biotype on whole potted the Kenyan *O. engelmannii* lineage based on the high reductions in chlorophyll content, reduction in cladode thickness and biomass of the host plant (Appendix IV). This biotype was sourced from an *O. engelmannii* lineage in USA, which has the same morphological characteristics as the Kenyan *O. engelmannii* lineage, both consisting of cladodes with yellow spines and brown bases. This could represent an old association (Hokkanen and Pimentel, 1989; Dennill and Hokkanen, 1990) since there is possibly a shared evolutionary history between the cochineal biotype and its host, enabling the cochineal to evolve feeding strategies that counteract the host's immune response (War *et al*., 2012). The morphological similarity between the Kenyan and USA *O. engelmannii* lineages matches the one described by Mbonani, (2019), between the Kenyan and Lm *O. engelmannii* lineages, suggesting that they might be coming from the same species of *Opuntia*. *Dactylopius opuntiae* biotype from collection site 66 was the most effective biotype on whole potted EC *O. engelmannii* lineage (Appendix V), making it a potential biocontrol agent for this *O. engelmannii* lineage. This biotype was collected from a white-spined *O. engelmannii* lineage in the USA, which is morphologically different to the EC *O. engelmannii* lineage. This could represent a new association (Hokkanen and Pimentel, 1989; Schulz *et al*., 2019) since there is probably no shared evolutionary history between the cochineal biotype and its host (Harris, 1991). In addition, recent work suggests that the EC *O. engelmannii* lineage is a South American species of *Opuntia* (Mayonde, unpublish data).

Why would an old association and a new association (of *D. opuntiae* biotypes on both Kenyan and EC *O. engelmannii* lineages) kill the plants?

It is suggested that the cochineals might have gone through an enemy release in their introduced range (Prior and Hellmann, 2014). The Enemy release hypothesis suggests that the success of

the biocontrol agents in introduced range is probably because the agents are 'released' from their specialized predators and parasites that limit their population in their native range (Prior and Hellmann, 2014; Schulz *et al*., 2019). In this case, the biological control agent thrived, possibly because of a lack of top-down pressure from natural enemies and a lack of bottom-up pressure from host plant that do not have well-developed defense mechanisms and favourable growing conditions found in the quaritntine facility (Heimpel and Mills, 2017; Schulz *et al*., 2019). This then has important implications for the biological control of *Opuntia* with the release of two cochineal predators, *Hyperaspis trifurcate* (Coccinellidae) and *Leucopina bellula* (Diptera: Chamaemyiidae) against *D. opuntiae* species which now feed on *O. ficus-indica* in Israel (Mendel *et al*., 2020). There are many predators that feed on *D. opuntiae* species in its native range (El Aalaoui *et al*., 2019; Mendel *et al*., 2020). These include: *Laetilia coccidivora* (Comstock) (Lepidoptera; Pyralidae), *Sympherobius barberi* (Banks) (Neuroptera; Hemerobiidae), *Chilocorus cacti* (Linnaeus) and *Cryptolaemus montrouzieri* Mulsant (Coleoptera; Coccinellidae), and *Baccha Fabricius (*Diptera: Syrphidae) (Mazzeo etal., 2019; El Aalaoui *et al*., 2019). In Africa, there are few introduced predators of *D. opuntiae* (e.g., *Exochomus flaviventris* Mader in South Africa) (Morrison, 1984). Nonetheless, if many of these natural enemies of *D. opuntiae* are released in Africa, it is predicted that it will reduce the effectiveness all biocontrol programmes using cochineals because these predators are not host-specific.

Synthesis

The main aim of this study was to determine which of 10 *Dactylopius* biotypes collected in the US were best suited as biocontrol agents for the Kenyan and the EC (South Africa) lineages of *O. engelmannii*. This chapter highlights some of the most important findings obtained from this study and the implications they have on the biocontrol control of *O. engelmannii* in Kenyan and South Africa. The objectives are outlined below with the major findings and a brief discussion.

(1). Evaluate the acceptability and suitability Kenya and Eastern Cape lineage of *Opuntia engelmannii* to ten cochineal biotypes collected from United States of America, using detached cladodes.

An *O. engelmannii* lineage (Kenya or Eastern Cape lineage) was considered as acceptable to the *Dactylopius* biotypes because the female cochineals settled on the detached cladodes of the host. The level of acceptability was addressed by the proportion of female cochineals settling on the detached cladodes of the host plants. An *O. engelmannii* lineage was regarded as suitable because several *Dactylopius* biotypes were able to settle, develop to adult stages and produce offspring on their host plants. The level of suitability of was best addressed by the fitness indices (FI). The Kenyan *O. engelmannii* lineage was the most acceptable and suitable host for the *D. opuntiae* biotype from site 21. This biotype comes from an *O. engelmannii* lineage that has the same morphological characteristics as the Kenyan *O. engelmannii* lineage (i.e., both have yellow- spined cladodes with brown bases). The morphological resemblance between the Kenyan and the USA *O. engelmannii* lineages resonate with those described by Mbonani, (2019), between the Kenyan and LM *O. engelmannii* lineages, suggesting that they are of the same species of *Opuntia*. This might represent an old association because of the possible shared evolutionary history between the *D. opuntiae* biotype and the Kenyan *O. engelmannii* lineage. The EC *O. engelmannii* lineage showed high acceptability and suitability to all ten *Dactylopius* biotypes. However, the EC *O. engelmannii* lineage was most suitable to *D. opuntiae* biotype from site 37. This biotype comes from a white-spined *O. engelmannii* lineage, which is morphologically different to the Eastern *O. engelmannii* lineage. This is thought to represent a new association since it is unlikely that there is a shared evolutionary history between the *D. opuntiae* biotype and the EC *O. engelmannii* lineage. The white-spined

and yellow-spined *O. engelmannii* lineages are geographically isolated (Figure 4.1) and all are morphologically different to the EC *O. engelmannii* lineage, suggesting that the EC *O. engelmannii* lineage might be a different species of *Opuntia* (Mayonde, unpublished data). The Kenyan *O. engelmannii* lineage was not a suitable host for any of the four *D. confusus biotypes*. The best performing *D. opuntiae* biotypes on both the Kenyan and EC *O. engelmannii* lineage had very high fitness indices in comparison with those observed in other species of *Dactylopius* (Mathenge *et al*., 2009a; Mathenge *et al*., 2010). However, the FI of *Dactylopius* biotypes on the EC *O. engelmannii* lineage were the highest in comparison with that observed on the Kenyan *O. engelmannii* lineage and on other species of *Opuntia* (Mathenge *et al*., 2009a; Mathenge *et al*., 2010).

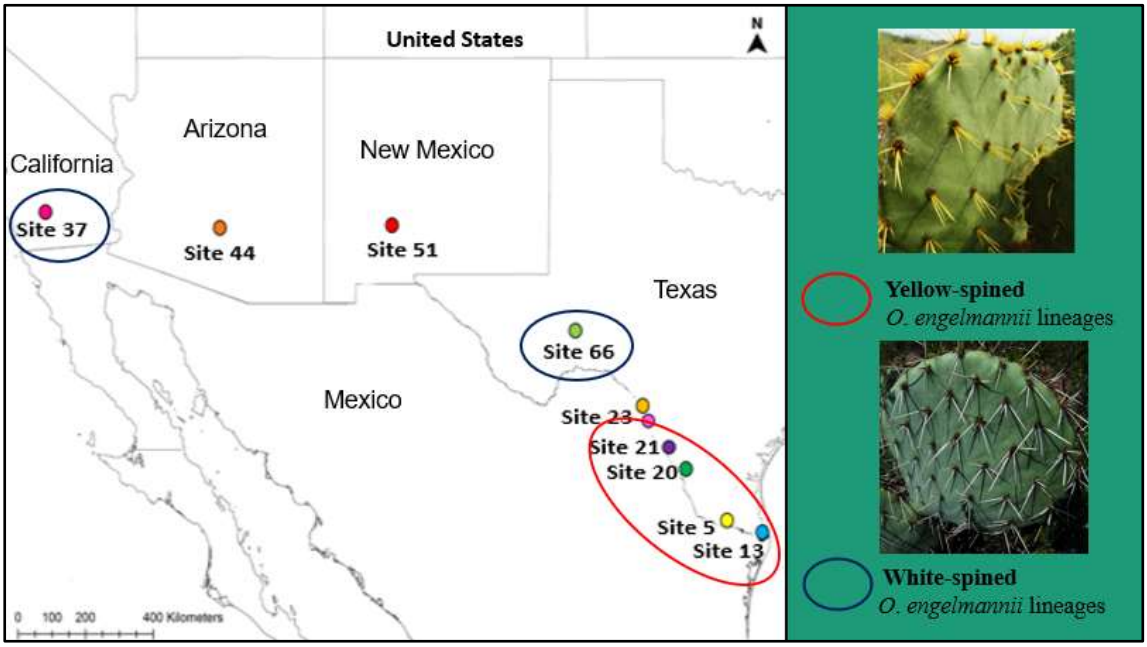

Figure 4.1: Geographically separated *Opuntia engelmannii* lineages in United States of America. The best performing biotypes on the Kenyan *Opuntia engelmannii* come from yellow-spined *Opuntia engelmannii* lineages (red circle), while the best performing biotypes on the Eastern Cape *Opuntia engelmannii* lineage come from white-spined *Opuntia engelmannii* lineages (blue circles). (Source N Venter).

(2). Assess the impact of the four best cochineal biotypes on whole Kenyan and Eastern Cape *Opuntia engelmannii* potted plants.

The four best cochineal biotypes (i.e., those with highest fitness indices) were selected for whole, potted Kenyan and EC *O. engelmannii* plant trial to assess which cochineal biotype would result in the most rapid host mortality. The physiological responses of the plants to cochineal herbivory were assessed by measuring chlorophyll content, cladode thickness, insect

percentage cover and biomass of the plants. The results show that the *D. opuntiae* biotype from site 21was the most effective biocontrol agent on the Kenyan *O. engelmannii* lineage based on the high reduction in chlorophyll content, cladode thickness and biomass. The *D. opuntiae* biotype from site 66 was the most effective on the EC *O. engelmannii*. The *D. opuntiae* biotype from site 21 comes from a yellow-spined *O. engelmannii* lineage, while the *D. opuntiae* biotype from site 66 emanates from a white-spined *O. engelmannii* lineage. Again, the sites from which these biotypes were collected are very far apart (approximately 200 km apart) (Figure 4.1). The *D. confusus* biotypes performed poorly in as much as they did not kill the plants, on both the Kenyan and EC *O. engelmannii* lineages. The best performing biotype on the Kenyan *O. engelmannii* might represent an old association between the insect biotype and its host, meanwhile the best performing biotype on the EC *O. engelmannii* might represent new association, based on morphological similarities or differences between O. *engelmannii* lineages and the insect-host interactions. The reasons as to why would an old association or new association might kill the plants are summarised below.

Factors contributing to Failures and successes of *Dactylopius* biotypes on host plant

There are many suggested reasons that explain why a biocontrol agent fail to kill its host plant. One of the most well-known, but poorly understood concept is the plants inducible defense mechanisms (Agrawal and Karban, 1999; Van Zandt and Agrawal, 2004). Plants will either resist or tolerate the damage caused by herbivory (Van der Putten, 2003; Cowie *et al*., 2018). In the case of the Kenyan *O. engelmannii* lineage, the host plants appeared to be resisting the *D. confusus* biotypes, possibly by releasing volatile chemicals that could repell or kill or deter the development of the cochineal insects (Preisser and Bastow, 2005; War *et al*., 2012). Whereas in the case of the Eastern *O. engelmannii* lineage, the host plants could be able to tolerate the *D. confusus* biotypes, as suggested by the enemy tolerance hypothesis (Schulz *et al*., 2019). The success of the biocontrol agents in killing their host could be attributed to mainly the enemy release hypothesis (Chun *et al*., 2010). Lack of specialized predators and parasites of biocontrol agents in the introduced range, enable them to successfully kill their host plants (Schulz *et al*., 2019). For example, the North American weevil *Stenopelmus rufinasus*, Gyllenhal (Coleoptera: Curculionidae) was able to kill its host *Azolla filiculoides* Lamarck (Azollaceae) ('water fern') in the United Kingdom (Reeder *et al*., 2018). Another example involves the killing of *Cylindropuntia fulgida* var. mamillata (Schott ex Engelm.) ('boxing-glove cactus') by the '*cholla*' biotype of *D. tomentosus* in South Africa (Klein *et al*., 2020).

Conclusion and recommendations

This research forms part of a bigger study of the biological control of *O. engelmannii* lineages in Africa. Findings from this research provides a framework for current biocontrol efforts and for future searches for appropriate agents for the relevant *O. engelmannii* lineages in Africa. Our predictions were proven to be valid, the high diversity of the *O. engelmannii* lineages in the Southwest US, as well as the morphological similarities of these lineages to Kenyan *O. engelmannii* have yielded at least one effective biocontrol agent from the collection; *D. opuntiae* biotype from site 21 (Figure 4.2). Meanwhile, *D. opuntiae* biotype from site 66 was the most effective biocontrol agent on the EC *O. engelmannii*, despite not having any morphological similarities with the EC *O. engelmannii* lineage and the USA *O. engelmannii* lineages (Figure 4.2). However, repeats of the experiments for the EC *O. engelmannii* should be considered given that the proportion of females settling on detached cladodes were used as a measure of best performance of cochineal biotypes instead of their fitness index. This proves the proportion of settled females on host is not always a good measure for insect performance on host plants. The incorporation of some of the measures of plant's physiological response (e.g., chlorophyll content using CCM-300 and insect percentage cover, using ImageJ) offers additional procedures for assessing the effectiveness of a biocontrol agent on its host. The results also suggest that there might be two distinct taxonomic varieties of *O. engelmannii* in Africa, one being the Kenyan *O. engelmannii* and the second one is yet to be confirmed). A multidisciplinary approach will be essential to fully understand the host-insect interaction (Figure 4.2). For instance, the response of a host plant to an insect's herbivory can affect the physiology, behaviour or even the gene expression of the insects (Musengi, 2018). Also, understanding how an invasive plant responds when attacked by biocontrol agents can be useful in understanding the invasive alien plant's ecology and its control. To promote the success of biological control agents, we also recommend that biocontrol practitioners must ensure that the introduced biocontrol agents are free from any of their own natural enemies/predators prior to introduction in the introduced range.

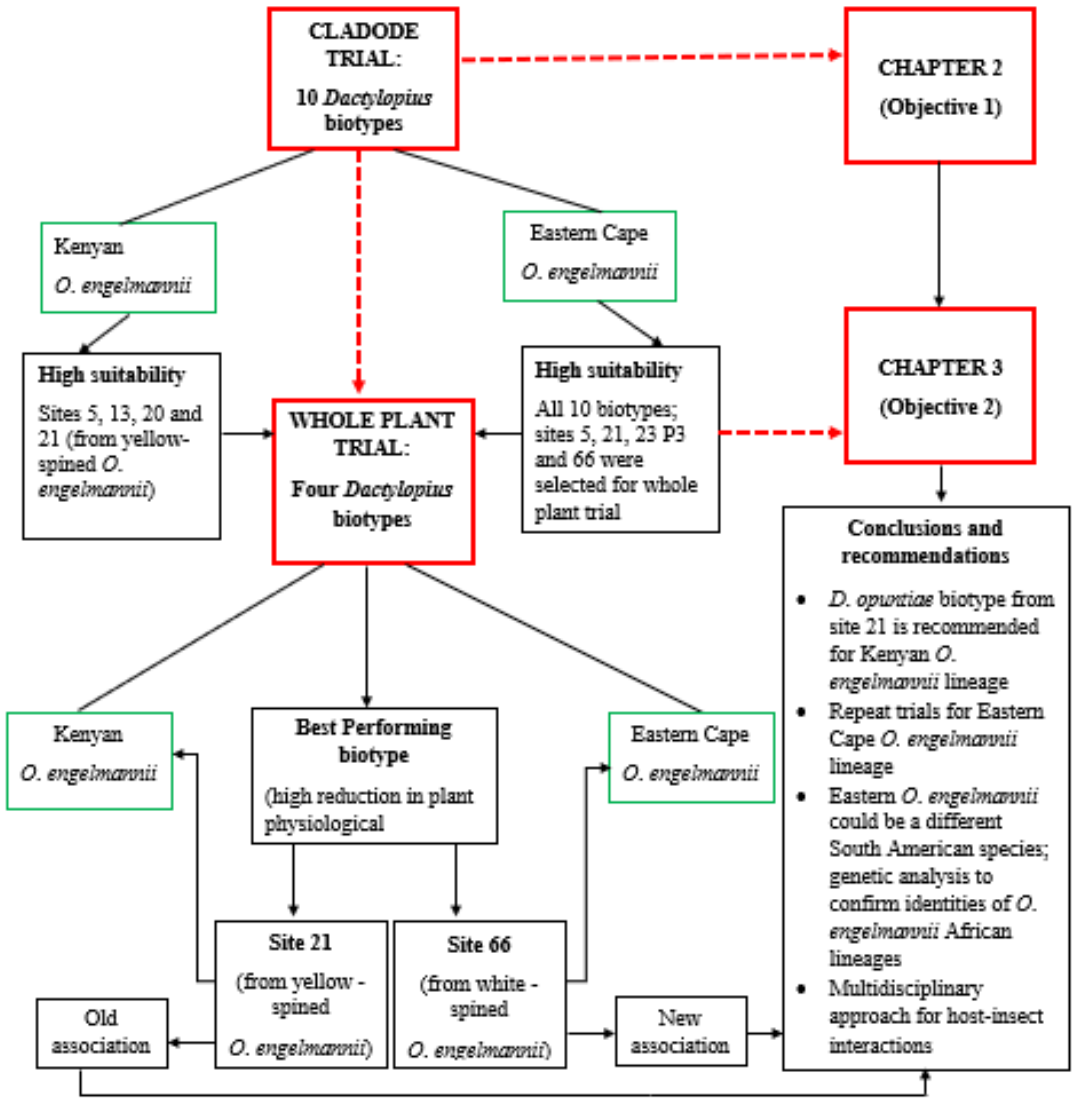

Figure 4.2: Summary of the findings and recommendations from this study evaluating the different biotypes of *Dactylopius opuntiae* and *Dactylopius confusus* species for the biological control of the invasive *Opuntia engelmannii* lineages in Kenya and South Africa (Source: ZH Machimane).

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APPENDICES

Appendix I

Table 1.1: Categories of fitness indices of *Dactylopius* biotypes (adopted from Mathenge *et al*., 2009a).

Category	Fitness index (FI)	Description
1	FI ≥ 1	Biotypes thrived on their host plant
2	FI < 1 but > 0	Biotypes survived on their host plant
3	FI = 0	Biotypes dying on their host plant

Appendix II

Table 1.2: *Dactylopius* biotypes used for the whole plant trials for the Kenyan and EC *Opuntia engelmannii* lineages. (**x**)- biotypes selected for the Kenyan *Opuntia engelmannii* lineage. (√)- biotypes selected for the EC *Opuntia engelmannii* lineage.

Site	Biotype	Kenyan *O. engelmannii* lineage	EC *O. engelmannii* lineage
5	*D. opuntiae*	**x**	√
13	*D. opuntiae*	**x**	
20	*D. opuntiae*	**x**	
21	*D. opuntiae*	**x**	√
23 P3	*D. confusus*		√
66	*D. opuntiae*		√

Appendix III

Figure I: The EC *Opuntia engelmannii* lineage forming new shoots towards the end of the experiment indicating that the plants were less susceptible to *Dactylopius confusus* biotype. Pictures taken on the 23rd of January 2020. (Source: ZH Machimane).

Appendix IV

Figure II: The damage caused by the best performing *Dactylopius opuntiae* biotype from site 21 (A) and poor performing *Dactylopius confusus* biotype from site 13 (B) on whole potted plants of the Kenyan *Opuntia engelmannii* lineage. (Source: ZH Machimane).

Appendix V

Figure III: The damage caused by the best performing *Dactylopius opuntiae* biotype from site 66 (A) and poor performing *Dactylopius confusus* biotype from site 23 P3 (B) on whole potted plants of the EC *Opuntia engelmannii* lineage. (Source: ZH Machimane).