THE BIOLOGICAL CONTROL OF CACTI (CACTACEAE: OPUNTIOIDEAE) IN SOUTH AFRICA: BASIS OF HOST SELECTION IN THE 'STRICTA' BIOTYPE OF *DACTYLOPIUS OPUNTIAE* (COCKERELL) (HEMIPTERA: DACTYLOPIIDAE)

by

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DECLARATION

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

(Signature of candidate)

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ABSTRACT

The cochineal insect, *Dactylopius opuntiae* (Cockerell), plays a major role in the control of *Opuntia* species. Host preferences of the 'stricta' biotype of *D. opuntiae* are only partially known, as it has not been tested against all *Opuntia* species; however, it was released 22 years ago in South Africa against *Opuntia stricta*. The 'stricta' biotype of *Dactylopius opuntiae* can potentially be used against one or more *Opuntia* species. I investigated the basis of host selection in this cochineal insect.

Firstly, I assessed the reproductive performance of the 'stricta' biotype of *D. opuntiae* on *Opuntia stricta*, *Opuntia humifusa* and two *Opuntia engelmannii* lineages. The life-history parameters recorded were crawler development time, crawler survival, female development time, female weight and number of crawlers produced by females. Results revealed large differences in the developmental biology and reproductive performance of the 'stricta' biotype of *D. opuntiae* between the *Opuntia* hosts. There was a significant difference between the hosts in the number of days taken to reach the first moult by the 'stricta' biotype of *D. opuntiae*. There was evidence that the 'stricta' biotype of *D. opuntiae* exhibited varying degrees of acceptability on the *Opuntia* hosts. The most acceptable and suitable hosts for the 'stricta' biotype of *D. opuntiae* were *O. stricta* and *O. humifusa*.

Secondly, I investigated some factors that might determine whether the 'stricta' biotype of *D. opuntiae* accepts or rejects a certain *Opuntia* as its host. The morphological and phytochemical aspects of the *Opuntias* were studied. Light microscopy revealed no significant differences in the thickness of the cuticle, epidermis and hypodermis of the *Opuntia* hosts but revealed many red particles, presumed to be tannins in the *O. engelmannii* lineages. The tannins, titratable acidity and pH of the *Opuntia* hosts' cladodes were studied. To determine the titratable acidity, cladode samples were collected every 3 hours from 0800hrs and for pH determination cladodes were harvested between 1300hrs and 1400hrs. Estimation of the tannins was done by the Folin-Denis' Method. The tannins and pH were significantly different between the *Opuntias.* Tannins ranged between 3.1-8.4 mg Tannic acid/g and were in the following rank order: *O. engelmannii*-Limpopo lineage > *O. engelmannii*-Kenya lineage > *O. humifusa* > *O. stricta*.

The pH ranged between 5.1-7.4 and cladodes from the most acceptable hosts of the 'stricta' biotype of *D. opuntiae* had organic acids that were acidic compared to the slightly alkaline ones on the least acceptable hosts - *O. engelmannii* lineages. These results suggest that tannins and pH of the *Opuntias* may be the basis of host selection in this cochineal insect. The impact of the 'stricta' biotype of *D. opuntiae*'s herbivory on the physiology of the acceptable and suitable hosts were examined by monitoring chlorophyll concentration changes and the diurnal changes in photochemical efficiency of the *Opuntias*. The chlorophyll concentration and the photochemical efficiency of PS II decreased as *D. opuntiae* herbivory increased. These physiological results show that the 'stricta' biotype of *D. opuntiae* could result in the successful biological control of *O. humifusa* in South Africa.

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LIST OF ABBREVEATIONS

ARC-PPRI	Agricultural Research Council-Plant Protection Research Institute
CAM	Crassulacean Acid Metabolism
CARA	Conservation of Agricultural Resources Act
F_o	Instantaneous fluorescence emission
F_m	Maximum fluorescence emission
F_v	Variable fluorescence emission
K_D	Rate constant for radiationless energy dissipation
NEMBA	National Environmental Management: Biodiversity Act
PEPC	Phosphoenolpyruvate carboxylase
PFD	Photon Flux Density
PS I	Photosystem I
PSII	Photosystem II
Q	Primary electron acceptor of photosystem II
q_{NP}	Non-photochemical fluorescence quenching
q_P	Photochemical fluorescence quenching
SAPIA	Southern African Plant Invaders Atlas
TA	Titratable acidity
VOCs	Volatile Organic Compounds

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Chapter 1

1.1 General Introduction and Outline

The movement of species to places far away from their native ranges has been going on for a long time, and now alien species are components of many ecosystems (van Kleunen et al. 2015). These alien species can be invasive and cause negative environmental impacts. Invasive species in their native range cause little harm due to constraints that restrict them from spreading excessively, but they can damage many ecosystems when outside their native range. The ever growing branch of invasion biology is characterised by the mushrooming of words to outline many notions about invasion. There is misuse and confusion on the existing terms perhaps as a result of the frequent evoking of anthropocentric concepts of the notion of 'invasion'. Many researchers have disputed the relative merits of many words in invasion biology (Richardson et al. 2000). Invasive plants are defined as alien plants that yield large quantities of reproductive offspring that can spread long distances away from parent plants, whilst weeds are plants, not necessarily aliens, which grow in sites where they are not wanted, usually having detectable economic and environmental effects (Richardson et al. 2000). Many cactus species are considered as members of the most relevant alien invasive species all over the world (Weber, 2003).

The distribution of the cactus family (Cactaceae; 'cacti') is from South America to North America (Edwards et al. 2005). The widespread horticultural trading of cacti is the main cause of the numerous cacti species mushrooming in many countries (Walters et al. 2011). Cacti are invasive in most of their introduced range where they are also often grown as either ornaments and/or crops. The Cactaceae has three recognized subfamilies: Pereskioideae, Opuntioideae and Cactoideae (Novoa et al. 2014). Of the 1922 cactus species, 57 have been classified as invasive (Fig. 1.1). The photosynthetic pathway, crassulacean acid metabolism (CAM), of cactus species leads to higher water-use efficiency and this increases their ability to survive in wide ecological ranges that may have extremes of heat and cold. The first non-native cacti were introduced to South Africa early in the 18th century (Annecke and Moran 1978). Since then more than 200 cactus species have been introduced into South Africa, mainly for their value as ornaments (Novoa et al. 2015).

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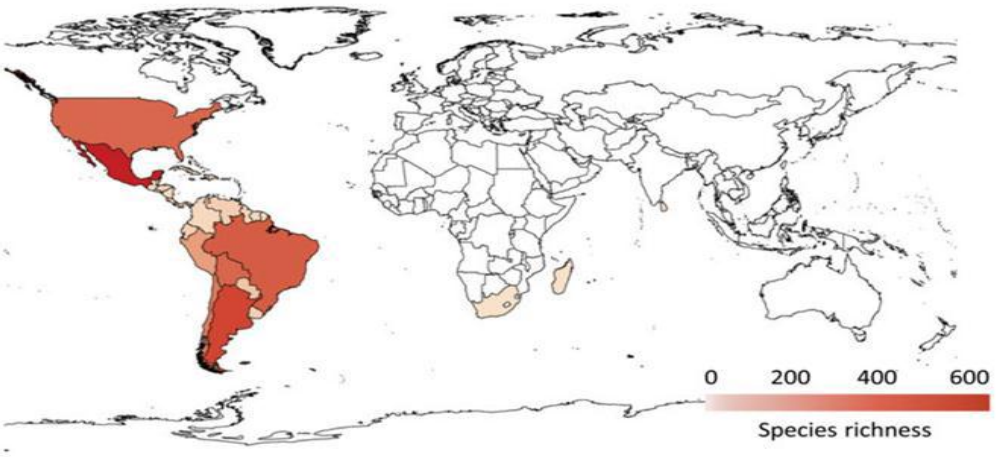

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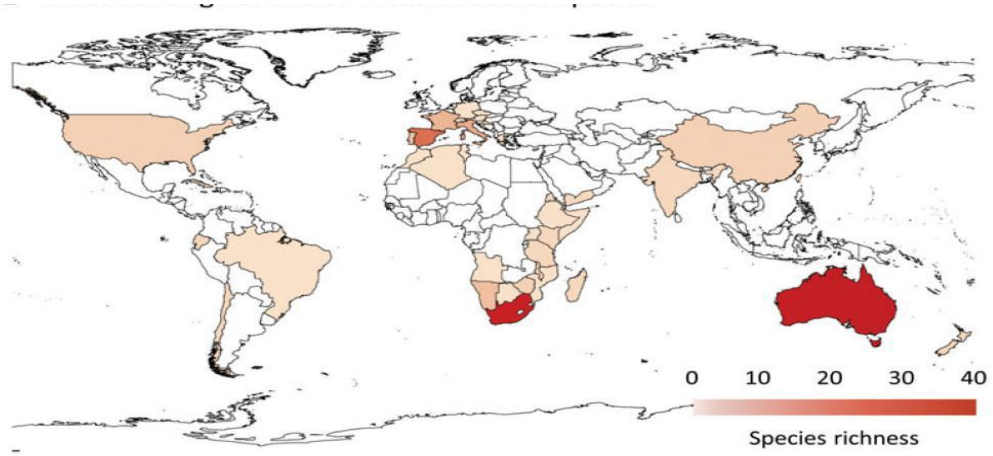


Figure 1.1. Reproduced with permission from the authors (Novoa et al. 2014). (a) Cactus species richness across the native (1922 species) and (b) invasive range (57 invasive cactus species). Shading indicates the number of taxa per country. Lighter colours correspond to less taxa".

Cacti form a part of the most extensive category of plants that are invasive in South Africa (van Wilgen et al. 2012; Kaplan et al. 2017). Their ease of growth, beautiful flowers, edible fruits and the artistic positioning of spines are some of the features that make them irresistible to plant collectors. South Africa's interior arid region provides favourable conditions for species that are adapted to drought, such as cacti, and is one of the main reasons why South Africa is a global hotspot for invasion by cacti (Kaplan et al. 2017).

1.2 Impacts of cacti

The cactaceae has several species that are a source of food thus giving this family economic importance (Stintzing and Carle, 2005; Feugang et al. 2006). Several species of cacti are used for medicinal purposes, as forage and as a source for natural colours. However, the extensive use of cacti is mainly restricted to their countries of origin (Mohamed-Yasseen et al. 1996; Vigueras and Portillo, 2001). In South Africa they have important socio-economic benefits, and approximately 300 cacti species are brought yearly into South Africa from other countries for ornamental horticultural reasons (Novoa et al. 2017). Many species of cacti also play a huge role in commercial agriculture for food and fodder for livestock. These drought-tolerant crops can cause the productivity of marginal land to increase significantly (Brutsch and Zimmermann 1993). However, cacti also have high negative environmental and socio-economic impacts (Novoa et al. 2016). The capacity of cacti to adapt and spread in many environments causes problems as the cacti form dense invasive stands that compete with other vegetation and this causes decrease in the productivity of commercial rangelands. Most species of cacti have spines that damage wildlife and livestock (Walters et al. 2011). According to Novoa et al. (2016), the highest negative impacts of cactus in South Africa are associated with animal production.

1.3 The *Opuntia* genus

“Treasure under its spines”, “Sacred plant” and “fruit for the poor” are some of the epithets used for *Opuntia* species and their fruits (Arias Jiménez, 2013). The importance of the Opuntias in the lives of people is conveyed in these names. The genera, *Opuntia* and *Cylindropuntia*, have the most widely introduced, cultivated and invasive species in the cactaceae family (Novoa et al. 2014). The Opuntias’ native range is from southern Canada to southern South America. Many Opuntias such as *O. monacantha* (Haw.), *O. stricta* (Haw.) Haw, *O. engelmannii* Salm-Dyck and *O. ficus-indica* (L.) Miller have spread to different parts of the world including South Africa. These species were mainly introduced as ornaments or sources of fodder (Brutsch and Zimmermann, 1993). *Opuntia* species can be used to make fodder for livestock and as live fences (Griffith, 2004).

However, invasive *Opuntia* species are changing productive land into unusable impenetrable thickets (Klein et al. 2015). Dense infestations of *Opuntia* displace native flora which results in disastrous ecological and economic impacts in the country. The genus *Opuntia* has approximately 180 species and is made up of mainly platyopuntias, meaning they have flattened-jointed stems known as "cladodes" (Cortázar and Nobel, 1992). Three characteristics distinguish the Opuntias from other cacti: growth of stems as recognizably different jointed segments; their areoles have short prickles known as glochids (whether or not they have regular spines) and new joints have rudimentary leaves (Fig 1.2). The cladodes are made up of a core tissue which is composed of a white medulla parenchyma and the cortex tissue which has photo-synthetically active parenchyma (Stintzing and Carle, 2005). The cortex tissue is covered with spines and multicellular hairs. Both the spines and the multicellular hairs form the areole.

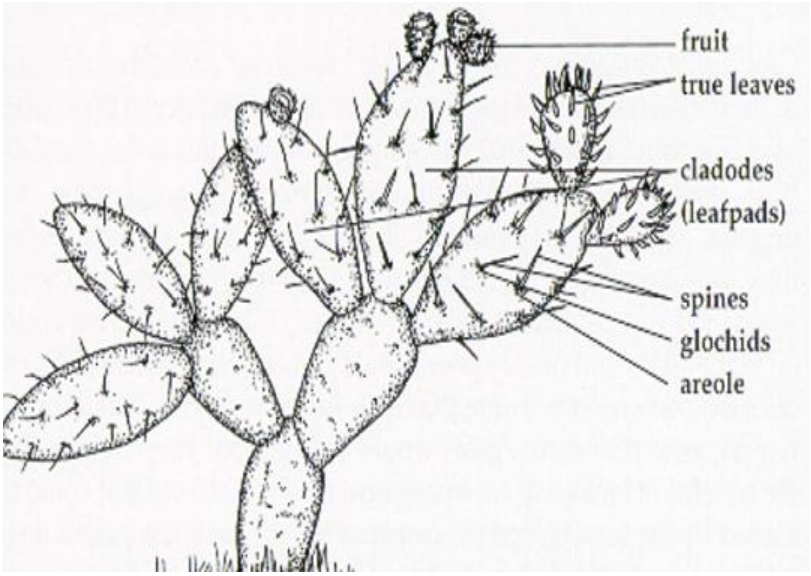


Figure 1.2 Typical platyopuntias (Cortázar and Nobel, 1992).

The taxonomy of the Opuntias has undergone repeated revision and is not totally clear. This renowned taxonomic difficulty is caused by the interspecific hybridization, polyploidy and variability in the morphology (Majure et al. 2012). For example, *Opuntia engelmannii* Salm-Dyck is diverse in its native range, the United States of America, where most of the accepted intraspecific taxa such as *O. engelmannii* var. *engelmannii*, *O. engelmannii var. linguiformis* and *O. engelmannii* var. *lindheimeri* (=subsp. *lindheimeri*) are found.

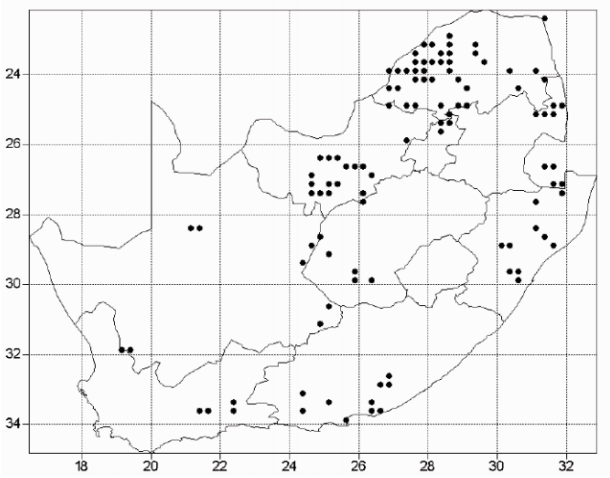
These varieties have been spread to different parts of the world including South Africa, Kenya, Spain and Australia (Majure et al. 2012). The morphological variability of *O. engelmannii* in South Africa and in Kenya has led to the use of the term lineage in this thesis based on where they are found. The three South African lineages of *O. engelmannii* which differ morphologically (Table-1.1) are found in the provinces of Eastern Cape, Northern Cape and Limpopo (Klein, 2015).

Table 1.1 Lineages of *O. engelmannii* (personal observations and unpublished data from Sipho Mbonani and Hildegard Klein)

Lineage	**Introduced range**	**Morphology**
Limpopo	Mokopane – South Africa	Green cladodes with large surface area. Long spines and glochids present, pointing sideways, especially on the top margins of the cladode. Spines and glochids are yellow.
Eastern Cape	Bedford district- South Africa	Cladodes are usually large. Long spines pointing downwards that are shiny and dark green. Glochids not noticeable on top margin of cladode.
Northern Cape	Douglas- South Africa	Cladodes are dark, greyish green with dark brown glochids especially noticeable on top margin of cladodes. Spines pointing downwards and are few.
Kenya	Loisaba	Small cladodes with relatively long and pointy spines. The spines point upwards and are clustered next to the glochids.

At least ten species of *Opuntia* have become troublesome weeds in South Africa (Smith et al. 2011). They invade savanna and grassland areas in many of South African provinces (Figure 1.3). *Opuntia ficus-indica* made a large contribution to making many parts of the arid karroid interior of South Africa almost useless for agricultural purposes approximately 100 years ago (Smith et al. 2011). The dense infestations of *O. engelmannii* lineages and *O. humifusa* (Raf) Raf decrease the current grazing potential of land and reduce access to livestock (Henderson, 2001). The spines on the cacti can injure livestock and they also irritate them in such a way that they are unable to feed.

a)



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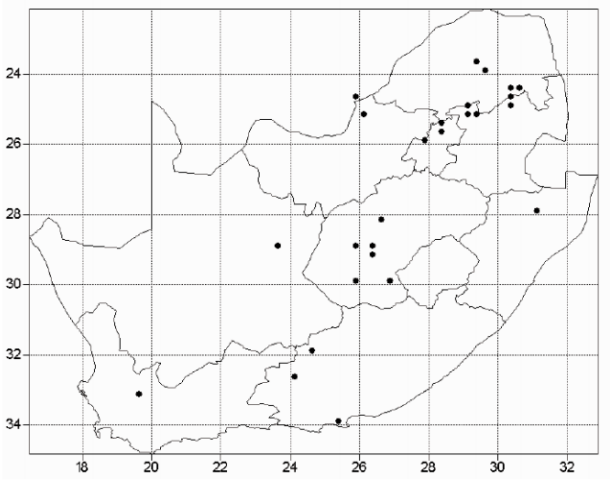

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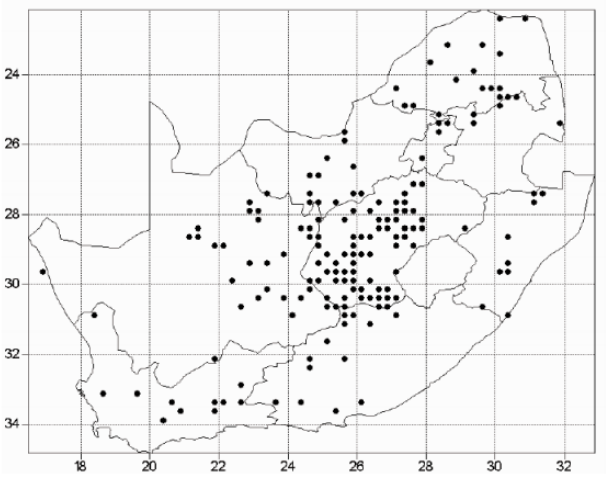


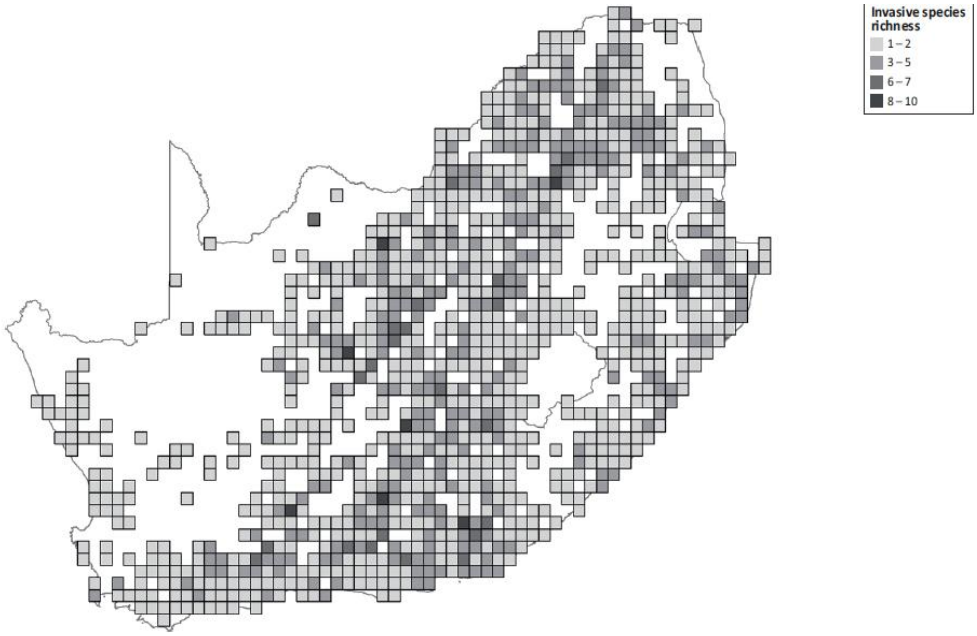
Figure 1.3 Distribution of a) *O. stricta*, b) *O. humifusa* and c) *O. engelmannii* lineages in South Africa. Prime degrees of longitude east and latitude south are represented by grid lines (Henderson, 2001).

1.4 Controlling invasive Opuntias

There is need to appropriately manage biological invasions to mitigate the negative environmental and socio-economic impacts that are caused by these invasions (Simberloff et al. 2013). In South Africa, the Conservation of Agricultural Resources (CARA) Act, which was introduced in 1984, assessed alien plants in terms of how invasive they are and attempted to find a sustainable solution to environmental problems caused by invasive species. The CARA regulations have been substituted by the National Environmental Management: Biodiversity Act (NEMBA) (Act 10 of 2004) - Alien and Invasive Species (AIS) Regulations which was promulgated on 1 October 2014. The purpose of NEMBA is to provide for the management and conservation of South Africa's biodiversity within the framework of the National Environmental Management Act (107 of 1998). NEMBA does not consider the morphological variability of *O. engelmannii*, and all the lineages of *O. engelmannii* in South Africa are not classified according to where they found. But nonetheless, *O. engelmannii* is on the NEMBA list.

Thirty five cacti species are already listed as invaders under NEMBA and *O. stricta*, *O. engelmannii* and *O. humifusa* are also listed as invaders (Fig 1.4).

a)



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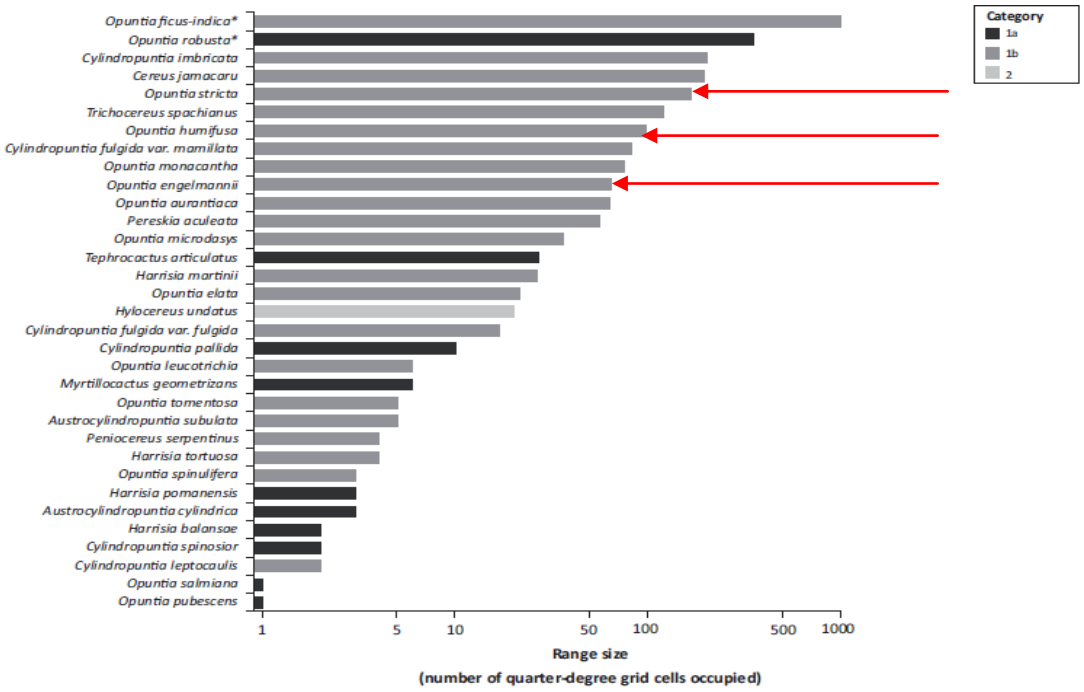


Figure 1.4 The extent of invasions by cactus in South Africa, representing (a) species richness of invasive cacti listed per quarter degree grid cell and (b) the range sizes of the invasive cacti. Source: Southern African Plant Invaders Atlas (SAPIA) database,accessed June 2017, the shading in (b) represents categories in the regulations of NEMBA. *O. stricta, O. humifusa* and *O. engelmannii* (shown by arrows) are in category 1b where all invasive species must be controlled and whenever possible, removed and destroyed. *, Excludes spineless cultivars.

The first line of defence in weed control is usually the use of chemicals but the high costs of herbicides prevent them from being used over the long term (Jones et al. 2015). Zengeya et al. (2017) also reported that some people are against the wide scale use of chemicals. Physical removal can be a cheaper option; unfortunately it can be dangerous because of the spines, when removing cacti specifically, and requires correct disposal (Holtkamp, 2012). Biological control (Biocontrol) of *Opuntia* species is a cost-effective method that has been used since 1913 and has been successful on several species in the cactus family (Zimmermann et al. 2004; Klein, 2011).

Biological control includes the use of parasites, predators and herbivores in keeping a weed or insect population at lower average densities than would be found in their absence (DeBach, 1964). One of the major steps in all biological control programs is the establishment of the biological control agent in the field. An agent is considered established only when it forms self-sustaining populations on its target plant (Coombs et al. 2004). Factors such as predators, release efforts, climate (e.g. van Klinken, 2004) and compatibility

of the biological control agent and target weed (e.g. Thomas and Ellison, 2000) may all contribute to the successful establishment of the agent. Once an agent has successfully established, the damage it causes must be assessed.

South African biological control scientists have developed a ranking system for the perceived degree of damage that individual biological control agents inflict on their target weed. The ratings are sometimes based on formal measurements and otherwise are subjective visual assessments (Klein, 2011) and are as follows: "Extensive"- very high levels of damage and few plants survive with no seed production; "Considerable"- high levels of damage and some plants may survive but seed production is decreased by more than 50%; "Moderate"-when there is perceivable damage but many plants survive and there is reduction of production of seeds by less than 50%; "Trivial"- some damage but the growth, survival and production of seeds is close to normal; "Unknown"- when the agent has just been released or when no evaluation has been done.

The combined impact of all agents on a particular target weed is categorized by estimating the extent to which the importance or impact of the target weed has been decreased by the agents. The assessments depend on the extent of reduction in the use of other optional control methods such as the use of chemicals (Klein, 2011) and are as follows: "complete control"- in areas where the agent has established and no other methods of controlling the weed are necessary to reduce it to acceptable levels; "substantial"- other management options can still be considered to strengthen the biocontrol, but the management efforts would be less compared to what was required before the biocontrol agent was introduced; "Negligible"- although there is damage caused by the agents, entire reliance on the implementation of other management options to control the weed is still there; "Not determined"- no evaluation of the programme has taken place.

In South Africa, the first weed biocontrol programme in 1913 targeted *O. monacantha* (Wild.) Haw (drooping prickly pear). This programme was a success and, within a few years, the weed's density had been reduced to negligible levels (Paterson et al. 2011). Other noteworthy success has included the biocontrol of *O. aurantiaca* Lindl (Moran and Zimmermann, 1991), *O. stricta* (Haw) Haw (Hoffmann et al., 1998), *O. ficus-indica* (L.) Mill (Zimmermann and Moran, 1999). The insects used on Opuntias for biological control are

native to North and South America. These insects are collected extensively by entomologists looking for the Opuntias' natural enemies. This led to the emergence of annotated catalogues of insects associated with the Opuntias and reports on the outcome of biological control projects (Moran, 1980). A total of 122 specialist plant-feeding species of insects on 119 *Opuntia* species was reported in 1979 (Moran, 1980). These insects are found in five orders of *Opuntia*-feeding insects: Coleoptera, Lepidoptera, Hemiptera, Diptera and Hymenoptera. The number of species per taxon is shown below (Fig 1.5).

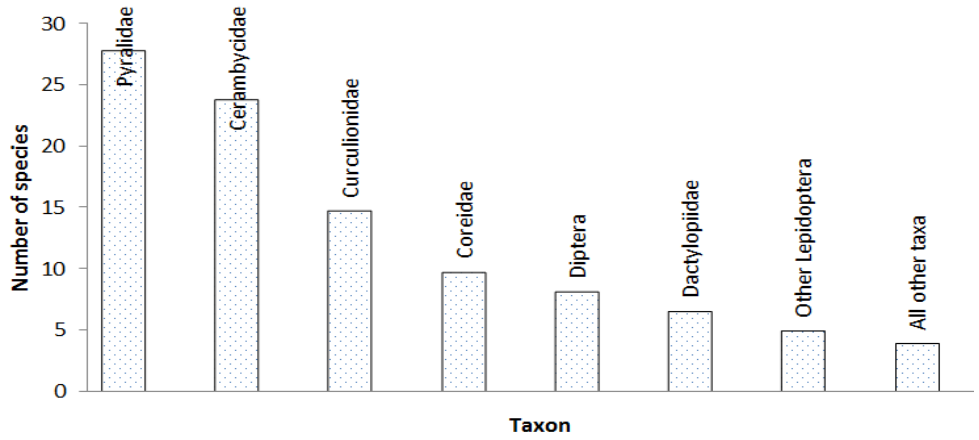


Figure 1.5 Number of host plant species of *Opuntia* on which the 122 insect species were recorded (Moran, 1980).

The summary of the recorded host range of the *Opuntia*-feeding insects are shown in Figure 1.6. The cochineal insects, *Dactylopius ceylonicus* Green and *D. opuntiae* Lichtenstein are good examples, as they were noted on thirty-nine and twenty-six hosts species respectively (Moran, 1980). Association structure of the insects recorded on Opuntias show that 22 % of these insects are sap-suckers (Moran, 1980).

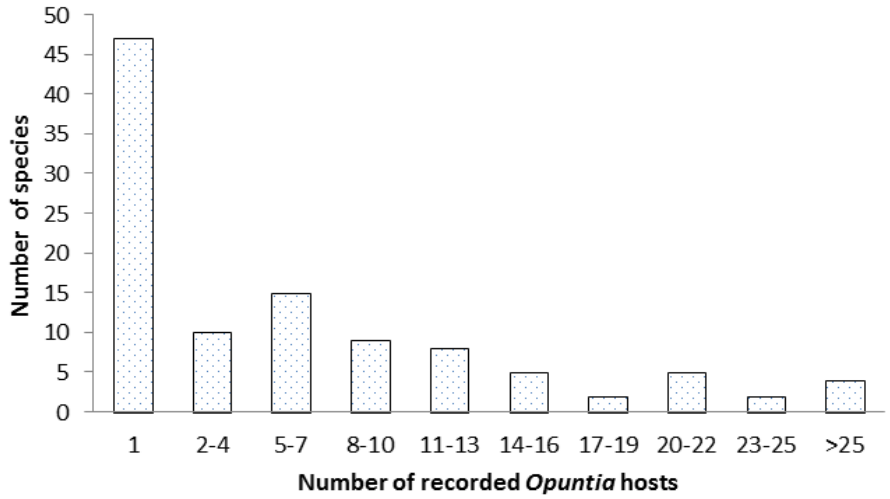


Figure 1.6 Noted hosts for the 122 insects species recorded on *Opuntias* (Moran, 1980).

To the best of our knowledge there has been no attempt to closely study the physiological interactions of the Opuntias and insects. The understanding of these interactions between *D. opuntiae* and the Opuntias could have implications for the way agents are selected for the biological control of cactus or other weeds. Understanding how invasive plants respond when attacked by biocontrol agents is beneficial in understanding the ecology of the invasive plant and its control (Pearson and Callaway, 2003). Part of this thesis looked at "non-preference", a term first coined by Painter in the 1950s (Browne and Withers, 2002). This term represents plant characteristics and responses by insects that cause or prevents the use of a particular plant.

A chain of steps involved in host selection for feeding and oviposition are as follows: host finding; host recognition; host acceptance and host suitability (Kumarasinghe and Jepson, 2003). It is still unknown what cues *D. opuntiae* insects use to recognize their host plants, and what causes them to accept or reject certain plants as host plants. Acceptance or rejection of the plant by an insect is influenced by many factors which can be intrinsic to the particular host or depend on the state of the insect (Courtney et al. 1989). If a host has a high intrinsic acceptability then that host will be accepted more readily; low acceptability will cause the host to be acceptable only when the state of the insect has changed. For example, when the insect's most preferred host is not available or no longer available, the insect may move onto a less preferred host. This will lead to an apparent hierarchy of acceptability, with potential hosts ranked in a particular order (Courtney et al. 1989). A critical assumption can then be

made that an individual which accepts a low ranking host will also accept all hosts above that in the rank-order.

I assessed insect-host interactions between the cochineal insect, *D. opuntiae*, and three *Opuntia* species: *O. stricta*, *O. engelmannii* and *O. humifusa*. The primary aim was to investigate the basis of host selection in *D. opuntiae* to discover how these *Opuntia* species protect themselves against herbivorous insects and which of these defences are effective in preventing attack by *D. opuntiae*. Assessing the interaction between these three *Opuntia* species and *D. opuntiae* will contribute new knowledge to the basis of host selection in *D. opuntiae* that was introduced into South Africa more than 22 years ago (Mathenge et al. 2010). Questions of whether the selection of the host by *D. opuntiae* or suitability of the host is influenced by the chemical and physical defences of the host species were investigated.

Plant-insect interactions are dynamic systems, undergoing continual change and variation (Mello and Silva-Filho, 2002). For example, plants have developed different defence mechanisms such as induction of defensive proteins to reduce attack by insects. The biochemicals of plants that have unfavourable effects on the feeding behaviour of insects may decrease the chances of survival (Appel and Schultz, 1992) especially for insects like *D. opuntiae* that have females that are sessile once they have settled. I focused on understanding the Opuntias' defences against herbivory by *D. opuntiae* and their effects on *D. opuntiae*. This is important as it could shed more light on how to utilize host acceptance to find suitable agents to control particular lineages of invasive plants. So there is potential value in being able to predict the suitability of a host to a biological control agent and this helps to determine if a biological control agent will have an effect on an invasive plant or not.

It is known that defensive components of plant quality have direct effects on reproductive performance of their herbivore insects (Awmack and Leather, 2002). Four major groups of plant chemicals are involved in resisting insect herbivory (Schoonhoven et al. 1998): compounds that contain nitrogen (such as alkaloids), cyanogenic glycosides and glucosinolates, terpenoids and phenolics (tannins, lignins, and polyacetates). I looked at the group of plant chemicals known as the phenolics, which includes tannins. The *Opuntia* hosts were first examined for their ability to support development and reproduction of *D. opuntiae* in Chapter 2. Effectiveness experiments were done on *Opuntia* hosts which sustained the

growth of an average of four or more individual insects from neonates to reproducing adults in order to determine if the feeding of the insects can reduce the vigour or kill the target species.

Chapter 3 compared the defence properties of these *Opuntia* hosts and determined whether any relationship exists between levels of defence properties measured and the ability of the *Opuntia* hosts to support development of *D. opuntiae*. Knowledge of the key physiological factors of the invasive species which are negatively impacted by a biological control agent can help us determine if a biological control agent will have an effect on its host plant. Chapter 4 dealt with the physiological responses of the host plants to *D. opuntiae* infestation to determine how *D. opuntiae* damages, reduces the vigour or results in the death of the host.

Chapter 2

Comparative studies on the biological control of three *Opuntia* species by the 'stricta' biotype of *D. opuntiae*

2.1 Introduction

Many potential hosts may be readily available for herbivorous insects, but the insects do not eat all of them. Most of these herbivorous insects are specialized to some extent; they feed on fewer hosts than are available. The group of plant species on which larval development, adult feeding and oviposition of an insect occurs in nature is known as the host range of the insect. There are three categories of feeding type in insects: monophagous, oligophaogus and polyphagous; these categories are based on the number of hosts they feed on (Solter and Maddox, 1998). Monophagous insects have a very narrow host range and develop on one or two host plants in the same genus whilst oligophagous insects develop on three or more host plants in the same genus or family (Pilson, 1999). Polyphagous insects develop on a wide host range made up of many host plants from different families.

The insects that are extreme specialists are the ones that fascinate entomologists (Pilson, 1999), because there are many potential hosts for these insects but why is their feeding limited to few potential hosts? Insects that are extreme specialists are the best biological control agents as they have negligible non-target effects. Insect host range is divided into two: physiological host range and ecological host range. The physiological range is the set of plants that insects have the ability to feed and develop on under artificial (laboratory) conditions in the absence of choice. If the insects feed and develop under natural conditions where they have the freedom to choose, it is known as the ecological host range (Solter and Maddox, 1998). The ecological host range is broad, for example, the Eucalyptus weevil (*Gonipterus* "*scutellatus*" Gyllenhal) can feed and develop on 13 *Eucalyptus* species in South Africa under natural conditions (Newete et al. 2011).

Cochineals are sap-sucking insects that feed only on cactus species. The cochineal insects belong to the family Dactylopiidae. This family has a single genus known as *Dactylopius* which has nine species that are natives of North or South America. *Dactylopius* species like *D. austrinus* De Lotto (Hosking, 1984) and *D. opuntiae* Cockerell (Flores-Hernandez et al. 2006) show similar life history characteristics and general structure. Eggs are laid singly and most of the eggs hatch within a day into first instar crawlers which are pink in colour. Phenotypic differences between the males and females manifest from the late first-instar phase onwards (Mathenge et al. 2009). The first instar female crawlers are covered with stiff bristles that aid them to disperse on the wind. The female becomes sessile once she has settled and inserted her mouthparts and never moves again. Thus, dispersal in females is limited to the first-instar nymphal stages.

The female nymphs will moult twice as they grow into adults (Mathenge et al. 2009). Some of the nymphs settle and develop on the hosts that they hatch on but others make use of the waxy filaments that develop on the nymphs to make them buoyant and are easily lifted in the air streams to other hosts (Foxcroft and Hoffmann, 2000). The females are ready to mate after the second moult and they start laying eggs approximately 21 days later (Mathenge et al. 2009). Although male crawlers have shorter bristles than females, they can also be wind dispersed. The male crawlers secrete a waxy coating as soon as they start feeding and the waxy coating is removed during the first moult leaving them with a slight wax covering. They keep on feeding after this moult for a few days and form a hollow "pupal" cocoon by secreting more wax. Three further moults occur in the cocoon where they finally emerge as winged adults that fly away to find a female to mate with.

Cochineal species feed on one or a few related species in the Cactaceae family (Guerra and Kosztarab, 1992; Portillo and Vigueras, 2006) and are therefore considered to be host specific. The traits of cochineal insects that are assumed to support a high level of specialization in a host include the sedentary female adult stage and low ability to disperse (Gullan and Kosztarab, 1997). Isolated populations of insects that arise as a result of low mobility and the resulting inbreeding causes the build-up of genetic characteristics that make them suitable to particular host plants or species. For example, Glynn and Herms (2004) found evidence of adaption to the scot pines (*P. sylvestris* L.) by the pine needle scale, *Chionaspis pinifoliae* (Fitch). Host specificity variation between populations may result in

clades that cannot be distinguished morphologically but that can interbreed and can only be identified by their distinct host choices for feeding (Dres and Mallet, 2002). Sometimes the host ranges of cochineal insects exhibit specialization at a sub-specific level and there are distinct host adapted populations associated with *Cylindropuntia* and *Opuntia* species that are closely related (Guerra and Kosztarab, 1992; Portillo and Vigueras, 2006).

Dactylopius opuntiae is the most important cochineal insect for biological control of *Opuntia* and has the widest distribution in South Africa (Karny, 1972; Zimmermann et al. 2004). *Dactylopius opuntiae* helped the control of *O. stricta* in Australia (Hosking et al. 1994) and its introduction into South Africa to control *O. ficus-indica* was highly successful (Zimmermann and Moran, 1999). It survived poorly when it was transferred to *O. stricta* in South Africa. Researchers later found that there are at least two host-adapted populations of *D. opuntiae* which are called "biotypes". The *D. opuntiae* "biotype," that has been in South Africa since 1937, gives satisfactory control of *O. ficus-indica* and is called the "ficus" biotype, whilst the one that has been successful in controlling *O. stricta* was brought to South Africa from Australia in 1996 (Table 2.1) and is known as the "stricta" biotype (Volchansky et al. 1999; Klein, 2011). These biotypes cannot be morphologically recognized but can be distinguished by their feeding and development choices (Volchansky et al. 1999; Hoffmann et al. 2002; Mathenge et al. 2010).

Cross breeding experiments of the two biotypes of *D. opuntiae* were undertaken to assess whether the two biotypes are closely related. The biotypes interbred freely and the first generation hybrids developed well on both *O. ficus-indica* and *O. stricta* (Hoffmann et al. 2002). Mathenge et al. (2009) studied the biology of the cochineal insect, *D. tomentosus* (Lamark) on *Cylindropuntia fulgida* (Engelmann) F.M. Knuth var. fulgida (Engelmann) F.M. Knuth and *Cylindropuntia. imbricata* (DC.) F. Knuth. They observed that one of the *D. tomentosus* provenances thrived on *C. fulgida* but had longer nymphal period, decreased adult survival and reproduction on *C. imbricata*. This is probably another example of a biotype.

Table 2.1 Cochineal insects (*Dactylopius* species) which were introduced into South Africa to control invasive cacti. Year of introduction in brackets after the name of the biological control agent. Ratings of perceived degree of damage on the target weed (Klein, 2011): "complete"- in areas where the agent has established and no other methods of controlling the weed are necessary to reduce it to acceptable levels; "substantial"- other management options can still be considered to strengthen the biocontrol, but the management efforts would be less compared to what was required before the biocontrol agent was introduced.

Cochineal species	**Host plants**	**Damage**	**Source**
D. ceylonicus (1913)	*O. monacantha* (smooth prickly pear)	Complete control	Lounsbury (1915) and Zimmermann et al. (2004)
D. austrinus (1935)	*O. auarantica* (jointed cactus)	Substantial control	Zimmermann et al. (2004)
" ficus" biotype of *D. opuntiae* (1937)	*O. ficus-indica* (sweet prickly pear)	Substantial control	Klein (2011)
" stricta" biotype of *D. opuntiae* (1996)	*O. stricta* (Australian pest pear)	Complete control	Volchansky et al. (1999)

Hildegard Klein (personal communication, 2015) observed that the 'stricta' biotype of *D. opuntiae* thrived on *O. humifusa*. However, the 'stricta' biotype did not develop well on *O. engelmannii* – Limpopo lineage and the *O. engelmannii* – Kenyan lineage. This raises the question of what causes the *D. opuntiae* to survive on particular Opuntias and what makes a suitable host for a particular biotype of *D. opuntiae*. *Opuntia humifusa* and the *O. engelmannii* lineages still need a biological control agent in South Africa and they have shown the greatest increase in range in the South African Plant Invaders Atlas 2000-2016 (Henderson and Wilson, 2017). These *Opuntia* species are also in category 1b of the NEMBA regulations; species in this category must be controlled and whenever possible, removed and destroyed.

2.2 Insect responses to plant hosts

It is important to assess how a biological control agent responds when introduced to a host. There are two sides to the question of whether a plant can be a host for a biological control agent. The first side of the question is called acceptability and the second one is known as suitability, these two terms are discussed below.

2.2.1 Host acceptability

When a phytophagous insect finds a host it may not be able to establish on it; the amount of energy required to cut leaf tissues and the amount of dry matter in the leaf may hinder the insects. Acceptability is the ability of a biological control agent to feed and or lay eggs on the host plant (Browne and Withers, 2002). The chances that an insect will feed or oviposit on a certain host individual rely on the 'acceptability' of the host to the insect. This is affected by both innate tendencies and past experience. Many such influences usually operate at the same time thus increasing or decreasing the chances of acceptance (Courtney et al. 1989). When an insect encounters a host it seems as though many factors are in the balance. There is acceptance of the host if the net effect of the factors that influence acceptance is positive and rejection if the net effect is negative (Browne and Withers, 2002). This view has resulted in the interpretation of host acceptance as a threshold character and these thresholds are known to vary with time (Courtney et al. 1989). For example, it has been found that old females of *Dacus tyroni* (Froggatt) (Diptera: Tephritidae) will accept hosts that were rejected earlier in life when there is a shortage of their preferred host (Fitt, 1986).

The general consensus is that the thresholds of host acceptance (the lowest strength of a stimulus that induces a positive response) of insects can differ due to the physiological condition (Barton Browne, 1993), development stage (Barton Browne, 1995), and previous experience (Vet and Dick, 1992; Turlings et al. 1993) of the insects. The behaviour of insects is a product of physiological processes in the endocrine, muscular and nervous systems (Barton Browne, 1993). Therefore the physiology of insects has a major role in the acceptability of the host plant. The development stage of an insect affects the rate of food intake (e.g. the amount of sap fed on by the cochineals). There are temporal changes in the rate of food intake within and between larval instars (Barton Browne, 1995).

These changes are probably generally the same in all species (Barton, 1995). Studies have shown that in some species after a moult the rate of food intake first increases (Chapman et al. 1990; Reynolds et al. 1986) and then decreases. The ability of insects to change their responses to cues of foraging based on experience appears to be a characteristic of many species (Turlings et al. 1993). The learning of profitable cues can take place both during the immature stage and adult stage. Upon contact with the host plant, the insect naturally recognizes host-derived unconditioned stimuli and the insect relates these stimuli with the surrounding stimuli to which they originally showed no or less responsiveness (Vet and Dick, 1992; Turlings et al. 1993).

It is known that phytophagous insects exhibit varying degrees of association with host species (Unni et al. 1996). For example, *D. opuntiae* developed more rapidly on *Opuntia tardospina* Griffiths and *O. ficus-indica* than on *Opuntia megacantha* Salm-Dyck (Karny, 1972). Klein (personal communication, 2013) assessed the efficacy of the 'stricta' biotype of *D. opuntiae* on *O. humifusa* and *O. engelmannii* species in the laboratory and it showed different acceptance of these hosts. The most acceptable host was *O. humifusa* and the *O. engelmannii* lineages were the least acceptable hosts. The time taken by a significant number of the 'stricta' biotype of *D. opuntiae* crawlers to settle on *O. stricta* and *O. humifusa* were shorter compared to those that settled on the *O. engelmannii* lineages. The 'stricta' biotype of *D. opuntiae* crawlers are considered to have settled when they insert their sucking mouthparts into a host and start feeding fixed at one site. The reasons why the insect takes a shorter time to settle on a particular species of *Opuntia* and not on others have never been investigated or explained.

2.2.2 Host suitability

An organism's success is commonly assessed in terms of the quantity of viable offspring that it has contributed to the next generation (Barton Browne, 1993). For an insect to be regarded as a "success", it must utilize resources and avoid death before contributing viable offspring to the next generation. It is also known that a biological control agent is considered established only when it forms self-sustaining populations on its target plant (Coombs et al. 2004). This focuses on the second side of the question: whether a plant can be a host for a biological control agent or not? There is need to check whether the plant supports

development of the biological control agent from neonate to a reproducing adult and this is known as suitability (Browne and Withers, 2002). The successful utilization of a host plant by an insect relies on the availability of a 'proper fit' between characteristics of the plant and insect. The physical and physiological characteristics of both the plant and insect are subjected to temporal changes. Even when a host has been utilized by an insect to some extent, there are chances that the host will prove to some degree to be unsuitable and the insect will not be able to develop. For example, *O. ficus-indica* supported the development of the 'ficus' biotype of *D. opuntiae* from neonate to a reproducing adult (suitability) but the 'ficus' biotype did not develop well when introduced onto *O. stricta*. The 'ficus' biotype initially utilized *O. stricta* but at a later stage barely survived and did not persist on *O. stricta* in South Africa (Volchansky et al. 1999) probably as a result of the differences in chemical defences of *O. ficus-indica* and *O. stricta*. Klein (personal communication, 2013) suggested that *O. engelmannii* lineages might not be suitable as hosts (cannot support development from neonate to a reproducing adult) of the 'stricta' biotype of *D. opuntiae*. The reasons why the 'stricta' biotype of *D. opuntiae* may not reproduce or take longer to reproduce on a particular species of *Opuntia* and not on others have never been investigated or explained.

2.3 Plant responses to insect herbivory

Painter (1951) defined resistance of plants to insect herbivory as the number of inherent qualities that a plant has which play a role in the ultimate extent of damage done by the insect. Painter divided the types of plant resistance to insect herbivory into three main groups: Anti-xenosis (non-preference), antibiosis and tolerance (see Fig 2.1). The group of plant characteristics and responses of an insect that cause the insect to reject a particular plant is known as non- preference. Characteristics of plants such as texture and taste might be part of this type of plant resistance (Painter, 1951). These characteristics reduce the desirability of the plant to the insect. There has been divergence of opinion for a long time when it comes to the fundamental basis of insect-plant relationships (Beck, 1965). One theory is that the insect's primary host in its native range has the specific nutrients and ecological environment that are not found on other plant species. Then the insect recognizes the host by a 'botanical instinct' (Beck, 1965). It was suggested that this 'botanical instinct' may just be intense sensitivity to complex chemical and physical stimuli that emerge from the plant. Non-

preference plant characteristics can also fail to supply the attractive stimuli to the insect (Bjorkman et al. 1997).

The detrimental effects of these plant characteristics on the insect feeding on a resistant plant are known as antibiosis. So obviously high-resistant plants (not or less acceptable to insects) have high antibiosis. These detrimental effects include: death of insects at different instar stages, unusual length of life stages, smaller size and low fecundity. The suggested physiological explanations for the detrimental effects on the life cycle of insects are as follows: deleterious effects of toxins on the insects; the absence of specific food materials in the plants eaten; presence of the food material but for some reason it is not available to the insects (Painter, 1951; Bjorkman et al. 1997; Awmack and Leather, 2002). Tolerance is when a resistant plant grows well although it is supporting an infestation similar to that damaging a susceptible host (Beck, 1965).

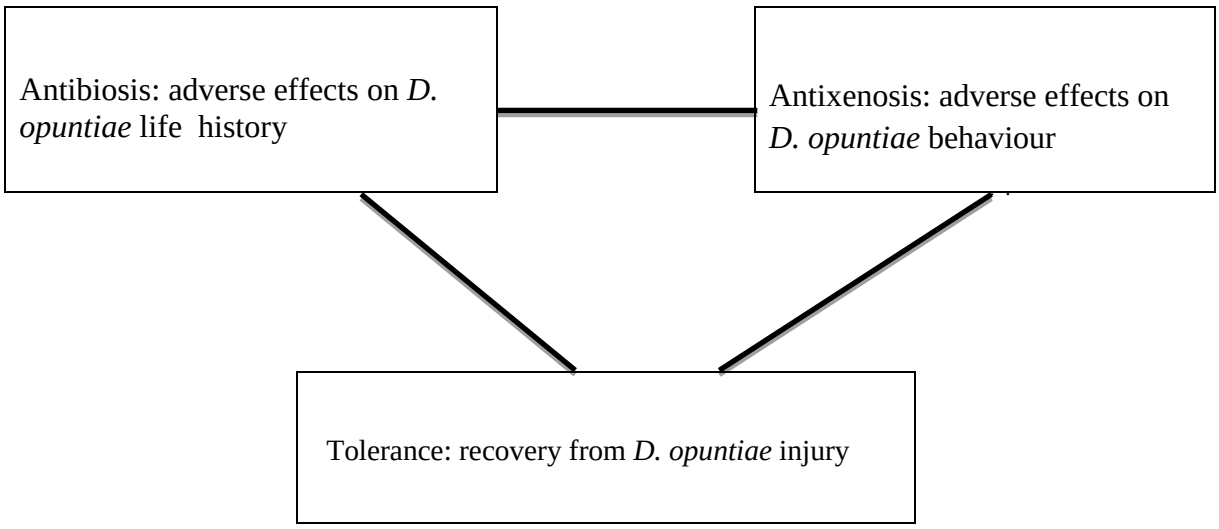


Figure 2.1 Schematic of resistance based on Painter's (1951) concept.

Studies on plant resistance have been done almost entirely on their implications for agriculture and, therefore, the emphasis is on the negative economic effects caused by the insects when they damage agricultural crops. Here I investigated plant resistance and its implications for the biological control of invasive alien plants. The 'stricta' biotype of *D. opuntiae* was introduced into South Africa 22 years ago to control the spread of *O. stricta*. So host specificity was done against one target species, *O. stricta*, and there is now a need to know if the 'stricta' biotype of *D. opuntiae* can be used to control the spread of other invasive Opuntias in South Africa.

Since the reasons why the 'stricta' biotype of *D. opuntiae* has low acceptability on particular *Opuntia* hosts compared to others and the reasons why a particular *Opuntia* is not a suitable host of this cochineal are not known, it is necessary to investigate them. In order to do that there is need to assess host specificity and reproductive performance of the 'stricta' biotype of *D. opuntiae* on the Opuntias. Therefore, the aims of this chapter were (i) to assess the acceptability of the 'stricta' biotype of *D. opuntiae* on *O. stricta*, *O. humifusa*, *O. engelmannii*-Limpopo lineage and *O. engelmannii*-Kenyan lineage; (ii) to assess the suitability of *O. stricta*, *O. humifusa*, *O. engelmannii*- Limpopo lineage and *O. engelmannii*-Kenyan lineage as hosts of the 'stricta' biotype of *D. opuntiae* and (iii) to assess the effectiveness of the 'stricta' biotype of *D. opuntiae* as a biological control agent of *O. stricta*, *O. humifusa*, *O. engelmannii*- Limpopo lineage and *O. engelmannii*- Kenyan lineage.

2.4 Materials and methods

The acceptability and suitability laboratory trials were conducted at the Wits insectary, on the University of the Witwatersrand (Wits) east campus, Johannesburg.

2.4.1 Plant culture

Ten potted plants each of *O. stricta*, *O. humifusa*, *O. engelmannii*- Limpopo lineage and *O. engelmannii*- Kenyan lineage (Table 2.2) were obtained from the ARC-PPRI in Pretoria. Four cladodes were harvested from each potted plant and planted into pots at the Wits insectary to make a total of forty potted plants per species. Clones from the same plant were noted and plants of the same clone were not used in the same trial. This decreased variability between plant replications, since plants of the same clone would have similar genetic make-up and are expected to have the same degree of cochineal resistance when grown under the same conditions.

Table 2.2 *Opuntia* hosts used in the study

Genus/species	**Common name for South Africa**
O. stricta	Australian pest pear (Henderson, 2001)
O. humifusa	Creeping prickly pear (Henderson, 2001)
O. engelmannii	Small round-leaved prickly pear (Henderson, 2001)
Limpopo lineage	No common name
Kenyan lineage	No common name

2.4.2 Insect culture

The population of the "stricta" biotype of *D. opuntiae* was obtained from the ARC-PPRI in Pretoria and was maintained on cladodes of *O. stricta*, which is a suitable host of this biotype of *D. opuntiae*. The following conditions, known to be conducive for the development, survival and reproduction of *Dactylopius* species (Sullivan, 1990) were maintained: constant room temperature at 27±2°C and 70±10% relative humidity including a 14-h daylight cycle. To maintain the *D. opuntiae* culture, which was kept in sealed plastic containers (270 mm × 180 mm × 110 mm) with perforated lids to allow ventilation, a fresh *O. stricta* cladode was placed in each container every two weeks and the cladodes that had shrivelled and died were removed.

2.4.3 Comparison of the reproductive performance and survival of the 'stricta' biotype of *D. opuntiae* on the three *Opuntia* species

Ten mature cladodes of comparable size of each of the three *Opuntia* species were harvested randomly from the potted plants of *O. stricta*, *O. humifusa*, *O. engelmannii* –Limpopo lineage and *O. engelmannii* –Kenyan lineage. Isolated cladodes which are detached from the plant can survive for many months and even develop buds (Hosking, 1984; Sullivan, 1990). All cladodes that were used in the experiments were washed thoroughly to remove unwanted organisms before the crawlers of *D. opuntiae* were placed onto them. Mature females were harvested from the "stricta" biotype rearing populations and removal of wax covering the

females was done by rolling the wax coating off the insect onto a pin, to allow harvesting of crawlers from the females (Mathenge et al. 2010). De-waxed females were placed in 90 mm diameter plastic Petri dishes and the following day the mixed crawlers produced by these different females were used in the trials. All the crawlers used were less than a day old and the tests were 'no-choice' as the crawlers were placed directly onto the cladodes of the respective *Opuntia* species in the individual containers.

Cladodes were placed in plastic containers with perforated lids that allowed ventilation, excluded other insects and prevented crawlers from escaping. The cladodes were supported by four pins embedded into a polystyrene block to suspend them in the air and ensure that the crawlers were able to settle anywhere on the cladode. A fine paint brush was used to transfer the crawlers singly to the cladodes until there were 30 on each cladode. The sex-ratio in *D. opuntiae* is approximately 50% females (Hoffmann et al. 2002). So the random selection of the 30 crawlers was expected to represent the prevailing sex ratio on each of the clean cladodes. Once the transfer of 'stricta' biotype crawlers onto these cladodes was done the cladodes were monitored every three days for 86 days using a stereo microscope to record the survival and development of the nymphal stages. Moulting was confirmed by the presence of a discarded white exuviate near the insect (Karny, 1972).

Recording of the following was done: number of settled crawlers; date of first and second moult; number that completed first and second moult. The number of females and males were also noted. Individuals were given the chance to develop to the adult stage without any disturbance. The time (in days) taken to undergo the first and second moult by the nymphs was recorded to determine the pre-oviposition time for the resulting females. The reproductive output of the insects which reached maturity was measured by counting the crawlers produced by the adult females. As soon as new crawlers were seen around any individual female, that particular female was gently dislodged from the cladode. The wax-coating of the mature female was carefully removed by rolling it onto a pin and the mass of the female was measured using a KERN ABT-NM balance which weighs to four decimal places. These mass measurements represented the female's condition at maturity. Female fecundity was measured by placing the females into separate vials and counting the number of progeny they produced in the vials until their death.

In this study the host acceptance level was considered as high when more than 50% (Jones et al. 2015) of the 'stricta' biotype of *D. opuntiae* crawlers had settled, whereas if less than 50% had settled they were regarded as having low acceptability. Host suitability was when more than 50% of the 'stricta' biotype of *D. opuntiae* crawlers developed to a point where they started producing progeny and, if less than 50% of the original cohort produced progeny, then the host is regarded as not suitable for the 'stricta' biotype of *D. opuntiae*. The reproductive performance data was found to be normally distributed using the Shapiro-Wilk W test. The data were checked for heteroscedasticity. Analysis of variance (ANOVA) was used for the reproduction and survival trials to compare the development of the 'stricta' biotype between host plants with regards to the duration of stages and mortality rates. All the statistical tests were done using R statistical package.

2.5 Effectiveness of the 'stricta' biotype of *D. opuntiae* as a biological control agent on the *Opuntia* hosts

Simple efficacy trials were done as a complementary test to the 'stricta' biotype of *D. opuntiae* acceptability and host suitability laboratory trials. These efficacy trials were done to determine if the 'stricta' biotype can kill the host plants. These trials had two parts: (i) to assess if the 'stricta' biotype of *D. opuntiae* colonies could form self-sustaining populations on the *Opuntia* species and (ii) to assess if the 'stricta' biotype colony can kill the *Opuntia* species. Abundance of all life phases of the 'stricta' biotype of *D. opuntiae* over successive generations was monitored by recording the number of individuals and their stages of development over time to assess the suitability of the *Opuntia* test species to sustain the 'stricta' biotype of *D. opuntiae* colonies. In the second generation the number of nymphs seen and settling represents the fecundity of the biological control agent as well as the host species suitability to support the colony of the biological control agent (Jones et al. 2015).

Five potted plants of each host species were used to assess if the 'stricta' biotype of *D. opuntiae* can kill the host plants. Two mature females which were about to lay eggs were placed at the bottom cladode of a healthy potted plant of each of the test species which was placed into an organza mesh-screened cage. After the females had produced nymphs they were given the chance to settle and develop to maturity. Every fortnight, each plant was observed to assess colony establishment. This was done by assessing the number of individuals and their stages of development over time. The establishment of colony relied on

the amount of second generation nymphs. The colony was regarded as established when at least 50 second generation nymphs were settled (Jones et al. 2015). Monitoring was done until the infestation killed the plant or until the colony of the 'stricta' biotype of *D. opuntiae* died.

2.6 Results

2.6.1 Settling of crawlers - acceptability

A significantly larger number of crawlers settled on *O. stricta* and *O. humifusa* (Fig 2.2) compared to those numbers that settled on *O. engelmannii* Limpopo lineage and *O. engelmannii* Kenya lineage ($F = 76.11$; $df = 3$; $P < 0.05$). The number of crawlers of the 'stricta' biotype of *D. opuntiae* that settled on *O. stricta* and *O. humifusa* were more than double those on the other two *O. engelmannii* lineages, with about 80% of the crawlers settling on *O. stricta* and *O. humifusa*.

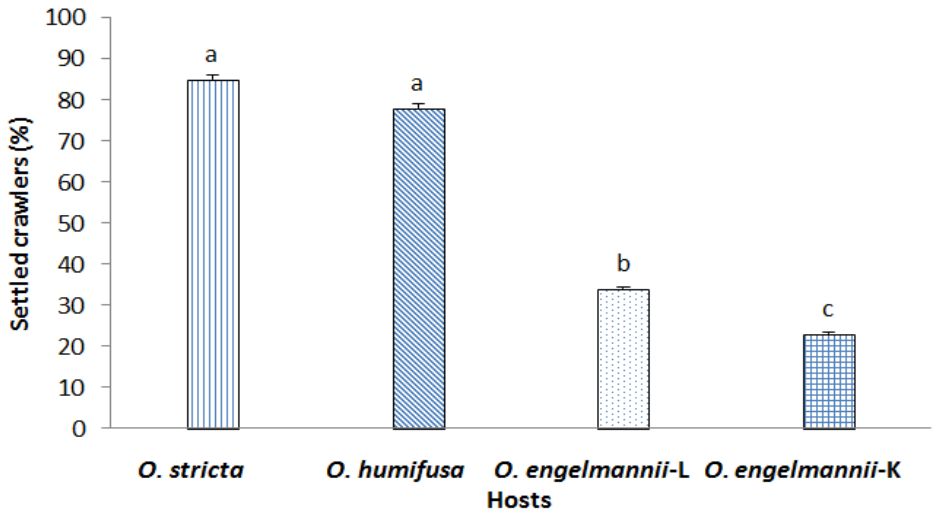


Figure 2.2 Percentage of 30 'stricta' biotype crawlers of *Dactylopius opuntiae* that settled on the various *Opuntia* hosts. Bars (mean± SE), means with identical letters do not differ significantly ($P > 0.05$). L- Limpopo lineage, K-Kenyan lineage.

2.6.2 Moulting and female fertility-suitability of the hosts

The number of 'stricta' biotype nymphs on *O. stricta* and *O. humifusa* were significantly the same at all life cycle stages as were those on both *O. engelmannii* lineages even though they had significantly different numbers of settled crawlers. The mean number of nymphs that

reached the first moult differed significantly (Fig 2.3) between host species (F = 102.7; df = 3; P < 0.05) and the mean number of nymphs that reached the second moult differed significantly between host species (F = 73.38; df = 3; P < 0.05).

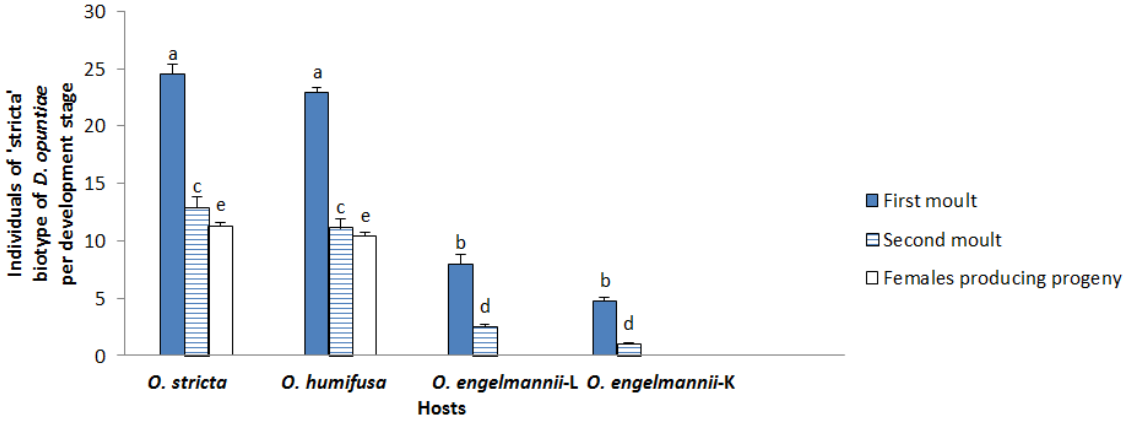


Figure 2.3 Mean numbers of 'stricta' biotype of *D. opuntiae* individuals reaching particular stages in the life cycle of the 'stricta' biotype of *D. opuntiae* on different *Opuntia* hosts. Bars (mean± SE) with identical letters do not differ significantly (P > 0.05). L- Limpopo lineage, K-Kenyan lineage.

The 'stricta' biotype progressed to the adult stage on all hosts, and there was a significant difference in the mean number of males that developed on each host (F = 33.78; df = 3; P < 0.05- Fig 2.4). The females that developed through to adults on both the *O. engelmannii* lineages did not produce any progeny (Fig. 2.3).

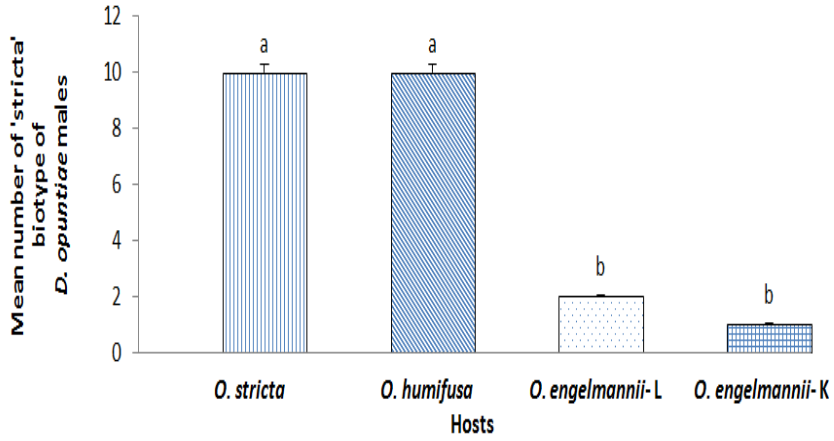


Figure 2.4 Mean numbers of 'stricta' biotype males of *D. opuntiae* on different *Opuntia* hosts. Bars (mean± SE), means annotated with the same letters do not differ significantly (P > 0.05). L-Limpopo lineage, K-Kenyan lineage.

There was a significant difference between the host plants in the number of days it took the 'stricta' biotype of *D. opuntiae* to reach the first moult (F= 36.11; df = 3; P < 0.05- Fig. 2.5).

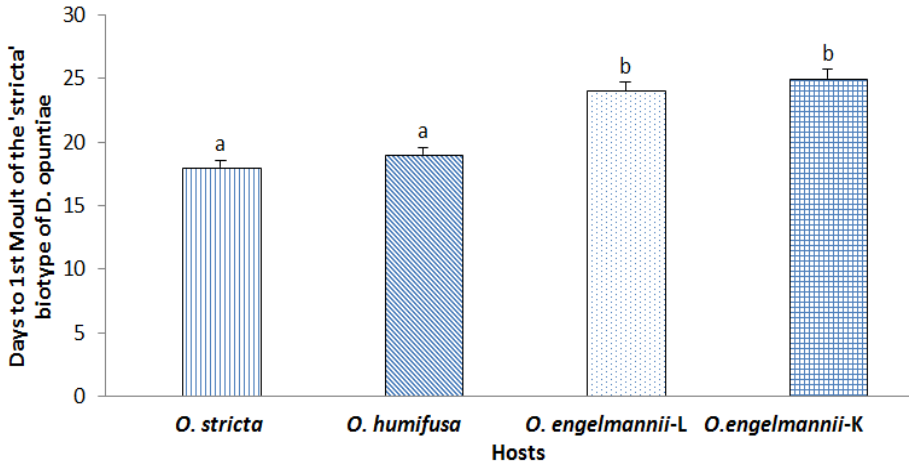


Figure 2.5 Number of days to first moult of the 'stricta' biotype of *D. opuntiae* on different *Opuntia* hosts. Bars (mean± SE), annotated with the same letters do not differ significantly (P > 0.05). L- Limpopo lineage, K- Kenyan lineage.

There was no significant difference in the mean number of females that produced progeny (P = 0.4777- Table 2.3), or in the number of progeny produced (P = 0.3129) by the females that developed on *O. stricta* and *O. humifusa*.

Table 2.3 Mean (±1 S.E.) time to develop of the 'stricta' biotype of *D. opuntiae* (from settling to 2nd Moult); female mass (n = 7); at first offspring production, and number of progeny per female of the 'stricta' biotype on different *Opuntia* hosts that supported development. F-values are for one-way ANOVA to compare means within columns. Means with identical letters in the same columns do not differ significantly according to the t-test (P > 0.05). MNM: mass not measured as they did not reach maturity; NPP: no progeny produced; K: Kenyan lineage; L: Limpopo lineage.

Insect/host combination ('stricta' biotype of *D. opuntiae*/*Opuntia* host)	**Period from settling to 2nd Moult (days)**	**Female mass (mg)**	**Progeny (n)**
'stricta' on *O. stricta*	42 ± 0.7 [a]	14.4 ± 0.5 [a]	431 ± 8 [a]
'stricta' on *O. humifusa*	45 ± 0.6 [b]	13.7 ± 0.5 [b]	417 ± 11 [b]
'stricta' on *O. engelmannii* L	55 ± 0.4 [c]	MNM	NPP
'stricta' on *O. engelmannii* K	56 ± 0.6 [c]	MNM	NPP
	$F_{(3)} = 113.4$		

There was a significant difference in the number of days taken to start offspring production between the females that developed on *O. stricta* and *O. humifusa* (P = 0.001- Fig. 2.6).

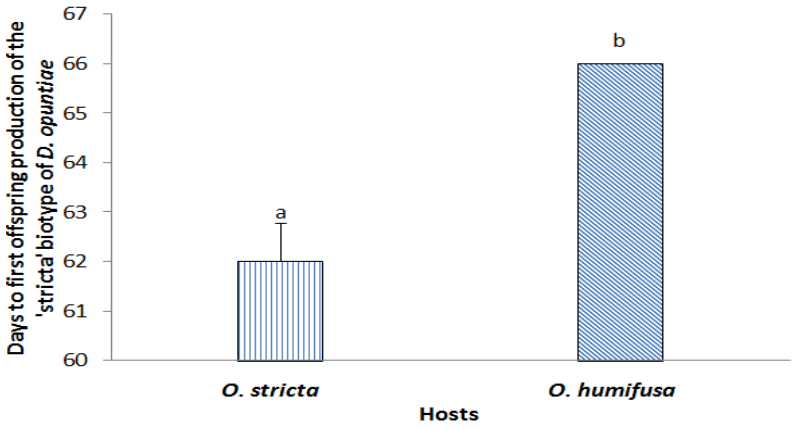


Figure 2.6 Number of days to first offspring production of the 'stricta' biotype of *D. opuntiae* on different *Opuntia* hosts. Bars (mean± SE) annotated with the same letters do not differ significantly (P > 0.05).

2.7 Effectiveness of the 'stricta' biotype of *D. opuntiae*

There were high numbers of first generation nymphs of the 'stricta' biotype of *D. opuntiae* on *O. stricta* and *O. humifusa* potted plants (Table 2.4) compared to those on *O. engelmannii* lineages.

Table 2.4 Number of first generation nymphs on the *Opuntia* host plants two weeks after placing the mature females of the 'stricta' biotype of *D. opuntiae* on the plants.

***Opuntia* host**	**Number of 'stricta' biotype nymphs of *D. opuntiae***
O. stricta	>50
O. humifusa	>50
O. engelmannii-Limpopo lineage	16
O. engelmannii-Kenya lineage	7

The 'stricta' biotype nymphs of *D. opuntiae* that developed on *O. humifusa* had high establishment rate (Table 2.5) since the nymphs established on the same week as those on *O.*

stricta. Populations of the 'stricta' biotype of *D. opuntiae* on *O. stricta* and *O. humifusa* caused the death (discoloured, dry and damaged cladodes falling to the ground) of Opuntias in almost the same week. The first generation nymphs of the 'stricta' biotype of *D. opuntiae* settled on the *O. engelmannii* lineages but did not kill the plants (Table 2.5).

Table 2.5 Time taken for the establishment of the 'stricta' biotype of *D. opuntiae* colonies of on each *Opuntia* host. NE: no establishment; - host not killed

***Opuntia* host**	**Time taken for establishment of colonies (weeks)**	**Time taken to kill the host (weeks)**
O. stricta	8	14.5
O. humifusa	8	15
O. engelmannii-Limpopo lineage	NE	_
O. engelmannii-Kenyan lineage	NE	_

2.8 Discussion

The results show that the 'stricta' biotype of *D. opuntiae* exhibits varying degrees of acceptance of different *Opuntia* host taxa, with the least acceptable hosts being the *O. engelmannii* lineages from both Limpopo and Kenya. *Opuntia stricta* and *O. humifusa* are the most acceptable and suitable hosts (support development of the biological control agent from neonate to a reproducing adult) of the 'stricta' biotype of *D. opuntiae*. The 'stricta' biotype of *D. opuntiae* showed small differences in its ability to develop and survive on these two hosts. The 'stricta' biotype nymphs of *D. opuntiae* that developed on the most acceptable and suitable hosts reached the adult stage within the shortest time which was 44 days. This has also been observed in other *Dactylopius* species, for example, Mathenge et al. (2009) reported that the time to develop to the adult stage in *D. tomentosus* is usually approximately 43 days.

The prolonged development time of the 'stricta' biotype of *D. opuntiae* on the *O. engelmannii* lineages and failure of the females to produce progeny shows that they are not suitable hosts of this cochineal. In the effectiveness experiments, the time needed for the

emergence of second generation nymphs and for the settling of the nymphs on the most acceptable hosts were major indicators of the 'stricta' biotype of *D. opuntiae*'s capability of controlling *O. humifusa*. Settling of these second generation nymphs and their subsequent effects on the health of the most acceptable host plants provided further evidence that this cochineal can be effective in controlling *O. humifusa*. The discolouring and drying of the damaged cladodes of *O. stricta* and *O. humifusa* plants became more apparent in successive weeks as the development of the second generation nymphs progressed.

Studies reported that the 'stricta' biotype of *D. opuntiae* is specific to *O. stricta* with potential to inflict damage on other *Opuntia* species (Volchansky et al. 1999; Hoffmann et al. 2002). A recent study by Rule and Hoffmann (2018) reported that *O. humifusa* is a suitable host of the 'stricta' biotype of *D. opuntiae*; our results mirror this recent study. These results confirm that the 'stricta' biotype of *D. opuntiae* can persist and reproduce on *O. humifusa*. Thus, it has potential to be a biocontrol agent of *O. humifusa* but it does not persist on the *O. engelmannii* lineages. The different acceptability shown by the 'stricta' biotype of *D. opuntiae* on the various *Opuntia* hosts could be as a result of different traits such as the level of amino acids, kinds of secondary metabolites such as defensive proteins, morphological and phytochemical characteristics, for example, tannins (El-Mostafa et al. 2014), pH and amount of acid in the cladodes (Meraz-Maldonado et al. 2012) of the hosts. For example, the first-instar nymphs have to pierce the cuticle and epidermis of the cactus cladodes before they can start feeding so the mechanical structures of the plants, such as thickness of the wax coating on the cladodes may influence the settling and development of the nymphs.

There is variation in chemical compounds between cactus species and the genera of cactus have been distinguished based on the variation in chemical compounds (Wallace and Gibson, 2002). The role played by chemicals that are found in cacti on insect-host interactions has been recorded, for example, alkaloids are poisonous to some *Drospholia* species and not to others (Danielson et al. 1995). So there is need to investigate the basis of the differences in acceptance shown by the "stricta" biotype of *D. opuntiae* on the *Opuntia* hosts to explain the reasons underlying these results. Nearly 23 years after its introduction into South Africa, the 'stricta' biotype of *D. opuntiae* is still effective in controlling its target weed, *O. stricta* and can also be effectively used to control *O. humifusa*. Conclusion can be drawn that if the durations of development of an insect on a standard host plant, in this case 'stricta' biotype of

D. opuntiae on *O. stricta* and a plant to be tested are compared, the difference can be regarded as a measure to express acceptability or resistance.

Chapter 3

Morphological and phytochemical aspects of the *Opuntia* hosts

3.1 Introduction

Plants are plentiful in relation to insect herbivores; but an important question to ask in insect/plant ecology is why all insects do not have the capability to eat all plants (Abe and Higashi, 1991). Plant defences have been cited as a mechanism controlling host selection and performance of herbivore populations (Zangerl and Berenbaum, 1993; Harvey et al. 2011). The co-evolution of plants and herbivores has resulted in plants having defense mechanisms against herbivores such as birds, mammals and insects. Mechanisms of defense against herbivory have been evolving for many years, and these mechanisms are shared across most plant families (Stotz, 1999). The co-evolutionary theory suggests that some insects have been successful in counteracting some plant defenses, and those defenses may then be used as feeding stimulants for those species which specialize on the plant (Chown and Nicolson, 2004). The term, defence, is reserved for scenarios where plants that have some resistance character are said to have higher fitness than those lacking that character (Agrawal, 1999). These defence mechanisms can be divided into two groups: pre-formed (constitutive) defences and inducible defences.

3.2 Inducible defences

Inducible defences are those that are activated after insect attack, and there are two types of inducible defences: direct and indirect. Any plant traits that by themselves affect the vulnerability of the host plant to insect attacks are called direct defences (Kessler and Baldwin, 2001). Indirect defences increase the chances of attracting inherent enemies of the herbivorous insect. The period between first attack of the plant by the herbivore and defense activation makes inducible defences inherently inferior to constitutive defences (Baldwin, 1998). This period between first attack and activation of defense makes the plant vulnerable for hours or even days until the defense is activated. The inducible direct defences are divided mainly into two groups: anti-nutrition and toxicity (Figure 3.1).

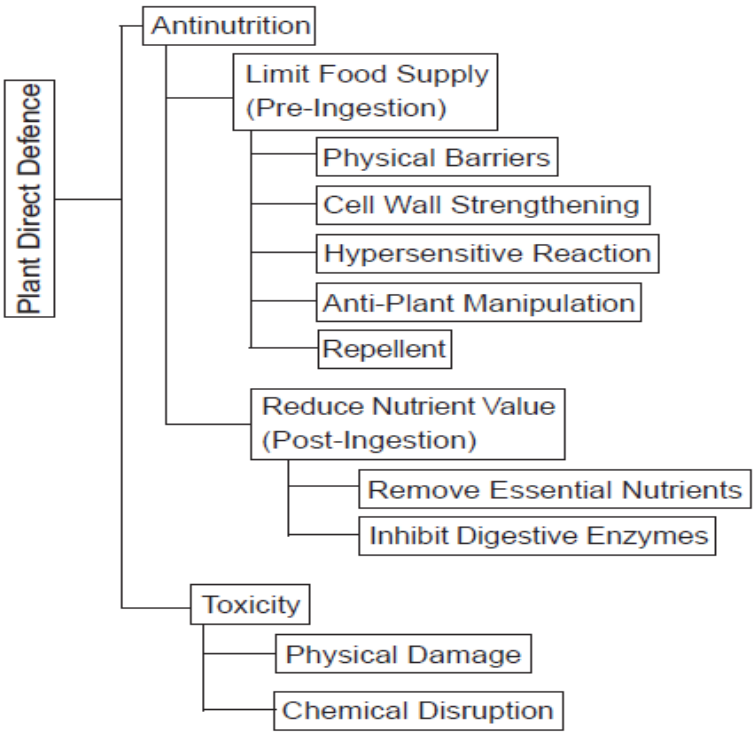


Figure 3.1 Groups of inducible direct defences against herbivorous insects: anti-nutrition and toxicity- physical damage to the insect and chemical disruption on the insect (Chen, 2008).

3.2.1 Anti-nutrition

Anti-nutrition can take place before ingestion to reduce the supply of food, and after digestion to decrease the usefulness of nutrients to the attacking insect (Chen, 2008). A range of mechanisms can be used by host plants to limit the supply of food to insects before ingestion. Cell wall strengthening can reduce food supply to insects as this further improves the quality of physical barriers to insect feeding, mostly for sap sucking insects like the 'stricta' biotype of *D. opuntiae*. One major way of resisting insect herbivory by plants is through hypersensitive responses (Goodman and Novacky, 1994). Hypersensitive response causes the swift death of cells at the attacked site thus stopping further feeding of the insect by starving it (Heath, 2000). This type of defence normally works well against insects that settle and feed on one spot such as the cochineals.

Anti-plant manipulation of host plants is also a vital process of anti-nutrition for herbivorous insects that change the local surrounding to get nutrition (Danks, 2002), for example, galling insects. When galling insects start feeding they form a region of "metabolic habitat

modification" inside the tissue of a plant commonly known as a gall (Goethals et al. 2001). In this gall region, the insect has increased nutrition as a result of a nutrient sink formation (Stone and Schönrogge, 2003; Zhu et al. 2008). Resistant plants can somehow prevent gall formation thus depriving nutrition to the attacking insect (Zhu et al. 2008).

Some plants synthesise and release complex mixtures of volatile organic compounds (VOCs) when they are attacked by insects, these VOCs attract the natural enemies of the herbivorous insects (Kessler and Baldwin, 2001; Kost and Heil, 2006). This is an indirect defense, but some VOCs can act as direct defense by acting as repellents to the insects that attack. After indigestion, anti-nutrition can also still take place by eliminating vital nutrients or hindering the digestion. Some enzymes of the plant can survive in the insect's gut and destroy nutrients that the insect could have used (Chen, 2008). Digestion can be inhibited by different protein inhibitors that are produced by the plant when it is attacked by an insect, these protein inhibitors hinder the action of digestive enzymes in insects such as amylases (Koiwa et al. 1997). This reduces insect growth and development because the inhibition of the digestion enzymes decreases the value of nutrients that have been ingested.

3.2.2 Toxicity

Plants can also produce chemicals that physically damage the insect. For example, the insect's structural proteins can be digested by proteases which are produced in excess by plants when they are attacked. The digestion of the structural proteins will take place after the proteases have been ingested and are in the gut of an insect thus causing physical damage (Pechan et al. 2000). Chemical defences can also cause damage to herbivorous insects. Regulatory enzymes of insect growth and development can be inhibited by chemicals from plants (Pechan et al. 2000). Chemical damage occurs when these regulatory enzymes are inhibited resulting in stunted growth or even death of the insects.

3.3 Constitutive defences

Constitutive defences are those that exist before insect attack and can be divided into two main groups: physical barriers and chemical barriers.

3.3.1 Physical barriers

The utilizability of plants as insect hosts is undoubtedly affected by their physical form and structure of the tissues. The surface of the plant is surrounded by a cuticle which is the first contact in the interaction of plants and insects (Bernsys et al. 1976). The cuticle is a continuous layer which has pores of stomata as the only gaps. Understanding the physical nature of the cuticle can help us to assess cues on the outer surface that insects use. For instance, as early as in the 1970s, Bernsys et al. (1976) found out that grasshoppers can differentiate between susceptible and non-susceptible plants by simply having their chemoreceptors being in contact with the surface wax of *Poa annua* leaves. There is another anatomical barrier beneath the cuticle called the epidermis. A study by da Silva et al. (2010) showed significant differences in the thickness of the cuticle and epidermis of *O. undulata* Griffiths and *O. ficus-indica*. The range of the cuticle thickness was 10 μm to 39 μm and for epidermis thickness it was 117 μm to 220 μm. According to da Silva et al. (2010), the epidermis is the major anatomical hindrance which provides formidable resistance to insects.

Under the epidermis there is a hypodermis, and it usually has at least one cell layer in the cladodes of Cactoideae and the Opuntioideae (Mauseth, 1999). The hypodermic cells appear collenchymatous, with thick cell walls. These features are related with wall functions, as mechanical support and barrier against pathogens (da Silva et al. 2010). Rigidity and xeromorphic adaptations of the cladodes can be attributed to cell wall thickness and the number of layers of the hypodermis (Terrazas-Salgado and Mauseth, 2002). Morphological and morphometric analysis of the anatomical structure of cactus epidermis can shed more light on the important mechanisms for the selection of effective biocontrol agents.

All plant cells are composed of a primary cell wall to give structural support. The primary cell wall is made up of crude fiber which is mainly composed of cellulose, a long polymer of chains consisting hundreds of glucose monomers which give strength and flexibility to the cell wall (Freeman and Beattie, 2008). Rodriguez and Cantwell (1988) reported a significant difference in the crude fiber of *O. amyclaea*, *O. ficus-indica* and *O. inermis*. The crude fiber ranged from 11.2 to 17.6 (% dry weight). Martin (1991) stated that most phytophagous insects do not digest cellulose and this limits the consumption of plants. Cell wall fortification as a result of high quantities of cellulose enhances physical barriers to herbivorous insects; mostly for insects like *D. opuntiae* which have to pierce the cell wall

first before they can start sucking. Insects that digest cellulose are mainly specialists on wood and, as a result, have made associations with different symbionts (Higashi et al. 1992). This inability of most insects to break down cellulose found in cell walls suggests that they may be more vulnerable to the mechanical defense of plants than what was previously believed. There is a possibility that the varying degrees of accepting the three *Opuntia* hosts shown by the 'stricta' biotype of *D. opuntiae* in Chapter 2 could be as a result of the difference in the physical barriers of the *Opuntia* hosts or in the amount of crude fiber in the *Opuntia* cladodes.

3.3.2 Chemical barriers

The chemistry of the plant has been attributed as the primary reason in limiting host use and choice among insect herbivores by many studies (Hochuli, 1996). However, the focus has been mainly on plant secondary metabolites, and there has been no through consideration of plant primary metabolites. The metabolic chemicals that are needed for normal growth and development of plants are known as primary metabolites. The photosynthetic pathway, Crassulacean acid metabolism (CAM), found in Cactaceae is a unique physiological adaptation that leads to the accumulation of organic acids. CAM is a short-term CO_2 concentrating process with CO_2 from the atmosphere being fixed at night through phosphoenolpyruvate carboxylase (PEPC). There are four phases of CAM which are as follows: Phase I is characterized by the uptake of CO_2 at night through open stomata and the storage of malic acid in the vacuoles (Lüttge, 2004). It is known that the amount of light that a CAM plant receives has a great impact on the balance between fixation of CO_2 and organic acids accumulation at night (Barker and Adams, 1997; Lüttge, 2004).

Phase II is the complex metabolic transition at the start of the light period early in the morning which is often characterised with the highest point of CO_2 uptake. Phase III is a time when malic acid is remobilized and decarboxylated and the resulting CO_2 is fixed again and is incorporated in the Calvin cycle. In phase IV, there is predominant CO_2 fixation and this is determined by the opening of the stomata during the last part of the light period. The increase of organic acids during metabolism of succulents by CAM refers to an increase of titratable acidity in green tissues at night and its decrease during the day when the tissues are photosynthesizing (Figure 3.2). Decarboxylation of malate happens during the day releasing CO_2 in the photosynthetic tissues (Bronson, 2011).

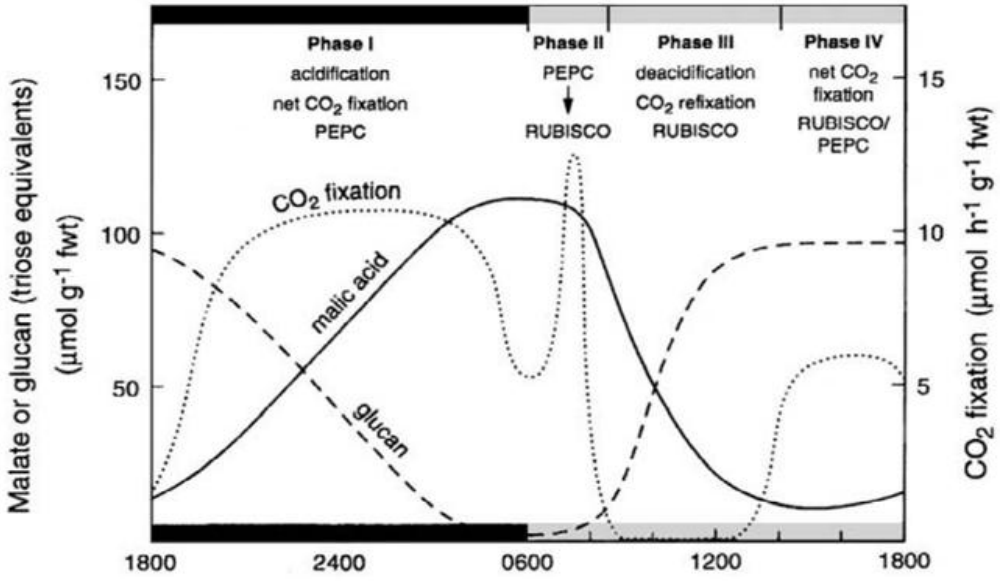


Figure 3.2 Phases of CAM showing the daily patterns of carbon dioxide fixation and the corresponding malic acid (Titratable acidity). The malic acid increases during metabolism of succulents by CAM. (Black and Osmond, 2003).

Titratable acidity (TA) is a measure of the amount of acid present in the tissues and pH is defined as the measure of the strength of acid in the tissues. The noticeable changes in the TA of plant tissues are associated with normal fluctuating intensities of light and temperature. There is plasticity in the aspects of the four phases of CAM and this is a ubiquitous feature of many CAM plants (Dodd et al. 2002). CAM is closely associated with the environment; therefore, it can be disturbed by the water status, temperature and level of light. At the plant species level, the phases described above offer a framework within which to chronicle CAM, but it should be highlighted that large variation in the patterns of diel CAM photosynthesis are frequent (Dodd et al. 2002). This makes generalizations about this unique physiological adaptation difficult, so there is a possibility that the *Opuntia* species in this study have different, variable levels of the components involved in CAM and therefore have different functioning of CAM pathways.

Variability between four different *Opuntias* has been reported in the content of soluble solids, TA and pH (Betancourt-Domínguez, 2006). The range of values reported by Betancourt-Domínguez (2006) for TA was between 0.12 to 0.87% and for pH it was 4.05 to 4.55. The TA and pH of the Opuntias under this study need to be compared.

Organic compounds that are not necessary for normal growth and development of a plant and are usually made as by-products during the manufacture of primary metabolites are known as plant secondary metabolites (Herbert, 1989). Plant secondary metabolites are thought to act as chemical defense against herbivores (Bennett and Wallsgrove, 1994; Mao et al. 2007). The secondary metabolites that often play a part in plant defense usually belong to one of the following large classes: alkaloids (e.g. sinalbin), phenolics (e.g. tannins, flavonoids and lignin) and terpenoids (e.g. saponin). These different plant secondary metabolites reduce the growth of the insect herbivores using different mechanisms. Some phenolics can be anti-nutritional by strengthening cell walls (Schroeder et al. 2006) and can also repel herbivorous insects along with other volatiles from oviposition (DeMoraes et al. 2001).

This chapter looked at the phenolics, specifically the tannins. Tannins can be found in a variety of forms and are often divided structurally into two functional groups: hydrolysable tannins and condensed tannins (Ayers et al. 1997). Within these groups there is a great variation in oxygenation patterns, degree of polymerisation, stereochemistry and identity of side chains (Clausen et al. 1990). So there is complexity in the interactions between tannins and insects because tannins are a collection of heterogeneous compounds. Researchers think that there may be a great natural variation in tannin-herbivore interactions as a result of structure specificity in the effects of tannins (Van Altena and Steinberg, 1992) and to physiological variation in herbivore response to tannins (Hagerman and Robbins, 1993). There is common association of increased tannin content of leaves with reduced water content and increased hardness (Bernays, 1981). It has also been shown that hydrolysable tannins cause insect feeding responses that vary from negative to positive depending on hydrolysable tannin concentration and on the insect species (Bernays, 1981).

Since all aspects of insect-tannin relationships show great variation, there is a difficulty in making generalizations (Bernays, 1981). The functional characteristic that defines tannins is their ability to bind to proteins. These tannin-protein interactions appear to be the ones that influence herbivore food selection and physiology (Clausen et al. 1990). When food protein is bound to tannins, it is made unavailable for digestion (Feeny, 1970). A study by Feeny (1969) showed that enzymes can be rendered inactive when they are exposed to tannins. This negative effect of tannins on the insects' digestion will have an adverse effect on the growth of the insects. For example, the sap-sucking insect, the greenbug *Schizaphis graminum* L grew poorly and eventually died as a result of the toxicity of tannins in the barley it was

feeding on (Todd et al. 1971). The effects of tannins on biological control agents such as the sap-sucking cochineal insects are not known.

Many parts of *Opuntia* are rich in flavonoids and phenolic acids (El-Mostafa et al. 2014; Allai et al. 2017). The tannins in *Opuntia* are rich in phenolic groups so assays for phenolic groups can be used for the analysis of tannins. The Folin and Prussian blue are the most common phenolic assays which can detect both hydrolysable and condensed tannins. However, Ayres et al. (1997) stated that methodological problems have been experienced on the research of tannins and in some cases different techniques seem to lead to different conclusions when assessing the anti-herbivore activity of tannins. It is known that some polyphenols are only produced by cladodes of some *Opuntia* species (El-Mostafa et al. 2014). Therefore, the aims of this chapter were to compare the (i) anatomical structures of the *Opuntia* cladodes; (ii) the amount of crude fiber in these Opuntias and relate these morphological phytochemical aspects to the degrees of acceptability exhibited by the 'stricta' biotype of *D. opuntiae* on the *Opuntia*.

3.4 Material and methods

3.4.1 Plant material

The following potted Opuntias (seven for each host) were placed in the glasshouse part of the insectary at Wits University: *O. stricta*, *O. humifusa*, *O. engelmannii*- Limpopo lineage and *O. engelmannii*- Kenya lineage. Each potted plant had at least five cladodes and these *Opuntias* had been shown to exhibit different degrees of resistance to the 'stricta' biotype of *D. opuntiae*. The species considered susceptible were *O. stricta* and *O. humifusa*; both *O. engelmannii* lineages were regarded as resistant to *D. opuntiae*. The potted plants were acclimatized to the glasshouse part of the insectary for six weeks. This section of the insectary received natural light; the maximum daily photosynthetic photon flux density (PPFD) was 1800 μmol m^{-2} s^{-1}. The maximum/minimum temperatures were 25 °C and 17 °C respectively and the daytime range was between 19 °C and 25 °C. The potted plants were watered every three days at field capacity to maintain soil moisture.

3.4.2 Morpho-anatomical and morphometric analysis

Secondary samples of the *Opuntia* cladodes (approximately 9 cm^2) were taken from the middle portion of the cladodes. These samples were then immersed in a fixative (50% ethanol: formalin: glacial acetic acid, 90:5:5) with the quantity of vegetal tissue to fixative volume being approximately 1: 20-30 (Johansen, 1940; da Silva et al. 2010) for 24hrs. Dehydration of the seven pieces per *Opuntia* clone was done through a graded series of ethyl alcohol (see Appendix 1) based on Johansen's (1940) methodology. After embedding in paraffin wax, the pieces were cut into 12 µm cross sections using a microtome. Staining of the sections was done with Safranin-Fast Green (see Appendix 2). The cross sections were mounted on labelled slides. The morphometric analysis experimental design was randomized, having seven replicates, one cladode as the experimental unit of each *Opuntia* clone. An Olympus BX63 OFM microscope was used for the morphometric analysis of the tissues with image analysis software program (Media Cybernetics). Measurement of the thickness of cuticle, epidermis and corresponding hypodermis was done on the images. ANOVA and comparison of mean values was done using the Tukey test after data were checked for heteroscedasticity using R statistical package.

3.4.3 Crude fiber determinations

An *Opuntia* cladode from each potted plant (7 plants per *Opuntia* species) was chopped into small pieces approximately 1 cm^2 and air dried. Crude fiber (acid-detergent) analysis was performed by grinding the dry material until it could pass through a 1 mm mesh screen. 1 gram of each dried sample (10 replicates for each species) was used to measure the acid-detergent fibre using the Van Soest procedure (AOAC, 1990). The crude fiber results were analysed by Bartlet's Test for homogeneous variances and ANOVA using R statistical package to compare the amount of crude fiber between the *Opuntia* hosts.

3.4.4 Measurement of pH and titratable acid

To determine the malic acid levels of the *O. stricta*, *O. humifusa*, *O. engelmannii*- Limpopo lineage and *O. engelmannii*-Kenyan lineages samples were collected every three hours, starting from 08h00 and continuing for 15 hours. A cork borer 8 mm in diameter was used to take the samples from the middle part of the cladodes (seven replicates per species). The samples were weighed and placed into 10 ml of 60% ethyl alcohol and frozen. A modified

version of the Zotz and Andrade (1998) protocol was used to determine organic acids concentration. Sixty % ethyl alcohol was poured into the samples until the 20 mL mark was reached. After boiling for 5 minutes the samples were titrated with 0.015 N NaOH (Hernández-González and Villarreal, 2007) to an end point (pH) of 8.2 that was measured with phenolphthalein indicator.

Titratable acidity (TA) was calculated using the following formula:

% acid = [ml NaOH used] x [0.015 N NaOH] x [milliequivalent factor] x [100] ÷ grams of sample

The predominant acid in cactus cladodes is malic acid so a milliequivalent factor of 0.067 was used (Meraz-Maldonado et al. 2012).

Whole cladodes for pH determination were harvested in the afternoon between 1300hrs and 1400hrs well after nocturnal acid accumulation. To determine pH, the cladode samples (seven replicates for each *Opuntia* host) were ground and distilled water was added to the sample to give an 8:1 (Perez- Harguindeguy et al. 2013) volume ratio of water to cladode sample. The samples were shaken in laboratory shaker for 1 hour and centrifuging at 2,000 rpm using the Rotofix 32A machine was done until there was a clear separation of the sediment and the supernatant. The pH was then measured from the supernatant using the HQ430d flexi pH meter.

3.4.5 Determination of tannins

A cladode from each potted plant (seven potted plants per species) was chopped into small pieces approximately 1 cm^2 and air dried. The air dried material was ground to pass through a 1 mm mesh screen. To extract tannins 0.5 g of the powdered material of each species was transferred to a conical flask and 75 ml of distilled water was added. The flask was heated gently to boil for 30 minutes and then centrifuged at 2,000 rpm for 20 minutes. The supernatant was collected into a 100 ml volumetric flask and distilled water was poured into the volumetric flask until it was full. 1ml of the sample extract was transferred to a 100 ml volumetric flask which had 75 ml distilled water. Estimation of the tannins was done by the Folin-Denis' Method (Saxena et al. 2013).

Phosphotungstomolybdic acid is part of Folin-Denis' reagent and is reduced by tannin-like compounds to form a highly coloured blue solution and the intensity of this solution is proportional to the quantity of tannins. 5 ml of Folin-Denis' reagent and 10 ml of sodium carbonate solution was added to the sample extract and dilution by distilled water to 100 ml was done. After shaking well the absorbance was read on a dr 3900 spectrophotometer at 755 nm after 30 minutes against a blank. The tannins of the sample (cladodes of *Opuntias*) were shown as being equal to mg of tannic acid by g of extract using a standard graph that was prepared using tannic acid. One-way ANOVA with Tukey's simultaneous test was also be used to assess if there is any significant differences in the phytochemical aspects between the host plants.

3.5 Results

3.5.1 Morpho-anatomical and morphometric aspects of the cladodes

All the *Opuntias* in this study had epidermises that are uniseriate (just one layer of cells) and there was no significant difference in the thickness of the cuticle, epidermis or the hypodermis, between the *Opuntias* (Table 3.1).

Table 3.1 Thickness of the cuticle, epidermis and hypodermis of the *Opuntia* hosts. Mean values (n = 7) followed by equal letters in the same column do not differ significantly (Tukey test, 5% significance). L-Limpopo lineage, K-Kenya lineage.

***Opuntia* hosts**	**Cuticle thickness (µm)**	**Epidermis thickness (µm)**	**Hypodermis thickness (µm)**
O. stricta	$7.21^{a} \pm 0.10$	$18.68^{b} \pm 0.08$	$168^{c} \pm 0.13$
O. humifusa	$7.23^{a} \pm 0.04$	$19.00^{b} \pm 0.06$	$170^{c} \pm 0.09$
O. engelmannii-L	$7.19^{a} \pm 0.07$	$18.70^{b} \pm 0.1$	$164^{c} \pm 0.06$
O. engelmannii-K	$7.20^{a} \pm 0.09$	$18.98^{b} \pm 0.04$	$170^{c} \pm 0.03$

The hypodermis cells of all *Opuntia* hosts had reddish particles that are assumed to be tannins and these particles were dominant in *O. engelmannii* lineages (Figure 3.3).

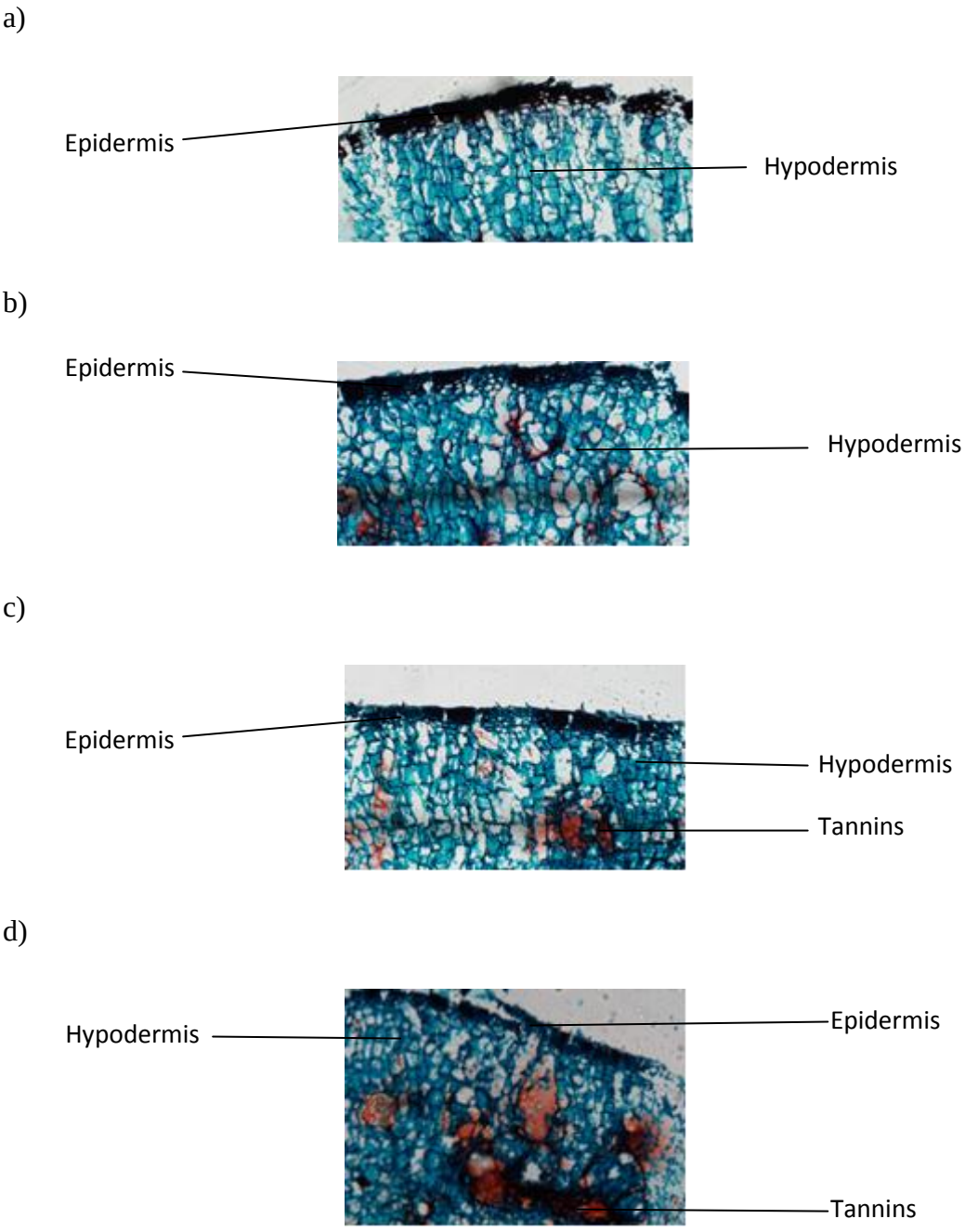


Figure 3.3 Cell contents of the epidermis and hypodermis of a) *O. stricta*; b) *O. humifusa*; c) *O. engelmannii*-Limpopo lineage; d) *O. engelmannii*-Kenya lineage. Scale: 100 µm.

3.5.2 Crude fiber of the *Opuntia* cladodes

There was no significant difference in the crude fiber content between the *Opuntia* species (F = 0.86; df = 3; P> 0.05 - Table 3.2).

Table 3.2 Crude fiber content of cladodes from four *Opuntia* hosts. Mean values (n = 7) annotated with the same letters do not differ significantly (P > 0.05).

***Opuntia* hosts**	**Crude Fiber (% dry weight)**
O. stricta	40.7 [a]
O. humifusa	40.9 [a]
O. engelmannii- Limpopo lineage	40.6 [a]
O. engelmannii-Kenyan lineage	40.8 [a]

3.5.3 pH and Titratable Acidity

The pH of the tissues in the *Opuntias* was significantly different among the *Opuntia* hosts (F = 3076; df = 3; P < 0.05); the most suitable hosts of *D. opuntiae* had organic acids that were acidic compared to the least suitable hosts which had organic acids which were slightly basic (Figure 3.4).

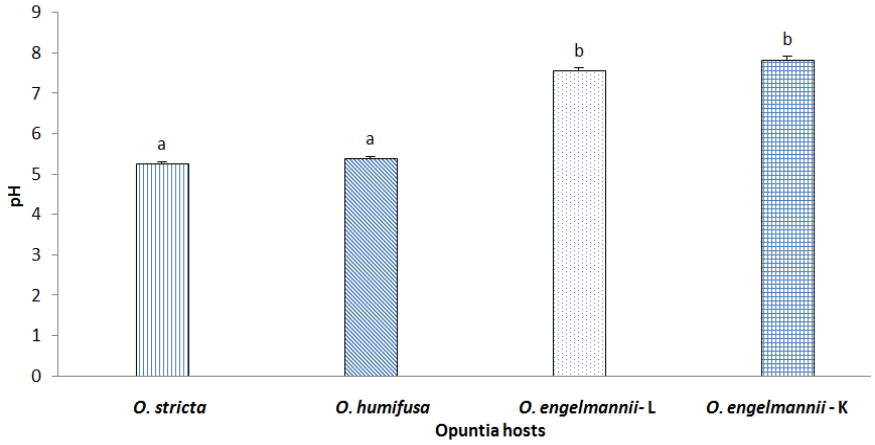


Figure 3.4 Difference in pH of selected *Opuntia* cladodes measured in tissue collected between 1300 hrs and 1400hrs. Bars (mean± SE) mean annotated with the same letters do not differ significantly (P > 0.05). n = 7. L-Limpopo lineage, K-Kenyan lineage.

All the hosts accumulated organic acids at night typical of CAM (Fig 3.5). Cladodes of all the hosts showed significant variability in the maximum value of acid accumulation (F=35.16; df =3, P < 0.05); *O. stricta* and *O. humifusa* recorded the highest acidity values.

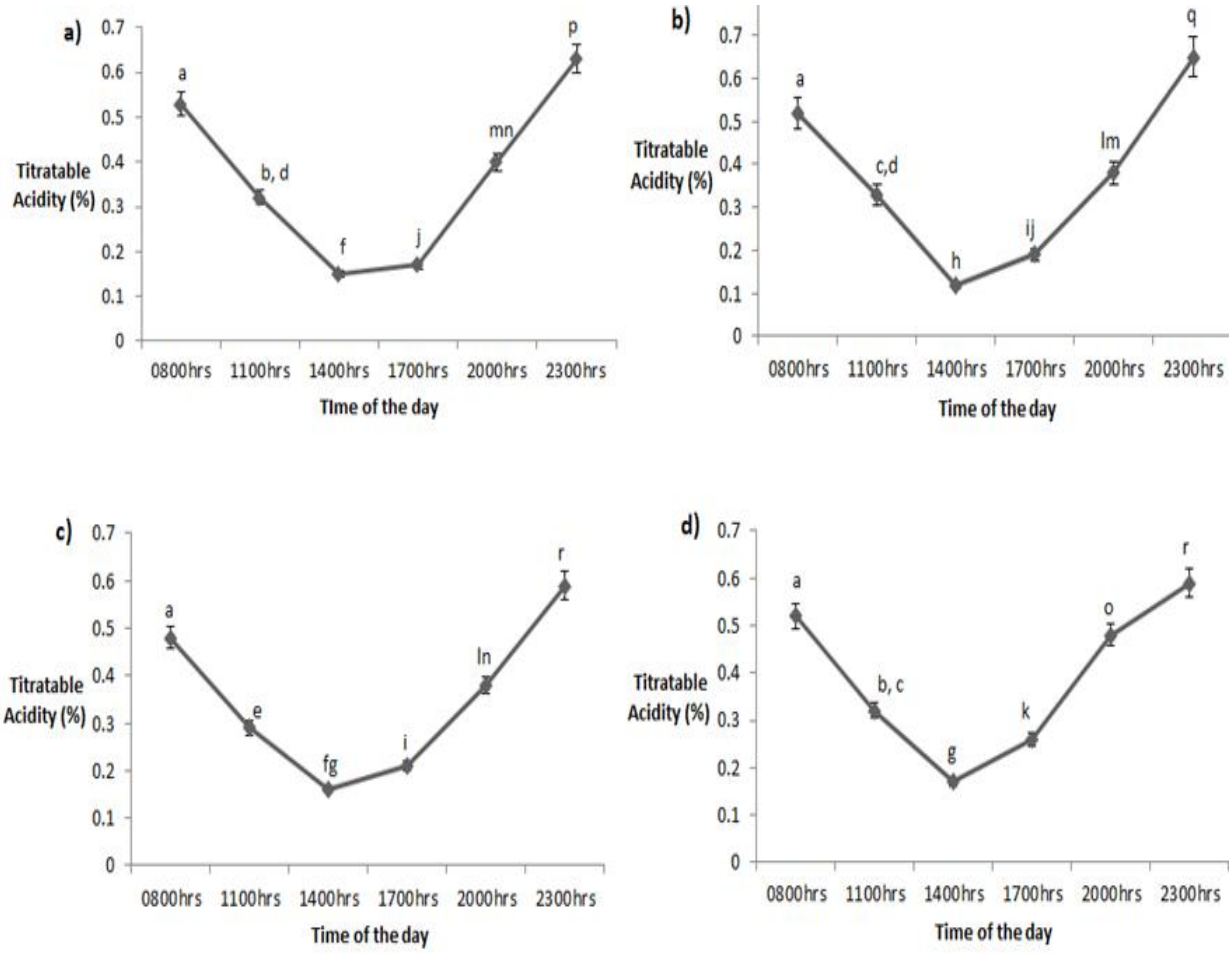


Figure 3.5 Titratable acidity fluctuations in a) *O. stricta*; b) *O. humifusa*; c) *O. engelmannii* - Limpopo lineage; d) *O. engelmannii* – Kenyan lineage. Data is based on 5 replicates of 7 samples of each *Opuntia* per sample period. Bars (mean± SE) mean annotated with the same letters in the same sampling period do not differ significantly (P > 0.05).

3.5.4 Tannins

Tannin levels in the *Opuntia* hosts were significantly different from each other (F = 4208; df = 3; P < 0.05) and were in the following rank order: *O. engelmannii*-Limpopo lineage > *O. engelmannii*-Kenyan lineage > *O. humifusa* > *O. stricta* (Table 3.3).

Table 3.3 Different tannin concentrations of the *Opuntia* hosts. Data are shown as mean ± SD. Values given show the means of 5 determinations. Means with identical letters do not differ significantly according to Tukey HSD t-test (P > 0.05).

***Opuntia* hosts**	**Tannins (mg Tannic acid/g)**
O. stricta	3.1 ± 0.15^{a}
O. humifusa	3.3 ± 0.05^{b}
O. engelmannii-Limpopo lineage	8.8 ± 0.11^{c}
O. engelmannii-Kenya lineage	8.4 ± 0.08^{d}

3.6 Discussion

The Opuntias under study did not exhibit any significant differences in cuticle, epidermis and hypodermis thickness. There was also no significant difference in crude fiber content of the cladodes between the *Opuntia* hosts. This shows that the utilizability of these *Opuntias* as hosts of the 'stricta' biotype of *D. opuntiae* is not affected by cuticle, epidermis and hypodermis thickness. The results suggest that the different phytochemical aspects of the *Opuntias* may be responsible for the varying degrees of acceptance shown by the 'stricta' biotype of *D. opuntiae* on the different *Opuntia* species. The most acceptable hosts of the 'stricta' biotype of *D. opuntiae*, *O. stricta* and *O. humifusa*, had low acidity in their organic acids compared to alkaline organic acids found in *O. engelmannii* lineages. It is known that green-leaf tissue pH is an important predictor of palatability to insect herbivores (Harguindeguy et al. 2013).

The difference in pH of the organic acids between the most acceptable hosts and least acceptable hosts suggest that pH is important in the palatability and suitability of the Opuntias as hosts of the 'stricta' biotype of *D. opuntiae*. It appears as if this cochineal insect prefers a low acidic environment to an alkaline environment so that it can effectively extract liquid contents of the host. Betancourt-Domínguez (2006) reported Opuntias' pH values from 4.05 to 4.55; the values in this study were higher than this range. Sap-sucking insects such as

D. opuntiae are primary consumers of phloem but it is difficult to get to this phloem sap as it is protected by plant defences such alkaline organic acids, especially in *O. engelmannii* lineages. So it is possible that the metabolic costs of processing phloem sap in an alkaline environment might be very high for the 'stricta' biotype of *D. opuntiae*.

There were differences in the maximum amount of organic acids between the acceptable hosts and least acceptable hosts, *O. engelmannii* lineages. The differences in the maximum amount of organic acids in these Opuntias may be caused by genotypic factors inherent to these *Opuntia* species. Generally, *Opuntia* cladodes have several organic acids such as malic, oxalic, citric, malonic, succinic, tartaric and pscidic (Jianqin et al. 2002). So, since titratable acidity is a parameter that estimates the amount of organic acids it is possible that the acceptable hosts might have organic acids that are needed by the 'stricta' biotype of *D. opuntiae* in high quantities compared to the less acceptable hosts.

The maximum amount of organic acids results are in agreement with Hernández-González and Villarreal (2007) who reported that species of cacti can differ in the maximum amount of organic acids that they can accumulate. Their results varied from 0.28 to 0.71 % and the results here varied from 0.18 to 0.67%. Variability between four different Opuntias has also been reported in the content of organic acids by Betancourt-Domínguez (2006); the organic acids were between 0.12 to 0.87%. This variability reported by Betancourt-Domínguez (2006) can result in different acceptability by *D. opuntiae* on the different Opuntias. The fluctuation in the organic acids of the Opuntias studied corresponds to the proposed definition for CAM which is the uptake of CO_2 at night through open stomata and the storage of malic acid in the vacuoles. Fluctuation of organic acids is denoted by an increase of the organic acids at night and a decrease during the day (Roberts et al. 1997; Lüttge, 2002, Lüttge, 2004).

The tannin tests showed that less acceptable hosts, *O. engelmannii* lineages had higher levels of tannins than the acceptable hosts. There is a known association of increased tannin content of leaves with increased physical strength (Bernays, 1981), so this physical strength of the cladodes' surfaces may also be affecting the sucking ability of the 'stricta' biotype of *D. opuntiae* on the *O. engelmannii* lineages. Tannins, like any other chemicals in the host plant, may be perceived by *D. opuntiae* through its peripheral chemoreceptors and it may either accept or reject the host; this may be adverse for or favourable to *D. opuntiae*.

The negative effect of tannins on the reproductive performance of the 'stricta' biotype of *D. opuntiae* shown in this study mirror a study by Todd et al (1971), in this study the sap-sucking insect, the greenbug *Schizaphis graminum* L had poor reproductive performance on barley as a result of tannin toxicity. These phytochemical results could have implications for the way agents are selected for the biological control of cactus or other weeds.

It should be noted that acceptance or rejection of the plant by an insect is influenced by many factors which can be intrinsic to the particular host. For example, the *O. engelmannii* lineages that have been shown to be least acceptable hosts of the 'stricta' biotype of *D. opuntiae* could be synthesising and releasing a lot of complex mixtures of volatile organic compounds which act as repellents to the cochineal. Although there are known problems with the methodologies in trying to quantify phytochemical aspects of the Opuntias, for example, quantification of tannins because of their heterogeneous nature, these quantitative differences in other phytochemicals between the *Opuntia* hosts play a pivotal part in the 'stricta' biotype of *D. opuntiae*'s reproductive performance thus affecting its impact as a biological control agent.

Chapter 4

The physiological impacts of *Dactylopius opuntiae* 'stricta' biotype on *Opuntia stricta* and *Opuntia humifusa*

4.1 Introduction

Biological control agents can kill or injure their host plant directly, or can weaken the plant so that it is less competitive in its environment (Klein, 2011). Prediction and assessment of the effectiveness of a candidate biological control agent is important. Assessment of the effectiveness is done to ascertain the ability of a biological control agent to significantly damage the host plants (Conrad and Dhileepan, 2007). In the physiology of plants, photosynthesis is the key function and its functional state is considered an important physiological activity when assessing the health of a plant. Herbivorous insects can affect many aspects of plant performance and knowledge of the key physiological factors which are negatively impacted by a biological control agent can help us determine if that biological control agent will have a great impact on its host plant. Biochemical and physiological changes in host plants are induced by the feeding of herbivorous insects and this affects processes such as photosynthesis (Gomez et al. 2004).

Plants offer various physiological parameters that can be used in different fields of plant science to study the effects of abiotic and biotic stresses on the growth of plants. Many quantitative assessments of the effectiveness of a biological control have been done (Conrad and Dhileepan, 2007; Marlin et al. 2013; Venter et al. 2013; Cowie et al. 2016). The following parameters have been measured in biological control to assess insect efficacy: root length, shoot length, number of leaves, seed production, carbon dioxide exchange, leaf chlorophyll concentration and leaf chlorophyll fluorescence. Analysis of chlorophyll concentration, chlorophyll fluorescence and carbon dioxide exchange by Cowie et al. (2016) showed that the biological control agent, *Gargaphia decoris* (Hemiptera: Tingidae), caused metabolic impairment which decreased photosynthetic rates of *Solanum mauritianum* Scop. (Solanaceae). It took 18 days for the metabolic impairment to cause leaf senescence.

Conrad and Dhileepan (2007) evaluated the effectiveness of *Carvalhotingis visenda* (Heteroptera: Tingidae), a leaf-sucking bug, on *Macfadyena unguis-cati* (Bignoniaceae) by assessing shoot length, root length and leaf chlorophyll concentration. The number of *C. visenda* per plant significantly increased, reducing chlorophyll by 60 - 90% over a period of 6 weeks. The overall results suggested that this leaf-sucking bug can significantly decrease chlorophyll concentration, leading to decrease in leaf biomass and the height of plant. This research represents an initial effort to describe the effect of a biological control agent on chlorophyll loss and photosynthetic performance in *Opuntias*.

4.2 Photosynthesis and pierce-sucking insects

The damage that is caused to plants by insects with piercing-sucking mouthparts is usually less evident than the damaged caused by insects with chewing mouthparts (Meyer, 1993). The extent of the damage caused by pierce-sucking insects can vary greatly as they can feed on xylem sap, phloem sap or other plant cells (Walters, 2015). The feeding on sap, for example by cochineal insects, is thought to cause greater physiological change such as photosynthetic stresses close to where the insect is feeding than to mechanical leaf feeding (Meyer, 1993). This is because sap feeding results in the removal of leaf compounds such as photosynthates, chlorophyll and water which strain the plant when it replaces these compounds in response to sap feeding (Gonda-King et al. 2014). Sap feeding insects are usually associated with decreases in chlorophyll content and rates of photosynthesis (Bondada et al. 1995; Cabrera et al. 1994; Schaffer and Mason, 1990; Cowie et al. 2016). These biochemical and physiological changes in response to insect feeding can be useful in investigating the resistance mechanisms of invasive plants.

4.3 Chlorophyll concentration

Chlorophyll concentrations differ with species, and this difference can be as a result of various kinds of stresses on the plant (Gitelson and Merzylak, 1997; Witkowski et al. 2009). However, it is generally expected that healthy plants have higher chlorophyll content than plants having some kind of stress such drought. When chlorophyll levels change as a result of different stresses, for example insect feeding, there is a possibility that these changes can help in investigating plant's resistance mechanisms to insect herbivory. To quantify chlorophyll

content, two ways have been embraced: chlorophyll meters and conventional chemical methods of measuring the chlorophyll level. Conventional chemical methods involve destructive sampling and take a lot of time during the laboratory analyses, whilst chlorophyll meters are simple and can be used in the field without destroying anything. Successful use of chlorophyll meters to estimate the concentration of chlorophyll in many plant species such *Eucalyptus nitens* is known (Netto et al. 2005; Pinkard et al. 2006). To observe the chlorophyll content in a sample when using the portable meter, two reflective wavelengths, 650 nm and 940 nm are used. Chlorophyll content decreases in stressed vegetation leading to the decrease in the absorption of light (Zarco-Tejada et al. 2000).

4.4 Chlorophyll fluorescence

Evaluation of the health of internal apparatus by analysing chlorophyll fluorescence parameters in the leaf is important (Clark et al. 2000), as this a quick and reliable way to detect and measure the tolerance of plants to stress (Li et al. 2006) such as feeding by biological control agents. Changes in chlorophyll fluorescence may take place before any physical signs of deterioration are seen in the plant, so stress can be identified before the start of physical damage (Lichtenthale et al. 2007). The primary photochemistry of photosynthesis is carried out by two functional and structural units of protein complexes: photosystem I (PSI) and photosystem II (PSII). These functional levels of photosynthesis can be studied by using chlorophyll fluorescence. Chlorophyll fluorescence is the re-emitted red/ far red light by chlorophyll molecules (Kalaji and Guo, 2008). The light energy absorbed by chlorophyll molecules goes through one of three fates: the energy can be used to drive photosynthesis; surplus energy can be dissipated as heat or be re-emitted as light: chlorophyll fluorescence. These processes compete with each other so an increase in one will lead to a decrease in the yield of the other two. So the yield of chlorophyll fluorescence emission can give us information on the modification in productivity of photosynthesis.

The changes in chlorophyll fluorescence yield were first detected as early as 1960 by Kautsky and colleagues (Kautsky et al. 1960). They discovered that transferring a photosynthetic apparatus from the dark into light causes increase in chlorophyll fluorescence yield within 1 second. This rise is caused by the decrease in electron acceptors, plastoquinone (Q_A), downstream of PS II (Maxwell and Johnson, 2000). When PS II absorbs light and acceptance of an electron by Q_A has been done, there will not be acceptance of another electron until Q_A

has passed the first electron onto the next carrier. During this phase, the reaction centre is known as ‘closed’. There is an overall decrease in productivity of the photochemistry and a corresponding increase in fluorescence yield due to a number of closed reaction centres (Maxwell and Johnson, 2000).

There is progressive closing of photosystem II reaction centres when a leaf is moved from darkness into light, and this leads to an increase in the production of chlorophyll fluorescence (Maxwell and Johnson, 2000). After this, however, the fluorescence level starts to decrease again and this phenomenon is known as fluorescence quenching. Fluorescence quenching can be explained in two conditions. Firstly, the rate of electron transportation away from PS II increases because of the light-incited activation of enzymes that play a role in metabolism of carbon and the opening of stomata. This quenching is known as ‘photochemical quenching’. Simultaneously, the efficiency of energy conversion to heat increases and this is known as ‘non-photochemical quenching’ (NPQ). These changes in the two processes will end after approximately 20 minutes and a steady-state is achieved, although this time to reach the steady-state can differ significantly between species of plants (Maxwell and John, 2000).

It is necessary to differentiate between the non-photochemical and photochemical contributions to quenching, so that information on the photosynthetic performance from chlorophyll fluorescence yield measurements is gained. Chlorophyll fluorescence induction can be measured by a continuous excitation fluorimeter (see fig 4.1 below). Shielding of the sample from ambient light is necessary to block the red/far red part of daylight from influencing the measurements. To avoid this, the sample could be dark adapted in a dark room with low light. Thorough dark adaptation is required to get the Fo parameter, which is the emission by excited chlorophyll a molecules in PS II (Kalaji and Guo, 2008). The maximum fluorescence parameter (Fp or Fm) is recorded when the light is fully saturating for the sample and there is full reduction of the electron acceptor Q_A. The variable part of the recording which is related to the highest potential for photochemical quenching is called the Fv parameter (Fv = Fm - Fo). The best explanation for the calculation of these parameters is probably illustrated in Figure 4.1.

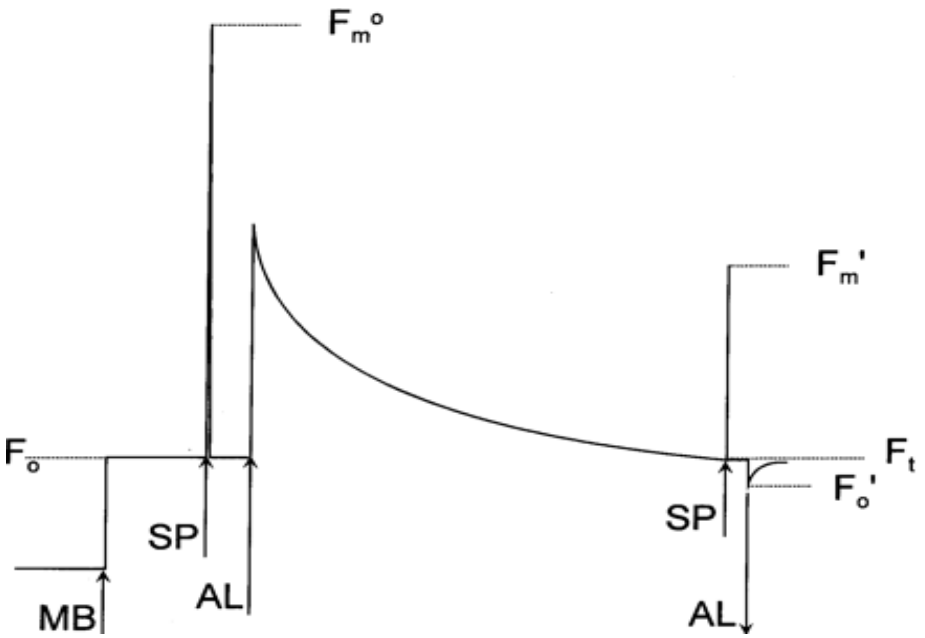


Figure 4.1 Typical fluorescence trace sequence (Maxwell and Johnson, 2000). Switching on of measuring light (↑MB) and measurement of the zero fluorescence level is done (F_o). Saturating flash of light is applied (↑SP) to measure the maximum fluorescence level Fm ^{o}. Light (↑AL) to drive photosythesis is then introduced and after some time another saturating light flash(↑SP) allows measurement of the maximum fluorescence in the light (F_m^i). F_t is the level of fluorescence just before the saturation flash. Zero level of fluorescence 'in the light' is estimated by turning off the actinic light (AL).

The ratio of variable to maximal fluorescence (Fv/Fm), when assessed in the dark adapted state represents the potential quantum yield of PS II. Bilger et al. (1995) stated that this ratio can be used to assess the decrease of PS II activity due to acute stress and if this ratio is below 0.83 it shows that there is negative impact on photochemistry. Reduction of Fv/Fm under natural conditions (Figure 4.2) is a result of photo-inhibition caused by photosynthetic photon flux density (PPFD) in excess (Long et al. 1994). Many Opuntias are known to show high levels of energy dissipation when exposed to direct sunlight in the afternoon compared to the morning when the same cladode surfaces are getting matching levels of light (Adams et al. 1989). This response has been attributed to differences in the speed of photosynthetic electron transport impacted by differences in the quantity of CO_2 that is generated internally (Adams and Demmig-Adams, 1996). The movement of electrons through PS II represents, under various conditions, the overall rate of photosynthesis (Maxwell and Johnson, 2000). It is also known that disturbance to PS II is often the first sign of stress in a leaf. Fluorescence gives insight into the capability of a plant to withstand stresses from the environment and into

the extent of damage done to the photosynthetic apparatus by those stresses. Baker and Adams (1997) made studies on *Opuntia macrorhiza* that offer a very good example of using chlorophyll fluorescence for assessing photochemical efficiency. An investigation was done of the photochemical efficiency in cladodes oriented differently which received different PFD and temperature during the day. All cladodes showed a midday decrease in NPQ coincident with malic acid decarboxylation.

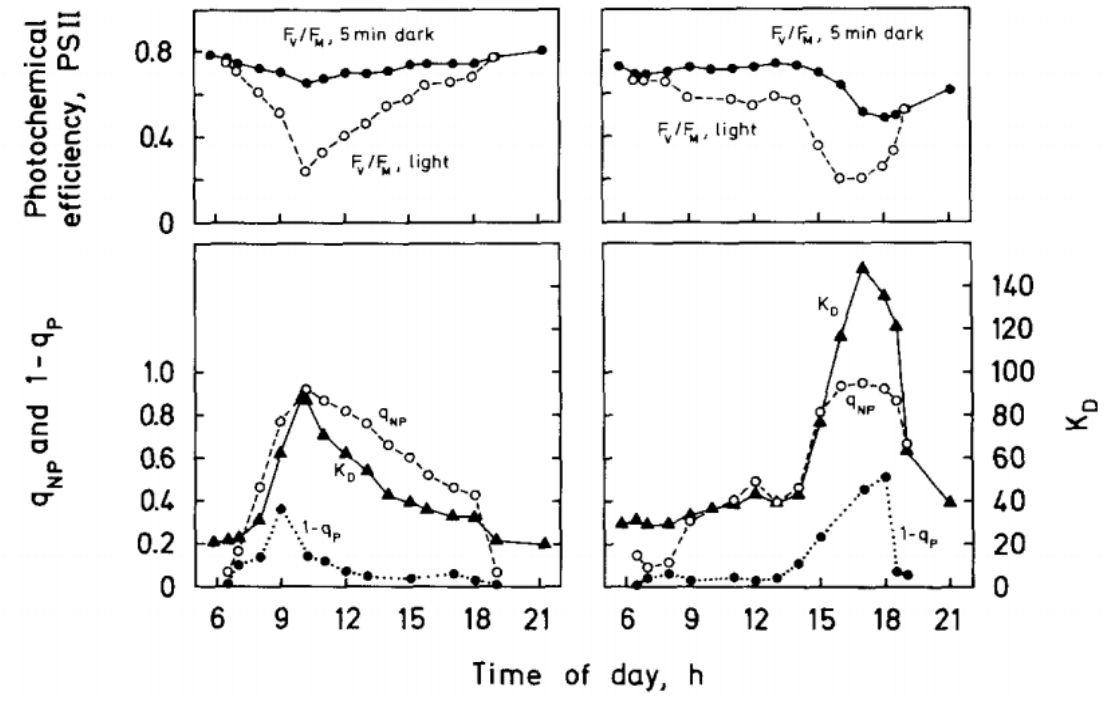


Figure 4.2 Diurnal measurements of photochemical efficiency (Fv/ F_M), the reduction state of Q (1-qp), the rate constant for radiationless energy dissipation (K_D), and non-photochemical fluorescence quenching (q~p) for a cladode of *O. ficus-indica* on 2 consecutive days (Adams et al. 1989).

Chlorophyll fluorescence is a non-destructive tool that was used in this research to study the physiological state of the host plants. When chlorophyll fluorescence is combined with gas measurements it gives a full picture of the plants' response to the environment. Unfortunately, many attempts were made to assess the CO_2 exchange without success. This chapter represents an initial effort to describe the effect of *D. opuntiae* 'stricta' biotype on plant pigment loss and photosynthetic performance changes in the host species. This chapter deals with one of the most important aspects of assessing the effectiveness of a biocontrol agent, namely, understanding the mechanism by which damage to the plant is caused by the

biological control agent and the response of the plant to the damage. The aim was to assess the impact of the 'stricta' biotype of *D. opuntiae* on the chlorophyll concentration and chlorophyll fluorescence of *O. stricta* and *O. humifusa*.

4.5 Materials and methods

4.5.1 Plant material

Potted plants of each species (*O. stricta* and *O. humifusa*), which supported the development of an average of at least four insects to maturity during the reproduction and development trials, were placed in the glasshouse part of the insectary at Wits University. These potted plants had been grown for over two years. The potted plants were acclimatized to the glasshouse part of the insectary for six weeks. Seven plants of each species were made controls (no *D. opuntiae*) and seven plants of each species as treatments (those that will have *D. opuntiae*).

4.5.2 Insects

The 'stricta' biotype of *D. opuntiae* females were obtained from cultures that were maintained on *O. stricta* in the same facility. Mature females were harvested from the "stricta" biotype rearing populations and removal of the wax was done by rolling the wax coating onto a pin off the insect so that it was easy to harvest crawlers from the females (Mathenge et al. 2010). 30 crawlers were placed in each potted plant (treatments) and a fecund female with her related large egg cluster (age unknown) were placed at the base of the bottom cladode of a healthy potted plant of each of the test species.

4.5.3 Feeding impact of the 'stricta' biotype of *D. opuntiae* on chlorophyll concentration

Chlorophyll concentration of the cladodes (infested and uninfested as control) was assessed with a portable CCM-300 meter (Opti-sciences, Inc. USA). This meter has a light source and two solid state detectors, one sensitive to infrared radiation and the other to red light. This enables the measurement of relative yield of fluorescence when there is background illumination. Assessment of the chlorophyll content was done at different developmental stages of the crawlers: settling of crawlers, first moult and second moult.

Four readings (four points 30 mm from the settled crawlers) on the cladode were averaged for each cladode and were regarded as one observation. The results are the average measurements for seven cladodes on seven plants of each species that are experiencing chlorosis. The experiment was stopped after 100 days when the treatment cladodes were senescing.

4.5.4 Impact of the 'stricta' biotype of *D. opuntiae* on chlorophyll fluorescence

Chlorophyll fluorescence was determined by using a OS1p Modulated Chlorophyll Fluorometer (OptiSciences, Inc. NH 03051 USA). Before the measurements, dark adapting of the cladodes was done for 24 hrs by layers of black plastics. The following parameters were measured: the initial fluorescence (F_0), maximum fluorescence (Fm) and quantum efficiency of open photosystem II centres-quantum yield (Fv/Fm). Measurements were done every 3 hours from 0600hrs – 2100 hrs. Low intensity modulated light was used to obtain F_0 so as to not induce any effect in the fluorescence variable (Kalaji and Guo, 2008). Fluorescence was measured during two consecutive days.

4.6 Data Analysis

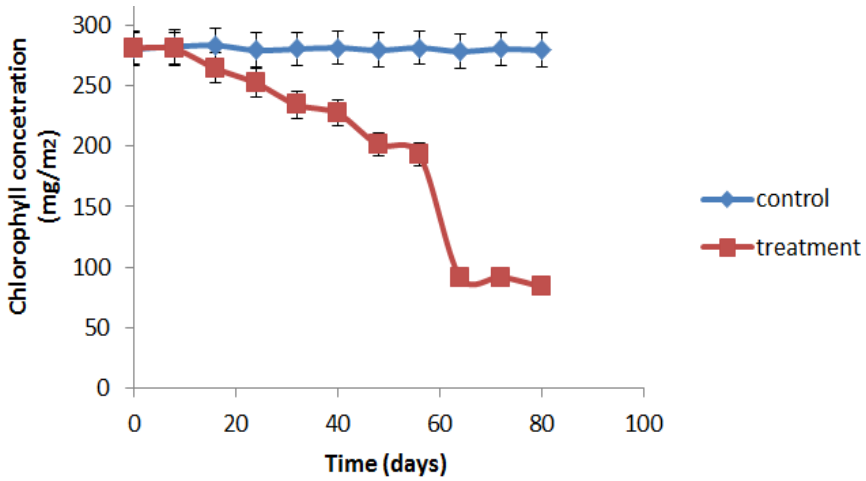
The comparisons of chlorophyll concentration (CCM-300 meter units) between treatment plants with *D. opuntiae* and control plants without *D. opuntiae* were subjected to unpaired and two-tailed student's t-test. One-way repeated measures ANOVA in R statistical package with Tukey's simultaneous test were used to compare the changes (in plants colonised by insects and those that are insect free) in the assessed physiological effects in the hosts.

4.7 Results

4.7.1 Feeding impact of the 'stricta' biotype of *D. opuntiae* on chlorophyll concentration

There was a significant decrease in the chlorophyll concentration of the treatment plants of *O. stricta* and *O. humifusa* over time compared to their controls (Figure 4.3). No significant difference in the decrease of chlorophyll concentration between the treatment plants of *O. stricta* and *O. humifusa* ($P = 0.06$).

a)



b)

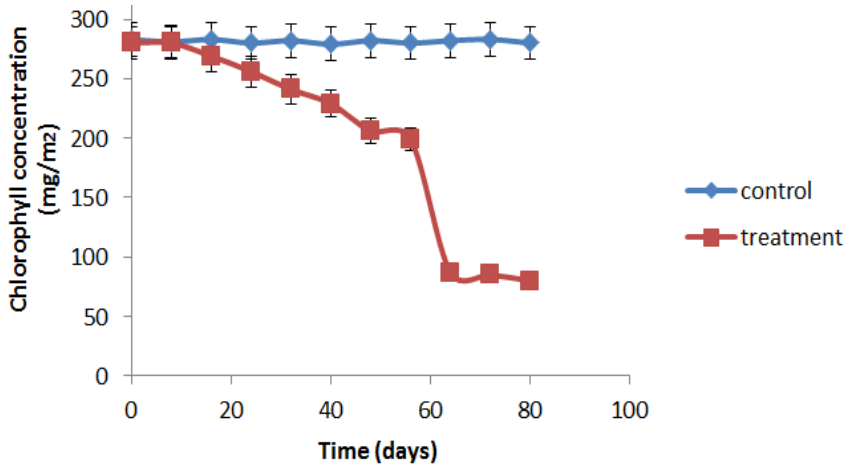


Figure 4.3 Changes in chloropyll concentration with cochineal (treatment) and without cochineal in a) *O. stricta* b) *O. humifusa* over a period of 80 days. For each data point (mean±SE) n = 6 plants. a) *O. stricta*- $F_{3.30}$ = 25.8; P = 0.0001 b) *O. humifusa*- $F_{3.30}$ = 27.2; P = 0.0001.

4.7.2 Impact of the 'stricta' biotype of *D. opuntiae* on chlorophyll fluorescence

There was a significant decline in photochemical efficiency (F_V/F_M) of treatment plants of *O. stricta* and *O. humifusa* over time compared to their controls during the first moult (Figure 4.4). The overall differences for the control and treatments for *O. stricta*: $F_{3.30}$ = 7.3; P = 0.0039; for *O. humifusa*: $F_{3.30}$ = 8.1; P = 0.0041.

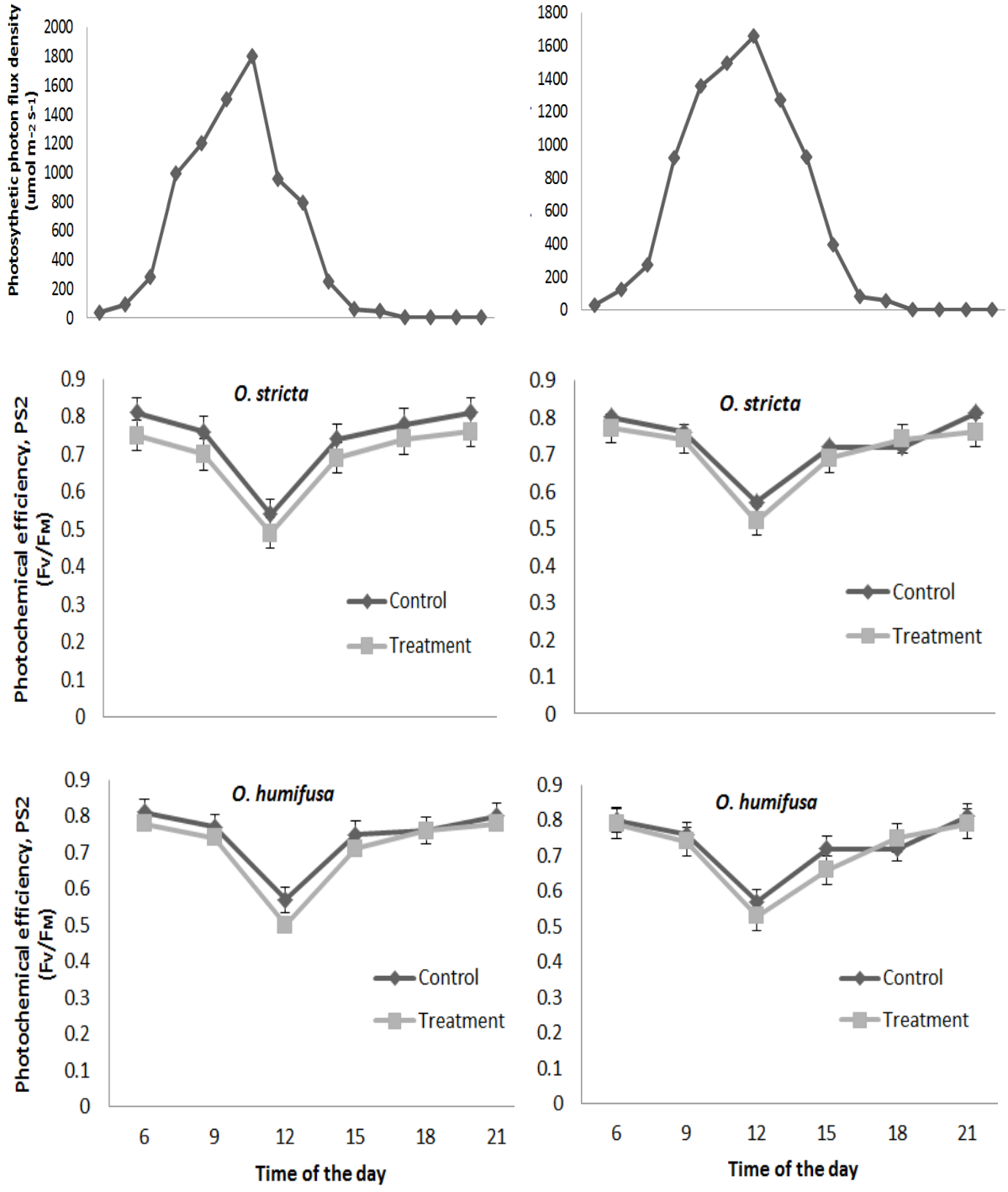


Figure 4.4 Natural light (Photosythetic photon flux density) and PS II maximum quantum efficiency (F_V/F_M) for two consecutive days (first day on the left and second day on the right) with and without 'stricta' biotype of *D. opuntiae* herbivory at first moult (18 days after placing the insects). For each data point (mean±SE) n = 6 plants.

The photochemical efficiency of *O. stricta* and *O. humifusa* decreased significantly over time during the second moult (Figure 4.5). The overall differences for the control and treatments for *O. stricta*: $F_{3.30} = 5.8$; P = 0.0021; for *O. humifusa*: $F_{3.30} = 6.2$; P = 0.0019.

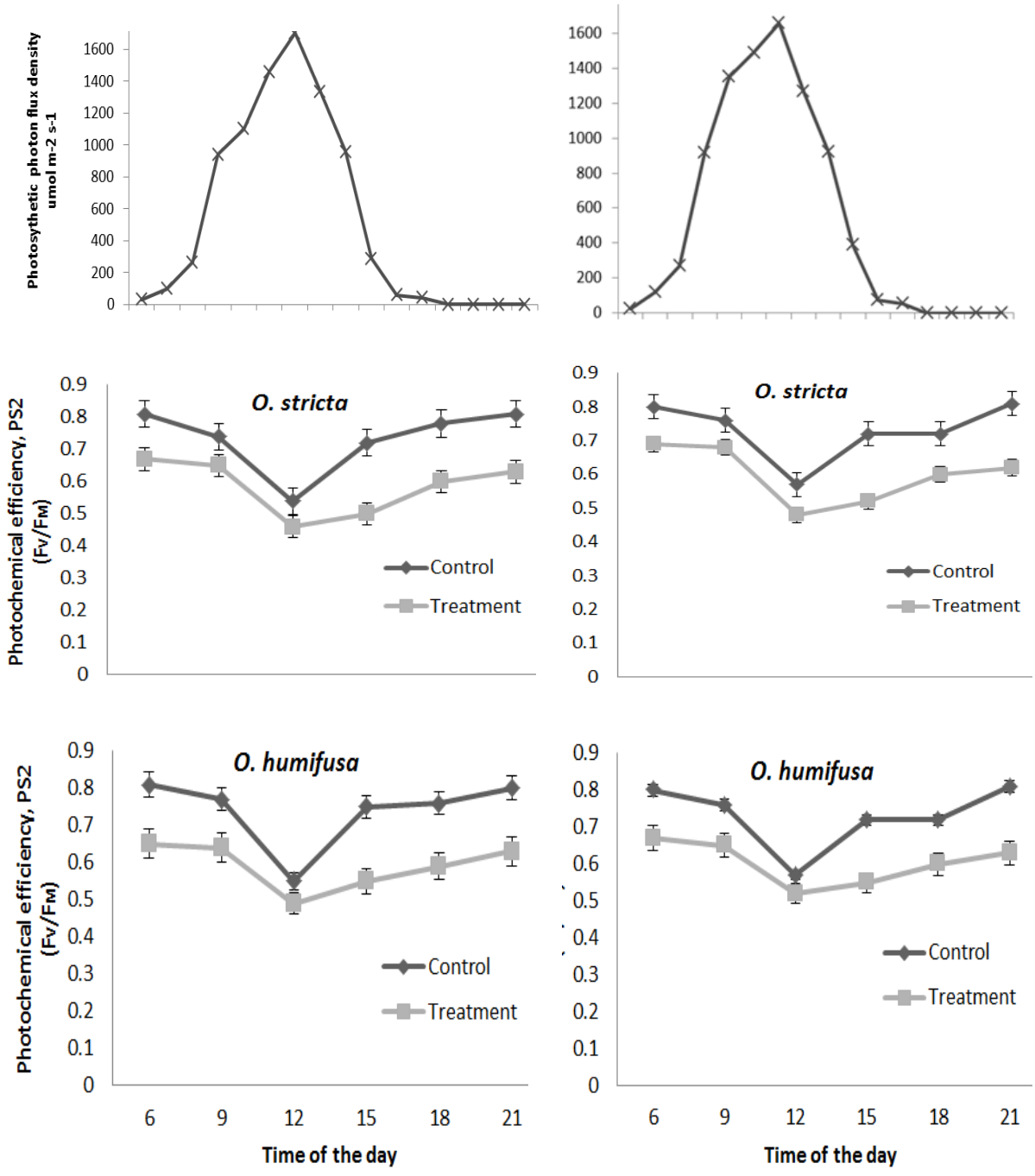


Figure 4.5 Natural light (Photosythetic photon flux density) and PS II maximum quantum efficiency (F_V/F_M) for two consecutive days (first day on the left and second day on the right) with and without 'stricta' biotype of *D. opuntiae* herbivory at second moult (45 days after placing the insects). For each data point (mean±SE) n = 6 plants.

4.8 Discussion

The results show the degree to which the 'stricta' biotype of *D. opuntiae* impacts the physiological variables of the Opuntias and if these impacts can account for the successful control of both *O. stricta and O. humifusa*. The results clearly showed that the feeding of the 'stricta' biotype of *D. opuntiae* can negatively impact the chlorophyll concentration as well as the maximum quantum efficiency of photosystem II consequently reducing photosynthesis of *O. stricta* and *O. humifusa* host plants. Overall photosynthesis in *O. stricta* and *O. humifusa* plants was significantly reduced as there was removal of chlorophyll and this limits the photosynthetic rate of damaged leaves. Nagaraj et al. (2002) showed a similar pattern in the reduction of photosynthetic rate in *Sorghum bicolor* (L.) Moench damaged by a sap-sucking insect, *Schizaphis graminum* (Rondani). Cowie et al. (2016) also showed that the biological control agent, *Gargaphia decoris* (Hemiptera: Tingidae), caused metabolic impairment which decreased photosynthetic rates of *Solanum mauritianum* Scop. (Solanaceae).

Herbivory by the 'stricta' biotype of *D. opuntiae* directly and negatively impacted the efficiency of photosystem II (PS II). This was shown by the decrease in photochemical efficiency (Fv/Fm) below 0.83, a ratio below which indicates a negative impact on photochemistry (Baker, 2008). The decrease in photosynthesis is evidenced by the decline in the photochemical efficiency of the PS II. Chlorophyll concentration and photochemical efficiency of PS II in *O. stricta* and *O. humifusa* plants decreased at increasing rates as the nymphs of the 'stricta' biotype of *D. opuntiae* developed. The decrease in chlorophyll content and photochemical efficiency of PS II was more profound after the second moult of the 'stricta' biotype of *D. opuntiae* probably due to the insects sucking more sap in preparation to lay their first eggs.

Many studies have shown that sap-sucking insects cause significant feed-induced leaf chlorosis which results in reduced photochemical efficiency (Buntin et al. 1993; Gomez et al. 2004; Ripley et al. 2006; Conrad and Dhileepan, 2007; Marlin, 2013; Cowie et al. 2016). The cell content-feeding spider mites were reported to have dehydrated the spongy mesophyll leading to closure of stomata, which, in turn, would have decreased photosynthesis and alter primary metabolism (Bondada et al. 1995). According to Conrad and Dhileepan (2007) the number of the leaf-sucking bugs, *C. visenda*, per plant significantly increased reducing the chlorophyll by 60 - 90% over a period of 6 weeks. The results of this study showed that after

a period of 6 weeks the ‘stricta’ biotype of *D. opuntiae* had reduced the chlorophyll by 40 – 50%. Conrad and Dhileepan (2007) used five males and five females of *C. visenda* per plant at the start of the experiment whereas in this study only one female of *D. opuntiae* was used. It is also known that the effects of insect feeding usually vary by plant species (Golan et al. 2015) and chlorophyll concentration differs with plant species and environment.

It should be noted that the decline in photochemical efficiency (Fv/Fm) of PS II cannot be attributed solely to herbivorous feeding, as the photochemical efficiency of the control plants decreased during the day. This is as a result of photo-inhibition caused by photosynthetic photon flux density (PPFD) in excess (Long et al. 1994) or could be as a result of non-photochemical quenching processes (Murchie and Lawson, 2013). It is also known that many cacti exhibit energy dissipation levels that are high when exposed to direct sunlight in the afternoon (Adams et al. 1989). Increase in photosynthesis and radiationless dissipation of energy as a result of an increase in PFD was enough to keep the primary acceptor of electrons in PS II in a reduced state in the morning. However, this was not the case in the afternoon when malic acid was used up. Furthermore, herbivory stress caused even more high levels of energy dissipation in the afternoon presumably as a result of decrease in photosynthetic activity. In conclusion, the ‘stricta’ biotype of *D. opuntiae* was documented to decrease the photosynthetic output of *O. humifusa* plants subsequently decreasing the plants’ resistance to other environmental stresses.

Chapter 5

5.1 General discussion and conclusion

This research explored the insect-host interactions between the "stricta" biotype of *D. opuntiae*, and four *Opuntias*: *O. stricta*, *O. humifusa*, *O. engelmannii*- Limpopo lineage and *O. engelmannii*- Kenyan lineage in order to investigate the basis of host selection in this cochineal insect. The response of the 'stricta' biotype of *D. opuntiae* on the *Opuntia* hosts can be summarised into two categories: (i) flourishing on their host- the insects settle swiftly, develop quickly and have high fecundity; (ii) dying on the host- failure to settle by most of the insects and none become fecund. There was correlation of host suitability with the speed of development of the 'stricta' biotype of *D. opuntiae*. The host plant species on which the 'stricta' biotype of *D. opuntiae* developed to the adult stage quickly were regarded as the most suitable hosts. The 'stricta' biotype had high acceptability on *O. stricta* and *O. humifusa* proving to be the most suitable hosts of this cochineal. The 'stricta' biotype showed low acceptability on both *O. engelmannii* lineages and therefore poor hosts for the 'stricta' biotype of *D. opuntiae*.

The 'stricta' biotype of *D. opuntiae* reduced the photosynthetic performance of *O. humifusa* showing that it has a great potential to be an effective biological control agent of *O. humifusa*. The results clearly showed that the feeding of the 'stricta' biotype of *D. opuntiae* can negatively impact the chlorophyll concentration as well as the maximum quantum efficiency of photosystem II consequently reducing photosynthesis and vigour of *O. stricta* and *O. humifusa* host plants. The photochemical efficiency results are consistent with previous research (Conrad and Dhileepan, 2007; Marlin, 2013; Cowie et al. 2016) which have shown that herbivorous insects reduce photochemical efficiency of the hosts.

Chapter 3 of this research explored the primary metabolites and secondary metabolites of the Opuntias. The results showed variability in tannins and pH of the organic acids found in the cladodes of the Opuntias. There was low acidity in the organic acids of the most acceptable hosts, *O. stricta* and *O. humifusa*, of the 'stricta' biotype of *D. opuntiae*. The *O. engelmannii* lineages which are the least acceptable hosts had alkaline cladode tissues. The pH of the host is known to play an important role in the palatability of the host (Harguindeguy et al., 2013). These pH results are in agreement with other previous studies that show that the acceptability

and suitability of a host can be affected by the pH of the host. According to Schultz and Lechowicz (1986), the diet pH and time since last feeding have an impact on the larvae midgut pH of late instar gypsy moth, *Lymantria dispar* (Erebidae: Lymantria).

The results of this study showed that these Opuntias have different amounts of tannins. This information is consistent with information in other studies. Clausen et al. (1999) showed that it is possible for congeneric species to have different amounts of tannins. There was a clear negative correlation between the amount of tannins in the Opuntias and the acceptability of the 'stricta' biotype of *D. opuntiae*. The least acceptable hosts of the 'stricta' biotype of *D. opuntiae* had higher quantities of tannins compared to the most acceptable hosts. This suggests that tannins may be responsible for acceptability of the different *Opuntia* species to the 'stricta' biotype of *D. opuntiae* and thus affect its efficacy as a biocontrol agent. The high quantities of tannins in the least acceptable hosts also suggest that tannins may be negatively affecting the 'stricta' biotype of *D. opuntiae*. This suggestion is broadly in line with those of other researchers for instance, Todd et al. (1971) reported that the sap-sucking insect, the greenbug *Schizaphis graminum* L grew poorly and eventually died as a result of the toxicity of tannins in the barley it was feeding on. The pH and tannins in the Opuntias have a major role in host selection in the 'stricta' biotype of *D. opuntiae*.

Barbenhenn et al. (2003) reported that insects may differ in the way they respond to tannins as a result of their level of adaptation to polyphenolics. The activity of phenolics requires oxidation which is affected by many abiotic and biotic factors (Apel, 1993). Phenolics are easily oxidized by oxidants and by enzymes found in leaves and the digestive tracts of herbivores (Felton and Duffey, 1991). So it is possible that these *Opuntia* species oxidize tannins differently and the 'stricta' biotype of *D. opuntiae* is adapted to the oxidation state of tannins found in *O. stricta* and *O. humifusa*. The most commonly accepted theories for adaptations to tannins include the alkaline gut pH of some insects (Appel and Schultz, 1992; Appel, 1993) and high levels of antioxidants (Barbenhenn et al. 2001, 2003), which maintains the tannins in a state where they cannot bind any proteins.

The resistance to the 'stricta' biotype of *D. opuntiae* feeding shown by the *O. engelmannii* lineages may arise from the failure of the *O. engelmannii* hosts to release the required stimuli for some elements of the feeding sequence or by having characteristics such as volatile organic compounds that negatively affect the feeding activities directly. Some of these

volatile organic compounds can act as direct defense by acting as repellents to the 'stricta' biotype of *D. opuntiae*. Only the proper association of the extrinsic releasing stimuli (characteristics of plant) and intrinsic threshold level of response (insect characteristics that can be modified physiologically) can lead to the natural behaviours of an insect, for example, feeding behaviour. Feeding behaviour is made up of the following steps: recognition and orientation of host pant; initiation of feeding; continuation of feeding; and cessation of feeding. So fundamental information on all the stages of feeding behaviour on a plant is needed.

A series of behavioural events are also involved in oviposition and different characteristics of the plants may play a part in the initiation and ending of each of these events (Thorsteinson, 1960). Other characteristics of plants such as alkaloids can hinder oviposition as there is failure to give the proper stimuli for releasing some of the oviposition behavioural components or by releasing stimuli that prevents oviposition behavioural release. In this study the *O. engelmannii* lineages' resistance to oviposition by the 'stricta' biotype of *D. opuntiae* could be as a result of *O. engelmannii* lineages failure to release the required stimuli for some stages of the oviposition sequence.

It is clear that behaviour and development of insects cannot be fully separated as they are dependent on each other. Assessment of the different insect performance traits at different life stages is a more integrative way of studying insect-host relationships. Host plant effects on herbivorous insects need to be comprehensively understood as changes to the performance traits that have an impact on behaviour, physiology or gene expression, for example; selective feeding and digestive metabolism can be crucial factors that cause the resistance or vulnerability of an insect to the defences of the host plant. Lastly, the identification of molecular processes that cause variations in the response of the insect provides a good platform for finding out which genes are targets of natural selection. There are many excellent questions on tannins in the interactions of plants and herbivores. For instance, the reasons why plants manufacture so many distinct structures of tannins are not known. A more focused question would be: ''what are the functional differences between different tannin structures as deterrents, antimicrobials and toxins?". There is need to explore the impacts of these different tannin types on different types of insects.

Plant resistance to insects is a complex subject since it is impossible to explain resistance basing on one straightforward characteristic of the plant. The various factors that have influence on insect-plant relationships prevent the formulation of all-inclusive generalizations that are meaningful. The comprehensive understanding of host-insect interactions can only be achieved via a multidisciplinary approach. The approach and findings of our study provide a framework for future studies on physiological studies on Opuntias and basis of host selection in the 'stricta' biotype of *D. opuntiae*. The results of this study will provide more comprehension of the effects of the 'stricta' biotype of *D. opuntiae* on *Opuntia* hosts. Understanding how invasive plants respond when attacked by biocontrol agents is beneficial in understanding the ecology of the invasive plant and its control. The conclusions and applicability of the knowledge gained from studying host-insect interactions cannot be overstated.

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Appendix 1

Table A1. Tertiary butyl alcohol (TBA) series

TBA	95% ethyl alcohol (ml)	Absolute ethyl alcohol (ml)	TBA (ml)	Distilled water (ml)	Paraffin oil/wax (ml)	Time (min)
1	50	0	10	40	0	40
2	50	0	25	30	0	40
3	50	0	35	15	0	40
4	50	0	50	0	0	40
5	0	25	75	0	0	60
6	0	0	50	0	50	60
7	0	0	10	0	90	Overnight

Appendix 2

Table A2. Staining with Safranin-Fast Green

1	Xylol 1	5 minutes
2	Xylol 2	5 minutes
3	Abs. ETOH	1 minute
4	95% ETOH	1 minute
5	70% ETOH	1 minute
6	50% ETOH	1 minute
7	1% safranin	15 minutes
8	50% ETOH	5 seconds
9	70% ETOH	30 seconds
10	95% ETOH	30 seconds
11	1% Fast green	15 seconds
12	95% ETOH	3 seconds
13	100% ETOH	10 seconds
14	Xylol 3	Dip