Host Specificity in South African Mistletoes

Desale Yosief Okubamichael

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PREFACE

The research presented in this thesis was carried out at the School of Life Sciences, University of KwaZulu-Natal (UKZN), Pietermaritzburg, South Africa, from 2009 to 2012, under the supervision of Prof. David Ward and co-supervision of Dr. Megan E. Griffiths.

This thesis, submitted for the degree of Doctor of Philosophy (PhD), is the product of the candidate's original work in substance, with due acknowledgment and credit given and cited in appropriate sections of the text and not having been submitted in whole or in part to be examined for any degree, nor is it being submitted in candidate for any other degree.

The thesis was written as papers and follows the format of the journal where each paper has been/will be submitted for publication, except the Introduction (Chapter 1) and Conclusions (Chapter 7). As a result the thesis does not follow a consistent format and tables and figures are at the end of each paper as they were/will be submitted to the respective journal.

…………………………..
Desale Y. Okubamichael (**Candidate**)
June 2013

I certify that the above statement is correct

DMWard

…………………………..
Professor David Ward (**Supervisor**)
June 2013

Megan E Griffiths Ward

…………………………..

Dr. Megan E. Griffiths (**Co-supervisor**)
June 2013

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DECLARATION 1 – PLAGIARISM

I, Desale Yosief Okubamichael, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

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DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis. At the time of submission, this thesis contains one published paper, one paper that has been provisionally accepted for publication and three papers submitted to peer-reviewed journals.

Author contributions:
DYO conceived all studies presented in the thesis, collected and analysed the data and wrote all the papers. MEG and DW contributed substantially to experimental design, data analysis and manuscript preparation.

Publication 1: Okubamichael DY, Griffiths ME and Ward D. Host specificity in mistletoes: a South African perspective. Submitted to *Annals of Botany*.

Publication 2: Okubamichael DY, Griffiths ME and Ward D. Host specificity, nutrient and water dynamics of the mistletoe *Viscum rotundifolium* and its potential host species in the Kalahari of South Africa. *Journal of Arid Environments* 75: 898–902.

Publication 3: Okubamichael DY, Griffiths ME and Ward D. Reciprocal transplant experiment suggests host specificity of a mistletoe *Agelanthus natalitius* in South Africa. Provisionally accepted for publication by *Journal of Tropical Ecology*.

Publication 4: Okubamichael DY, Griffiths ME and Ward D. Light and moisture improve initial seedling growth and survival of mistletoes. Submitted to *International Journal of Plant Sciences*.

Publication 5: Okubamichael DY, Griffiths ME and Ward D. Mistletoes control water loss through stomatal closure. Submitted to *Journal of Experimental Botany*.

Signed:………………………………………………
Desale Yosief Okubamichael
June 2013

CONFERENCE CONTRIBUTIONS

Poster: Okubamichael DY, Griffiths ME and Ward D. **Host specificity in a savanna mistletoe, *Viscum rotundifolium* (Viscaceae)**. 20–24 July 2009, 44th Annual Congress of the Grassland Society of Southern Africa (GSSA), UNISA Campus, Roodepoort.

Poster: Okubamichael DY, Griffiths ME and Ward D. **Rooipoort mistletoes on the De Beers diamond route**. 9–10 November 2010, 1st Annual Diamond Route Research Conference, De Beers, Johannesburg.

Oral presentation: Okubamichael DY, Griffiths ME and Ward D. **Host specificity of mistletoes**. 19–23 July 2012, 45th Annual congress Grassland Society of South Africa (GSSA), Kimberley.

Oral presentation: Okubamichael DY, Griffiths ME and Ward D. ***Viscum rotundifolium* is very specific on *Ziziphus mucronata* in the Kimberley area**. 4 September 2012, 9th Kimberley Biodiversity Research Symposium (KBRS), Kimberley.

Oral presentation: Okubamichael DY, Griffiths ME and Ward D. **Do mistletoes close their stomata and respond to abscisic acid?** 17–19 October 2012, Arid Zone Ecology Forum (AZEF), Worcester.

RELATED PUBLICATIONS

Okubamichael DY. 2009. Host specificity of the hemiparasitic mistletoe *Agelanthus natalitius*. MSc thesis, University of KwaZulu–Natal, Pietermaritzburg, South Africa.

Okubamichael DY, Rasheed MZ, Griffiths ME and Ward D. 2011. Avian consumption and seed germination of the hemiparasitic mistletoe *Agelanthus natalitius* (Loranthaceae). *Journal of Ornithology* 152: 643–649.

ABSTRACT

Chapter 1: Mistletoes intimately connect to their host trees with a haustorium that allows them to access nutrients and water. Mistletoes in South Africa vary greatly in their degree of host specificity. Most species occur on a wide range of host families, while others are restricted to a single host family or—at the extreme—to a single host species. Mistletoes that are host generalists at a larger spatial scale may become host-specific at a local scale. One of the challenges in mistletoe biology is determining the factors that maintain local host specificity. Birds potentially reinforce the mistletoe–host interactions by direct dispersal. However, many mistletoe species coexist while parasitising different co-occurring host species. This suggests that host trees may impose more selection pressure than birds in determining host specificity. Thus, my thesis examines the role of host trees as ecological and physiological filters that influence the infection patterns and determine host specificity of mistletoes in South Africa.

The second chapter of this thesis synthesises the literature on host specificity in mistletoes. I then present the results of four field and laboratory experiments that were used examine the features affecting host specificity in representatives of two families of mistletoes (Loranthaceae and Viscaceae) in South Africa. My main research objectives focus on host abundance and morphology, host compatibility, host water and nutrient content, abiotic influences on mistletoe seedling survival and growth and mistletoe–host stomatal morphology in relation to water potential that affect nutrient acquisition by mistletoes from their host trees.

Chapter 2: The geographic mosaic approach was explored as a potential explanation for the mistletoe–host interactions that direct host specificity in mistletoes. I synthesised the available literature on the mechanisms and factors that direct mistletoe host specificity. This was supported by data analysed from South African herbarium collections, books describing the South African flora and field observations in South Africa. I suggest that host abundance (host availability through

time and space) and host compatibility (as determined by genetic, morphological, physiological and chemical factors) play a primary role in determining host specificity in South African mistletoes, while differential bird dispersal strengthens or weakens mistletoe–host interactions. Analysis of the network structure of mistletoe–host interactions at different levels (e.g., at the level of population, species and genus) followed by genetic and reciprocal germination experiments may reveal the patterns and mechanisms of host specificity in mistletoes.

Chapter 3: I quantified the mistletoe–host composition, height of potential host trees and nutrient and water content of mistletoes and their hosts at Pniel Estates. Surveys of the study site revealed a single mistletoe species, *Viscum rotundifolium,* parasitising only *Ziziphus mucronata* and *Ehretia rigida.* Both parasitised host species were not the most abundant trees, were not the tallest trees and did not have the highest water or nutrient content of trees in the area, although these factors have been found to be good predictors for mistletoe parasitism in other studies. Subsequently, I tested mistletoe–host compatibility by conducting a germination experiment in the greenhouse by inculcating seeds of *V. rotundifolium* on freshly cut branches of nine available potential host trees. I found that mistletoe seeds had a greater chance of attachment and subsequent survival on branches of *E. rigida* and *Z. mucronata* as compared with seeds on co-occurring *Acacia* and other potential host species. This suggests that host compatibility plays a role in directing the host specificity of *V. rotundifolium* at Pniel Estates.

I found that individuals of *V. rotundifolium* had more negative water potentials than their host trees and, by doing so, they passively maintain the flow of nutrients. In addition, I found evidence that the mistletoe uses active uptake to access nutrients from host phloem because the leaf tissue of a mistletoe had a nitrogen-to-calcium ratio (N:Ca) >1. Conventionally, a high N:Ca ratio (>1) in the leaf tissue of a mistletoe is taken as evidence of active uptake from host phloem because N is highly phloem-mobile while Ca is a large molecule and is phloem-immobile. This method has shortcomings discussed at greater length in the chapter but my findings suggest that the mistletoe *V. rotundifolium* uses a combination of passive and active nutrient uptake.

Chapter 4: I quantified the mistletoe–host community composition and host physical features (height and diameter at breast height) in two sites in KwaZulu-Natal, South Africa—Highover and Mtontwane. The mistletoe *Agelanthus natalitius* (Loranthaceae) is common at both sites, parasitising the most abundant host species—*Acacia karroo*—and the second most abundant host tree—*Acacia caffra*. Prevalence of mistletoe infection (percentage of trees parasitised) was positively correlated with tree size (height and diameter at breast height). The two host species did not differ significantly in height. At Highover the host species *A. caffra* and *A. karroo* had a similar prevalence of mistletoe infection but at Mtontwane a significantly higher percentage of *A. caffra* trees was parasitised in comparison with *A. karroo*. However, the intensity of mistletoe infection (mean number of mistletoes per tree) was lower for *A. caffra* (Highover: 0.66 ± 0.01, Mtontwane: 0.89 ± 0.04) than for *A. karroo* (Highover: 0.73 ± 0.04, Mtontwane: 1.03 ± 0.64). There were two highly infected big trees in Highover and one in Mtontwane where many mistletoe-dispersing birds were nesting which inflated the numbers for intensity of mistletoe infection in *A. caffra*, however.

I tested mistletoe–host compatibility by conducting a reciprocal transplant experiment in the two study sites. I applied a paired design, using one local and one non-local mistletoe seed in each pair, with seed pairs placed on the two main host species at the different sites. Except in Highover where an unidentified pathogen retarded growth and survival, mistletoe seeds placed on the same substrate and in the same site as their source host grew a longer hypocotyl and had greater survival. Regardless of source, mistletoes placed on *A. karroo* had longer hypocotyls and greater survival than mistletoes on *A. caffra*. These results suggest that there may be adaptation of the mistletoe *Agelanthus natalitius* to the most frequently encountered host species, *Acacia karroo*.

Chapter 5: To simulate the conditions encountered by mistletoes during the dry and cold South African winter, mistletoe seedlings were monitored at different levels of microclimate (light, temperature and moisture) in a growth chamber. I found that higher light availability (20% and 40% shade versus 80% shade), cool temperatures (15°C and 20°C versus 25°C) and continuous moisture availability improved seedling development and subsequent survival of two mistletoe species (*Viscum rotundifolium* and *Agelanthus natalitius*).

Chapter 6: I studied the leaf stomata of two host–mistletoe pairs (*Acacia karroo–Agelanthus natalitius* and *Vitex obovata–Erianthemum dregei*) using a scanning electron microscope to investigate some of the underlying mechanisms that enable mistletoes to maintain more negative water potentials than their host trees and at the same time control water loss. In addition, I examined the response of mistletoes to the application of abscisic acid (ABA), a plant growth regulator that controls stomatal closure. I found that the mistletoes had a higher density of stomata and had larger stomata than their host trees. In addition, both mistletoe and host leaves closed their stomata during midday and in response to exogenous ABA. The ability of mistletoes to control water loss in this way may be one reason why mistletoes rarely kill their host trees, which would be maladaptive.

Chapter 7: The mistletoes used in my studies are known to be host generalists at a larger spatial scale but I found that they were host specific at a local scale. The results of my research suggest that host abundance and compatibility play a role in directing host specificity, while host nutrient and water status have little effect on host specificity at this local scale. The interactions between the generalist mistletoes used in my studies and their hosts are likely to vary over the geographic ranges of the mistletoe and alternate among different hosts. This may create multiple locally host–specific mistletoe populations and produce a complex geographic mosaic of mistletoe–host combinations across space and time. I suggest that mistletoe populations in South Africa may comprise numerous lineages incapable of parasitising the full range of host species, which could potentially lead to the formation of distinct host races over time.

In the future, it would be interesting to document the infection patterns of these generalist mistletoe species across their entire geographic ranges in southern Africa, with particular focus on the patterns of mistletoe infection in places where the host abundance changes among sites. Host preferences may vary with changes in host frequency and host community composition. This could be paired with reciprocal transplant germination experiments in several sites to ascertain whether the mistletoe species have higher fitness on the most locally abundant hosts.

TABLE OF CONTENTS

CHAPTER 5:

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CHAPTER 1

Introduction

Mistletoes are a polyphyletic group of aerial parasitic plants that evolved from root parasites (Mathiasen et al. 2008, Vidal-Russell and Nickrent 2008a,b). During this evolutionary process the root system of mistletoes was modified into a haustorium (from the Latin *haurire* = to drink) through which they obtain water and nutrients from the vascular tissue of the host (Kuijt 1969, Riopel and Musselman 1979, Hibberd and Jeschke 2001, Vidal-Russell and Nickrent 2008a,b, Westwood et al. 2010). Mistletoes (ca. 1600 species in 88 genera) belong to four families in the order Santalales. There is evidence that mistletoe groups evolved independently five times within this order (Vidal-Russell and Nickrent 2007, 2008a,b).

Mistletoes are widely distributed in all major biomes and climate types, absent only from extremely cold regions (Norton and Carpenter 1998, Norton and de Lange 1999, Watson 2001). The majority of mistletoe species infect a wide range of host trees (generalist mistletoes), while some mistletoes species are restricted to a particular host species (specialist mistletoes) (Norton and Carpenter 1998, Polhill and Wiens 1998). However, some generalist mistletoes show a preference for a subset of host species in a given locality (Clay et al. 1985, Norton and Carpenter 1998, Norton and de Lange 1999, Press and Phoenix 2005). Generalist mistletoes can even show variable performance among populations of the same host species in different parts of their geographic range (Press and Graves 1995, Rödl and Ward 2002, Okubamichael et al. 2011a).

In this study we focus on generalist mistletoes and investigate their local specificity, a term which refers to the restricted use of available host species at a local scale. Investigation of host specificity in mistletoes requires a fundamental understanding of the developmental processes of mistletoes, as well as the ecological filters that exert selection at different stages in the mistletoe life cycle—during establishment, growth and reproduction. Several biotic attributes of the host influence specificity in mistletoes: host compatibility (López de Buen and Ornelas 2002, Fadini 2011), abundance (Norton and Carpenter 1998), morphology (e.g., spinescence as described by Aukema and Martínez del Rio 2002a,b), success of mistletoes on source versus non-source host trees (Rödl and Ward 2002), twig or branch size (Sargent 1995), bark thickness and smoothness (Arruda 2006, Fadini 2011), chemical compatibility (haustorium-inducing factors as described by Cannon et al. 2011), host quality (nutrients and water as described by Dean et al. 1994) and the ability to access nutrients and water from the vascular bundle of the host tree (Hoffmann et al. 1986, Yan 1993). Abiotic factors such as light, temperature and moisture are also important filters at many stages of the mistletoe life cycle that potentially influence host specificity in mistletoes (Shaw and Weiss 2000).

Mistletoes lack true ovules and therefore do not have true seeds, but functionally the dispersal unit is a seed (Vidal-Russell and Nickrent 2008a) and the term seed will be used to describe mistletoe dispersal units throughout this thesis. Most mistletoe species rely on birds for dispersal of their seeds (Godschalk 1983, Aukema 2003, 2004, Green et al. 2009, Okubamichael et al. 2011b). However, dwarf mistletoe seeds are dispersed explosively (Hawksworth and Wiens 1996, Smith 1973), *Misodendrum* spp. are dispersed by wind (Vidal-Russell and Nickrent 2007) and *Tristerix* spp. are dispersed by marsupials (Amico and Aizen 2000). Birds consume mistletoe fruits and subsequently wipe their bills, regurgitate or defecate the seeds on host tree branches (Aukema 2003, 2004, Roxburgh 2007, Green et al. 2009, Okubamichael et al. 2011b). Birds usually initiate germination of mistletoe seeds by removing the fruit cover (exocarp), which otherwise inhibits germination (Okubamichael et al. 2011b). They also expose the sticky viscin surrounding seeds (Paquet et al. 1986), enabling the seeds to firmly attach to the host trees.

Dispersal of mistletoe seeds by birds may involve a high degree of coevolution between the mistletoes and the birds (Reid 1989, 1990, Medel et al. 2004). Birds differentially perch on and disperse mistletoe seeds to tall, large trees that have been previously infected (López de Buen and Ornelas 1999, Aukema and Martínez del Rio 2002a,b, Carlo and Aukema 2005). Therefore, mistletoes are usually aggregated at a locality or on individual trees (Donohue 1995, Reid and Stafford Smith 2000, Carlo and Aukema 2005, Robinson and Geils 2006, Ward and Paton 2007, Okubamichael et al. 2011b). Mistletoe–bird interactions play an important role in determining mistletoe distribution (Liu et al. 2011). However, this is not the focus of this thesis because recent studies (Okubamichael 2009, Okubamichael et al. 2011b) have examined the role of birds in directing host specificity of mistletoes in South Africa.

Mistletoe seed germination is substrate-insensitive (Lamont 1983, Yan 1993, Yan and Reid 1995), but some studies indicate that microclimate (water, oxygen, temperature and light) may affect establishment success in mistletoes (Lamont 1983, Room 1971, 1973, Baskin and Baskin 1998). Microclimate can also be an important filter that acts at different stages of the mistletoe life cycle and determine mistletoe distribution at a small scale (distribution within the tree canopy) and could influence the larger-scale distribution within a tree community. Survival of mistletoe seedlings is often very low (Lamont 1983). Little is known about how long mistletoes can remain autotrophic before they start to access resources from their host trees (Lamont 1983, Hawksworth and Wiens 1996), although work by Reid (1987) on *Amyema quandang* suggests that seedlings of this species were parasitic by 6 months of age and Yan (1993) found that *Amyema preissii* and *Lysiana exocarpi* had sufficient food reserves to grow for up to a year before attaching to the vascular tissue of the host. Mistletoes may respond to divergent selection pressures imposed by different environmental conditions, as well as to geographic isolation and host adaptations that potentially promote host race formation (Jerome and Ford 2002). Microhabitat suitability or habitat preference may vary among mistletoe species. In this thesis I examine the extent to which microhabitat (i.e., light, temperature and moisture) influences the seedling growth for two South African mistletoe species.

Mistletoes depend on host trees for water and mineral nutrition from the xylem (Ehleringer et al. 1985, Der and Nickrent 2008, Nickrent and García 2009). Xylem-tapping mistletoes are usually partially heterotrophic because they take up nitrogen in the form of amino acids in the xylem stream and therefore assimilate host carbon (the carbon stem of the amino acid) at the same time (Raven 1983). Mistletoes can access nutrients from the host trees using either active or passive uptake or a combination of both (Bowie and Ward 2004, Okubamichael et al. 2011a). Mistletoes of the same species may even use different uptake mechanisms on different host species (Bowie and Ward 2004, Okubamichael et al. 2011a). Mistletoes need to maintain negative water potentials to passively uptake nutrients from the xylem. In contrast, active uptake does not require water potential adjustment because the mistletoes take up nutrients from the phloem. Passive uptake is the most common mechanism reported in studies on mistletoes (Raven 1983, Ehleringer et al. 1985, Bowie and Ward 2004) but there are some mistletoe species that approach complete dependence on active uptake from the phloem of the host tree for their carbon source (Hull and Leonard 1964, Ehleringer et al. 1985, Marshall et al. 1994, Amico et al. 2007, Der and Nickrent 2008, Nickrent and García 2009). Stomata morphology is an important trait that determines the ability of mistletoes to passively uptake nutrients from host trees and at the same time control water loss.

Aims and objectives of the study

Little is known about host specificity (a measure of coevolution) in mistletoes (see also Amico et al. 2007). The main aim of this thesis is to determine whether some generalist mistletoes in South Africa display local host specificity and to investigate the mechanisms responsible for directing local host specificity. Generalist mistletoes can vary in their infection patterns among hosts and produce host races (Amico et al. 2007). Mistletoe host races are taxa that associate with different principal hosts but show no consistent morphological and physiological differences that separate them from other members of the same species. Investigation of host race formation is important because it can serve as the basis of speciation. Thus, I investigate host specificity in selected species of mistletoe in two families (Loranthaceae and Viscaceae) in South Africa.

The objectives of this study are to:

- synthesise information on host specificity in mistletoes based on the published literature, field observations and herbarium data, with an emphasis on southern African mistletoes,
- determine the infection patterns and host specificity of selected mistletoes in South Africa by quantifying host attributes (community composition, abundance and morphology),
- investigate host compatibility by performing laboratory and field reciprocal transplant experiments,
- evaluate the role of microhabitat in the early growth and survival of mistletoes
- investigate the morphology of leaf stomata in relation to water and nutrient uptake mechanisms.

Study sites and species

The work presented in this thesis summarises several field investigations and laboratory studies carried out at the School of Life Sciences on the Pietermaritzburg campus of the University of KwaZulu-Natal from 2009 to 2012. Study sites were selected in different areas of South Africa: two sites in KwaZulu-Natal (Mtontwane near Colenso and Highover near Richmond), two in the Northern Cape (Pniel Estates near Barkly West and Rooipoort near Schmidtsdrift in the Kimberley area) and one in Gauteng (Walter Sisulu National Botanical Garden, Johannesburg). Several representatives of the two largest mistletoe families (Loranthaceae and Viscaceae) occur in these areas. One survey was conducted on *Agelanthus natalitius* (Loranthaceae), which is commonly found at the two sites in KwaZulu-Natal. *Erianthemum dregei* (Loranthaceae)—another species of mistletoes found occurring naturally at Mtontwane—was also used for mistletoe–host stomatal studies. Another survey was conducted to investigate host specificity in the mistletoe *Viscum rotundifolium* (Viscaceae) in the Northern Cape. In addition to the above three species of mistletoes, *Viscum combretum* (Viscaceae) and *Tapinanthus rubromarginatus* (Loranthaceae) were also recorded at the Walter Sisulu National Botanical Garden in Johannesburg. The last two species were not investigated in detail but they were extremely host-specific, each parasitising a separate single host species at the study site.

Thesis structure

Chapter 1 is a brief introduction of the thesis that outlines the key questions, objectives, study sites, species and structure of the thesis. **Chapter 2** provides a review of the existing literature on mistletoe host specificity. It also presents data from field observations and herbarium collections.

It considers the possible explanations for mistletoe host specificity and identifies gaps in current knowledge. It highlights the mechanisms of host specificity and summarises the processes that determine mistletoe host specificity. In addition, the geographic mosaic theory (Thompson 1994) is described and presented as an explanation for patterns in mistletoe host specificity.

Chapter 3 presents a field survey on the influence of the community composition, abundance, water and nutrient status of host trees for the mistletoe *Viscum rotundifolium* at Pniel Estates, Northern Cape. In addition to the field survey, the chapter presents results from an inoculation experiment conducted to test the degree of host compatibility. The main focus of this experiment was the mistletoe–host relationship with regard to water and mineral nutrients. I investigated the role of nutrient uptake by comparing a highly phloem-mobile element, nitrogen (N), to a phloem-immobile element, calcium (Ca). N:Ca ratio is an index that shows the means of nutrient access by the mistletoes either from the xylem (passively, if N:Ca $\leq$ 1) or phloem (actively, if N:Ca > 1) of the host trees (Panvini and Eickmeier 1993, Bowie and Ward 2004).

This PhD thesis builds on the studies that I carried out for my MSc degree. Particularly, **Chapter 4** of this thesis links to my MSc thesis by including some of my initial results. New data on survival over a longer period were incorporated into this chapter. Furthermore, the study was made more comprehensive during my PhD studies. The chapter additionally surveys the mistletoe *Agelanthus natalitius* that I did not publish in my MSc studies. Moreover, a reciprocal transplant germination experiment was applied at the two sites (Mtontwane and Highover) and on the two commonly infected host species (*Acacia karroo* and *A. caffra*). This study tested whether host race evolution has occurred in mistletoes by conducting cross-infection experiments (e.g., Clay et al. 1985, Glazner et al. 1988, Rödl and Ward 2002). I tested whether mistletoe seeds perform better on the host species that their parent plants grew on than when transferred to non-source host species. To do this, I quantified the hypocotyl and haustorium formation as bases of mistletoe host specificity, which may indicate that host specificity initiates at an early stage of mistletoe development (Rödl and Ward 2002, Okubamichael et al. 2011a). The chapter presents the survival of mistletoes over 6 mo. The effects of the initial results of germination and growth are keys to the final results of survival and were analysed to determine the relationship between hypocotyl growth and survival. This also helps to understand the host specificity observed in previous mistletoe studies (**Chapter 3**).

Chapter 5 addresses the effects of different levels of shade, moisture and temperature on mistletoes grown in a growth chamber. Early seedling growth of two species of mistletoes—*Agelanthus natalitius* (Loranthaceae) and *Viscum rotundifolium* (Viscaceae)—in terms of hypocotyl, radicle, holdfast and haustorium formation were explored as important growth traits that promote mistletoe survival.

Chapter 6 explains the role of stomatal control by mistletoes on water use by these plants. The leaf stomata of two mistletoe species—*Agelanthus natalitius* and *Erianthemum dregei*)—that parasitise two different host species of different families—*Acacia karroo* (Fabaceae) and *Vitex obovata* (Lamiaceae)—were directly investigated using a scanning electron microscope. In this chapter the size and number of leaf stomata on mistletoe leaves were compared with leaves from their host trees. The chapter also presents data on diurnal stomatal regulation and stomatal condition after abscisic acid treatment (which leads to closure of stomata) of both mistletoes and their host trees. These experimental data are presented along with water potential measurements of mistletoes and their host trees in the field. Previous studies on this topic were mainly based on indirect evidence, resulting in exaggerated conclusions of a lack of stomatal control by mistletoes. This study substantially advances our understanding of the role that stomatal control plays in the water relations of mistletoes and their hosts.

Chapter 7 provides a general discussion of the main features of each of the preceding data chapters. Most importantly, it describes our current understanding of mistletoe–host specificity and ecophysiology and suggests further investigations.

All the chapters in the thesis with the exception of the Introduction (**Chapter 1**) and the Conclusions (**Chapter 7**) have been published or submitted for publication in peer-reviewed journals. The data chapters follow the format of the targeted journals. As a result, some repetition and inconsistency of format is unavoidable. The submitted or published chapters are as follows:

Chapter 2: "Host specificity in mistletoes: a South African perspective" has been submitted to Annals of Botany.

Chapter 3: "Host specificity, nutrient and water dynamics of the mistletoe *Viscum rotundifolium* and its potential host species in the Kalahari of South Africa" was published in *Journal of Arid Environments* 75: 898–902.

Chapter 4: "Reciprocal transplant experiment suggests host specificity of a mistletoe *Agelanthus natalitius* in South Africa" is provisionally accepted for publication by *Journal of Tropical Ecology*.

Chapter 5: "Light and moisture improve initial seedling growth and survival of mistletoes" has been formatted for *International Journal of Plant Sciences*.

Chapter 6: "Mistletoes control water loss through stomatal closure" has been submitted to *Journal of Experimental Botany*.

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CHAPTER 2

Host specificity in mistletoes: a South African perspective

Desale Y. Okubamichael, Megan E. Griffiths and David Ward*

School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa

**For correspondence. E-mail ward@ukzn.ac.za.*

- *Background* Mistletoes range from generalist species to those that are host specific at both local and larger geographic scales. However, many generalist mistletoes differ in their degree of host specialisation and can be locally host specific. As yet, the mechanisms that determine host specificity in mistletoes are not well documented or understood.
- *Scope* We used a geographic mosaic approach to explain mistletoe–host interactions and host specificity in mistletoes. We synthesised the available literature on the multiple factors that direct mistletoe host specificity. This was supported by data obtained from field observations and herbarium collections in South Africa. Host abundance and host compatibility (genetic, morphological, physiology and chemical) play a primary role in determining mistletoe host specificity, while differential bird dispersal strengthens or weakens the interactions of mistletoes and their host trees.
- *Conclusions* We conclude that a geographic mosaic approach can help to explain the patterns and processes directing host specificity in mistletoes. Analysis of the structure of mistletoe–host networks should be investigated in the future to document the patterns of host specificity at different levels. Reciprocal infection experiments, genetic analyses of confined mistletoe populations and comparative phylogenetic studies can also be used to determine the primary processes that influence host specificity in mistletoes.

***Key words**:* bird dispersal, coevolution, haustorium, haustorium-inducing factors, host compatibility, parasitic plants.

INTRODUCTION

Many mistletoe species in Africa (70%) are generalists, infecting a wide range of host trees (Polhill and Wiens, 1998). Mistletoes often experience selection for host-specific adaptations at a local level, as different hosts exert different selection pressures that ultimately drive local mistletoe specialisation (Norton and Carpenter, 1998; Norton and de Lange, 1999; Amico *et al*., 2007; Blick *et al*., 2012; Kavanagh and Burns, 2012). Therefore, mistletoes that are initially capable of utilising several host species can become restricted to a subset of available hosts (Barlow and Wiens, 1977; Amico *et al*., 2007; Okubamichael *et al*., 2011*a*, Kavanagh and Burns, 2012). In these cases, generalist mistletoe species are composed of distinct host-specific populations that are capable of forming new lineages and eventually speciating. Host specificity refers to the restricted host usage of available potential host species at a local scale (Thompson, 1988; Norton and Carpenter, 1998), while preference refers to the hierarchical ranking of host use (Thompson, 1988). These two concepts are not always synonymous; however for the purpose of this review, preference by mistletoes for particular hosts is a form of host specificity.

We synthesised the literature on mistletoe host specificity and host preferences, with particular reference to recent southern African studies. We hypothesise that host abundance and host compatibility determine host specificity in mistletoes. Host specificity in mistletoes can be dynamic in space and time, which we investigate using a geographic mosaic approach. While the geographic mosaic approach is used widely to explain the relationship between specialisation and coevolution—particularly host–parasite associations in animals (*sensu* Thompson, 1988; 1989; 1993; 1994; 1997)—it is rarely applied to mistletoe–host interactions. Thus, a geographic mosaic approach was introduced in this review to examine the variation in host use among species and populations of mistletoes.

GEOGRAPHIC MOSAIC MODEL

Thompson (1994) developed the geographic mosaic model to explain the relationship between specialisation and coevolution in a broad ecological and historical context. He argues that the coevolution between pairs of species or populations within a local scale must be maintained to eventually establish the interaction across a broader geographic range (Thompson, 1994). According to the model, coevolution is a reciprocal evolutionary change in interacting species at local, regional and global levels driven by natural selection, creating ever-changing geographic mosaics of species interactions with one another (Thompson, 1989; 1994). The geographic mosaic model proposes that coevolving interactions incorporate three components that collectively drive ongoing coevolutionary dynamics of global biodiversity: *geographic selection mosaics*, *coevolutionary hotspots* and *trait remixing* (Thompson, 1994; 1997).

Geographic selection mosaics: Genotype-by-environment interactions determine the fitness of interacting species. Natural selection acts on the variation of the interspecific interactions causing population specialisation in separate regions. *Coevolutionary hotspots* are subsets of communities in which much of the evolutionary change occurs. These are often embedded in a broader matrix of coevolutionary coldspots (Gomulkiewicz *et al*., 2000), where local selection is non-reciprocal. The geographic range of a parasitic species does not perfectly match that of its preferred host(s) and may only overlap at certain localities. In most mistletoe species, the perching behaviour and movement of frugivorous birds directly affect mistletoe distribution among host trees (Reid, 1991; Aukema and Martínez del Rio, 2002*a*,*b*). The three-way interaction between mistletoe–bird–host often results in a mosaic of coevolutionary hotspots in which certain local populations contribute greatly to the overall coevolution between the mistletoes and their hosts. *Trait remixing* occurs through changes in the genetic structure of coevolving species due to mutations, gene flow, random genetic drift and extinction of local populations. It is the continuous altering of the spatial distributions of potentially coevolving genes and traits that drives the processes of coevolution (Thompson, 1994; 1997).

It is quite plausible to suggest that mistletoes coevolve with their hosts; however there are few data to support this proposal (Marcos and Tomas, 2011; Kavangh and Burns, 2012). For example, Medel *et al*. (2010) recorded a coevolutionary arms race between mistletoe and host in Chile, where two cactus species (*Echinopsis chilensis* and *Euclchnia acida*) have developed extremely long spines to resist infection by the mistletoe *Tristerix aphyllus*. The mistletoe has reciprocally evolved a very long hypocotyl (the structure that protrudes as the mistletoe germinates and attaches to a host twig before forming a haustorium). However, such reports are scant in the literature on mistletoe–host coevolution. Therefore in this review we use host specificity as a potential measure of coevolution (Thompson, 1989).

GEOGRAPHIC PATTERNS OF HOST SPECIFICITY

Approximately 70% of mistletoes in Africa are generalists that parasitise several families, 12% are specific on trees of one family but sporadically parasitise a few genera of other families and 18% are specific to one or a few species of a single genus (Polhill and Wiens, 1998). Information on mistletoe species and their host genera were obtained from mistletoe books of southern Africa (Wiens and Tolken, 1979; Visser, 1981) and Africa (Polhill and Wiens, 1998). Analyses of Shannon-Wiener index (H') showed that the two main mistletoe families in southern Africa parasitise a high diversity of host genera (Loranthaceae parasitised 89 host genera with H' = 4.26, Viscaceae parasitised 65 host genera with H' = 4.05) while some are specialists (Fig.1). Many of the southern African mistletoe species in both the Loranthaceae and Viscaceae use *Acacia* and

Combretum as their main host plants. Few mistletoe species are very host specific and use only one host species over their entire geographic range (e.g., *Viscum minimum* parasitises only *Euphorbia horrida* and *E. polygona* in the Eastern Cape, South Africa) (Polhill and Wiens, 1998).

We used specimens from the Bews Herbarium at the University of KwaZulu-Natal, Pietermaritzburg to examine patterns of host use in southern African mistletoes. The collection includes 340 herbarium specimens of mistletoes from the Loranthaceae, encompassing 46 species that were collected from over 200 host species, and 179 herbarium specimens of mistletoes from the Viscaceae, including 14 species collected from over 70 host species. We found that *Acacia karroo* and *A. caffra* were the most commonly used host species by all mistletoes. This may be related to the availability of these *Acacia* species, as *A. karroo* is the most widely distributed *Acacia* species in South Africa (van Wyk and van Wyk, 1997). However, in areas where *A. karroo* is not the most abundant potential host species, many mistletoe species are found on other host species (Fig. 2). In the herbarium collection, *Viscum rotundifolium* was found parasitising the highest number of host species in KwaZulu-Natal but it was restricted to *Ziziphus mucronata* from the Free State to the Northern Cape provinces in South Africa (see also Okubamichael *et al*., 2011*a*). The same species was found on the more abundant species *Boscia albitrunca* and *B. foetida* in Namibia. These results suggest that mistletoes that are host generalists across the entire range may be specific to particular hosts on a local scale.

Many mistletoe species tend to have one primary host species and use other host species less frequently. Even in the most generalist mistletoes, not all available host genera are equally susceptible to infection by mistletoes at a given locality. Usually mistletoes have a primary host genus that they prefer or become host specific on. This may be linked to the existence of coevolutionary hotspots where the interactions between mistletoes and their hosts are strong. For instance, the mistletoes *Plicosepalus kalachriensis* and *P. undulatus* parasitise only *Acacia* species and are good examples of mistletoe coevolution. In addition, *Viscum menyharthii* parasitises predominantly *Acacia* and *Ficus* species, even though *Acacia* species are generally not the primary hosts for Viscaceae.

We also found that mistletoe species are less likely to share a single primary host genus, especially if they are from different families. A Sørensen index (*Sim*) was used to calculate the similarity in host genera use by the two major mistletoe families. This index is calculated as 2C/A+B, where A= number of species in sample A, B= number of species in sample B and C= number of species common in both A and B (see e.g. Magurran 1988). The Sørensen index comparing the host species used by mistletoes in the Viscaceae and Loranthaceae was low (*Sim* = 0.26; 20 host genera were shared between Loranthaceae and Viscaceae). This indicates that mistletoe species in Viscaceae parasitise mainly host genera that are not used by mistletoe species in Loranthaceae and *vice versa*. For example, *Euphorbia* and *Olea* are some of the most common host trees for Viscaceae but they are not common hosts for Loranthaceae. Additionally, even the most generalist species in the Loranthaceae (*Tapinanthus oleifolius*) and the most generalist in the Viscaceae (*Viscum rotundifolium*) had a low similarity index (*Sim* = 0.29). *Viscum rotundifolium* does not utilise all 32 species of *Acacia* that are reported to be parasitised by other mistletoes in southern Africa, only occurring on *A. erioloba* and *A. karroo*. These findings show clear trends for southern Africa that could be further tested by examining host ranges in these two mistletoe families in North America and Australia.

We have also observed that several mistletoe species in the Walter Sisulu Botanical Garden (near Johannesburg, South Africa, ca. 300 hectares) have a non-overlapping domain of host species. In this particular site, if a host species is parasitised by a particular mistletoe, it is unlikely to be parasitised by other mistletoe species occurring in the same habitat (Fig. 3). A negative co-occurrence pattern in mistletoe species that specialise on distinct suites of host species has been also reported in North America, New Zealand and Australia (Hawksworth and Wiens, 1972; Blick and Burns, 2009; Blick *et al*., 2012). Similarly, Fadini (2011) showed that three congeneric and sympatric mistletoe species (*Psittacanthus biternatus*, *P. eucalyptifolius* and *P. plagiophyllus*) specialise on different host species in the Amazon. Mistletoes that are host specific may have a competitive edge over non-specific mistletoes where several mistletoes coexist. For example, Jerome and Ford (2002) suggested that host specificity in *Arceuthobium americanum* reduces potential competition with other mistletoe species that may utilise the same host species at a given site. A pattern of non-overlap in mistletoe primary host use may be indicative of competitive exclusion and could contribute to a geographic mosaic of mistletoe–host interactions. Such a geographic mosaic could ultimately determine patterns of host specificity in mistletoes (*sensu* Blick and Burn 2009). Further investigation is warranted to quantify the degree of competition among mistletoe species and to determine the mechanisms that drive such interactions.

MECHANISMS THAT DETERMINE HOST SPECIFICITY

Mistletoe interactions with birds and host trees direct the generality or host specificity of a mistletoe at a given locality. Birds and host trees act at different stages in the mistletoe life cycle. Pollination and seed dispersal by birds initially affect the distribution of mistletoes on host trees.

Thereafter, diverse host traits influence the establishment and survival of mistletoes, further filtering the distribution of mistletoes among host trees (Fig. 4).

POLLINATION AND SEED DISPERSAL

Pollination of mistletoes is carried out by insects, birds and rarely by wind (Kirkup, 1998; Watson, 2001). Viscaceae flowers are highly reduced in size and monoecious (unisexual) (Vidal-Russell and Nickrent, 2008). Loranthaceae flowers are large, conspicuous and bright in colour and dioecious (bisexual). The Loranthaceae are mainly bird pollinated, whereas the Viscaceae are wind- and insect-pollinated (Aukema, 2003; Mathiasen *et al*., 2008). Self-compatibility in mistletoes limits outcrossing and enhances inbreeding with nearby individuals (Vaknin *et al*., 1996, Ladley *et al*., 1997). Amico *et al*. (1997) reported that peak flowering time and fruiting of *Tristerix aphyllus* and *T. corymbosus* do not overlap greatly, which limits interspecific pollen and seed transfer. Even *Arceuthobium americanum* which is an obligate outcrossing species has limited pollen dispersal (maximum 400–512 m) (Jerome and Ford, 2002). This limits pollen flow and increases population differentiation in *Arceuthobium* (Jerome and Ford, 2002). In addition, preferential pollen transfer among individuals growing on the same or nearby tree may limit pollen flow among host races. Therefore, pollination can act as an important mechanism isolating mistletoe populations. On the other hand, Stebbins (1950) argued that even a limited amount of long-distance gene flow in plants can be a strong homogenising force in terms of restricting population differentiation. Work by Bernhardt (1983) on the mistletoe genus *Amyema* suggested that self-compatibility in mistletoes did not stop taxa from preferentially outcrossing.

Most mistletoe species rely on birds to direct dispersal of their seeds, although dwarf mistletoes (*Arceuthobium* spp.) have seeds that are dispersed explosively (Hawksworth and

Wiens, 1996), *Misodendrum* by wind (Vidal-Russell and Nickrent, 2007) and *Tristerix* by marsupials (Amico and Aizen, 2000). Birds consume mistletoe fruits and subsequently wipe their bills, regurgitate or defaecate the seeds on the branches of host species (Reid, 1991; Roxburgh, 2007; Green *et al*., 2009; Okubamichael *et al*., 2011*b*). By doing so, birds break the physical dormancy of the seed and initiate germination of mistletoe seeds by removing the fruit cover (exocarp), which otherwise inhibits germination (Roxburgh, 2007; Okubamichael *et al*., 2011*b*). Birds also expose the sticky viscin, enabling seeds to firmly attach to branches of host trees. Reid (1991) found that some mistletoe species have developed specific fruit-displaying characteristics that target specific frugivorous birds. These birds have, in turn, developed dietary and anatomic specialisation to process mistletoes fruits and they frequently disperse the seeds to suitable host braches where the mistletoes can easily establish. Thus, mistletoes and their dispersers can reciprocally coevolve, although the nature of this coevolution is often diffuse (see Reid 1991 and references therein).

Birds disperse more mistletoe seeds on the parental host tree than elsewhere. The probability of seed survival decreases as the dispersal distance increases (Overton, 1994; Okubamichael *et al*., 2011*b*). Mistletoes are usually aggregated at a locality (infestation patches) or on individual trees (intensification) and follow a negative binomial distribution at the population level (Robinson and Geils, 2006; Overton, 1994). Birds affect the frequency and level of mistletoe–host interactions across time and space, which in turn influences the geographic mosaic of mistletoes and their hosts. Birds could transfer mistletoe seeds across large distances and potentially disperse seeds to very distantly related hosts (Aukema and Martínez del Rio, 2002*a*,*b*), which could facilitate host switches. This may weaken the reciprocal evolutionary association between host and mistletoe. On the other hand, birds may enhance the interactions between mistletoes and their prospective hosts (e.g., if the host trees provide a reward such as fleshy fruits that are available at the same time as the mistletoe fruits) (Okubamichael *et al*., 2011*b*). In either case, birds act as an initial filter for host specificity in mistletoes.

Birds may learn through time to differentially visit specific host species (Godschalk, 1983, 1985). This may increase the chance of efficient dispersal to the appropriate host thereby facilitating host specificity (Martínez del Rio *et al*., 1995; Aukema and Martínez del Rio, 2002*a*). This may increase mistletoe seed dispersal on particular host trees and may limit dispersal to other host trees. Host specificity also enhances aggregation of individual mistletoes on trees of the specific host, which makes their pollination frequent and easy (Watson, 2011). In this regard, mistletoe species with high host specificity could be selected over those that are host generalists. Future research should investigate seed dispersal strategies of host generalist and host specialist mistletoes by investigating fruit traits such as size, colour and nutritional quality. Specialist mistletoes would be expected to have fruit traits that target specific birds capable of directing fruit dispersal to the appropriate host, thereby increasing the mistletoe fitness.

HOST ABUNDANCE AND COMPATIBILITY

In plant communities where species diversity is high and there are few dominant species—such as in rain forests—mistletoes tend to be generalists (Barlow and Wiens, 1977). High host specificity is not likely to confer any selective advantage in such environments. Instead, there may be selection for traits that allow the mistletoes to infect and grow on a wide range of host species (Press and Graves, 1995; Downey, 1998).

In less diverse temperate forests and semiarid savannas—where dominance of one or a few tree species is typical—mistletoes are more likely to be specific to one genus or even to a single host species (Norton and Carpenter, 1998; Okubamichael *et al*., 2011*a*). In these environments selection favours close physiological adaptations of the mistletoes for the predominant host species (Barlow and Wiens, 1977; Dean *et al*., 1994; Downey *et al*., 1997; Downey, 1998). There are many long-term associations of hosts and mistletoes that evolve in a restricted, unidirectional way that result in extremely host-specific mistletoes (Norton and Carpenter, 1998; Barlow and Wiens, 1977). However, there are also instances where mistletoes parasitise uncommon host trees as a result of host compatibility at the genetic, mechanical, physiological and biochemical level (Yan, 1993*a*; Yan and Reid, 1995; Okubamichael *et al*., 2011*a*; Fadini, 2011). This may create a geographic mosaic of several mistletoe–host combinations across the landscape.

Usually the ever-changing composition of plant communities creates opportunities for new interactions between species (Thompson, 1999). Thus specialisation may be a dynamic state capable of changing rapidly rather than a static endpoint. Mistletoes have shorter generation times and higher reproductive rates than their host trees (Norton and Carpenter, 1998). Therefore, mistletoes may adapt quickly to a shift in host abundance in the ecosystem. The adaptation to host genotypes would also be faster than the emergence of new resistant host genotypes, which is consistent with the Red Queen Hypothesis or the evolution of an "arms race" between the mistletoe and its host (Jokela *et al*., 2000; Thompson 2005*a*,*b*). When hosts develop resistance, selection favours traits in the mistletoe that increase virulence or otherwise allow them to overcome host resistance. Research on this topic in mistletoes would enhance our understanding of parasitism evolution in general and mistletoe host specificity in particular.

HOST QUALITY AND MORPHOLOGY

The evolution of a haustorium enabled parasitic plants to acquire water and nutrients from other plants. This adaptation may have evolved in arid environments where both resources are limited (Atsatt, 1973, 1977; Ehleringer *et al*., 1984; Bowie and Ward, 2004). Nitrogen is often a limiting nutrient in plants and mistletoes have been hypothesised to selectively parasitise host species that are high in nitrogen (Midgley and Joubert, 1991; Dean *et al*., 1994; Pennings and Callaway, 2002). Dean *et al*. (1994) found that mistletoe species richness was positively correlated with the average nitrogen level of the plant community in major vegetation types in South Africa. However, Griffiths *et al*. (submitted) used a phylogenetically independent analysis of the Dean *et al*. (1994) data and found that the area occupied by a host species was more important in determining mistletoe species richness than nitrogen. This suggests that the quality of host trees in terms of nitrogen content may not be as critical as previously thought in terms of driving host specificity in mistletoes.

In any parasite–host association a parasite develops traits that aid in effectively penetrating the host. In response, the hosts usually develop resistance to parasite infection (Thompson, 1994; Medel *et al*., 2010). In mistletoes, the haustorium encounters a range of resistance pressures by potential host trees, in which some individuals or host species are susceptible and some are resistant at various phases of haustorium penetration. The bark of many non-host plant species is resistant to haustorial penetration by mistletoes (Yan, 1993*a*). In this case, mistletoe infection is blocked before establishment can take place. Non-host species that lack bark resistance sometimes develop a wound periderm that blocks access to the xylem, thereby curtailing further establishment of mistletoes (Yan, 1993*a*). The primary host species of the mistletoes in a study by Yan (1993*a*) showed initial bark resistance, which may be an important evolutionary adaptation to reduce infection. However, none of the primary host species exhibited xylem resistance (Yan, 1993*a*).

Several other traits determine the degree of local host specificity in mistletoes. For example, mistletoe-dispersing Chilean mockingbirds (*Mimus thenca*) avoid perching on certain cactus hosts (*Echinopsis chilensis* and *Eulychnia acida*) with extremely long spines (Martínez del Rio *et al*., 1995). Hence, host individuals with longer spines have lower mistletoe infection rates than those with shorter spines (Martínez del Rio *et al*., 1995). Even if birds disperse mistletoe seeds to long-spined cacti, the seeds remain hanging on the spine and their hypocotyl dies before it can form a holdfast on the host. Similar selection pressure has contributed to the evolution of longer hypocotyls in populations of the mistletoe *Tristerix aphyllus* that parasitise long-spine cactus hosts (Medel *et al*., 2010).

HAUSTORIUM-INDUCING FACTORS

Mistletoe seeds germinate quickly and indiscriminately on any substrate (i.e., they are site- and host-insensitive) (Glazner *et al*., 1988; Yan, 1993*b*, Rödl and Ward, 2002). Mistletoe seeds are relatively large compared to seeds of root parasitic plants and some species have sufficient food reserves to allow the radicle to grow for up to a year while attaching to the vascular tissue of the host plant (Yan, 1993*b*). However, all parasitic angiosperms (including mistletoes) may require chemicals (haustorium-inducing factors, HIF) or a contact signal to initiate the development of the haustorium (Clay *et al*., 1985; Jamison and Yoder, 2001; Rödl and Ward, 2002). In root parasites, several chemicals have been identified as HIFs, including two flavonoids, xenognosin A and B, quinone 2, 6 dimethoxy-1,4-benzoquinone (DMBQ), phenolic acids and cytokinins

(Lynn *et al*., 1981; Steffens *et al*., 1982; Tomilov *et al*., 2006), but none are known for mistletoes.

Dodder (*Cuscuta pentagona*), a facultative aerial parasite, uses volatile chemicals released by the host to sense the location of hosts and cue haustorium development on preferred host species (Runyon *et al*., 2006). Tomilov *et al*. (2006), in their studies of root parasites, indicated that HIF may be species-specific and activate specific receptors in particular parasites or host plants that may produce several HIF with possible redundancy of active molecules. Cannon *et al*. (2011) performed a similar experiment and showed that the mistletoe *Phoradendron serotinum* (now *P. leucarpum*: Kuijt, 2003; Abbot and Thompson, 2011) seedlings respond to host volatile compounds. This suggests that volatiles may also be important in directing host specificity in mistletoes.

FUTURE DIRECTIONS

The geographic mosaic approach can be used to explain patterns of coevolution between mistletoes and their host trees. It would be useful to test the geographic mosaic model using reciprocal transplant experiments on a range of host species and sites to determine differences in mistletoe fitness on different hosts (such as hypocotyl length, degree of haustorium establishment, survival and reproduction). Reaction norms—the pattern of phenotypes produced by a given genotype under different environmental conditions—could then be used to determine the selection pressure in populations of mistletoes in different environments (Yan, 1993*b*; Lynch and Walsh, 1998; Rödl and Ward, 2002). Reciprocal transplant experiments on mistletoes tend to result in low establishment success (Rödl and Ward, 2002), which require using large sample sizes. Alternatively, molecular markers could be used to investigate genetic differentiation among populations. For example, host race speciation in *Tristerix* (*T. corymbosus* to cacti specific *T. aphyllus*) was confirmed using molecular phylogeny (Amico *et al*., 2007; Amico and Nickrent, 2009).

At present there is a need to understand the complex networks of mistletoe–host interactions. Ecological networks most frequently fit nested or modular patterns (Genini *et al*., 2012). Networks that are nested contain a few generalists that interact with one another and with specialist species, which allows for the persistence of specialists. In modular networks, generalist species form sub-groups (modules) that interact more with the species within their module than they do with species in other modules. Network analysis (a test of modularity and nestedness) could be used to examine the structure of patterns of mistletoe–host interactions at the population, species, genera and family levels (*sensu* Genini *et al*., 2012). This could be supplemented by a more comprehensive reciprocal transplant and a genetic study. Together these investigations could reveal the underlying processes that are responsible for the development and maintenance of mistletoe host specificity.

It would be interesting to test whether geographic specialisation of mistletoes on different hosts results from genetic divergence in preference hierarchies (phylogenetic host specificity) or ecological differences in the availability of hosts (specificity in geographic space). For example, it is well known in animal parasites that some populations exclusively parasitise one host for many generations but do not lose their ability to recognise other major hosts that they do not normally encounter (Poulin *et al*., 2010; 2011; Cooper *et al*., 2012). On the other hand, some animal parasites switch to new hosts and lose their ability to infect the host species that previously acted as a host. Currently, there are no data on this subject in mistletoes and it would be interesting to use a similar line of investigation to examine coevolution in mistletoes and their host trees.

As mistletoes switch among different host species, the haustorium probably requires adaptive plasticity so that they can access nutrients and water from the prospective host species (see Gonzáles *et al*., 2007). For example, haustorium initiation and function in root parasites exhibit strong selection pressure associated with host coevolution (Westwood *et al*., 2010). We suggest that the formation of haustoria that perform differently on different host substrates would be a good trait to study when examining adaptive phenotypic plasticity in mistletoes (see Medel *et al*., 2010). Research aimed at discovering the presence or absence of HIFs in mistletoes would provide a holistic insight into parasitic plant evolution, particularly the evolution of host specificity in mistletoes.

A phylogenetic comparison of mistletoes and their hosts could reveal the relative importance of cospeciation and host-switching events in mistletoe speciation. In addition, it should be determined whether mistletoes parasitise phylogenetically or biogeographically similar hosts. The combined results of these investigations would comprehensively test the geographic mosaic theory to explain the mistletoe–host interaction at local and at larger geographic scales.

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FIGURE CAPTIONS

FIG. 1. Mistletoe species and number of parasitised host genera (from Visser, 1981), summarising the pattern of infection of the common mistletoe species found in southern Africa of the two largest families of mistletoes, (A) Loranthaceae and (B) Viscaceae.

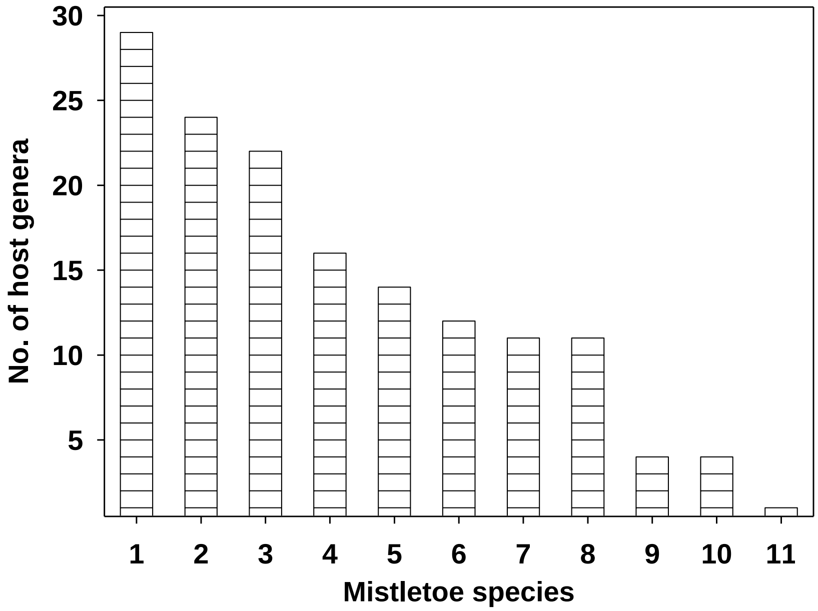
FIG. 2. *Acacia* host species utilisation by mistletoe species in southern Africa. *Acacia karroo* is the most abundant host tree in South Africa and many types of mistletoe species utilise this abundant species. However, in Namibia, *A. erioloba* and *A. mellifera* are quite common and mistletoe were found on these more common hosts. In Zimbabwe, *A. nigrescens* is common and highly utilised by mistletoe species (see van Wyk and van Wyk, 1997 for the distribution pattern of each *Acacia* species).

FIG. 3. We recorded four mistletoe species that differ from generalist to host-specific mistletoes at Walter Sisulu National Botanical Garden, Johannesburg, South Africa. *Viscum rotundifolium* parasitises at least six tree species, but it does not appear to parasitise tree species that are sole hosts for other co-occurring mistletoes. *Agelanthus natalitius* has a limited number of host species and predominantly parasitises *Acacia caffra* and is more rarely found on *Dombeya rotundifolia* and *Acacia karroo*. *Viscum combretum* mainly parasitises *Combretum erythrophyllum* and rarely is found on *Dombeya rotundifolia*. At the extreme end of host specificity, *Tapinanthus rubromarginatus* parasitises only *Protea caffra.* Dashed circles of host trees indicate that they are rare at the location. Dashed lines indicate that the associated mistletoe seldom parasitises those host trees. The broader and darker line indicates mistletoes that are specific to the indicated host trees. The triangle shows that the mistletoes range from host

generalist (indicated by the base of the triangle) to host specific (indicated by the pointed end of the triangle) species.

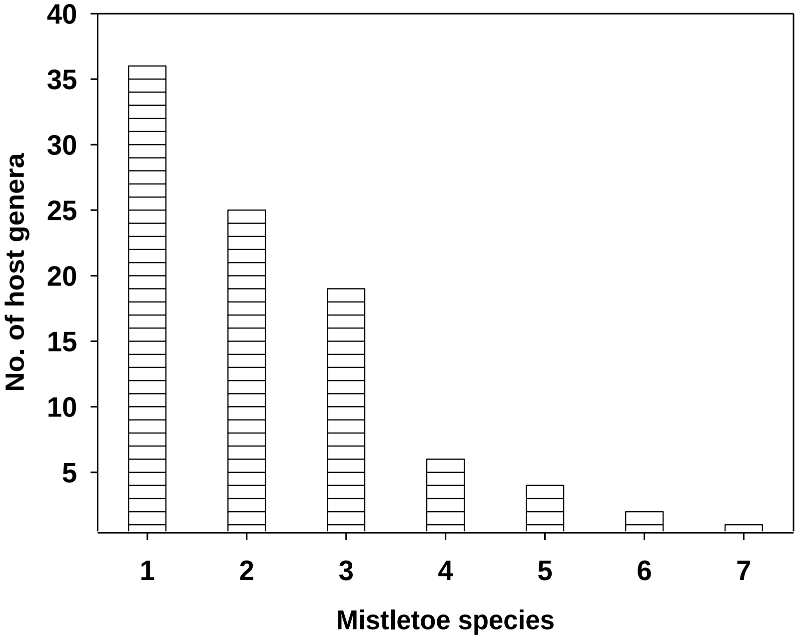
FIG. 4. A diagram representing the multifaceted processes that affect the infection patterns of mistletoes among available potential host species. These factors act at various levels of dispersal and at different developmental stages of mistletoes, thus filter mistletoes among available host species and influence mistletoe host specificity. The triangle shows generalist mistletoes filtering down by several processes to host specific mistletoes that ultimately initiate host race formation.

(A) Loranthaceae



(1=*Tapinanthus oleifolius*, 2=*T. quequensis*, 3=*T. kraussianus*, 4=*Agelanthus gracilis,*
5=*T. rubromarginatus*, 6= *Erianthemum ngamicum*, 7=*A. natalitius*,
8=*Moquiniella rubra*, 9=*T. forbessii*, 10=*Septulina glauca*,
11= *Plicosepalus amplexicaulis)*

(B) Viscaceae



(1=*Viscum rotundifolium*, 2=*V. capense*, 3=*V. combreticola*, 4=*V. tuberculatu*
5=*V. continuum*, 6=*V. crassulae*, 7=*V. minimum*)

FIG. 1.

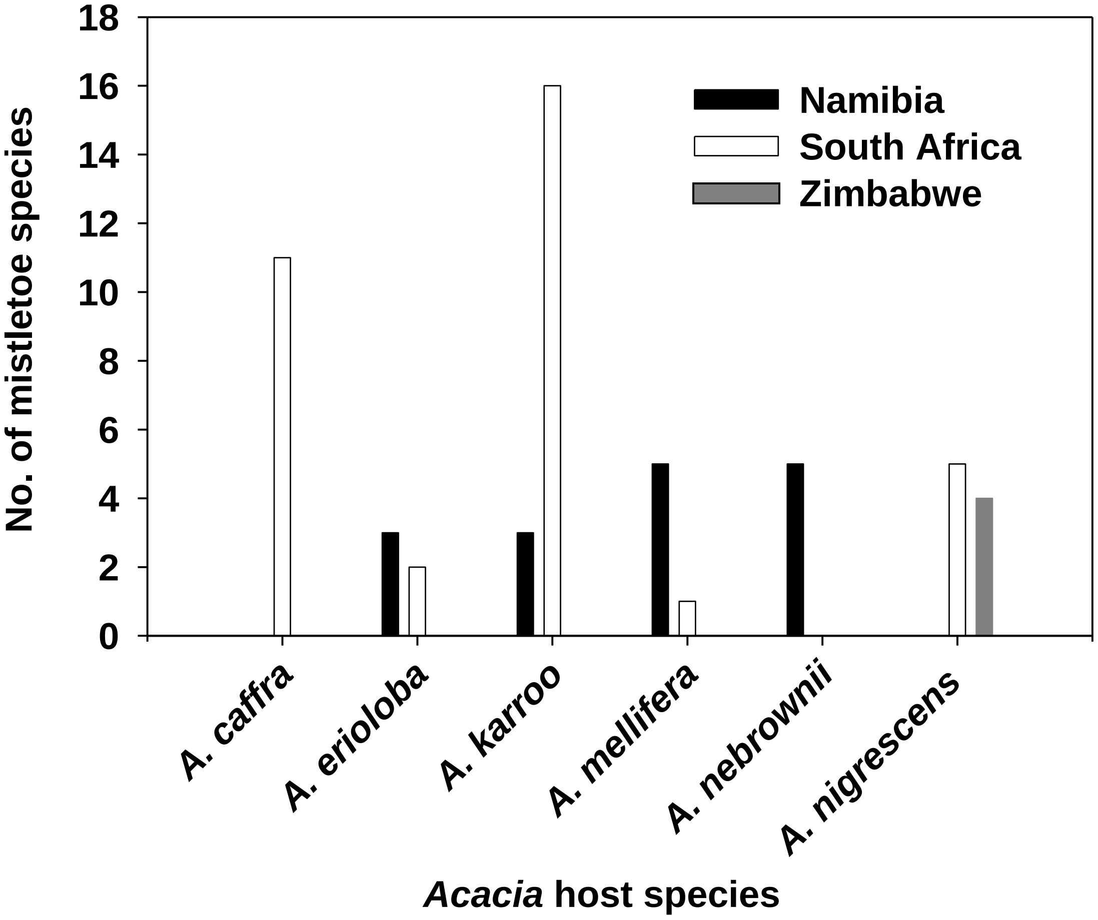

18
16
14
12
10
8
6
4
2
0
No. of mistletoe species
Namibia
South Africa
Zimbabwe
A. caffra
A. erioloba
A. karroo
A. mellifera
A. nebrownii
A. nigrescens
Acacia host species

FIG. 2.

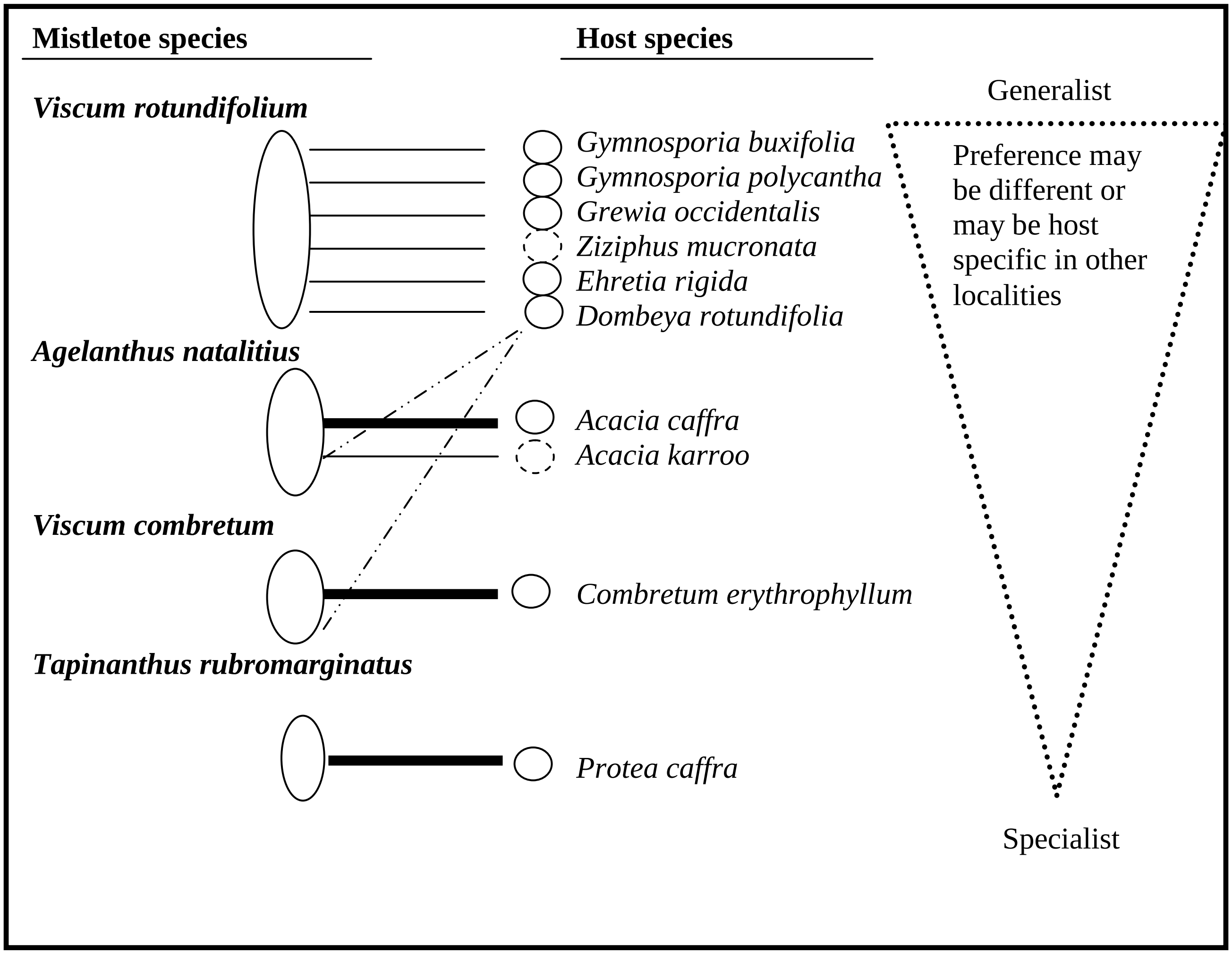
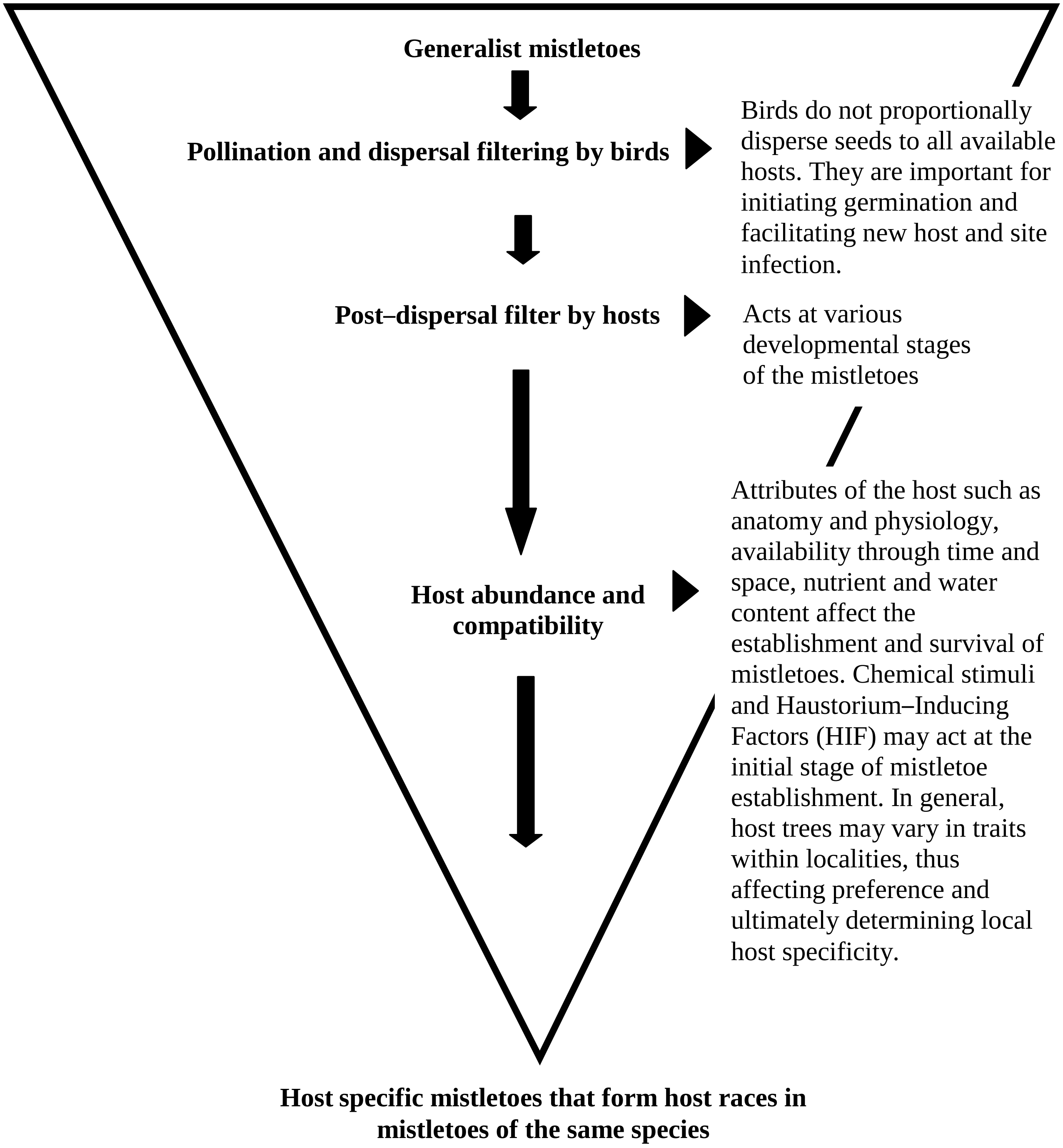

Mistletoe species
Host species
Viscum rotundifolium
Gymnosporia buxifolia
Gymnosporia polycantha
Grewia occidentalis
Ziziphus mucronata
Ehretia rigida
Dombeya rotundifolia
Agelanthus natalitius
Acacia caffra
Acacia karroo
Viscum combretum
Combretum erythrophyllum
Tapinanthus rubromarginatus
Protea caffra
Generalist
Preference may be different or may be host specific in other localities
Specialist

FIG. 3.



Birds do not proportionally disperse seeds to all available hosts. They are important for initiating germination and facilitating new host and site infection.

Acts at various developmental stages of the mistletoes

Attributes of the host such as anatomy and physiology, availability through time and space, nutrient and water content affect the establishment and survival of mistletoes. Chemical stimuli and Haustorium–Inducing Factors (HIF) may act at the initial stage of mistletoe establishment. In general, host trees may vary in traits within localities, thus affecting preference and ultimately determining local host specificity.

FIG. 4.

CHAPTER 3

Host specificity, nutrient and water dynamics of the mistletoe *Viscum rotundifolium* and its potential host species in the Kalahari of South Africa

D.Y. Okubamichael*, M.E. Griffiths, D. Ward

School of Biological and Conservation Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa

*Correspondence author. Tel.: +27 794522770; fax: +27 332605105.
E-mail address: dessu81@gmail.com (D.Y. Okubamichael).

Abstract

The mechanisms underpinning local host specificity in mistletoes remain elusive. We determined the degree of host specificity in the mistletoe *Viscum rotundifolium* at Pniel Estates, near Kimberley, South Africa. We found that *V. rotundifolium* parasitises only *Ehretia rigida* and *Ziziphus mucronata* at this site. Both commonly parasitised host species were not the most abundant trees, were not the tallest trees and did not have the highest water or nutrient content of trees in the area, although these factors are good predictors for mistletoe parasitism. Mistletoe seeds deposited on branches of *E. rigida* and *Z. mucronata* have a greater chance of attachment and subsequent survival, compared with those seeds deposited on co-occurring *Acacia* and other potential host species. The mistletoes had more negative water potentials than their host trees and by doing so they can passively maintain the flow of nutrients. In addition, the mistletoes had a N:Ca ratio >1, perhaps indicating active uptake from host phloem. Using both passive and active uptake may be a selective advantage in a nutrient-poor environment or on a nutrient-deficient host species.

Keywords: Active nutrient uptake; Germination; Host Compatibility; Hypocotyl; N:Ca ratio; Passive nutrient uptake

1. Introduction

Mistletoes are adapted to attach to branches of host trees and access the vascular tissue of host trees via a specialised structure known as the haustorium (Glatzel and Geils, 2009; Press and Phoenix, 2005). Mistletoes exclusively access nutrients and water from the host xylem. They photosynthesise their own carbon, but some mistletoe species access the photosynthetic product from the phloem of host trees (Glatzel and Geils, 2009). Mistletoe presence and abundance in a given area can be influenced by the distribution of suitable host species (Rödl and Ward, 2002), the degree of host specificity (Okubamichael, 2009), behaviour of avian dispersers (Aukema and Martínez del Rio, 2002), pollinators (Ladley et al., 1997), habitat fragmentation (Lavorel et al., 1999), fire (Kelly et al., 1997), herbivory (Ehleringer et al., 1986) and parasite–host chemical interactions (Tomilov et al., 2006; Yoder, 1999). Birds are the primary dispersers of mistletoe seeds and the influence of bird behaviour on the distribution of mistletoes has been studied more extensively than other determining factors (Aukema and Martínez del Rio, 2002; Lopez de Buen and Ornelas, 1999; Martínez del Rio et al., 1996; Okubamichael et al., 2011). Mistletoe distribution on a landscape scale is not well understood, although some models and predictions have been proposed (Aukema and Martínez del Rio, 2002; Mathiasen et al., 2008; Overton, 1994). On a local scale, most studies of mistletoes have determined that mistletoe infection is positively related to tree size, water and nutrient status, canopy cover and previous infection of a tree or a site (Aukema and Martínez del Rio, 2002; Okubamichael, 2009; Roxburgh and Nicolson, 2005).

Some mistletoe species parasitise a broad range of hosts but they can show local host preferences. Mistletoes may specialise on different co-occurring host species within different parts of their geographic range (Martínez del Rio et al., 1996; Norton and Carpenter, 1998) or even show differences in host preference between parasites in different parts of the same population (Gibson and Watkinson, 1989; Rödl and Ward, 2002). However, it is not clear why some mistletoes are generalists while others are specialists (Press and Phoenix, 2005). Hosts that most greatly enhance the growth, reproduction and fitness of the parasite population may be preferred; however, the mechanisms underpinning these responses are not well understood (Press and Phoenix, 2005).

The aerial habit of mistletoes enables them to minimise direct competition for soil resources with other plants and allows them to access more light (Vidal-Russell and Nickrent, 2008). Nonetheless, mistletoes must compete for water and nutrients from the branches of host trees. To do so, they maintain higher transpiration rates than their host trees by opening their stomata (Bowie and Ward, 2004; Glatzel, 1983; Ward et al., 2006). This usually enables them to maintain the flow of water and accumulate nutrients passively from the host xylem (termed “passive nutrient uptake”; Lamont 1983; Panvini and Eickmeier, 1993). However, mistletoes differ in their dependence on carbon from their host trees; some mistletoe species may actively uptake nutrients from host phloem.

Because nitrogen is highly phloem-mobile, while calcium is a large molecule and is phloem-immobile, a N:Ca ratio > 1 implies active uptake from host phloem and may suggest that water and nutrient acquisition are not tightly coupled (Bowie and Ward, 2004; Okubamichael, 2009; Panvini and Eickeier, 1993). This method has certain shortcomings. For instance, most of the N assimilated by the xylem-tapping mistletoe could be in the form of host amino acids transported in the xylem. If so, the imbalance may simply be due to the fact that cations other than Ca are transported in the xylem to balance the negative charge of the amino acids in solution. This method also ignores the possibility that Ca is preferentially stored in woody or senescent mistletoe tissue, explaining the imbalance between N and Ca in green leafy tissue. However, for the sake of this study we assumed the conventional method gives an estimate of what nutrients are obtained from the host vascular system.

Viscum rotundifolium parasitises hosts of at least 36 genera from several plant families including other mistletoes (*Tapinanthus* and *Viscum*) (Polhill and Wiens, 1998; Visser, 1981). However, *V. rotundifolium* seems to be host specific in some locations. Thus, the main objectives of this study were to: (1) determine infection patterns of *V. rotundifolium* and evaluate its local host specificity, (2) experimentally test mistletoe–host compatibility; (3) examine differences in water and nutrient availability among potential host trees in relation to *V. rotundifolium* infection (see Dean et al., 1994), (4) use the N:Ca ratio to determine whether this mistletoe species potentially accesses nutrients from xylem and/or phloem of host trees.

2. Methods

2.1. Survey

We conducted our survey in a semi-arid savanna in the Kalahari of South Africa, 30 km north of Kimberley at Pniel Estates, a site with a mean annual precipitation of 388 mm (Britz and Ward, 2007; Meyer et al., 2005). The area is dominated by black thorn (*Acacia mellifera*), umbrella thorn (*Acacia tortilis*) and camphor bush (*Tarchonanthus camphoratus*) (Britz and Ward, 2007; Meyer et al., 2005). We surveyed 23 randomly selected plots (50 m x 20 m) spaced at a minimum distance of 500 m. In each plot, we recorded the tree species composition and the number of *Viscum rotundifolium* in individual host trees. In addition, we randomly selected infected trees and counted the nearest 10 trees. We recorded the tree species and mistletoe infestation. This enabled us to quantify the number of neighbouring potential host species. We also measured the height of 15 trees selected randomly from each of the nine potential host species. We did not use one of the potential host species (*Grewia flava)* for our germination experiment (section 2.2) and for our leaf nitrogen analysis (section 2.4) because this deciduous species had dry branches and had no leaves during our field study.

2.2. Germination

We collected fresh branches of eight potential host species from Pniel: *Acacia erioloba*, *A. karroo, A. mellifera, A. tortilis*, *Searsia lancea*, *Tarchonanthus camphoratus*, *Ehretia rigida* and *Ziziphus mucronata*. For each species, we randomly selected five non-infested trees and from each tree we cut two branches. We also collected fruits randomly from 40 *V. rotundifolium* (20 on *E. rigida* and 20 on *Z. mucronata*). We transported the freshly cut branches and mistletoe fruits to the University of KwaZulu-Natal. The branches were placed horizontally in a shade house with a regular watering regime. Each branch was inoculated with 10 seeds of *V. rotundifolium* onto a total of 80 branches (eight potential host species x 10 branches from each species). We monitored the germination and the growth pattern of the hypocotyl for one month. A germinated *V. rotundifolium* may have one or two pairs of hypocotyls, thus the number of attachment points ranges from zero to four. The hypocotyl attachments were recorded as the number of points attached directly to the bark of the host branch.

2.3. Water potential (Ψ)

We measured predawn and midday leaf water potential (Ψ) of host species and the mistletoes using a Scholander pressure chamber (Scholander et al., 1965; Tyree and Hammel, 1972). We measured Ψ of the two main host species (*Z. mucronata* and *E. rigida*) and their mistletoes in September 2009. In addition, we selected two non-parasitised potential host species, *A. mellifera* and *A. tortilis*, for comparison. For each host species we selected 15 trees randomly and measured leaf Ψ for each tree at both predawn and midday. We measured Ψ in mistletoe–host pairs, using one leaf from the host and one from the mistletoe. All selected trees were similar in size and all leaves used for the measurements had a similar orientation in the canopy so as to minimise differences in microclimate. To ensure minimal water loss, we measured leaf Ψ immediately after the leaves were cut. The leaf petiole was examined under a simple 10× magnifying lens and readings were recorded with the first observation of water exuded on the surface of the petiole.

2.4. Nutrient status

We collected leaf samples from 15 randomly selected trees of each potential host species found at Pniel Estates. For *Z. mucronata* and *E. rigida*, we selected 15 infected trees with only a single infection by *Viscum rotundifolium.* We collected a pair of leaf samples for analysis of nutrient content, one from the host and the other from the mistletoe on the host. All samples were transported to the University of KwaZulu-Natal, oven-dried for 48 h at 70°C and then ground in a Wiley mill at 40 μm. Nitrogen (N; mg $gDWT^{-1}$) was analysed in a LECO FP2000 nitrogen analyser using the Dumas combustion method (AOAC International, 2000). Calcium (Ca; mg $gDWT^{-1}$) was analysed by the acid digestion method and measured by an atomic absorption spectrophotometer (AOAC International, 2000).

2.5. Statistical analysis

We tested the differences in frequency of germination success among potential host species using χ^2 tests. We used ANOVA with *post hoc* multiple comparisons to compare variation in: (1) the number of hypocotyl attachments among all the potential host species and (2) the nutrient and water content of the potential host species and their mistletoes (all analyses done in GenStat version 11). Student's t-tests were used to test the differences in nutrients between the mistletoe–host pairs. We used Pearson correlations to investigate how the mistletoes respond to the nutrient and water status of their host trees. We used 95% confidence limits to test if the N:Ca ratio was significantly different from 1. In all our analyses df = error degrees of freedom.

3. Results

3.1. Survey

The numbers of individuals of each potential host species in the transects and on the nearest ten host trees to infected host trees showed that at least nine potential host species of *V. rotundifolium* coexist at Pniel Estates (Fig. 1). However, *V. rotundifolium* was observed on only two species, *Ehretia rigida* and *Ziziphus mucronata* (n = 1553 trees in all the plots). Prevalence (% number of infected host trees per species) was higher for *Z. mucronata* (42/125 = 34%) than *E. rigida* (6/28 = 21%). Number of parasites per individual host tree was similar for *E. rigida* (66/28 = 2.36) and *Z. mucronata* (311/125 = 2.49). The mean height of trees was significantly different among potential host species ($F_{8,134} = 44.29$, $P < 0.001$); this showed that *E. rigida* and *Z. mucronata* are not the tallest trees in Pniel Estates (Table 1). *Z. mucronata* is approximately half the size of *A. erioloba*—the tallest tree species at Pniel—and shorter than three other potential host species (*A. karroo*, *S. lancea* and *A. tortilis*). *E. rigida* is only marginally taller than the shortest host tree, *A. mellifera*, with an average height of only one third that of *A. erioloba*.

3.2. Germination

Germination of *V. rotundifolium* seeds was 90% of 750 inoculated seeds and there was no significant difference in germination rate among seeds grown on the eight potential host species (χ^2 test). The number of hypocotyl attachments was significantly different among potential host species ($F_{7,749} = 4.94$, $P < 0.001$),with more hypocotyl attachments on *Z. mucronata* and *E. rigida*.

3.2. Nutrient and water status

Leaf water potential (Ψ) differed significantly among potential host species at predawn ($F_{3,39} = 31.42$, $P < 0.001$) and at midday ($F_{3,39} = 12.50$, $P < 0.001$). Interestingly, parasitised host species (*Z. mucronata* and *E. rigida*) were more water stressed than the non-parasitised potential host species we measured (*A. mellifera* and *A. tortilis*). We considered there was no difference in Ψ between non-parasitised and parasitised (by single mistletoe) host trees of *Z. mucronata* / *E. rigida*. Previous studies showed that slight mistletoe infestation did not reflect a difference in host water status (Bowie and Ward, 2004; Ward et al., 2006). The Ψ of the mistletoes on *Z. mucronata* were significantly more negative than the Ψ of their hosts at both predawn ($F_{1,29} = 7.08$, $P = 0.013$) and midday ($F_{1,29} = 19.67$, $P < 0.001$). Similarly, the Ψ of the mistletoes on *E. rigida* were significantly more negative than the Ψ of their hosts at both predawn ($F_{1,29} = 24.87$, $P < 0.001$) and midday ($F_{1,29} = 7.61$, $P = 0.01$). The Ψ of mistletoes on *Z. mucronata* was positively correlated with Ψ of their host trees at predawn ($r = 0.82$, $F = 27.54$, $P < 0.001$, $df = 30$) and midday ($r = 0.86$, $F = 37.74$, $P < 0.001$, $df = 30$). However, the Ψ of the mistletoes on *E. rigida* were not correlated with those of their hosts at either predawn ($r = 0.32$, $F= 1.48$, $P = 0.25$, $df = 30$) or midday ($r = 0.49$, $F = 4.11$, $P = 0.06$, $df = 30$).

Nitrogen (N) concentration was significantly different among potential host species ($F_{7,119} = 59.39$, $P < 0.001$). At least three *Acacia* species (*A. erioloba*, *A. karroo* and *A. mellifera*) were higher in N than the other host species in Pniel. However, they were not parasitised by *V. rotundifolium*. N concentration of the *E. rigida* hosts was higher than *Z. mucronata* hosts. The N concentration of mistletoes on *Z. mucronata* was significantly higher than their host trees ($t = 2.70$, $P = 0.017$, df = 30). In contrast, the N of mistletoes on *E. rigida* was significantly less than that of their host trees ($t = 5.60$, $P < 0.001$, df = 30). There was no significant correlation between the N concentration of the mistletoes and their host trees ($r = -0.24$, $F = 0.80$, $P = 0.39$, df = 30 for *Z. mucronata*; $r = 0.20$, $F = 0.54$, $P = 0.48$, df = 30 for *E. rigida*). The N:Ca value (mean ± SE) of mistletoes on *Z. mucronata* was 1.45 ± 0.036 and mistletoes on *E. rigida* was 1.61 ± 0.045.

5. Discussion

Viscum rotundifolium is the most widespread and least host-specific of all *Viscum* species in southern Africa (Visser, 1981; Polhill and Wiens, 1998). However, at Pniel Estates, *V. rotundifolium* parasitised only *Z. mucronata* and *E. rigida* among many other species that are hosts elsewhere (Visser, 1981), such as *A. erioloba, A. mellifera*, *A. karroo* and *A. tortilis*, *T. camphoratus*, *S. lancea* and *G. flava*. Why this mistletoe is locally host specific on *Z. mucronata* and *E. rigida* while many alternative potential hosts exist sympatrically is unknown. Parasites may perform better on hosts with a high nitrogen or water content, greater abundance, taller host species, or those with lower defence capacity (Seel et al., 1993; Ehleringer et al., 1985; 1986b). Contrary to what might be expected based on previous findings (Norton and Carpenter, 1999; Aukema and Martínez del Rio, 2002; Ward et al., 2006; Okubamichael, 2009), *V. rotundifolium* parasitises *Z. mucronata* and *E. rigida*, which have lower N concentration, more negative Ψ, are not the most common species and are not the tallest host species at Pniel.

We found that parasitised hosts did not have greater water content which may indicate that this mistletoe species does not select hosts on the basis of water status. Xylem-tapping mistletoes maintain more negative Ψ than their hosts, which enables them to passively obtain water and nutrients from the host (Bannister and Strong, 2001; Davidson and Pate, 1992; Press and Phoenix, 2005). To do so, mistletoes open their stomata and experience high water loss. Usually a higher transpiration rate enables the mistletoes to accumulate more minerals, especially nitrogen (Dean et al., 1994; Glatzel, 1983; Lamont and Southall, 1982; Okubamichael, 2009; Panvini and Eickmeier, 1993). This is consistent with our finding for *V. rotundifolium* on *Z. mucronata* and it confirms that mistletoes exceed the water potential of their host trees and access nutrients passively (Bowie and Ward, 2004; Glatzel and Geils, 2009). It is not clear why *V. rotundifolium* growing on *E. rigida* failed to concentrate more N than their hosts, although the hosts themselves had a higher nitrogen concentration than *Z. mucronata* (Bowie and Ward 2004 and Marshall et al. 1994). It could be argued that mistletoes require only a certain level of nitrogen and will not take up more N from the host than needed.

The N:Ca ratio of the mistletoes is used as an index of nutrient access from the host phloem or xylem (Bowie and Ward, 2004; Okubamichael et al., 2009; Panvini and Eickmeier, 1993). We found that N:Ca was significantly > 1 in mistletoes growing on both host species, which perhaps indicates active nutrient uptake. This implies that *V. rotundifolium* uses active nutrient uptake on both hosts and can access nutrients from the phloem. We suggest that to survive in a nutrient-poor environment and on nutrient-poor host species it is of great advantage for the mistletoes to use both passive and active uptake mechanisms.

Frugivorous birds affect the distribution of mistletoes by causing local aggregation on individual trees and in particular sites (Aukema and Martínez del Rio, 2002; Green et al., 2009; Lopez de Buen and Ornelas, 1999; Martínez del Rio, 1996). Earlier studies (e.g., Aukema and Martínez del Rio, 2002; Roxburgh and Nicolson, 2005; Ward et al., 2006) report a strong positive correlation between mistletoe parasitism and host tree height. Because birds perch on tall trees, *A. erioloba* would be expected to be preferentially parasitised. However *Z. mucronata* and *E. rigida* are among the smallest tree species in Pniel Estates (Table 1).

Mistletoes are also more numerous on hosts whose fruits ripen at the same time as the mistletoes, because the hosts themselves are an abundant fruit source for frugivorous birds. For example, in one mistletoe species, *Phoradendron hexastichum*, that parasitises the dioecious plant, *Cecropia schreberiana*, female hosts with fruits had twice the infection of male hosts with no fruits (Carlo and Aukema, 2005). Fruits of *Z. mucronata* and *E. rigida* ripen the same time as those of the mistletoe, while the other available potential host species do not have fruits that would attract birds. Thus, it can be inferred that birds perch preferentially and disperse mistletoe seeds on *Z. mucronata* and *E. rigida* because they are attracted by the fruits of these host species. However, it cannot be assumed that birds do not visit and deposit mistletoe seeds onto other potential host species. Absence of a single infection on all other potential host species is less likely to be explained by the effect of seed dispersers.

In the mistletoe life cycle, seed deposition/retention, germination and attachment are preconditions for survival success and every step may affect the survival of mistletoes. For example, the host specificity of the mistletoe *Struthanthus* aff. *polyanthus* was positively correlated to hosts with rough bark, which retain mistletoe seeds better than hosts with smooth bark (Arruda et al., 2006). *Z. mucronata* and *E. rigida* both have fairly smooth bark in comparison with other potential host species at Pniel Estates but *V. rotundifolium* was found to be host specific on both hosts. We found that *V. rotundifolium* has no specialised substrate requirements and germinated on all potential host species without significant differences.

Seeds may not establish or survive on some host species due to host incompatibility (Yan, 1993). Thus, differences in the frequency of mistletoe infections on different tree species may result from mistletoe–host compatibility. Our study clearly showed a significant difference in the number of hypocotyl attachments, a structure that gives rise to a haustorium. Mistletoe seeds that have more than one attachment point would likely attach more firmly to the branches of host trees and survive better than those seeds with only one hypocotyl. This indicates that host specificity is already operating at the stage of haustorium formation. Host specificity at the stage of haustorium formation has been demonstrated in reciprocal transplant experiments that found haustorium growth to be greater on source- or parent-host species (Rödl and Ward, 2002; Okubamichael, 2009), indicating that there is tight mistletoe–source coupling (Glazner, 1988; Clay et al., 1985). To be host specific, the parasite may need chemical cues from suitable hosts to trigger haustorial development, as studies in other parasitic species have reported (Matvienkoet al., 2001; Runyon et al., 2006). For host specificity to be heritable, preferences for such cues must be inherited and passed to the next generation (Jamison and Yoder, 2001), but this has never been investigated in mistletoes.

6. Conclusions

Viscum rotundifolium is host specific on *Z. mucronata* and *E. rigida* in our study area. Other studies have demonstrated that mistletoes prefer host species that are tall, abundant and nutrient- and water-rich. Mistletoe–host compatibility in this mistletoe was demonstrated by the fact that seeds form more cotyledons and hypocotyl attachments on *E. rigida* and *Z. mucronata* than seeds deposited on co-occurring host species. The ability of a mistletoe to recognise its host trees seems to appear shortly after germination and may be caused by genetic, phenological or chemical (e.g., bark chemistry) differences among host species that subsequently direct local host specificity. In addition, this mistletoe species uses both passive and active uptake, which may be a selective advantage in a nutrient-poor environment or on a nutrient-deficient host species.

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Table 1.

Height (Mean ± SE) of nine potential host species (n = 15 per species) of *V. rotundifolium* at Pniel Estates (South Africa). *Acacia erioloba*, *A. karroo*, *Searsia lancea* and *A. tortilis* are taller than the other four potential host species.

Potential host species	**Mean ± SE height (m)**
Acacia erioloba	5.73 ± 0.19
A. karroo	4.90 ± 0.32
Searsia lancea	3.65 ± 0.31
A. tortilis	3.29 ± 0.21
Ziziphus mucronata	2.83 ± 0.12
Tarchonanthus camphoratus	2.38 ± 0.14
Grewia flava	2.19 ± 0.06
Ehretia rigida	2.16 ± 0.15
A. mellifera	2.11 ± 0.11

Figure captions

Fig. 1. Frequency of the nine potential hosts of *Viscum rotundifolium* at Pniel Estates. In this locality only *Ehretia rigida* (Er) and *Ziziphus mucronata* (Zm) were parasitised hosts. The other seven potential host species which coexist in the area were not parasitised although they are hosts elsewhere: *Acacia erioloba* (Ae), *A. karroo* (Ak), *A. mellifera* (Am), *A. tortilis* (At), *Grewia flava* (Gf), *Searsia lancea* (Sl), *Tarchonanthus camphoratus* (Tc).

Fig. 2. Hypocotyl attachments (Mean ± SE) on the eight potential hosts (n = 10 seeds per host). Abbreviations as in Fig. 1.

Fig. 3. Mean N ± SE (mg $gDWT^{-1}$) of mistletoes and their potential hosts. Abbreviations as for Fig. 1. VrZm indicates *Viscum rotundifolium* on *Z. mucronata* and VrEr indicates *Viscum rotundifolium* on *E. rigida.*

Figures

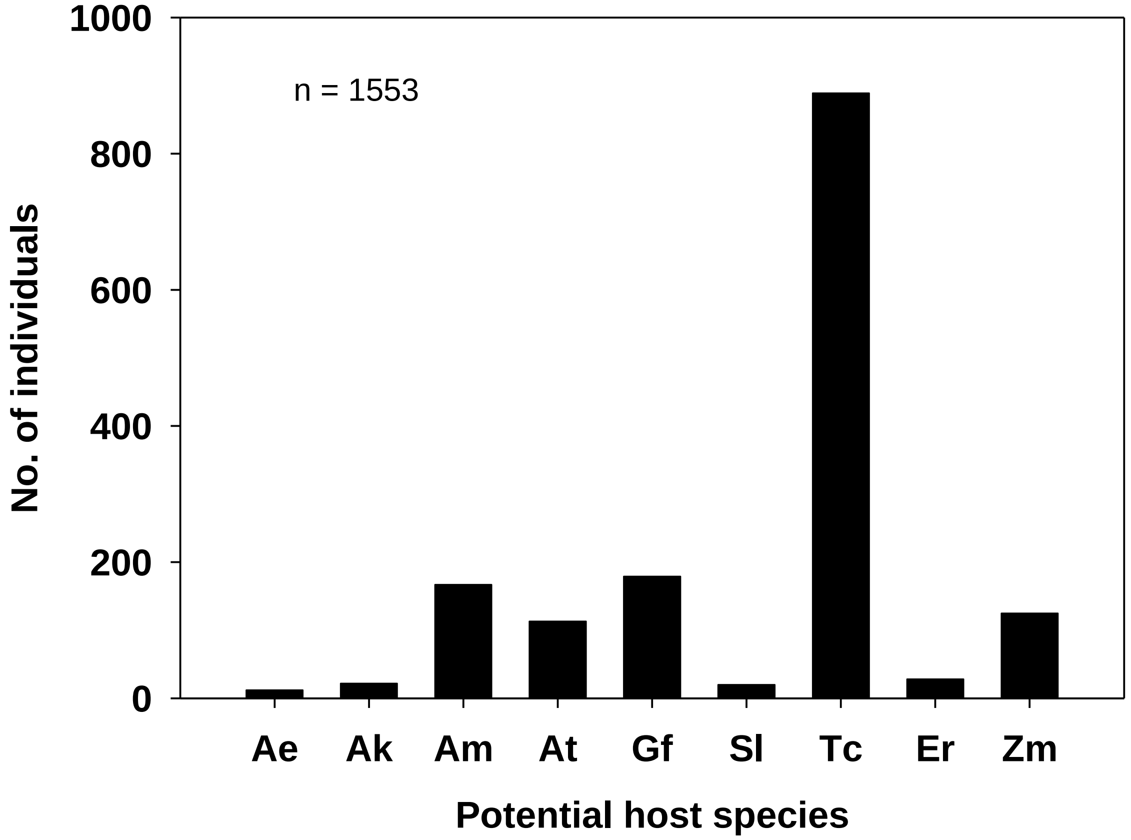


Fig. 1.

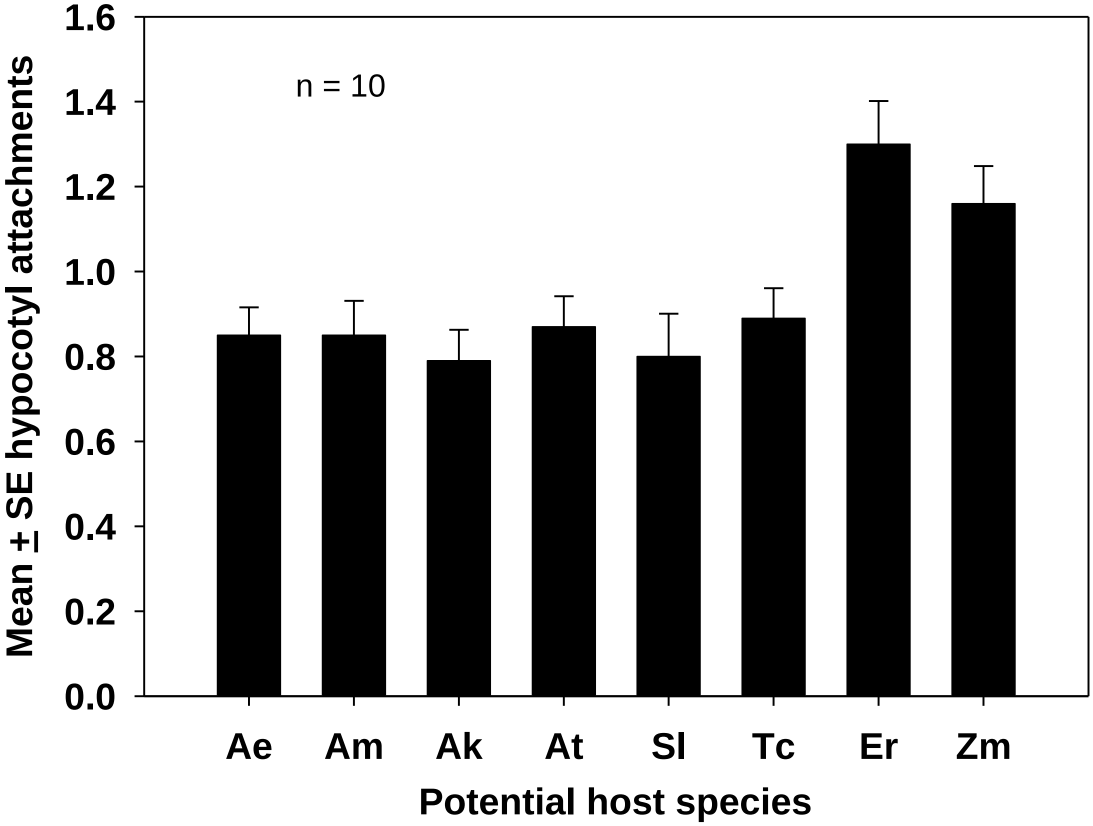
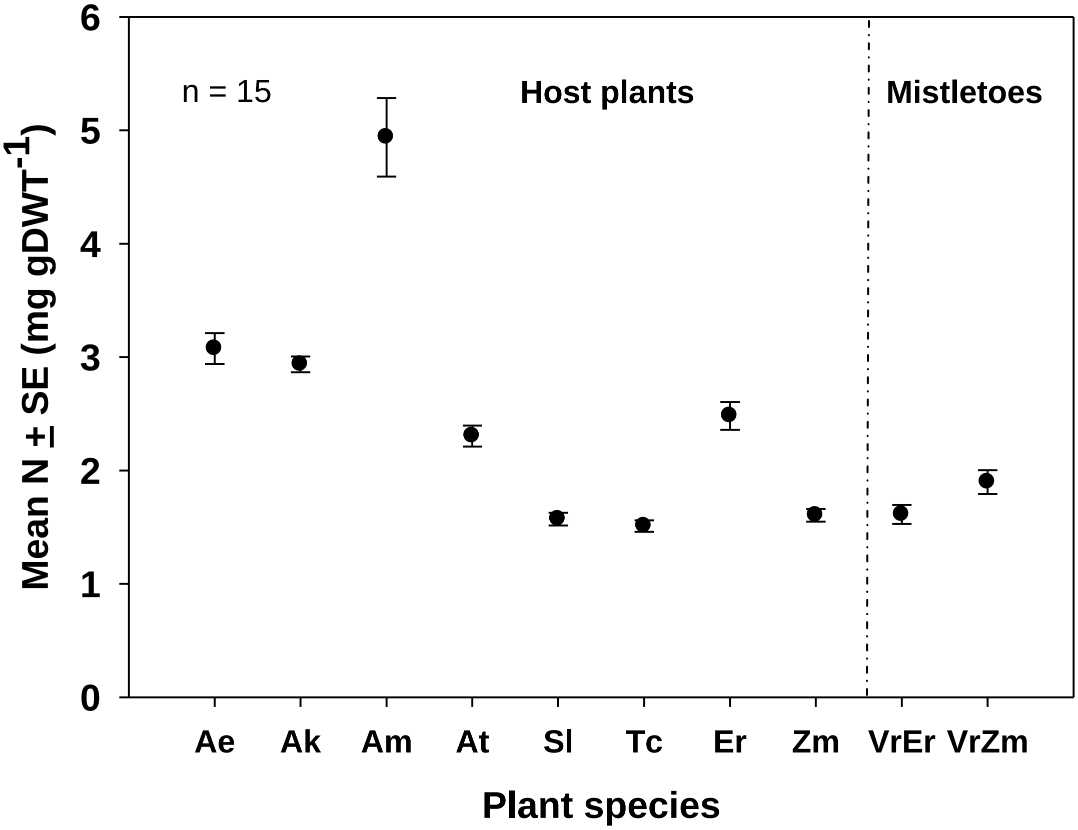

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Mean ± SE hypocotyl attachments
n = 10
Ae
Am
Ak
At
Sl
Tc
Er
Zm
Potential host species

Fig. 2.



6
5
4
3
2
1
0
n = 15
Host plants
Mistletoes
Mean N ± SE (mg gDWT^{-1})
Ae
Ak
Am
At
Sl
Tc
Er
Zm
VrEr
VrZm
Plant species

Fig. 3.

CHAPTER 4

Reciprocal transplant experiment suggests host specificity of the mistletoe *Agelanthus natalitius* in South Africa

Desale Y. Okubamichael*, Megan E. Griffiths and David Ward

School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209,
Pietermaritzburg, South Africa

Running title: Host specificity of a mistletoe *Agelanthus natalitius*

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*Corresponding author

Tel.:+27 794522770

Fax: +27 332605105

E-mail address: *dessu81@gmail.com*

Abstract: We surveyed the community composition of trees that host the mistletoe *Agelanthus natalitius* (Loranthaceae) at two sites in South Africa. We recorded five infected host species but we focused on the two most commonly infected species, *Acacia karroo* and *A. caffra*, because other host species were either rare or rarely infected in the study sites. *Acacia karroo* was the most abundant host species at both sites, followed by *A. caffra.* In Mtontwane prevalence (percentage of infected trees) was higher on *A. caffra* than *A. karroo*, but *A. karroo* had a higher intensity of infection (number of mistletoe infections per tree) at both sites. Prevalence and intensity of infection showed a significant positive relationship with tree size for both host species at both sites. We tested the genotype-by-environment interaction effects in this mistletoe by conducting reciprocal transplant experiments using *A. natalitius* seeds on the two host species in the field. Initial germination was not site-, substrate- or host-sensitive. However, a general pattern was found that hypocotyls of the germinated seeds grew longer when seeds were placed on the same host species as the parent plant within their own source locality. Consistent with this observation, mistletoes placed on their source host species generally had higher survival than those transferred to non-source host species after 6 mo. Overall, mistletoe seeds from parent plants on *A. karroo* and mistletoe seeds placed on *A. karroo* had the highest survival. This could be the result of an adaptation of the mistletoe to the most frequently encountered host species.

Key Words: adaptive phenotypic plasticity, *Acacia caffra*, *Acacia karroo*, *Agelanthus natalitius*, coevolution, G × E interactions, prevalence, intensity, host specificity, reaction norms

INTRODUCTION

Mistletoes comprise a diverse group of hemiparasitic flowering plants that have become specialised to accessing nutrients and water from host trees via a haustorium (Stewart & Press 1990). Mistletoes vary widely in their degree of host specificity, ranging from extreme specialists that parasitise a single host species to generalists that use many different host species with no apparent infection difference among host species (Dean *et al*. 1994, Norton & Carpenter 1998, Norton & de Lange 1999). In some mistletoe species, host infection varies geographically such that at a given location a mistletoe species may infect only part of its potential host set (Okubamichael *et al*. 2011a, Rödl & Ward 2002, Thorogood *et al*. 2009). It could be argued that mistletoes use the most abundant trees in the community simply because there are few alternatives (neutral interactions governed by species abundance) but a mechanism of host adaptation is still required. The factors that explain why mistletoe species only infect a subset of the available host species in a given locality are not clear.

Host specificity in mistletoes is a composite measure of relative abundance of mistletoes on the parasitised host species (Mathiasen *et al.* 2008). Differential parasitism of mistletoes of the available host species is often expressed as host preference (i.e., only a subset of host trees are infected). Host compatibility at the genetic, mechanical, physiological and biochemical level are most likely to affect the growth and survival of mistletoes on host trees, subsequently determining host preference in mistletoes (Fadini 2011, Okubamichael *et al*. 2011a, Yan 1993a, Yan & Reid, 1995). Host preference may not be necessarily driven by traits of host trees that directly affect the growth and survival of mistletoes. For example, birds may influence differential seed dispersal and thus affect host preference in mistletoes. It is therefore crucial to quantify the differential parasitism of mistletoes in the field and to test host compatibility.

Genotype performance across environments can be reflected by reaction norms tested by an interaction effect in analysis of variance (Lynch & Walsh 1998). If the genotypic responses to environmental changes of two or more reaction norms are non-parallel, it indicates a genotype by environment (G × E) interaction (Japhet *et al.* 2009). In this particular study, we tested the contribution of G × E interactions to local adaptation of mistletoes on host species at a specific site by means of reciprocal transplant experiments in two combinations of mistletoe–host populations, using hypocotyl length and survival as indices of growth performance. The growth of the hypocotyl, the structure that eventually forms the haustorium, is hypothesised to be an essential trait determining the establishment success of parasitic plants (Yoder 1999).

The aims of this study were to quantify the degree of host specificity and to evaluate the factors that determine the local distribution and specialisation of the mistletoe *Agelanthus natalitius* (Loranthaceae) in two populations in South Africa. We predicted that host recognition and preference occurs during the early developmental stages of germination and haustorium formation in mistletoes and would be reflected in the survival of mistletoes.

METHODS

Study sites and species

We surveyed the tree community and the population distribution of the mistletoe *Agelanthus natalitius* in two sites ca. 110 km apart in KwaZulu-Natal, South Africa: Highover (29˚ 54'S, 30˚ 05'E) and Mtontwane (28˚ 48'S, 29˚ 56'E). The vegetation of Mtontwane is characterised by *Acacia caffra, A. karroo, A. tortilis* and *A. nilotica* woodlands and thickets. The vegetation of Highover is characterised by *A. karroo, A. caffra* and *A. ataxacantha* woodlands and thickets, with a denser vegetation and steeper terrain than at Mtontwane.

Agelanthus natalitius is widely distributed throughout southern Africa (Polhill & Wiens 1998, Visser 1981). This mistletoe species parasitises at least 11 tree genera, including *Acacia*, *Carya*, *Citrus*, *Combretum*, *Dichrostachys*, *Dombeya*, *Grewia*, *Pterocarpus*, *Punica*, *Sclerocarya* and *Terminalia* (Visser 1981, Wiens & Tolken 1979). However, geographic variation in the infection patterns over the parasite's range suggests that *A. natalitius* may be locally specialised on particular host species.

Field survey

Host composition was assessed by quantifying the tree community in the two study sites (Highover and Mtontwane). We randomly selected and surveyed 64 plots (32 plots in each site) (20 × 50 m). We identified all tree species in each plot; any species that had at least one infected tree in the study site was recorded as host species. We counted the number of mistletoes on each tree and measured tree size (height and diameter) because it may be an important parameter that determines the infection pattern of mistletoes among host trees (Overton 1994, Roxburgh & Nicolson 2007, Ward *et al*. 2006). Tree height was measured with a measuring pole; if the tree was inclined or growing on a slope, trigonometric calculations were applied to determine tree height from the ground. Diameter at breast height (dbh) of the trunk was measured approximately 1.5 m above the base of the stem; in the case of multistemmed trees the following calculation was used: (dbh = SQRT [sum (stem diameter^2)]). Trees below 2 m in height and < 3 cm in diameter were excluded, because these were never parasitised by a mistletoe (pers. obs.).

Reciprocal transplant germination experiment

We carried out field reciprocal transplant germination experiments using seeds of mistletoes parasitising the two main host species, *A. karroo* and *A. caffra*, in our two field sites. First, we bagged unripe mature fruits using nylon mesh bags to protect fruits from bird consumption 1 mo prior to collection. To avoid pseudoreplication, we randomly selected 20 individual mistletoes in different host trees from each of the two main host species in both sites. We collected fully ripe and undamaged fruits by hand picking. For each seed used for the experiment, we manually removed the exocarp (pulp cover) and endocarp (the skin covering the seed). This is essential because the layers covering the seed can act as barriers to germination in mistletoes (Ladley & Kelly 1996, Okubamichael *et al*. 2011b). Furthermore, this enables the sticky viscin surrounding the seed to be exposed, which facilitates the temporary attachment of mistletoe seeds to host branches. We worked the viscin by hand to increase its stickiness (for a similar method, see Ladley & Kelly 1996, Sargent 1995).

We used non-parasitised individual trees in our experiment to avoid any effects of previous infection and susceptibility. Trees ranged in height from 2–6 m and were all located in open areas to avoid shade effects (Okubamichael *et al*. 2011b). We monitored a total of 64 individual trees that were marked at Mtontwane and Highover. For each host species in each site we had two groups: one group received seeds of mistletoes obtained from *A. caffra* and the other group from *A. karroo.* Each group consisted of eight trees. For each experimental tree, we selected two healthy branches at the same position within the canopy and of similar size (8–12 cm girth, although mistletoes are capable of growing on much smaller twigs). Twig size was based on the optimal size for seedling establishment based on previous studies in this particular mistletoe species. In addition, this host twig size enabled us to inoculate many seeds on a single twig. Most importantly, if many seeds had established during the experiment, this branch size would maintain many seeds better than smaller twigs that would not withstand high infection of mistletoes (Sargent 1995). Each branch received 10 seeds linearly orientated and placed 3 cm apart; of these, five seeds were from Highover and five seeds from Mtontwane. Each seed was marked with a distinctly coloured pin. We applied a paired design (one local and one non-local seed) for each pair to experience identical environmental conditions including the current host species, bark surface and branch diameter (Rödl & Ward 2002).

We monitored the seed germination, hypocotyl growth and survival after 1 wk, after 1 mo and after 6 mo at both sites. At each time period we recorded the condition of each seed as germinated (indicated by protrusion of the fresh green seed embryo), dead (where colour had changed to black and the seed had become dried and shrivelled) or lost *in situ*. Where germination occurred we measured hypocotyl length from the base of the viscin layer to the distal end of the protruded hypocotyl. Hypocotyls that curved towards the substrate and attached to the host substrate were considered to have successfully established because the haustorium will form in this position. However, if the hypocotyls grow away from the host bark they do not attach to the host and do not become successfully established. The experiment was designed to test the G × E interactions in response to site, source and current substrate while other abiotic factors were equivalently experienced by mistletoe seeds.

Statistical analysis

Host species and germination (expressed as the percentage of seeds that germinated) were analysed for differences in frequency using χ^2 tests (SPSS 18.0 for Windows). The difference in prevalence (i.e., the percentage of individual trees carrying at least a single mistletoe infection) between host species and correlations of prevalence with tree height and dbh were tested using binary logistic regression. We also tested prevalence of grouped host trees with height (1-m class width) and with dbh (10-cm class width) after prevalence was arcsine-square root-transformed. The relationship between intensity of infection and tree height and trunk dbh was further analysed with GLIM, as the frequency distribution of parasitism among the two host species followed a negative binomial distribution (Krebs 1989) (variance/mean = 6.30/0.79 and k = 0.16, N = 1464, χ^2 for goodness of fit = 9.17, df = 3, P = 0.027). We used ANOVA to test the differences (1) among host species in mean height and dbh, (2) among infected and uninfected trees in mean height and dbh. We used ANOVA to test the effect of site, source, current substrate and time on hypocotyl length in the mistletoe seedlings (SPSS 18.0 for Windows). Significant interactions in an ANOVA indicate that the reaction norms are not parallel (i.e., that there is a G × E interaction). We performed Kaplan-Meier survival analysis (Kaplan & Meier 1958, SPSS 18.0 for Windows). over 6 mo. When overall significance was confirmed we did a pairwise comparison and a new alpha value was computed to account for the Bonferroni correction. We fitted a sigmoid curve to determine the effect of hypocotyl length within 6 mo on survival of the mistletoe seedlings.

RESULTS

Field survey

At the two study sites, five host species were recorded as being parasitised by the mistletoe *Agelanthus natalitius*, namely *Acacia caffra*, *Acacia karroo*, *Acacia tortilis*, *Acacia nilotica* and *Leucaena leucocephala* (all Fabaceae; nomenclature after Van Wyk & Van Wyk 1997). *Acacia tortilis*, *A. nilotica* and *L. leucocephala* were excluded from further analyses because these species were either rare in the study sites or had few infected individuals. *Acacia tortilis* was absent in Highover and only one *A. nilotica* was recorded in the survey plots at that site. Similarly, only two individuals of *L. leucocephala* were recorded in a single plot at Highover, both of which were infected. At Mtontwane, only a few individuals of *A. nilotica* (N = 3) and *A. tortilis* (N = 9) were infected, each supporting a single *Agelanthus natalitius* individual. Thus, all statistical analyses were applied to the two most common host species, *A. karroo* and *A. caffra*, which grow abundantly at both sites and were recorded with many trees infected by *Agelanthus natalitius*.

We recorded a total of 1464 trees (*Acacia karroo* and *A. caffra*) hosting 1202 mistletoes (*Agelanthus natalitius*) in the 64 surveyed plots (20 × 50 m) from the two sites (Highover and Mtontwane). *Acacia karroo* was significantly more abundant than *A. caffra* at both sites; there were almost four times as many *A. karroo* as *A. caffra* at Highover and three times as many *A. karroo* as *A. caffra* at Mtontwane (Table 1). There was no significant difference in prevalence of mistletoe infection on the two host species at Highover, but a significantly greater percentage of *A. caffra* trees was parasitised at Mtontwane (Tables 2 and 3). Infection intensity (number of mistletoes per tree) was higher for *A. karroo* (0.73 ± 0.04 and 1.03 ± 0.64) than for *A. caffra* (0.66 ± 0.01 and 0.89 ± 0.035) at Highover and Mtontwane, respectively (Table 4). At Highover, infected trees of *A. karroo* had an average of 3 mistletoes per infected tree as compared to 2.5 per infected tree of *A. caffra.* At Mtontwane, infected trees of *A. karroo* had an average of 4 mistletoes per infected tree as compared to 2.5 per infected tree of *A. caffra*. If we had excluded the two highly infected trees at Highover and one at Mtontwane that many mistletoe-dispersing birds used for nesting, the number of mistletoes per infected tree of *A. caffra* would drop to 1.

There was no significant difference in tree height and trunk dbh between *A. karroo* and *A. caffra* trees in either site (Table 5). However, the mean height and trunk dbh of infected trees were significantly greater than for uninfected trees for both species in both sites (Table 6, Figure 1). The logistic regression analysis indicated that both height and dbh had a significant positive effect on the probability of infection (slopes for prevalence and height ranged from 0.38–0.85, range in Wald statistic = 12–40, $P < 0.001$, range in N = 157–622). A similar result was obtained for dbh, although there was a lower slope (slopes for prevalence and dbh ranged from 0.050–0.096, range in Wald statistic = 5–41, $P < 0.001$, range in N = 157–622). Prevalence of grouped trees was the proportion of infected trees in the given class. Prevalence was positively correlated with height (1-m class width) and with dbh (10-cm class width) (results after prevalence was arcsine-square root-transformed, height, range in r = 0.90–0.95, range in F = 17–40, $P < 0.05$, range in N = 157–622; and dbh, range in r = 0.90–0.97, range in F = 12–50, $P < 0.05$, N = 157–622). However, the dbh class of *A. caffra* in Highover was not significantly positively correlated with prevalence (r = 0.50, F = 1.00, P = 0.39, N = 157) (Figure 2).

The distribution of *Agelanthus natalitius* among host trees was strongly aggregated, meaning that most potential hosts were not infected, while a few individual host trees were highly infected and supported most of the parasites (e.g., we observed a single host tree with 56 mistletoes in our study). The GLIM analysis showed that the number of mistletoes per host tree (infection intensity) had a positive significant relationship with tree height (range in slopes = 0.30–0.70, $P < 0.001$, range in N = 157–622). A similar result was obtained for dbh, although with a lower slope (range in slopes = 0.024–0.032, $P < 0.001$, range in N = 157–622).

Reciprocal transplant germination experiment

Germination of *Agelanthus natalitius* seeds started within 1 d in both sites and reached 100% after 1 wk, independent of host substrate and site. Within the first mo, 7% of the germinated seeds of *A. natalitius* were unsuccessful (either they died or were lost in situ) and there was no significant difference in germination success whether they were placed on a source or non-source host species and whether they had been translocated to a different site or were germinated within their locality ($\chi^2 = 5$, $P = 0.78$, $N = 1280$). In this case, we compared eight combinations at each site (see Appendix 1 for the details of each combination). In contrast to germination success, hypocotyl length was significantly influenced by the three way interactions (site × source × current substrate). This is important to note because it reflects the presence of genotype × environment (G × E) interactions (Table 7).

At Highover, mistletoe seeds placed on the same current substrate as their source host within their site grew significantly longer hypocotyls than those transferred to non-source hosts, except for mistletoes on *Acacia karroo* (Figure 3a and Appendix 1a). We noticed an unidentified infection on mistletoe seeds placed on *A. karroo* in Highover and we suspect that this resulted in very short hypocotyl growth of these mistletoes (Figure 3a and Appendix 1a). As a result, mistletoes from *A. karroo* of Highover that were placed on *A. karroo* at Highover grew much shorter hypocotyl than mistletoes from *A. karroo* of Mtontwane and placed on *A. karroo* at Highover (Appendix 1).

In Mtontwane, mistletoe seeds placed on the same current substrate as their source host within the same site grew the longest hypocotyls (Figures 3b and Appendix 1b). Thereafter, mistletoe seeds obtained from *Acacia caffra* and placed on *A. karroo* within the same site had longer hypocotyls than those obtained from *A. karroo* but obtained from Highover (Figure 3b and Appendix 1b). Similarly, mistletoes obtained from *A. karroo* hosts but placed on *A. caffra* within the site grew longer hypocotyls than those obtained from Highover.

Overall, if substrate only was compared, mistletoe seeds placed on *A. karroo* had longer hypocotyls than mistletoe seeds placed on *A. caffra* in Highover and Mtontwane (Figures 3 and Appendix 1). This was the case because within a site if mistletoes were transferred, mistletoes from *A. karroo* grew less on *A. caffra* while mistletoes from *A. caffra* placed on *A. karroo* grew much longer hypocotyl in both sites.

The number of hypocotyls that curved towards and contacted the host substrate was also higher when mistletoe seeds were placed on the source host species ($\chi^2 = 97$, $P < 0.01$, $N = 309$). In this case, we also compared the eight combinations at each site (see Appendix 1 for the details of each combination). Even when we excluded source and site effects by considering current host substrate only hypocotyls attached better on *A. karroo* ($\chi^2 = 28$, $P < 0.01$, $N = 309$). Hypocotyl length over 6 mo was positively correlated with the probability of survival of mistletoe seedlings ($r = 0.95$, $F_{1, 8} = 57.55$, $P < 0.001$) (Figure 4).

Survival

Overall the survival curves showed significant differences across all 16 combinations over 6 mo (Table 8). Three groups out of four showed a significant difference in survival curves (Figure 5). Overall, survival was higher for mistletoe seeds on *Acacia karroo* than on *A. caffra*.

At Highover**,** mistletoes placed on *Acacia karroo* did not show any significant differences in survival regardless of what site or host species they were obtained from (Figure 5a). However, mistletoes placed on *A. caffra* at Highover showed significant differences (Figure 5a). Survival of mistletoes from *A. karroo* at Mtontwane placed on *A. caffra* at Highover (mkM × kH) was significantly lower than for the other three combinations at Highover.

At Mtontwane, mistletoes obtained from *Acacia karroo* at the same site had the highest survival on *A. karroo* (mkM × kM), which was followed by mistletoes obtained from *A. caffra* and placed on *A. karroo* at Mtontwane (mcM × kM) (Figure 5b). Mistletoes from *A. karroo* at Highover placed on *A. karroo* at Mtontwane had intermediate survival, while mistletoes obtained from *A. caffra* at Highover placed on *A. karroo* at Mtontwane had the lowest survival of all seeds inoculated on *A. karroo*. Mistletoes from *A. karroo* at Mtontwane placed on *A. caffra* at Mtontwane (mkM × cM) had the highest survival, followed by mistletoes of *A. caffra* from Mtontwane placed on *A. caffra* in Mtontwane (mcM × cM) (Figure 5b). Mistletoes of *A. karroo* from Highover placed on *A. caffra* at Mtontwane (mkH × cM) and mistletoes of *A. caffra* from Highover placed on *A. caffra* at Mtontwane (mcH x cM) had the lowest survival of seeds inoculated on *A. caffra* (Figure 5b). This demonstrates that mistletoe seedlings perform better on the parental host species and in the same site from which they were obtained.

DISCUSSION

Host abundance

Agelanthus natalitius parasitises several host species and its local distribution can be patchy. Our study found *Acacia karroo* to be the most compatible host species for *Agelanthus natalitius* in two field sites in KwaZulu-Natal, South Africa based on the fact that higher infection intensity (number of mistletoes per tree) was recorded on *A. karroo.* The reciprocal transplant germination experiment in the field also showed that overall the hypocotyls of *Agelanthus natalitius* seedlings grew better on *A. karroo* than *A. caffra*. This demonstrates a general preference by the mistletoe for the most abundant host species, *A. karroo*. These results were consistent with those of other studies demonstrating that host specificity can be influenced by host abundance, given that abundant host species are encountered most frequently and are more reliable hosts through space and time (López de Buen & Ornelas 2002, Norton & de Lange 1999, Zuber & Widmar 2000). In contrast, Roxburgh & Nicolson (2005) found no relationship between observed prevalence among host species and compatibility of the mistletoe *Plicosepalus kalachariensis* in Zambia.

Many bird species disperse *Agelanthus natalitius* seeds to the same tree as the maternal plant or on a nearby tree (Green *et al*. 2009, Okubamichael *et al*. 2011a, Roxburgh 2007). This may reduce colonisation of new sites, but might improve chances of landing in a safe site (Norton & Carpenter 1998, Norton & de Lange 1999, Okubamichael *et al*. 2011a). Thus adaptation of a mistletoe to the most abundant host species would facilitate dispersal efficiency. Rare host species have less chance of receiving mistletoe seeds by chance, except when birds differentially perch on those host species due to the presence of fleshy fruits or some other trait that would make them attractive to birds.

Host tree traits

Birds differentially disperse mistletoe seeds to tall trees (Aukema & Martínez del Rio 2002, Ward *et al*. 2006), so any difference in size among potential host species could result in differential distribution of mistletoes among host trees. However, *A. karroo* and *A. caffra* trees were not significantly different in size (height and dbh) in either site, so differences in mistletoe infection cannot be attributed to size differences in the host species. We found that infected trees were taller and had a greater trunk diameter than uninfected host trees for both host species in both sites. This result may be a consequence of the behaviour of dispersers, as birds differentially perch on tall trees and may deposit mistletoe seeds in the process. Moreover, if trees are tall, they are probably older and have had more time to become infected by mistletoes (Aukema & Martínez del Rio 2002, Donohue 1995, Overton 1994). Thus, tall and big trees are frequently observed with a greater number of mistletoe infections than short and smaller trees (Aukema 2004, Donohue 1995, Roxburgh & Nicolson 2007, Ward *et al*. 2006).

Overton (1994) explained the frequency of mistletoe infection as an accumulation function of infection with time as the tree gets older. As in previous studies (Aukema 2004, Donohue 1995, Roxburgh & Nicolson 2007), linear regression analysis in this study produced a weak correlation between height of tree and infection intensity. This analysis may be inappropriate, however, because the data in these other studies followed a negative binomial distribution. An alternative (GLIM) analysis explained the observed patterns better because the frequency distribution of the number of mistletoes per tree (infection intensity) is a good fit to the negative binomial distribution (i.e., most species are free of mistletoes and a few individuals are highly infected). Previous infection increases the likelihood of further infection and causes a clumped or aggregated distribution which is likely due to the limited dispersal distance of mistletoe seeds from their source (Aukema 2004, Overton 1994, Ward & Paton 2007).

There was a steeper slope of the regression of tree height to proportion of prevalence because birds differentially visit tall trees. Trees with big trunks are often tall but this is not always the case; this may explain why there is a weaker relationship between prevalence and dbh as compared to tree height. For example, at Highover, *A. caffra* trees were tall but they did not have big trunks. In addition, tall trees are usually more branched and provide more twigs with a suitable diameter than short trees (Sargent 1995). Mistletoes deposited on tall trees also have greater success because tall trees are less likely to be shaded by neighbours, thereby providing adequate light for mistletoes (Lamont 1982, Okubamichael *et al*. 2011a, Ward *et al*. 2006, Ward & Paton 2007). Tall trees may supply more nutrients and water to the mistletoes due to deeper and broader root systems (Ward *et al*. 2006). Tall trees also protect mistletoes from browsing by large herbivores (Roxburgh & Nicolson 2005, 2007). Mistletoes are often selected by herbivores over their host trees because the mistletoes have higher mineral and nitrogen concentrations and have few physical and chemical defence mechanisms. Thus, herbivores can limit mistletoes (Midgley & Joubert 1991). Associated with this, *Agelanthus natalitius* growing on *A. karroo* may be better protected against foragers because *A. karroo* has longer spines than *A. caffra* and is better defended against herbivores (see also Martínez del Rio *et al*. 1996).

Reciprocal transplant experiment

Our study supports previous findings that germination of mistletoes is independent of site and substrate provided that the pericarp is removed (Ladley & Kelly 1996, Lamont 1983, Rödl & Ward 2002, Roxburgh & Nicolson 2005). Hypocotyl length and growth form, however, showed crossed reaction norms, indicating that there were significant differences due to G × E interactions in this mistletoe species. The growth patterns of the hypocotyl varied based on site, source and the substrate on which they were placed. This implies that morphologically identical mistletoes may be genetically different, such that early seedling development is greatest when there is correspondence between maternal and seedling host species. Several studies also demonstrated that the development of the haustorium is more successful when mistletoe seeds are placed on their source host species (Arruda *et al*. 2006, Clay *et al*. 1985, Rödl & Ward 2002, Yan 1993b). Unfortunately, measuring lifetime fitness differences in reciprocal transplant experiments in long-lived perennial plants requires more time than was available for this study. For this reason a quantitative genetic analysis should be considered in future studies.

Host compatibility, host selection, host recognition, localisation of gene flow and spatial segregation of host species or populations promote host race formation in mistletoe populations (Glazner *et al*. 1988). Our findings show that differences in host utilisation of source and non-source host species may have a genetic basis, leading to differential success of individuals from one mistletoe population when grown on a different host species or in different areas. However, it is still not clear what mechanisms the mistletoes use to differentiate between hosts of different species and from different localities.

The haustorium is a distinct and unifying structure of parasitic plants. Thus, investigating haustorium formation and adaptation provides a holistic approach to study both infection patterns and host specificity in parasitic plants (Calvin & Wilson 2006, Thorogood *et al*. 2009). Although the chemical interactions between aerial parasites and their hosts are as yet unstudied, it has been found in root parasites that host-derived chemicals stimulate germination and haustorium formation (Bouwmeester *et al*. 2003, Chang & Lynn 1986, Matvienko *et al*. 2001, Tomilov *et al*. 2004, Yoder 1999). The results of this study suggest that chemical interactions between mistletoes and their host trees may occur during post-germination development and this may be the basis for the G × E interactions we observed at the early stage of the mistletoe growth (see also Runyon *et al*. 2006, Thorogood & Hiscock 2010).

Survival over 6 mo clearly showed that mistletoes transferred to non-source host species and to non-local sites had lower survival. This demonstrates that mistletoes have mechanisms that facilitate compatibility to their parental hosts. In addition, the fact that mistletoes on *A. caffra* did not show any difference in survival at Highover may indicate that this population of mistletoes uses *A. caffra* opportunistically and the mistletoes that can grow on *A. caffra* may not be as specific as those that grow on *A. karroo* at this site. We found that mistletoes had longer hypocotyls when grown on *A. karroo*, regardless of their parental host, which could reflect an adaptation to the most frequently encountered host tree. Similar findings were reported by Norton & Carpenter (1998) and by Rödl & Ward (2002). Further studies on mistletoe performance (in terms of hypocotyl growth, survival and reproduction) should focus on combinations of reciprocal transplant experiments. For example, differences in mistletoe performance should be tested on parental versus non-parental hosts and preferred versus non-preferred hosts to determine the underlying mechanisms that determine host compatibility.

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Table 1. Chi-square test on the frequency of two tree species (*Acacia karroo* and *A. caffra*) that are hosts of the mistletoe *Agelanthus natalitius* at two sites (Highover and Mtontwane). *Acacia karroo* was significantly higher in abundance than *A. caffra* in both sites (N = 1464). Significant values at P < 0.05 are indicated in bold.

Source of variation	df	Chi-Square	P
Site	1	4.8	**0.029**
Species	1	421	**< 0.001**
Species in Highover	1	278	**< 0.001**
Species in Mtontwane	1	150	**< 0.001**

Table 2. Generalised linear model test results for the prevalence of infection (% of trees with a mistletoe infection) of two host species (*Acacia karroo* and *A. caffra*) in two sites (Highover and Mtontwane) (N = 1464). See Table 3 for post-hoc comparisons. Significant values at P < 0.05 are indicated in bold.

Source of variation	df	Wald Chi-Square	P
Site	1	3.6	0.06
Species	1	5.5	**0.02**
Site × species	1	.6	0.43

Table 3. The prevalence of mistletoe infection (percent of trees with at least one mistletoe infection) on two host species at two sites (N = 1464). There was no significant difference in prevalence between *Acacia caffra* and *A. karroo* at Highover, but percentage of infected trees of *A. caffra* was significantly higher than of *A. karroo* at Mtontwane. The lowercase letters denote significant differences between groups.

Site	Species	Prevalence (% ± SE)	Multiple comparison
Highover	*A. caffra*	26 ± .04	ab
	A. karroo	22 ± .02	b
Mtontwane	*A. caffra*	34 ± .04	a
	A. karroo	25 ± .02	b

Table 4. Generalised linear model test results for the intensity of mistletoe infection (mean number of parasites per tree) for two host species at two sites. Intensity of infection was significantly higher on *A. karroo* than on *A. caffra* (N = 1464 trees parasitised by 1202 mistletoes).

Source of variation	df	Wald Chi-Square	P
Species	1	11.178	**0.024**
Site	1	0.047	0.829
Species × site	1	1.876	0.171

Table 5. Generalised linear model test for the height and diameter at breat height (dbh) of two host species (*Acacia karroo* and *A. caffra*) at two sites (Highover and Mtontwane). There were no significant differences in height and dbh of host species at both sites.

		height		dbh	
Source of variation	df	Wald chi-Square	P	Wald Chi-Square	P
Species	1	0.001	0.972	3.4	0.384
Site	1	0.074	0.786	6.3	0.227
Species × site	1	0.058	0.809	2.8	0.435
Error degrees of freedom	1464				

Table 6. GLIM of test for the height and diamater at breast height (dbh) of infected and uninfected trees (status) of two host species (*Acacia karroo* and *A. caffra*) at two sites (Highover or Mtontwane). Significant values at P < 0.05 are indicated in bold.

		height		dbh	
Source of variation	df	Wald Chi-Square	P	Wald Chi-Square	P
Species	1	0.2	0.697	0.2	0.642
Site	1	107.1	**< 0.001**	14.8	**< 0.001**
Status	1	63.7	**< 0.001**	68.6	**< 0.001**
Species × site	1	5.0	**0.025**	1.8	0.176
Species × status	1	0.3	0.584	2.6	0.109
Site × status	1	10.4	**0.001**	5.6	**0.018**
Site × species × status	1	0.2	0.656	0.1	0.706
Error degrees of freedom	1464				

Table 7. GLIM of the reciprocal transplant experiment of *Agelanthus natalitius*. Site = Highover or Mtontwane; Source = source (original) host species; Current substrate = host that the mistletoe was transferred to manually. Significant values at 95% are indicated in bold.

Source of variation	df	Wald-Chi Square	P
Current substrate	1	101.9	**.000**
Site	1	23.9	**.000**
Source	1	77.3	**.000**
Time	1	17.2	**.000**
Current substrate × site	1	3.9	.049
Current substrate × source	1	19.6	**.000**
Current substrate × time	1	.6	.451
Site × source	1	75.4	**.000**
Site × time	1	.1	.722
Source × time	1	.3	.559
Current substrate × site × source	1	53.6	**.000**
Current substrate × site × time		.1	.796
Current substrate × source × time	1	1.0	.339
Site × source × time	1	1.7	.195
Current substrate × site × source × time	1	.6	.446
Error degrees of freedom	1206		

Table 8. Log-rank test of survival for the reciprocal transplant germination experiment of the mistletoe *Agelanthus natalitius* over 6 mo. The mistletoe seeds were from two host species (*Acacia karroo* and *Acacia caffra*) and placed on source and non–source host species at two sites (Highover and Mtontwane) (4 × 4 = 16 combinations). For example, trees of *A. caffra* at Highover received seeds from: (1) mistletoes that parasitise A. *caffra* at Highover, (2) mistletoes that parasitise *A. caffra* at Mtontwane, (3) mistletoes that parasitise *A. karroo* at Highover and (4) mistletoes that parasitise *A. karroo* at Mtontwane.

Source of variation	Chi-square	df	P
Overall	51.3	15	**<.0001**
Mistletoes on *A. karroo* at Highover	10.6	3	**0.014**
Mistletoes on *A. caffra* at Highover	3.57	3	0.312
Mistletoes on *A. karroo* at Mtontwane	8.93	3	**0.030**
Mistletoes on *A. caffra* at Mtontwane	8.61	3	**0.035**

FIGURE CAPTIONS

Figure 1. Height (a) and diameter at breast height (b) (mean ± SE) of host trees *Acacia karroo* and *Acacia caffra* infected and uninfected by the mistletoe *Agelanthus natalitius* at Highover and Mtontwane.

Figure 2. Prevalence of the mistletoe *Agelanthus natalitius* against tree height and dbh of the two host species, *Acacia karroo* (solid circles with solid regression line) and *Acacia caffra* (hollow circles and dashed regression line). (a) Prevalence against tree height at Highover, (b) prevalence against tree height at Mtontwane, (c) prevalence against dbh at Highover and (d) prevalence against dbh at Mtontwane. All were significantly positively correlated with the exception of prevalence of *Acacia caffra*, which was not significantly correlated with tree dbh at Highover.

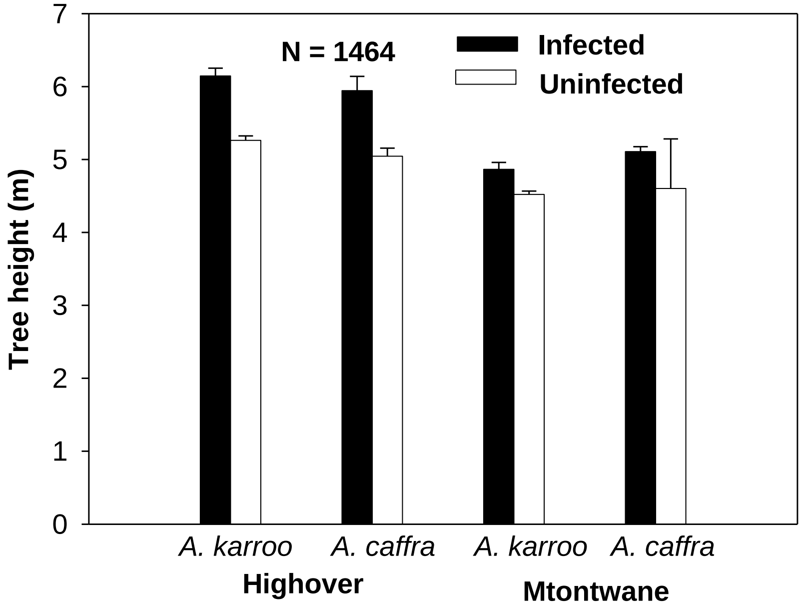
Figure 3. The hypocotyl length after 1 mo (white bar) and 6 mo (black bar) (mean ± SE) of the germinated mistletoe seedlings of *Agelanthus natalitius* in the reciprocal transplant experiments of all combinations of source host species × current substrate at both sites, Highover (a) and Mtontwane (b). Abbreviations: source host species, **mk** = mistletoe seedlings obtained from mistletoes grew originally (source) on *Acacia karroo* and **mc** = mistletoe seedlings obtained from mistletoes grew on *Acacia caffra* and current substrate; **k** = *Acacia karroo* and **c** = *Acacia caffra*; source host species with their respective site; mkH = mistletoes from *A. karroo* at Highover, mkM = mistletoes from *A. karroo* at Mtontwane, mcH = mistletoes from *A. caffra* at Highover, mcM = mistletoes from *A. caffra* at Mtontwane.

Figure 4. Relationship between hypocotyl length of mistletoe seedlings of *Agelanthus natalitius* and their survival.

Figure 5. Percentage survival over 6 mo of *Agelanthus natalitius* mistletoe seedlings. Two groups of mistletoes received from two different host species placed on two host species at two sites (= 16 combinations in total). (a) Mistletoes placed on *Acacia karroo* at Highover, (b) mistletoes placed on *Acacia caffra* at Highover, (c) mistletoes placed on *Acacia karroo* at Mtontwane and (d) mistletoes placed on *Acacia caffra* at Mtontwane. Abbreviations for mistletoe sources: mkH = mistletoes from *A. karroo* at Highover, mkM = mistletoes from *A. karroo* at Mtontwane, mcH = mistletoes from *A. caffra* at Highover, mcM = mistletoes from *A. caffra* at Mtontwane. Abbreviations for hosts: kH = *A. karroo* from Highover, kM = *A. karroo* from Mtontwane, cH = *A. caffra* from Highover and cM = *A. caffra* from Mtontwane. NS = not significant. The lowercase letters denote significant differences between groups of mistletoe seeds.

FIGURES

(a) Height



(b) Diameter at breast height

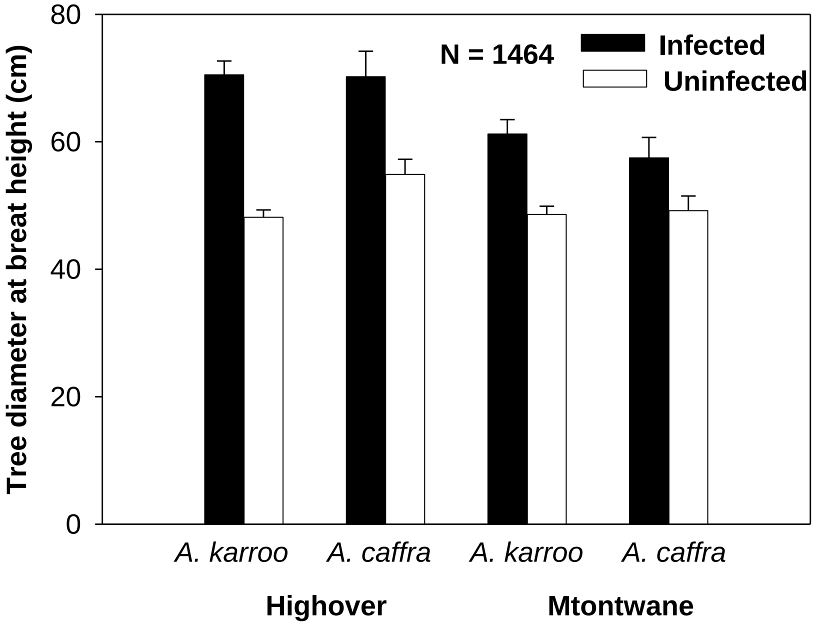
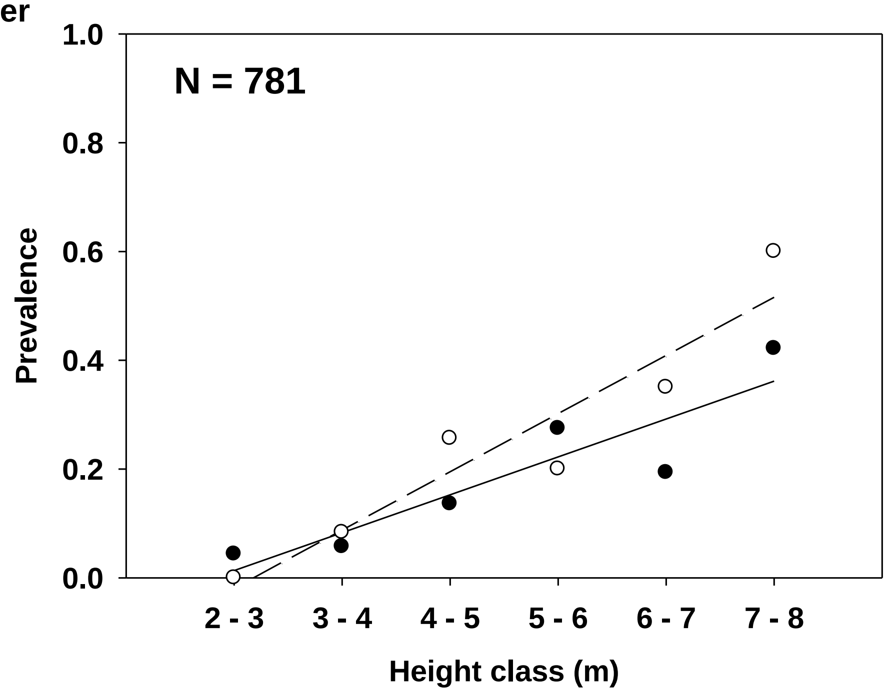
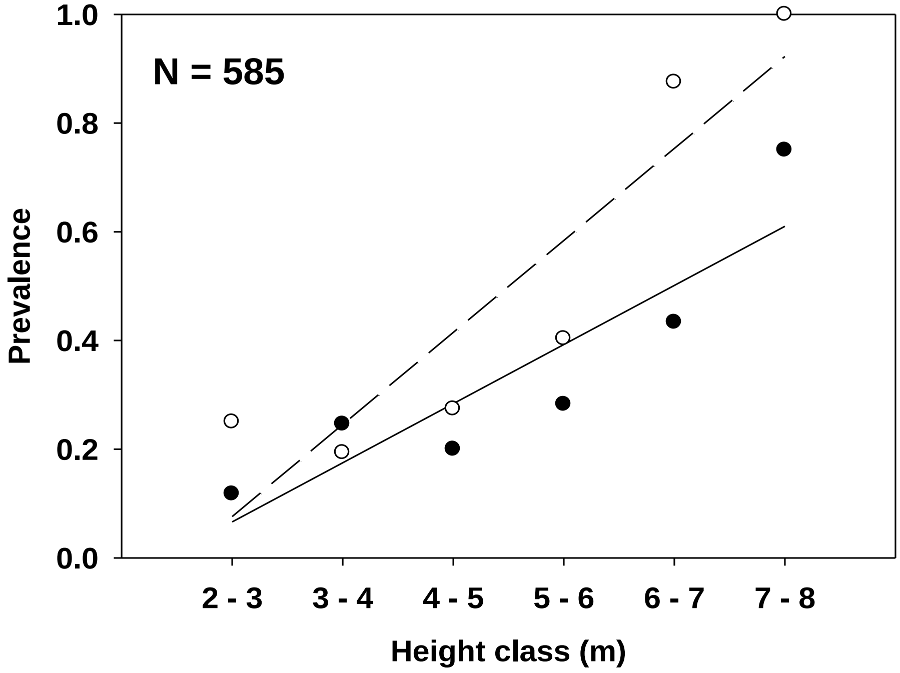


Figure 1.

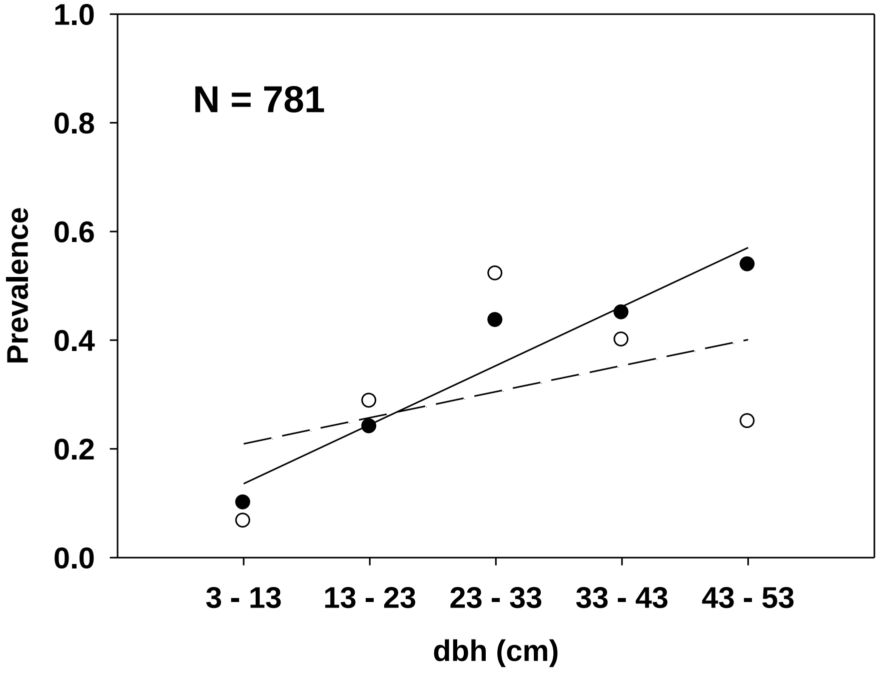
(a) Highover



(b) Mtontwane



(c) Highover



(d) Mtontwane

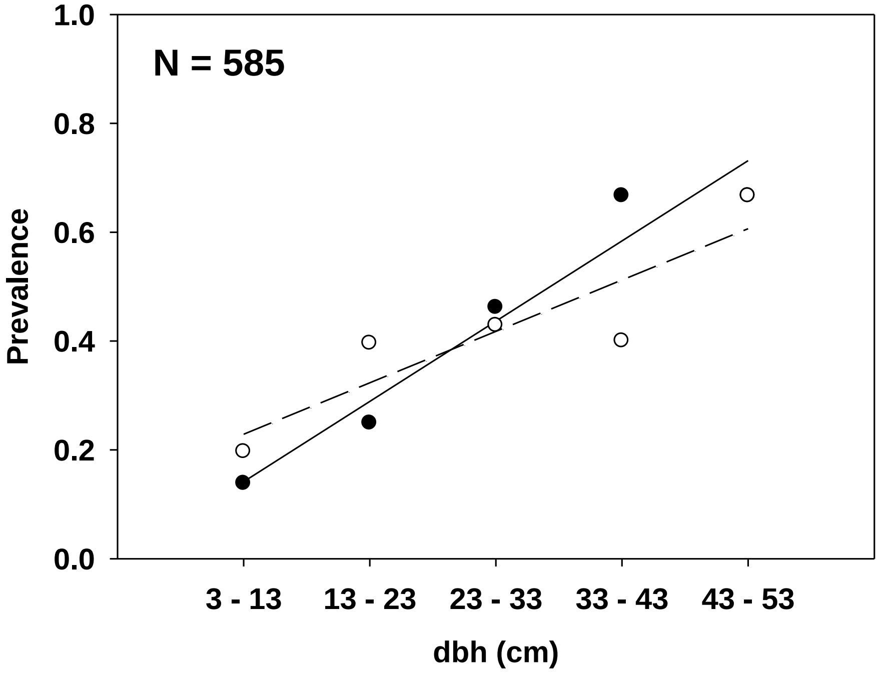
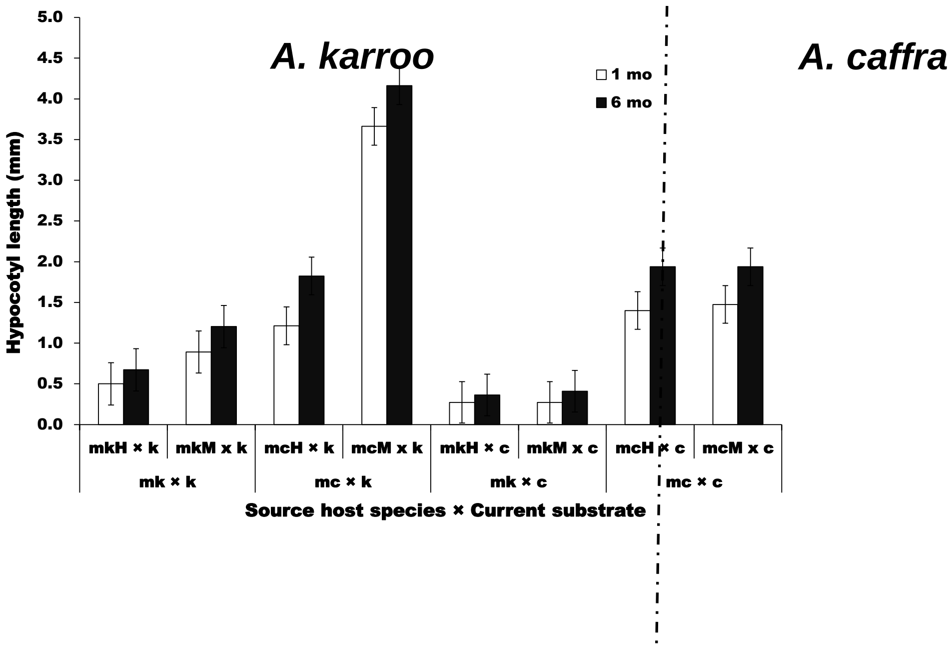


Figure 2.

(a) Highover



A. karroo
A. caffra
1 mo
6 mo
Hypocotyl length (mm)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
mkH × k
mkM x k
mcH × k
mcM x k
mkH × c
mkM x c
mcH × c
mcM x c
mk × k
mc × k
mk × c
mc × c
Source host species × Current substrate

(b) Mtontwane

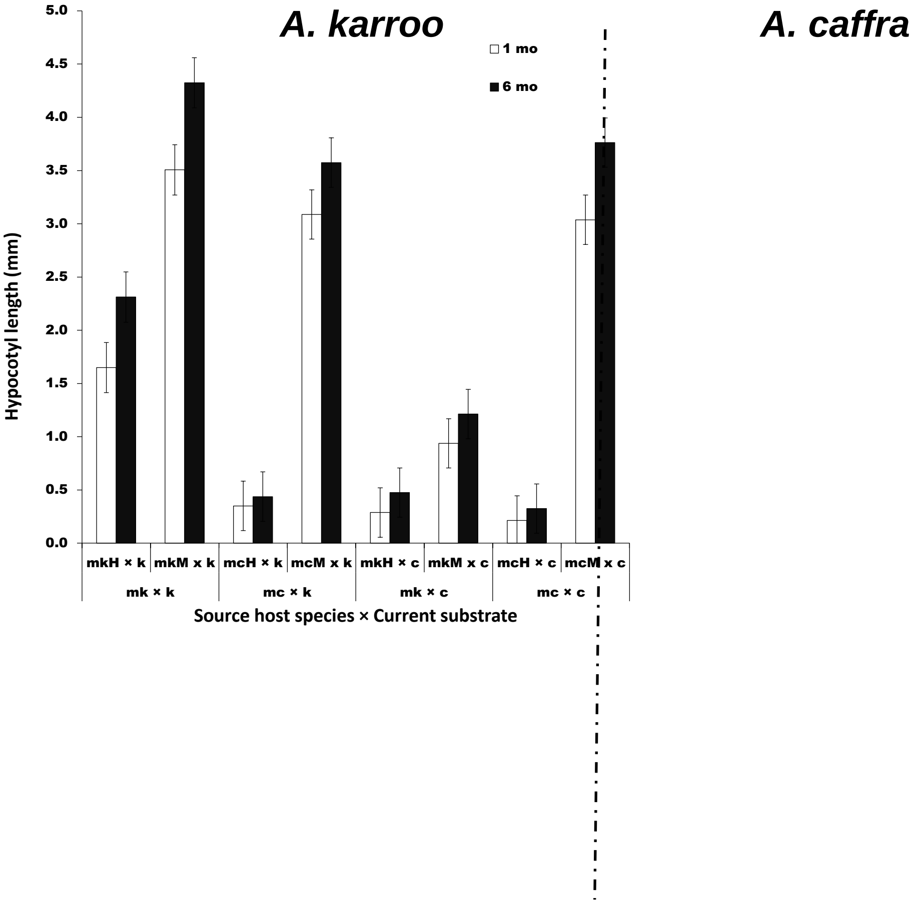


Figure 3.

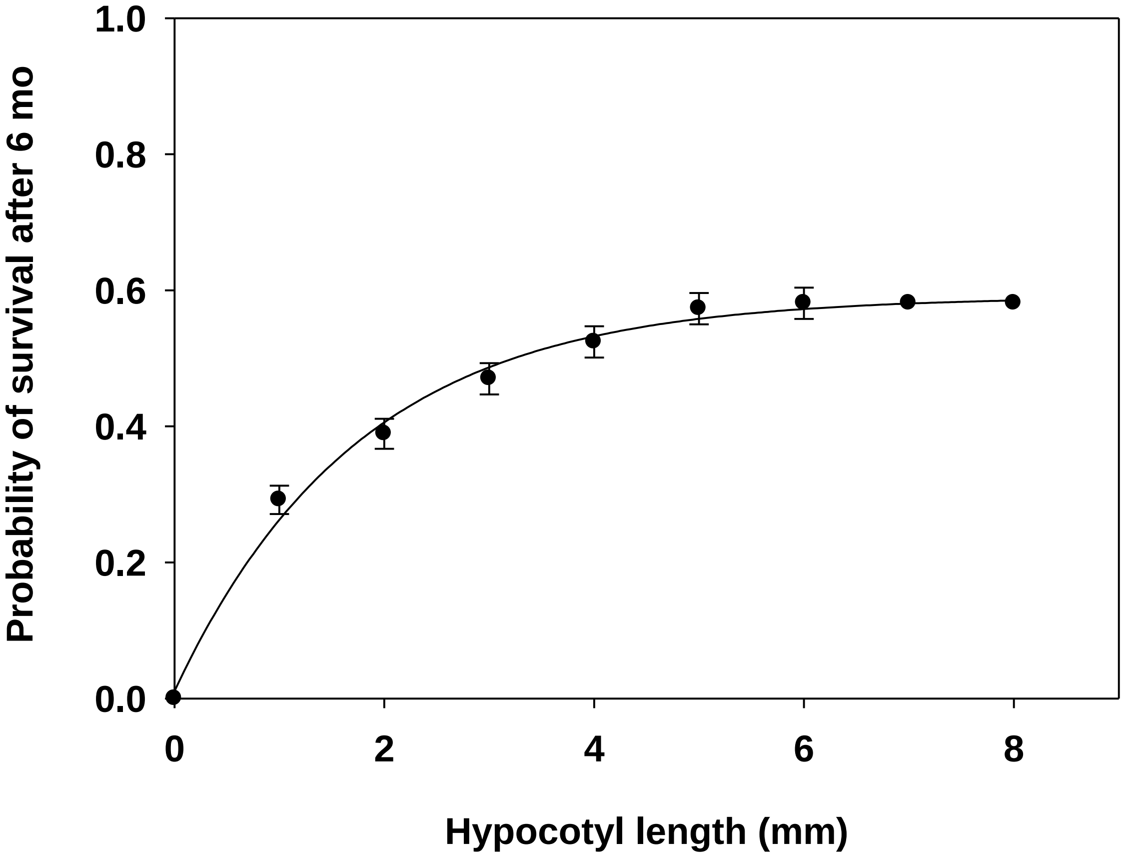
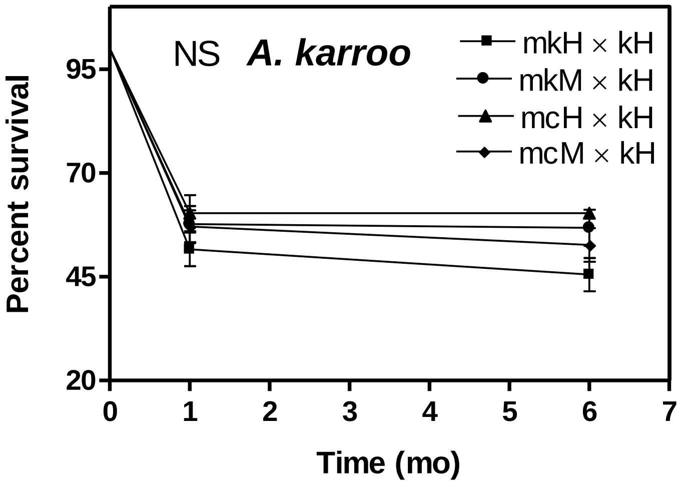
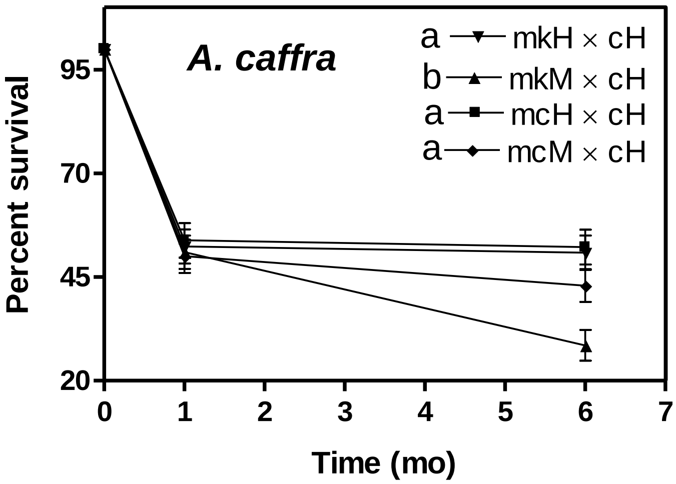

1.0
0.8
0.6
0.4
0.2
0.0
Probability of survival after 6 mo
0
2
4
6
8
Hypocotyl length (mm)

Figure 4.

(a) Highover


NS
A. karroo
mkH × kH
mkM × kH
mcH × kH
mcM × kH
Percent survival
95
70
45
20
0
1
2
3
4
5
6
7
Time (mo)


A. caffra
a mkH × cH
b mkM × cH
a mcH × cH
a mcM × cH
Percent survival
95
70
45
20
0
1
2
3
4
5
6
7
Time (mo)

(b) Mtontwane

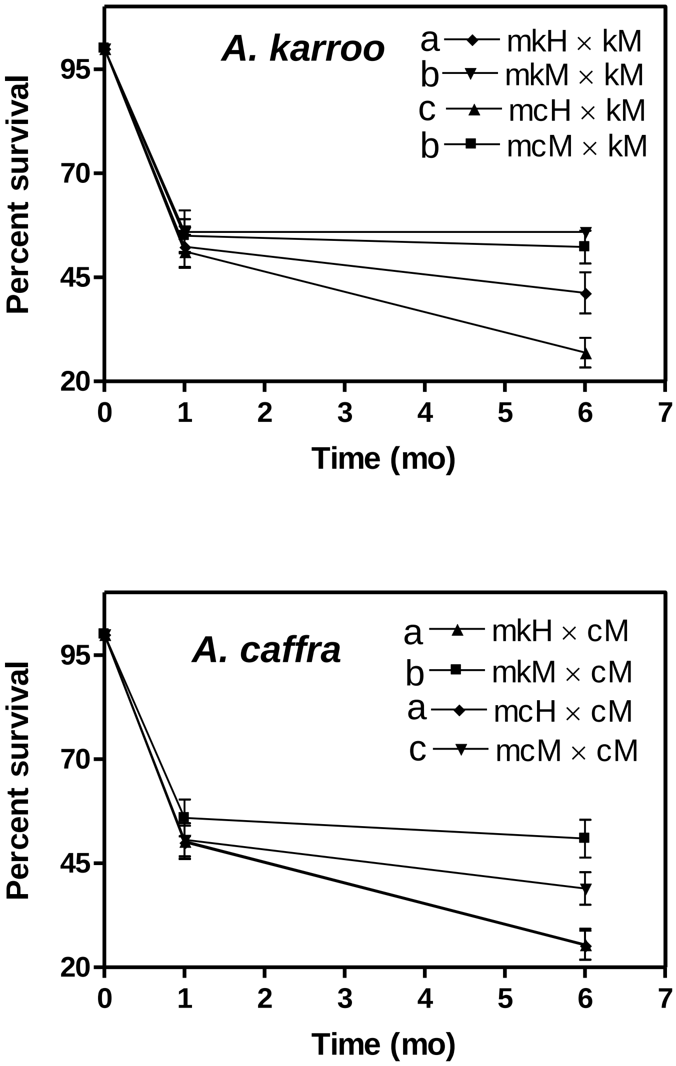
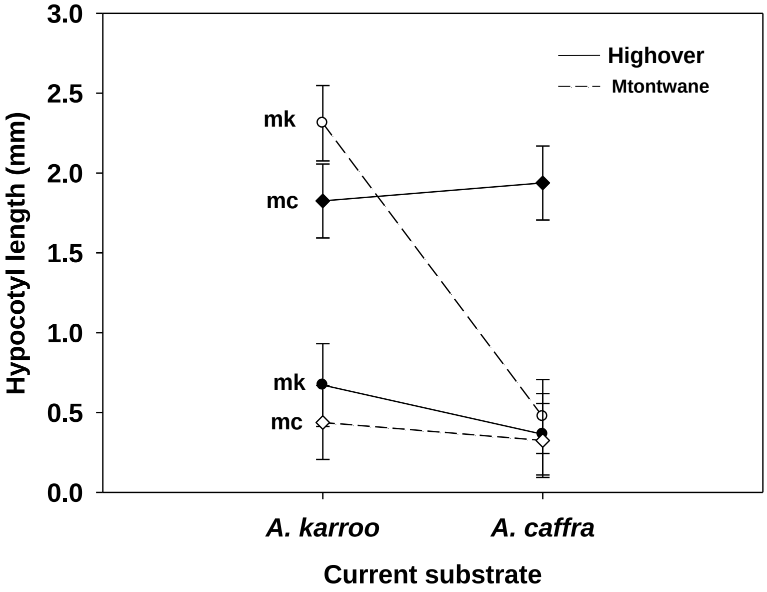


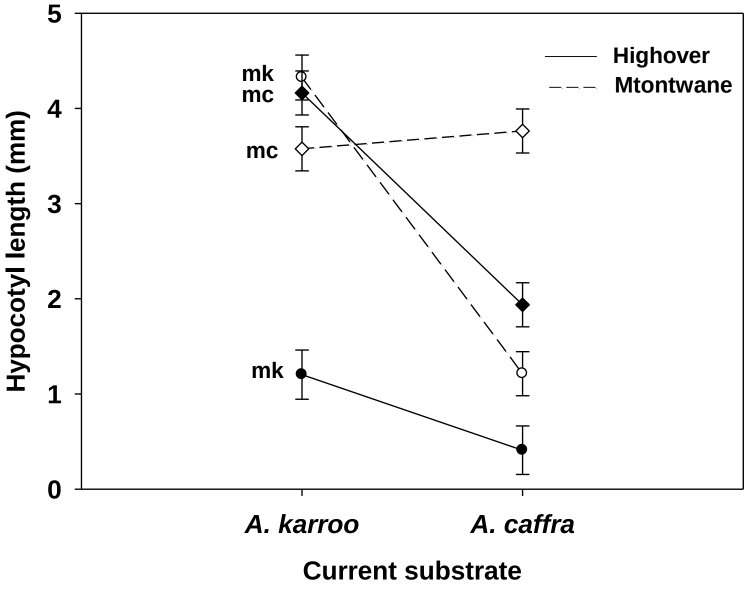
Figure 5.

Appendix 1. In the reciprocal transplant experiment there was a total of 16 combinations. These combinations were different based on source- and current-substrate (*Acacia karroo* and *A. caffra*) and site (Highover and Mtontwane). There were eight combinations at each site. The average hypocotyl length is shown for each combination at the two sites: (a) Highover and (b) Mtontwane.

(a) Highover



(b) Mtontwane


Hypocotyl length (mm)
0
1
2
3
4
5
mk
mc
mc
mk
Highover
Mtontwane
A. karroo
A. caffra
Current substrate

CHAPTER 5

LIGHT AND MOISTURE IMPROVE INITIAL SEEDLING GROWTH AND SURVIVAL OF MISTLETOES

Desale Y. Okubamichael, Megan E. Griffiths and David Ward*

School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa.

*Author for correspondence; e-mail: ward@ukzn.ac.za

Microclimate plays a key role in determining mistletoe distribution. Moisture and light are essential for seedling growth and survival in mistletoes, as seed carbohydrate reserves are insufficient to allow the hypocotyl to be connected to the xylem without photosynthesis. Mistletoes are more abundant at the edge of tree patches and rare in denser understory in African savannas. Mistletoes are also common on trees that grow in the vicinity of water in these habitats. We assessed the effects of abiotic factors (i.e., light, temperature and moisture) in controlling initial seedling growth of two South African mistletoe species, *Agelanthus natalitius* (Loranthaceae) and *Viscum rotundifolium* (Viscaceae). For both *A. natalitius* and *V. rotundifolium* we found that high light (20% and 40% shade versus 80% shade), cool temperatures (15°C and 20°C versus 25°C) and continuous moisture availability were required for seedling development and subsequent survival in a growth chamber. High light availability, low temperature and high moisture increased the hypocotyl length, haustorium formation, plumulary (embryonic shoot) leaf size and subsequent survival. These traits would help seedlings to survive during the long non-parasitic stages prior to host attachment. The results from our study suggest that the microclimatic conditions provided by different host trees contribute to patterns of mistletoe infection and determine their distribution in natural environments.

Key words: growth chamber, haustorium, hypocotyl, microclimate, physical dormancy, seeds.

Introduction

Mistletoe seeds adhere to, germinate, establish and grow on host trees (Kuijt 1969; Hawksworth and Wiens 1996; Polhill and Wiens 1998). As the seed germinates, the meristematic hypocotyl protrudes a few millimetres (Bhatnaga and Johri 1983; Bhandari and Vohra 1983; Baskin and Baskin 1998). The hypocotyl tip bends towards the substrate and swells to form a holdfast with a region of intense meristematic activity (Bajaj 1967; Kuijt 1969, 1977). The holdfast afterwards develops a penetration wedge known as a haustorium that exerts mechanical pressure on the cortex and penetrates through small twigs of a host tree (Atsatt 1973, 1977; Press and Graves 1995; Sargent 1995; Wilson and Calvin 2006). Finally, a connection is established between the xylem of the mistletoe and the host vascular system, which allows the mistletoe to access water and nutrients from the host plant (Fisher 1983; Lamont 1983; Sallé 1983; Ehleringer and Marshall 1995; Pate 1995; Reid et al. 1995).

Mistletoe seeds germinate as long as physical dormancy has been broken by removing the exocarp (usually done in nature by birds). However, mistletoe seedlings seldom survive long enough to develop into adult plants (Lamont and Perry 1977; Lamont 1982, 1983; Yan 1993; Overton 1994; Yan and Reid 1995; Ladley and Kelly 1996). Mistletoes can germinate on a range of biotic and abiotic substrates (Yan 1993; López de Buen and Ornelas 2002; Rödl and Ward 2002; Green et al. 2009). However, it is suggested that mistletoe seedling development is substrate-sensitive after germination has occurred (Clay et al. 1985; Hoffmann et al. 1986; Glazner et al. 1988; Rödl and Ward 2002). For example, seeds of mistletoe *Phoradendron tomentosum* (now *P. leucarpurn* ssp. *tomentosum*; Kuijt 2003; Abbott and Thompson 2011) grow better on the same species of host tree that supports the maternal plant (Clay et al. 1985; Hoffmann et al. 1986; Glazner et al. 1988). Similarly, Okubamichael et al. (2011*a*) and Rödl and

Ward (2002) found that mistletoe seeds developed haustoria and more quickly reach the host branch when they were placed on their source host trees than on non-source host trees. Although research on mistletoe ecology and biology has been the focus of many recent works (e.g., recent works (e.g. Runyon et al. 2006; Mathiasen et al. 2008; Fadini 2011; Okubamichael et al. 2011a,b), our understanding of the specific growth requirements of mistletoe seedlings is still lacking (see also Baskin and Baskin 1998). Little is known about how long mistletoes can remain autotrophic before they start to access resources from their host trees (Lamont 1983, Hawksworth and Wiens 1996), although work by Reid (1987) on *Amyema quandang* suggests that seedlings of this species were parasitic by 6 months of age and Yan (1993) found that *Amyema preissii* and *Lysiana exocarpi* had sufficient food reserves to grow for up to a year before attaching to the vascular tissue of the host.

Many researchers (Yan 1993; López de Buen and Ornelas 2002; Rödl and Ward 2002; Green et al. 2009) have shown that mistletoe seed germination is substrate insensitive but only a few (see Baskin and Baskin 1998) have investigated the role of microclimate (water, oxygen, temperature and light) on subsequent survival in mistletoe seedlings. Effects of microclimate on seedling growth and survival could determine mistletoe distribution at a small scale (within the tree canopy) and potentially affect larger scale distribution (within the tree community). Mistletoes respond to divergent selection pressures imposed by different environments, as well as to geographic isolation and host adaptations (Jerome and Ford 2002). Microhabitat requirements are likely to vary among mistletoe species and different host trees are likely to provide different microhabitat conditions. To investigate this, we assessed the effects of abiotic factors (i.e., light, temperature and moisture) in controlling initial seedling growth of mistletoes.

We specifically tested the effects of shade, temperature and moisture on the early stages of seeding development of two species of mistletoes (*Agelanthus natalitius* and *Viscum rotundifolium*) common in South Africa (Polhill and Wiens 1998). We selected these two species of mistletoes to represent the two most speciose families of mistletoes: Loranthaceae (± 950 species) and Viscaceae (± 450 species) (Polhill and Wiens 1998). They also infect a wide range of host species and grow in markedly different biomes in South Africa. We quantified hypocotyl elongation, holdfast formation, aerial shoot formation (plumulary leaves) and survival of the two species of mistletoes over six months in growth chambers.

Material and Methods

Mistletoe Fruit Collection

In winter 2011, mature fruits of the mistletoe *Agelanthus natalitius* (which parasitises *Acacia karroo* and *Acacia caffra*) were collected from two sites in KwaZulu-Natal province: Highover (29° 54'S, 30° 05'E) and Mtontwane (28°48'S, 29°56'E). Mean annual rainfall at Highover is 763 mm and at Mtontwane is 769 mm. Fruits of the mistletoe *Viscum rotundifolium* (which parasitises *Ziziphus mucronata*) were collected from three sites in the Northern Cape Province (mean annual rainfall = 360 mm): Pniel Estates (28°36'S, 24°28'E), Rooipoort (28°33'S, 24°10'E) and Schmidtsdrift (28°46'S, 23°59'E). In each site, we randomly selected 15 mistletoe-infested trees. From each tree, 20 healthy fruits were collected from each mistletoe and transported to the laboratory where they were kept at 5°C until they were used.

Germination of Mistletoe Seeds

To break the physical dormancy of the mistletoe seeds (Baskin and Baskin 1998), the exocarp of each fruit was manually removed. Thereafter, the endocarp was also removed to expose the viscin. The mistletoe seeds were placed in a row (2-3 cm apart) on a circular piece of filter paper (Whatman No.1) in disposable plastic petri dishes (65 mm in diameter). The viscin layer held the seeds on the filter paper. The filter paper was kept moistened with distilled water until germination occurred.

Shade, Temperature and Moisture Treatments

The standard experimental condition in the growth chamber was a 16 h photoperiod provided by fluorescent lamps (photosynthetic photon flux density, 90.5 μmol m^{-2} s^{-1}). Except where stated otherwise, seeds were incubated at alternative temperatures of 15/20°C and supplied with a one-quarter strength Hoagland's solution (Hoagland and Arnon 1950). We ensured that the filter paper was saturated every second day (approximately 5 mL of liquid).

We selected healthy seeds only with a green hypocotyl protruding and transferred them to the growth chamber; dead seeds were replaced. Following germination, 900 *A. natalitius* seeds and 675 *V. rotundifolium* seeds were transferred to the growth chambers to use for the experiment. We used five seeds per petri dish and replicated five times (25 seeds in five petri dishes) for the three levels of each treatment (shade, temperature and moisture).

To evaluate the effect of shade, petri dishes were covered with shade cloth (20%, 40% and 80%). The shade levels were selected on the basis of our previous field observations that found a positive correlation between mistletoe distributions and light availability (Okubamichael 2009). To evaluate the effect of different temperature regimes, seeds were incubated at three constant temperatures (15°C, 20°C and 25°C). The selected temperatures represent the field conditions experienced by mistletoes in South Africa, where there are cool winters and warm to hot summers. The nutrient supply/moisture treatment consisted of three categories: (1) petri dishes that were supplied with a quarter strength Hoagland's solution every second day, (2) petri dishes that were supplied with a half strength Hoagland's solution every second day and (3) petri dishes that were drenched with distilled water every day and supplemented with a quarter strength Hoagland's solution once every two weeks. In this experiment, the nutrient treatments were confounded by moisture but for the sake of simplicity, we refer to the treatment as moisture. We could not separate nutrients from moisture because the mistletoe seeds in the growth chamber would die without a nutrient supply. We examined the extension of the hypocotyl (to the nearest mm), radicle formation, shoot formation and survival of the seedlings for a period of six months (fig. 2). The traits that we measured were directly related to favourable conditions for survival and growth of mistletoes in the field. It takes six months from a mistletoe seed landing on a host substrate during the dry winter to mistletoe establishment during the wet summer in the field. Thus, we monitored mistletoe seedling growth for six months, which would be sufficient time to examine the effect of the microclimates imposed in the growth chamber.

Statistical Analyses

We used a Generalised Linear Model (GLIM) (SPSS version 18) to test the effects of the three independent variables: site (from where we collected the mistletoe fruits), host (from which host species the mistletoe fruits were obtained) and treatments (shade, temperature and moisture) on the influence of early growth traits of mistletoes. Hypocotyl length, haustorium formation and leaf size were treated as continuous variables in GLIM (normal distribution with identity link (i.e., untransformed) function). The probability of survival differences across treatment levels were treated as a binary response and thus incorporated with a logistic link function of binomial error. When overall significance was confirmed, a further pairwise comparison (to test for differences among the three levels within each treatment) was conducted using contrast analysis.

Results

In both mistletoe species (*Agelanthus natalitius* and *Viscum rotundifolium*), treatments of shade, temperature and moisture significantly affected mistletoe seedling hypocotyl length, haustorium formation, leaf size and survival (tables 1, 2). In the shade treatments, the growth (hypocotyl length, haustorium formation, leaf size) and survival of both mistletoe species was significantly influenced by the main effect of shade only (three levels of shade). However, the other main effects (site and host) and their interactions did not significantly influence the growth parameters in the shade treatments. Similarly, in the temperature and moisture treatments, the growth (hypocotyl length, haustorium formation) and survival of *V. rotundifolium* was influenced by the main effects of temperature and moisture, while site and interaction effects did not significantly influence the growth of the mistletoes. The three-way interaction (temperature × site × host) significantly influenced the hypocotyl length of *A. natalitius*. The other main effects (site and host) in the temperature treatments also influenced the haustorium formation of *A. natalitius*, but none of the interactions were significant. Leaf size of *A. natalitius* was not significantly affected by temperature or by other main or interaction effects. In the temperature treatments, survival was significantly influenced by temperature but not by other main or interaction effects. In the moisture treatments, hypocotyl length was significantly affected by moisture. However, haustorium formation in *A. natalitius* was affected by the three-way interaction (host × site × moisture treatment) and leaf size and survival were affected by the two-way interaction (host × moisture treatment).

Shade

Hypocotyl Length

Host, site and their interactions had no effect on seedling hypocotyl length in both mistletoes (tables 1, 2). For both mistletoes, shade had a significant influence on the hypocotyl length of the seedlings (tables 1, 2). Hypocotyls grew significantly longer in less shade than at high shade levels. *Agelanthus natalitius* seedlings produced longer hypocotyls at 20% and 40% shade than at 80% shade but there was no significant difference between 20% and 40% shade. In *V. rotundifolium*, hypocotyl length was significantly different at the different shade levels, 20% > 40% > 80% (fig. 3A).

Haustorium Formation

Host, site and interaction effects did not significantly influence seedling haustorium formation for both mistletoe species. Seedling haustorium formation was only significantly affected by the shade levels imposed (tables 1, 2). *Agelanthus natalitius* seeds initiated a haustorium from the second week and within two months all the seeds that had produced a haustorium had finished growing (fig. 4*A*). The earliest haustorium formation for *V. rotundifolium* seedlings started at three months and the latest was produced at the end of six months (fig. 4*A*). Many seeds of *A. natalitius* kept at 20% and 40% shade produced haustoria within a month after germination. There was no significant difference between 20% and 40% shade levels for *A. natalitius* in haustorium formation. All shade levels were significantly different in *V. rotundifolium*, with 80% shade having the least haustorium formation in both mistletoes (fig. 4*A*).

Leaf Size

None of the *Viscum rotundifolium* seedlings produced leaves over the study period but *Agelanthus natalitius* seedlings did. Site, host and any interactions did not significantly influence leaf size in *A. natalitius* (table 1). Seeds incubated at 20% and 40% shade produced bigger leaves than those at 80% shade, but leaves produced at 20% and 40% shade did not differ in size from each other (fig. 5*A*).

Survival

Host, site and any interactions had no effect on seedling survival in both species of mistletoes (tables 1, 2). Survival at low shade (20% and 40%) was higher than high shade (80%) in both species of mistletoes (fig. 6*A*). Survival of *Agelanthus natalitius* mistletoes from *Acacia caffra* was similar between 20% and 40% shade but survival of mistletoes from *Acacia karroo* at 40% was greater than at 20%. For *V. rotundifolium*, survival rates were similar between 20% and 40% shade but these were significantly higher than at 80% shade.

Temperature

Hypocotyl Length

The hypocotyl length of *A. natalitius* was significantly influenced by the three-way interaction of temperature × site × host (table 1). The hypocotyl length of *Viscum rotundifolium* was significantly influenced by the main effect of temperature (table 2). However, site with its interactions did not significantly influence the hypocotyl length of *V. rotundifolium* (table 2). Hypocotyls grew significantly longer at lower temperatures (15°C and 20°C) than at the highest temperature (25°C) in both mistletoe species (fig. 3*B*). Hypocotyl length of *V. rotundifolium* was inversely related to temperature (15°C > 20°C >25°C). *Agelanthus natalitius* mistletoes from *A. caffra* at Highover had similar hypocotyl length when incubated at 15°C and 20°C, but these were significantly longer than at 25°C. *Agelanthus natalitius* mistletoes from *A. caffra* at Mtontwane had the highest growth overall and hypocotyl length at 15°C was significantly higher than at 20°C. *Agelanthus natalitius* mistletoes from *A. karroo* at Highover had significantly longer hypocotyls than for those from *A. karroo* at Mtontwane and those grown at 15°C were longer than for those grown at 20°C. Site and host did not affect *A. natalitius* at 25°C (fig. 3*B*).

Haustorium Formation

In addition to temperature, the other main effects (site and host) influenced the haustorium formation of *A. natalitius* (table 1). However, *V. rotundifolium* haustorium formation was not affected by site or any interaction (table 2). Many seeds of *A. natalitius* incubated at 15°C and 20°C produced a haustorium within a month after germination (fig. 4*B*). There was no significant difference between 15°C and 20°C for *A. natalitius* in haustorium formation.

Leaf Size

None of the main effects (site, host and temperature) and their interactions significantly affected the size of *Agelanthus natalitius* seedlings (table 1) (fig 5*B*).

Survival

Site, host and any interaction had no significant effect on the survival of both mistletoe species in temperature treatments (tables 1 and 2). Survival to six months of germinated seeds of both mistletoes was significantly affected by temperature (tables 1 and 2). Survival of *V. rotundifolium* seedlings was higher at 15°C and 20°C than at 25°C. Survival of *Agelanthus natalitius* seedlings was significantly different at all temperatures (15°C > 20°C > 25°C) (fig. 6*B*).

Moisture

Hypocotyl Length

Host, site and their interactions had no effect on seedling hypocotyl length in both mistletoe species (tables 1, 2). Seedlings of both mistletoe species treated with continuous distilled water (CDW) produced significantly longer hypocotyls than those treated with Hoagland's solution (CDW > ¼ H > ½ H), although the differences between CWD and ¼ H were small compared to the difference between ¼ H and ½ H (fig. 3*C*).

Haustorium Formation

Haustorium formation in *A. natalitius* was affected by the three-way interaction of host × site × moisture treatment (tables 1, 2). However, only moisture significantly influenced the haustorium formation of *Viscum rotundifolium*. Only a few of the mistletoe seedlings produced a haustorium at the high concentration of nutrients (½ H) in both mistletoe species (fig. 4*C*).

Leaf Size

Leaf size was affected by the two-way interaction of host × moisture treatment in *Agelanthus natalitius* (table 1 and fig. 5*B*).

Survival

Survival was affected by the two-way interaction of host × moisture in *Agelanthus natalitius* (tables 1, 2). Seedlings of *A. natalitius* differed in their survival depending on the host species they were obtained from (*A. karroo* > *A. caffra*). Moisture significantly influenced the survival of *Viscum rotundifolium*. In both mistletoes, seedling survival was higher in CDW-treated seedlings compared to those treated with ¼ H and ½ H (fig. 6*C*).

Discussion

Unlike most seeds, mistletoe seeds lack a testa and cotyledons are vestigial (Hawksworth 1961; Bhatnagar and Johri 1983; Sallé 1983). The endocarp is surrounded with sticky viscin and the viscin cells absorb water and swell, which enables the seed to firmly attach to the substrate upon drying (Hawksworth 1961; Godschalk 1983a; Paquet et al. 1986). As the seed germinates, the hypocotyl develops and forms a primary haustorium (Bhatnaga and Johri 1983; Bhandari and Vohra 1983; Baskin and Baskin 1998). The first two plumular leaves emerge from the hypocotyl which forms between the two cotyledons. The part of the hypocotyl that remains outside the host forms the shoot of the mistletoe. Mistletoe strategies involve rapid growth (elongating the hypocotyl and forming the haustorium) and establishing a permanent connection with their host plants before the reserve of endosperm is exhausted (Dawson and Ehleringer 1991).

Favourable environmental conditions enhance traits that affect the success of the seedling at overcoming host resistance (Dawson and Ehleringer 1991). Such conditions increase the photosynthetic ability of the seedling and provide enough energy and strength for twig penetration of a host as quickly as possible (Lamont 1983; Polhill and Wiens 1998). This improves successful establishment of mistletoes. Inability or delay in finding the host may lead to significant seedling mortality due to desiccation, seed wash away from the branch by rain and wind, fungal attack and insect predation (Room 1973; Lamont 1983; Lichter and Berry 1991; Polhill and Wiens 1998).

Although there were a number of complicated results in this study, there were obvious differences in hypocotyl length and haustorium formation in seedlings of the mistletoe *Agelanthus natalitius* at different temperatures. In our series of experiments we found that high availability of light (20% and 40%), low temperatures (15°C and 20°C) and a moisture source with lower nutrients (CDW and ¼ H) improved seedling growth in the two species of mistletoes we studied. Hypocotyls are reported to continue linear growth until they make contact with an obstacle before initiating a holdfast (Hawksworth 1961; Scharpf and Parmeter 1967). In our experiment, the longer the hypocotyl grew the more quickly it attached to the filter paper and formed a haustorium. Thus, the factors that affect hypocotyl growth likely also affect the haustorium formation.

Shade

We found that sufficient light availability clearly promoted hypocotyl growth, haustorium formation, leaf size and the subsequent survival of mistletoe seedlings. In the 80% shade treatment, hypocotyls emerged but were unable to maintain growth. The site of collection and its interaction with all other factors did not influence the hypocotyl length, signifying that hypocotyl length was influenced by shading alone. Under the 80% shade treatment, only a few seeds produced haustoria. By contrast, under high light availability and low temperature relatively more seeds produced haustoria within a short period of time for *Agelanthus natalitius* (< 1 month) and over a longer period of time for *Viscum rotundifolium* (4–6 mo).

A number of studies have also shown that seeds of mistletoes germinate at a higher percentage in light than in dark (Scharpf and Parmeter 1962; Scharpf 1970; Knutson 1984; Bejaj 1967; 1968). Room (1973) showed that *Tapinanthus bangwensis* (Loranthaceae) germinated at a higher percentage and seeds were more likely to become established in light versus shade. Bonga and Chakraborty (1968) also found that radicle growth of dissected *Arceuthobium pusillum* embryos grew more in the light than in the dark. Rigby (1959), Lamont (1982) and Mauseth et al. (1985) reported that mistletoe seeds could germinate while they were still enclosed by the exocarp, although none of the hypocotyls could actually penetrate through the exocarp. Germination of mistletoe seeds is possible in dark conditions, but haustorium formation is impossible (Lamont 1982; Baskin and Baskin 1998). Simple sugars, which are produced photosynthetically, are a more efficient source of energy for hypocotyl growth than the complex carbohydrates typically used for storage in the endosperm (Lamont 1983). The mistletoe species studied here (*Agelanthus natalitius* and *Viscum rotundifolium*) both possess chlorophyllous seeds, thus light availability could enhance photosynthesis. The photosynthetic ability of germinating seeds can increase seed longevity beyond the limit of the stored nutrients, which increases the likelihood of survival while forming an attachment to a host (Scharpf 1970; Lamont 1982; Bhandari and Vohra 1983; Tocher et al. 1984; Norton and Ladley 1998; Norton et al. 2002).

Temperature

Temperature is one of the most important environmental factors controlling seed germination. However, available data on the temperature requirements of mistletoes is limited (Baskin and Baskin 1998). Germination of mistletoes occurs in a range of 5°C-35°C. The literature contains conflicting reports on favourable temperatures for mistletoe seedling growth (Beckman and Roth 1968; Lamont 1982, Sallé 1983). Results of this study demonstrate that low temperature promotes hypocotyl length, although for *A. natalitius* site and host also affected the hypocotyl length, haustorium formation and survival. However, leaf growth in *A. natalitius* was barely affected at the temperature range studied. The three-way interaction of site × host × temperature had a significant influence on hypocotyl length in *A. natalitius* whereas in *V. rotundifolium* none of these factors had a significant effect.

Mistletoe hypocotyls lack a root cap, making them more vulnerable to microclimate conditions than non-parasitic plants. In South Africa, mistletoe seed dispersal and germination occurs in winter (Lamont 1983; Okubamichael et al. 2011b) when the temperature is low (average of 19°C). Thus, we expected that mistletoe seedlings would perform better in cooler temperatures. Hypocotyl survival in our study was high (80–90%) at 15°C and 20°C but fell to 20–25% at 25°C. Temperature has also been shown to have an effect on the survival of hypocotyls of dwarf mistletoes (Delachiave and de Pinho 2003). At 25°C, the hypocotyls of dwarf mistletoes failed to grow or survive.

Moisture

Moisture availability affected the hypocotyl length but not haustorium formation in *Agelanthus natalitius*. Moisture also promoted leaf size and survival in this species. When a seed germinates as an epiphyte (glued to a branch), then the presence or absence of moisture, or fluctuation therein, is the first condition to determine whether the germination process will proceed or terminate (Baskin and Baskin 1998). Unlike other plants where pre-drying is a prerequisite for germination to occur (Baskin and Baskin 1998), mistletoe seeds cannot germinate if they dry out. Probably for this reason, when seeds were provided with a continuous supply of distilled water in our experiment, the hypocotyl length was greater. Light and moisture are required from the very beginning for photosynthesis to initiate. Thus, moisture usually enhances infection ability of the hypocotyl. Different nutrient supply treatments had no effect on haustorium formation of *A. natalitius*.

Viscin is an important trait that has evolved in mistletoes. Viscin contributes to the directed dispersal of mistletoe seeds by adhering to bird dispersers that then wipe the seeds onto appropriate microsites (Godschalk 1983a,b). Once the viscin dries out it attaches the seed to the twig and protects the mistletoe from heavy rainfall and the sun. It is also important for supplying a small amount of nutrients to the growing hypocotyl (Benzing 1990). Viscin is hygroscopic and retains moisture and protects the mistletoe seed from desiccation. Germination in mistletoes is a defined event followed by an extensive seedling stage, hence the possibility of dehydration is considerable (Benzing 1990). Mistletoes germinate during winter where it is very dry in the summer rainfall regions in the southern hemisphere and where there is little rain is available in the ecosystem. Even a favourable year can have dry spells and the likelihood that seeds with dry out is high.

Conclusions

The microclimate requirements of mistletoes at the seedling stage are almost unknown. Our research highlights the importance of microclimate conditions in determining mistletoe seedling growth and survival. The growth chamber experiment presented here demonstrates that shade, temperature, moisture, site and host species all have varying effects on mistletoes at the seedling stage. The results of this study suggest that mistletoe seeds in nature that do not land on an appropriate host tree with suitable microsite conditions will not succeed due to both biotic and abiotic factors (see e.g., Rödl and Ward 2002).

Mistletoe infection takes up to a year as the seeds change from an autotrophic to a hemiparasitic life form (Yan 1993). Even a seed that germinates on a potential host tree must be resilient to the microclimate during the prolonged post-germination stages (Norton and Ladley 1998; Norton et al. 2002). The positive correlation between the high prevalence of mistletoes and tree height (Reid and Stafford Smith 2000; Aukema and Martínez del Rio 2002; Ward et al. 2006) could be explained by the ability of mistletoes to survive better at high light intensities. Bigger trees probably provide better moisture than short trees as well. Microclimate interacting with chemical releases from the host could have an appreciable effect on the growth and success of mistletoes and determine host specificity in mistletoes.

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Table 1

The generalised linear model results of the effects of treatments (shade, temperature and moisture) on seedling growth (hypocotyl length, haustorium formation, leaf size and survival) of *Agelanthus natalitius*

		Shade	Temperature	Moisture
Source of variation	df	p	p	p
Hypocotyl length				
Host	1	0.79	0.45	0.79
Site	1	0.66	0.01	0.98
Treatment	2	<0.001	<0.001	<0.001
Host × site	1	0.43	0.01	0.70
Host × treatment	2	0.76	0.20	0.46
Site × treatment	2	0.17	0.16	0.96
Site × host × treatment	2	0.09	<0.001	0.99
Error degrees of freedom	288			
Haustorium formation				
Host	1	0.36	0.02	<0.001
Site	1	0.61	0.01	<0.001
Treatment	2	<0.001	<0.001	0.79
Host × site	1	0.48	0.69	<0.001
Host × treatment	2	0.97	0.51	<0.001
Site × treatment	2	0.24	0.88	<0.001
Site × host × treatment	2	0.26	0.88	<0.001
Error degrees of freedom	288			

Table 1
(Continued)

		Shade	Temperature	Moisture
Source of variation	df	p	p	p
Leaf size				
Host	1	0.32	0.33	0.13
Site	1	0.29	0.30	0.62
Treatment	2	0.003	0.10	<0.001
Host × site	1	0.90	0.58	0.99
Host × treatment	2	0.76	0.62	0.001
Site × treatment	2	0.90	0.87	0.94
Site × host × treatment	2	0.90	0.29	0.89
Error degrees of freedom	288			
Survival				
Host	1	0.55	0.99	0.002
Site	1	0.08	0.99	0.77
Treatment	2	<0.001	<0.001	<0.001
Host × site	1	0.99	0.99	0.99
Host × treatment	2	0.57	0.96	0.04
Site × treatment	2	0.96	0.46	0.96
Site × host × treatment	2	0.29	0.86	0.96
Error degrees of freedom	288			

Table 2

The generalised linear model results of the effects of treatments (shade, temperature and moisture) treatment on seedling growth (hypocotyl length, haustorium formation and survival) of *Viscum rotundifolium*

		Shade	Temperature	Moisture
Source of variation	df	p	p	p
Hypocotyl length				
Site	2	0.73	0.93	0.78
Treatment	2	<0.001	<0.001	<0.001
Site × treatment	4	0.96	0.74	0.95
Error degrees of freedom	216			
Haustorium formation				
Site	2	0.85	0.55	0.89
Treatment	2	<0.001	<0.001	<0.001
Site × treatment	4	0.90	.32	0.98
Error degrees of freedom	216			
Survival				
Site	2	0.51	1.00	0.07
Treatment	2	<0.001	<0.001	0.005
Site × treatment	4	0.32	0.84	0.68
Error degrees of freedom	216			

Figure Captions

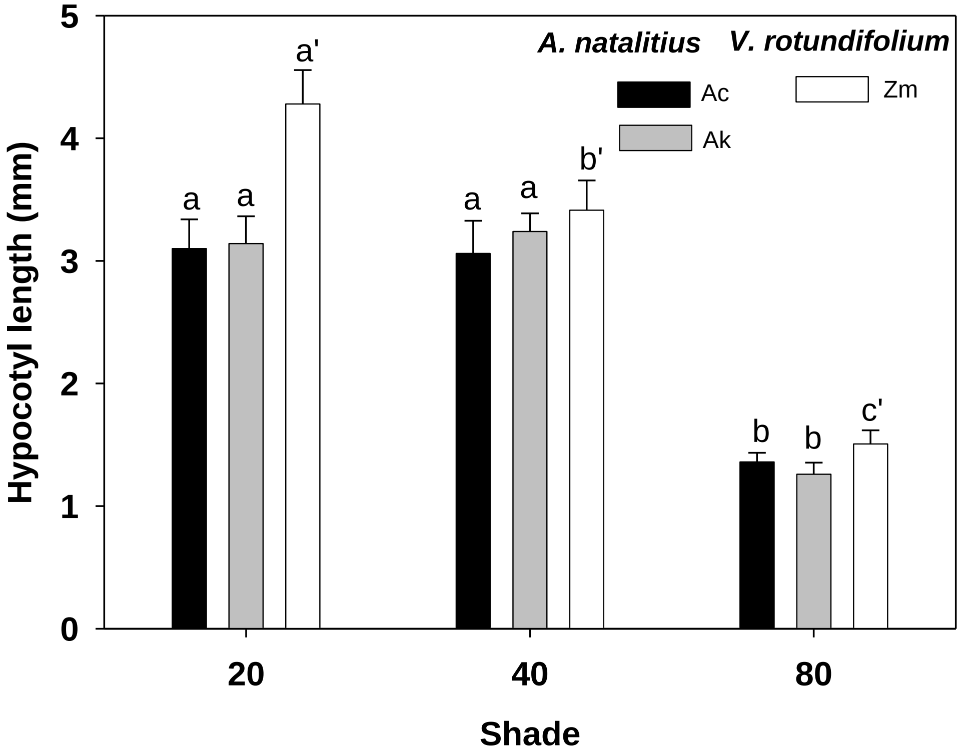
Fig. 1 Seedling hypocotyl length (mean ± SE) over six months in two species of mistletoes, *Agelanthus natalitius* and *Viscum rotundifolium.* The treatments were: **A** shade, **B** temperature and **C** moisture. In **C, 1** indicates the treatment of quarter strength Hoagland's solution every second day, **2** indicates half strength Hoagland's solution every second day and **3** indicates a continuous supply of distilled water every day supplemented with a quarter strength Hoagland's solution every two weeks. Seeds of *A. natalitius* were obtained from *Acacia caffra* (**AnAc**) and *Acacia karroo* (**AnAk**) and seeds of *V. rotundifolium* were obtained from *Zizphus macronata* (**VrZm**).

Fig. 2 Seedlling haustorium formation over six months in two species of mistletoes, *Agelanthus natalitius* and *Viscum rotundifolium.* The treatments were: **A** shade, **B** temperature and **C** moisture. Seeds of *A. natalitius* were obtained from *Acacia caffra* (**AnAc**) and *Acacia karroo* (**AnAk**) and seeds of *V. rotundifolium* were obtained from *Zizphus macronata* (**VrZm**).

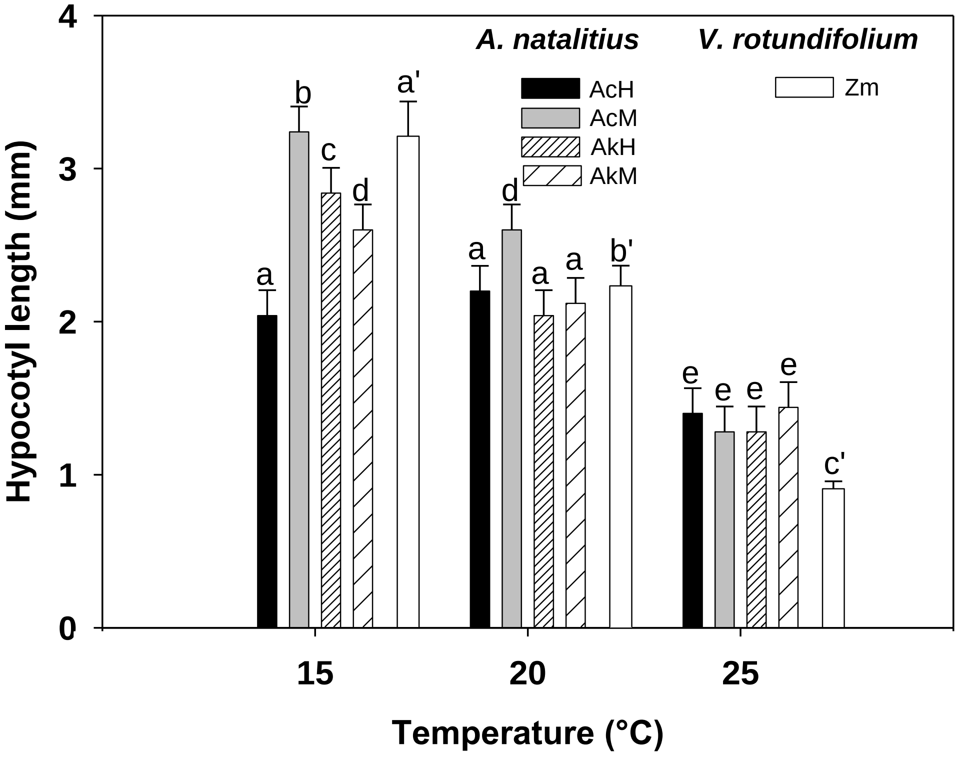
Fig. 3 Seedling leaf length (mean ± SE) of *Agelanthus natalitius* under the following treatments: **A** shade, **B** temperature and **C** moisture. None of the *Viscum rotundifolium* seedllings produced leaves within the six months. Seeds of *A. natalitius* were obtained from *Acacia caffra* (**AnAc**) and *Acacia karroo* (**AnAk**) and seeds of *V. rotundifolium* were obtained from *Zizphus macronata* (**VrZm**).

Fig. 4 Seedling survival frequency (%) over six months of the two species of mistletoes, *Agelanthus natalituis* and *Viscum rotundifolium* treated under the following treatments: **A** shade, **B** temperature and **C** moisture. Seeds of *A. natalitius* were obtained from *Acacia caffra* (**AnAc**) and *Acacia karroo* (**AnAk**) and seeds of *V. rotundifolium* were obtained from *Zizphus macronata* (**VrZm**).

(A)


A. natalitius
V. rotundifolium
Ac
Ak
Zm
Hypocotyl length (mm)
0
1
2
3
4
5
a
a
a'
a
a
b'
b
b
c'
20
40
80
Shade

(B)


A. natalitius
V. rotundifolium
AcH
AcM
AkH
AkM
Zm
Hypocotyl length (mm)
0
1
2
3
4
a
b
c
d
a'
a
d
a
a
b'
e
e
e
e
c'
15
20
25
Temperature (°C)

(C)

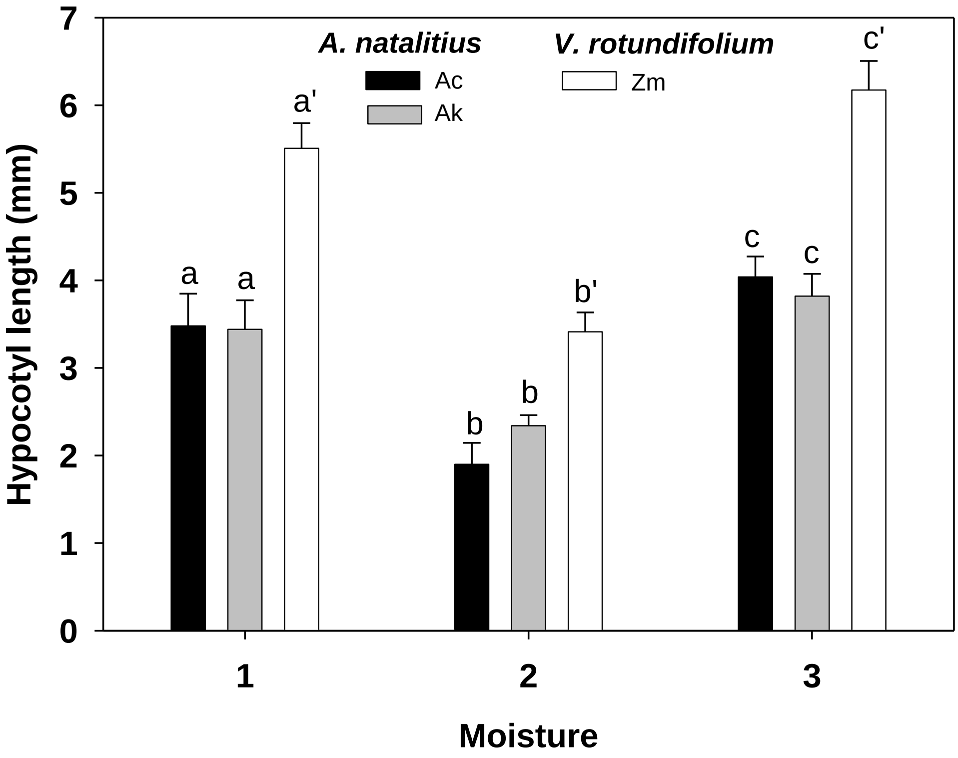
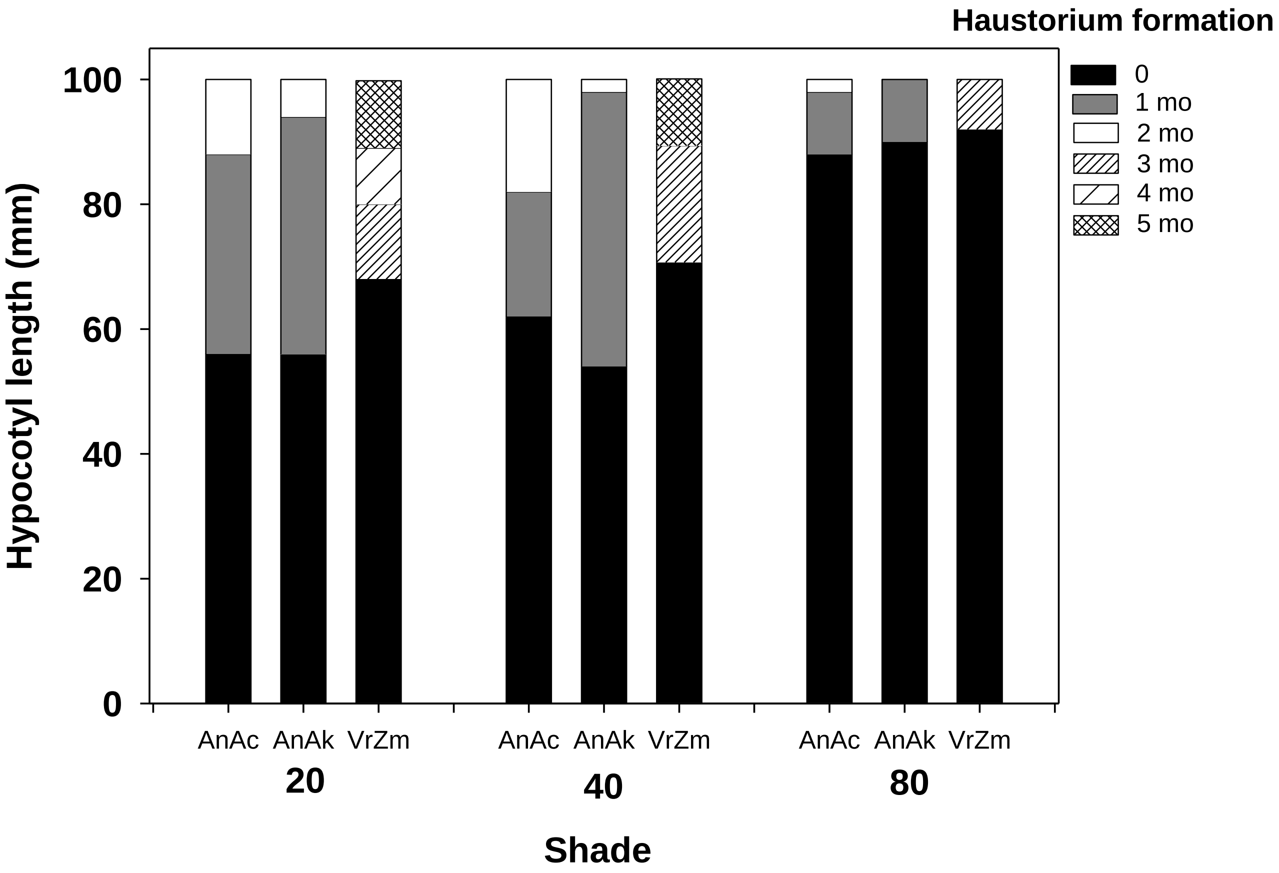
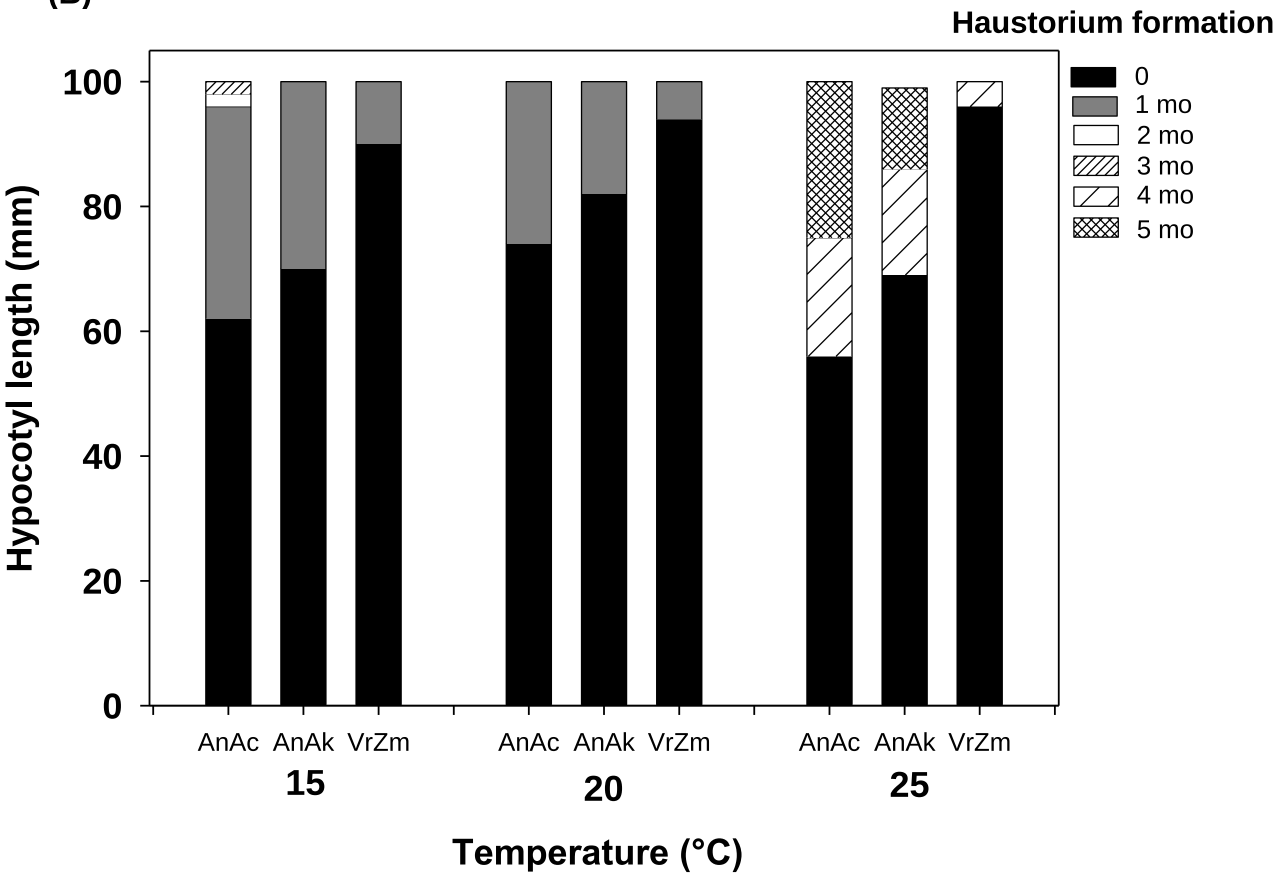

A. natalitius
Ac
Ak
V. rotundifolium
Zm
Hypocotyl length (mm)
0
1
2
3
4
5
6
7
Moisture
1
2
3
a
a
a'
b
b
b'
c
c
c'

Fig. 1

(A)


Haustorium formation
0
1 mo
2 mo
3 mo
4 mo
5 mo
Hypocotyl length (mm)
0
20
40
60
80
100
AnAc
AnAk
VrZm
20
40
80
Shade

(B)


Haustorium formation
0
1 mo
2 mo
3 mo
4 mo
5 mo
Hypocotyl length (mm)
0
20
40
60
80
100
AnAc
AnAk
VrZm
15
20
25
Temperature (°C)

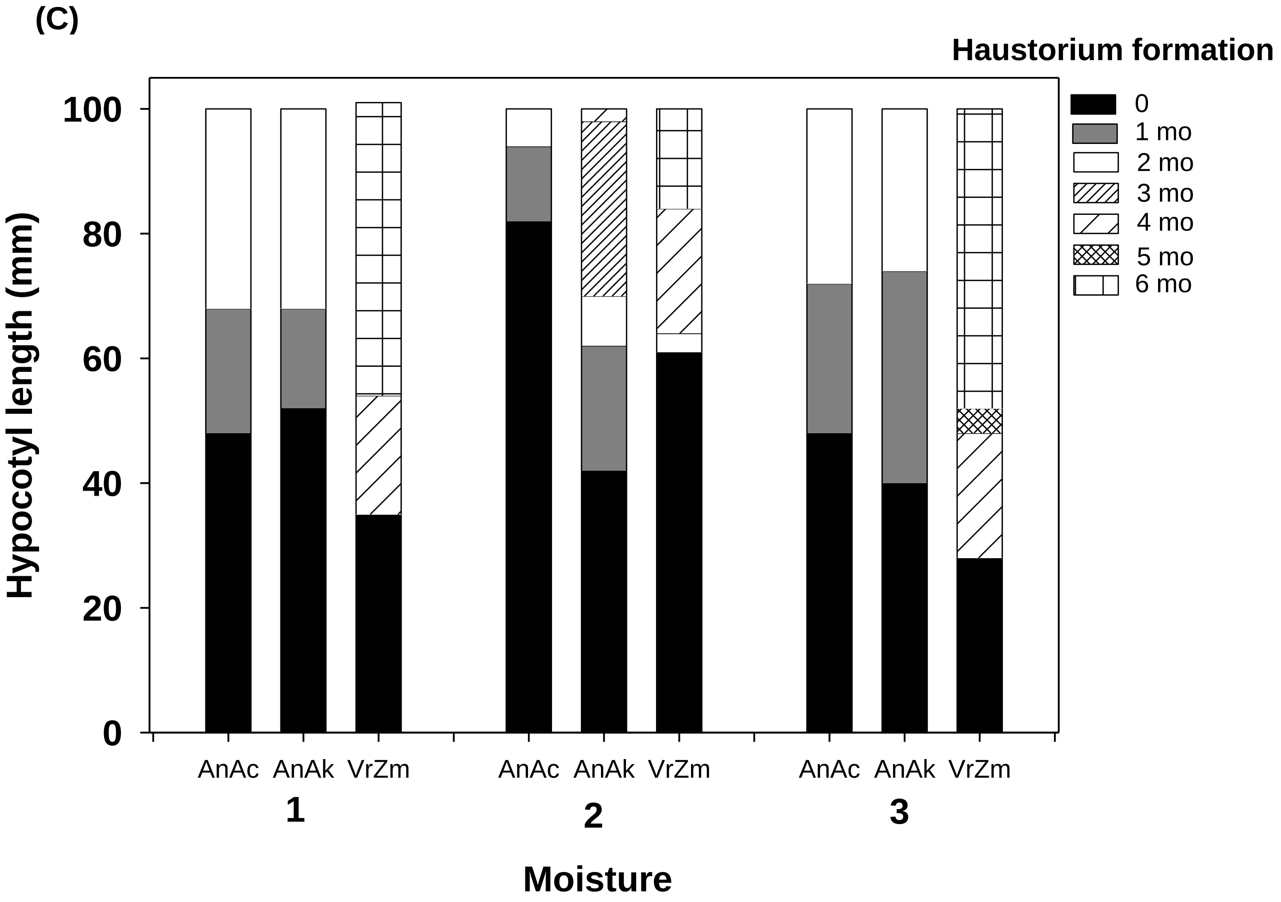
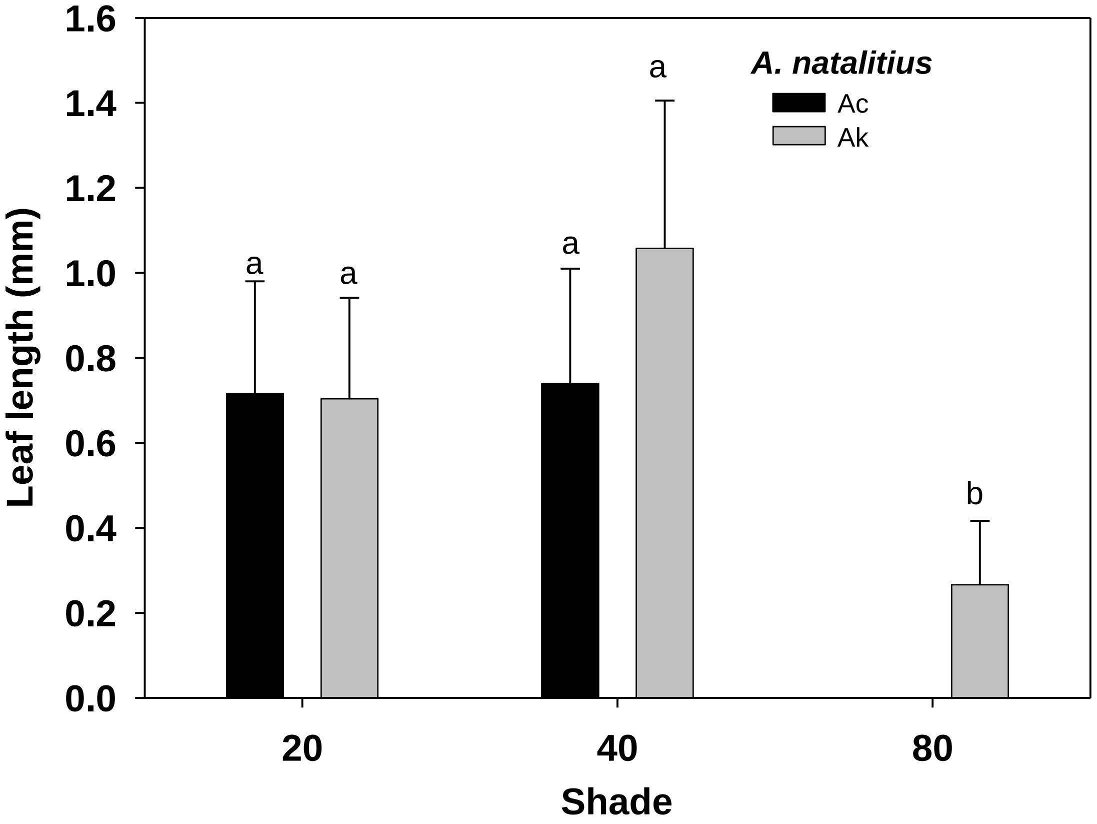

(C)
Haustorium formation
0
1 mo
2 mo
3 mo
4 mo
5 mo
6 mo
Hypocotyl length (mm)
0
20
40
60
80
100
AnAc
AnAk
VrZm
AnAc
AnAk
VrZm
AnAc
AnAk
VrZm
1
2
3
Moisture

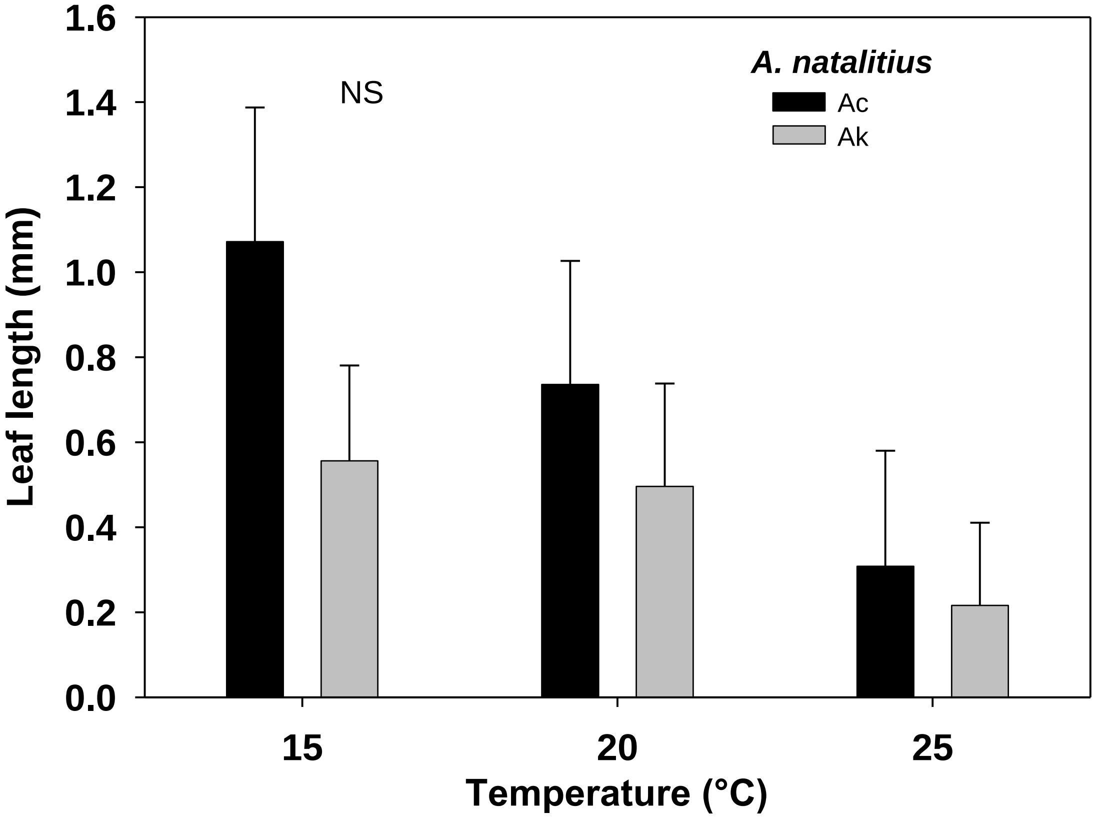
Fig. 2

(A)



Leaf length (mm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
A. natalitius
Ac
Ak
a
a
a
a
b
20
40
80
Shade

(B)



Leaf length (mm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
NS
A. natalitius
Ac
Ak
15
20
25
Temperature (°C)

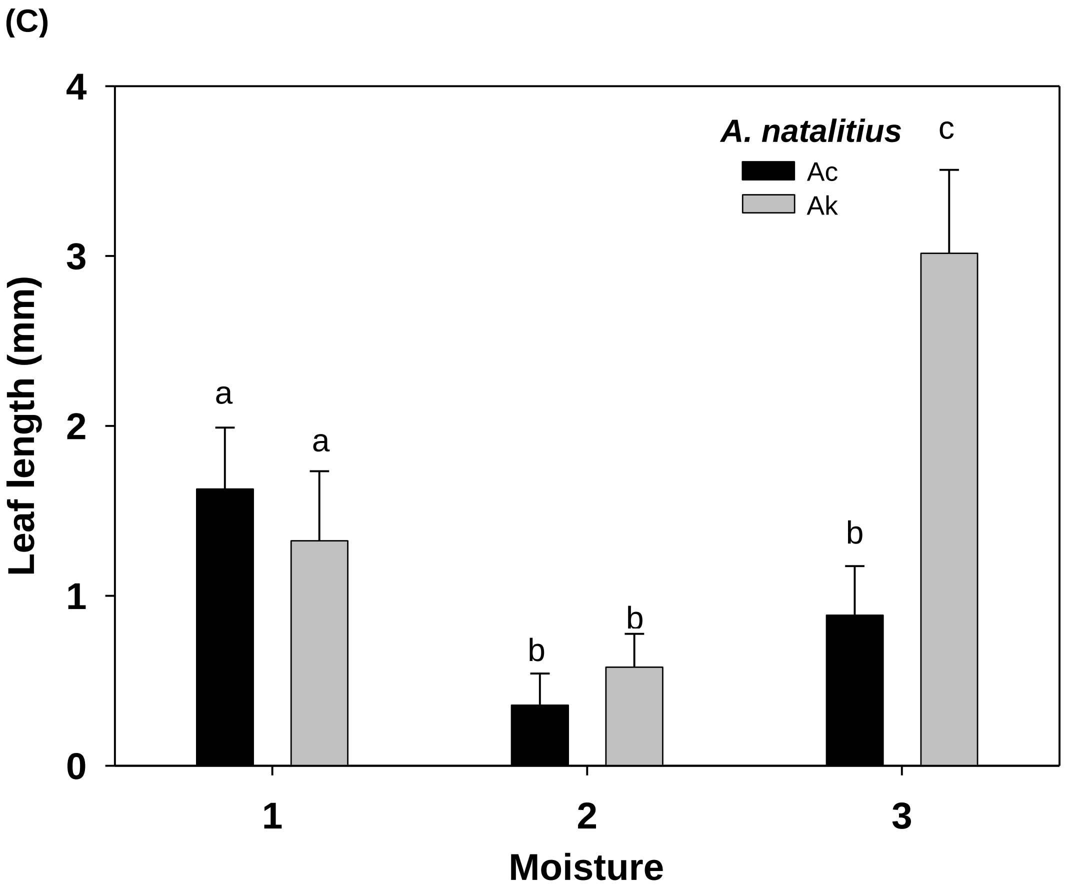
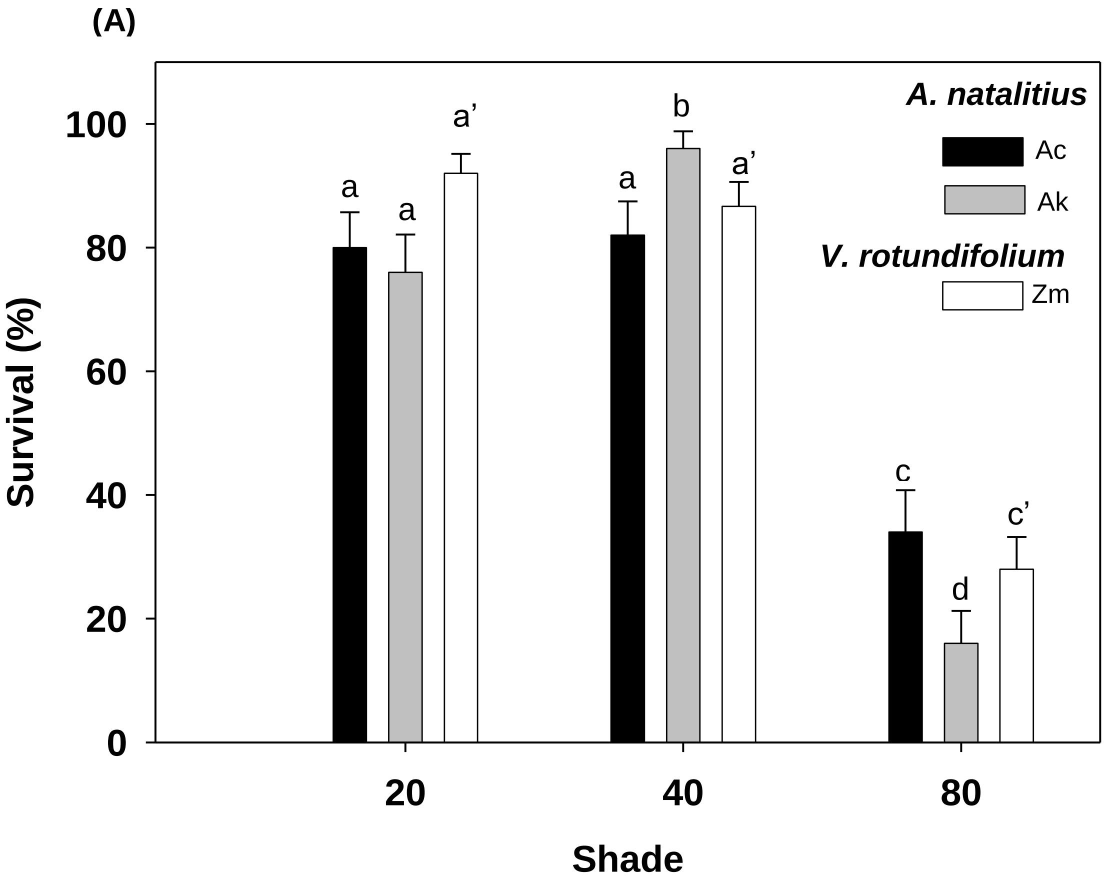
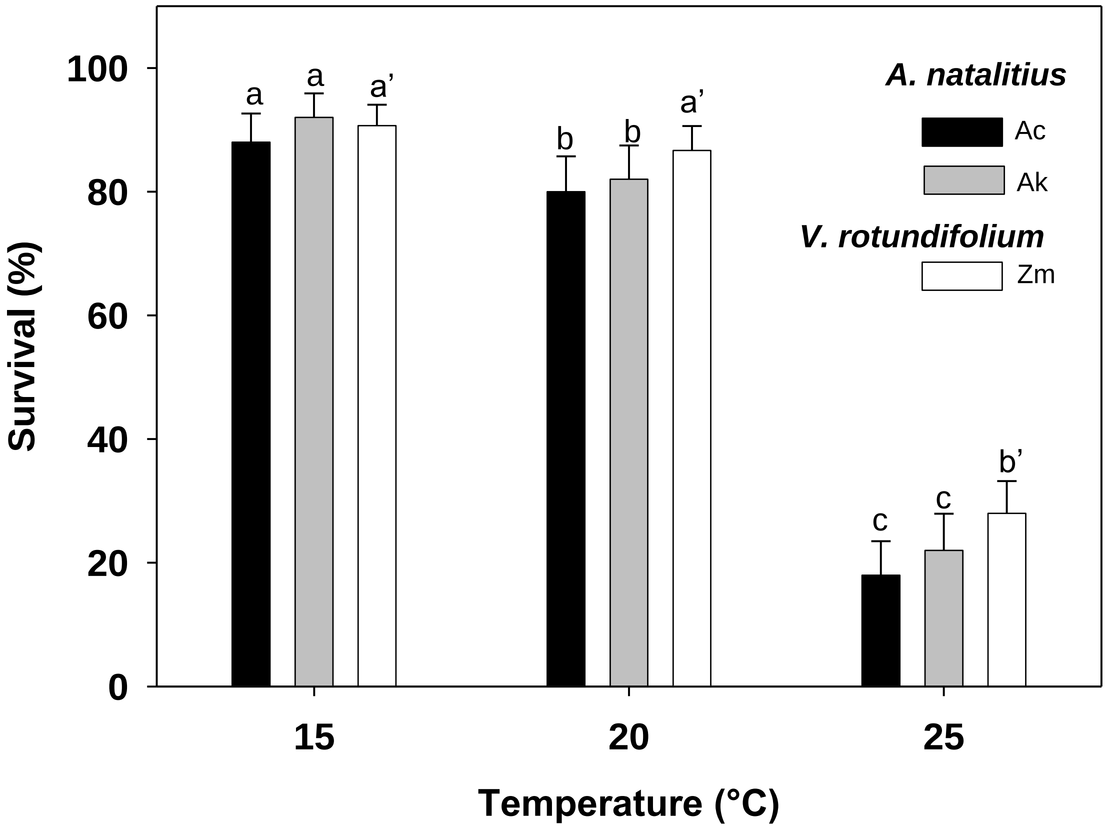

(C)
A. natalitius
Ac
Ak
Leaf length (mm)
0
1
2
3
4
a
a
b
b
b
c
1
2
3
Moisture

Fig. 3


(A)
Survival (%)
0
20
40
60
80
100
20
40
80
Shade
A. natalitius
Ac
Ak
V. rotundifolium
Zm
a
a
a'
a
b
a'
c
d
c'


Survival (%)
0
20
40
60
80
100
15
20
25
Temperature (°C)
A. natalitius
Ac
Ak
V. rotundifolium
Zm
a
a
a'
b
b
a'
c
c
b'

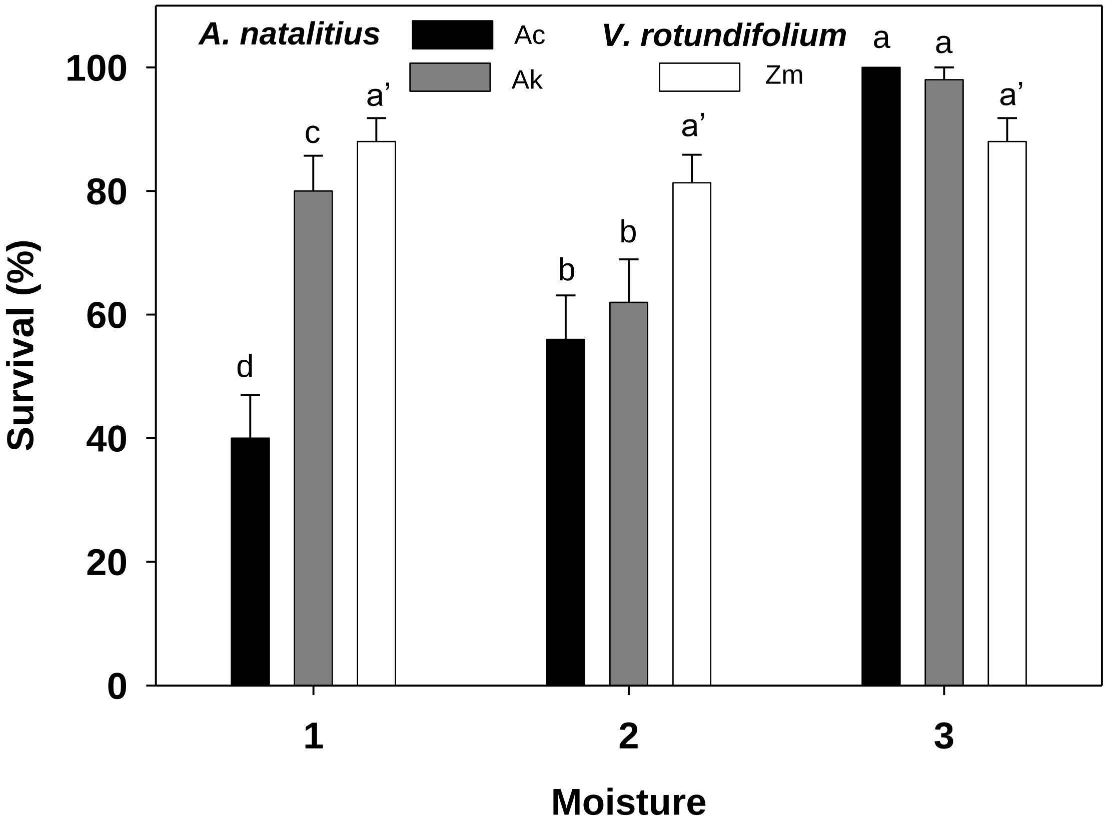

A. natalitius
Ac
Ak
V. rotundifolium
Zm
Survival (%)
0
20
40
60
80
100
1
2
3
Moisture
d
c
a’
b
b
a’
a
a
a’

Fig. 4

CHAPTER 6

Mistletoes control water loss through stomatal closure

Desale Y. Okubamichael, Megan E. Griffiths and David Ward*

School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa

To whom correspondence should be addressed author: Email: ward@ukzn.ac.za

Abstract

Mistletoes maintain higher transpiration rates and more negative water potentials than their host trees so that they can take up mineral nutrients. Little is known about the role that the stomatal morphology of mistletoes plays in controlling water loss. Mistletoes are also reputed to not respond to abscisic acid (ABA), which is a plant regulator that triggers stomatal closure during times of water stress. In the field, we investigated the water potential relationships of two mistletoes, *Agelanthus natalitius* and *Erianthemum dregei* (Loranthaceae), which parasitise *Acacia karroo* and *Vitex obovata*, respectively. Using plants grown in the greenhouse, we examined the stomata of the leaves of mistletoes and their hosts. We extracted one pair of leaves (one each for the dorsal and ventral surfaces) from each of seven replicates of plants which we sampled during the morning, midday and after treatment with abscisic acid. We examined the leaves using scanning electron microscopy and measured the size and number of stomata on leaves. In the field, we found that mistletoes had more negative water potentials than their host trees. The water potential of mistletoes was positively correlated with that of their host trees, particularly when they were highly stressed at midday. The micrograph images revealed that mistletoes have a higher density of larger stomata than their host trees. Mistletoes have more stomata on the dorsal and ventral surfaces of their leaves compared with their host trees, which mainly have stomata on the ventral side. Both mistletoes and host leaves close their stomata during midday and in response to ABA. These findings support the previous suggestions that mistletoes potentially lose more water than their host trees but can also control water loss. We suggest that this ability to control water loss is one of the primary reasons why mistletoe parasitism rarely kills host trees, which would be maladaptive.

Key words: leaf surface, host, parasite, scanning electron microscopy, stomata density, stomata length, water potential

Introduction

Mistletoes are aerial hemiparasites that obtain water and nutrients from their host plants (Schulze *et al*. 1984; Ehleringer *et al.* 1985; Sala *et al*. 2001; Glatzel and Geils 2009). Mistletoes usually maintain higher transpiration rates and more negative water potentials than their host so that they can passively uptake mineral nutrients (Schulze *et al*. 1984; Panvini and Eickmeier 1993). Mistletoes usually have higher mineral concentrations than their host trees (Lamont and Southall 1982; Lamont 1983; Panvini and Eickmeier 1993; Türe *et al*. 2010), which is enhanced by the predominantly one-way flow of nutrients from host to parasite (Glatzel and Geils 2009).

Some research has suggested that mistletoes do not close their stomata in response to abscisic acid (ABA) (Schulze *et al*. 1984; Escher *et al*. 2008). ABA is a plant regulator that causes leaf stomata to close when there is water stress to avoid dehydration and wilting (Radin *et al.* 1988; Franks and Farquhar 2001; Hussain *et al*. 2012). ABA synthesis occurs either in the leaf or in the root and the regulator is transported in the xylem and phloem (Davies and Zhang 1991). Root-borne ABA initiates water control, but if water shortage in the soil is prolonged the ABA concentration gradually builds in the transpiration flow. This is further enhanced by ABA production from old leaves during drought and is the reason that old leaves wilt before younger leaves (Hussain *et al*. 2012). Consequently, a high concentration of ABA enhances stomatal inhibition in young leaves. It has been suggested that the midday decline in stomatal conductance is due to increased sensitivity to ABA of the xylem, which is induced by negative leaf water potential in plants (Hussain *et al*. 2012). However, whether ABA plays a role in controlling mistletoe stomata is unknown.

There have been several studies on the water and nutrient relationships of mistletoes and their host trees (e.g., Glatzel 1983; Davidson and Pate 1992; Press and Graves 1995), but little is known about the underlying mechanisms of water and nutrient acquisition by mistletoes. Several studies (e.g., Davidson and Pate 1992; Strong and Bannister 2002; Bowie and Ward 2004) quantify the differences between mistletoes and host trees in terms of water potential, transpiration, stomatal conductance and carbon assimilation. This study aimed to investigate the water potential relationships of mistletoe–host pairs and explore the control of water loss in mistletoes by direct observation of their stomata. We measured water potential of the mistletoe–host pairs in the field and assessed the closing of the stomata of independently grown mistletoes and their host trees at different times during the day and after abscisic acid treatment in a greenhouse. We were particularly interested in testing the hypothesis that stomata of mistletoes remain open all the time and do not respond to ABA, thus lacking a control for water loss.

Materials and methods

Field water potential measurements

We measured the water potential of mistletoe–host pairs at Mtontwane (28° 48'S, 29° 56'E) in the KwaZulu-Natal midlands, South Africa. These pairs were the mistletoe *Agelanthus natalitius* and its preferred host *Acacia karroo* and the mistletoe *Erianthemum dregei* and its host *Vitex obovata*. We measured leaf water potential at predawn (during the time of maximum water uptake due to stomatal closure and lower ambient temperatures) and at midday (when there is maximum water stress and more negative water potentials due to maximum transpiration). We randomly selected ten mistletoe-infected host trees with fewer than three mistletoes per host for each mistletoe–host pair. We selected leaves with a similar position in the canopy and exposure to the sun. At predawn and midday for each of the 10 mistletoe–host pairs, we excised one leaf from the host and one leaf from the mistletoe with its petiole intact. We immediately measured the water potential for each leaf using a Scholander pressure chamber (modification of the model 3115 produced by PMS, Oregon, USA) and a ×10 magnifying lens to observe water exuded on the surface of the petiole. As soon as the first drop of the water was exuded the pressure was recorded.

Greenhouse experiments

Haustorium-intact seedlings of the mistletoes *Agelanthus natalitius* and *Erianthemum dregei* were detached from their preferred host trees—*Acacia karroo* and *Vitex obovata*, respectively—from

Mtontwane. Host seedlings (*A. karroo* and *V. obovata*) were collected from the same site (N = 30 plants from each species). Host and mistletoe seedlings were placed with their roots in jars of water and immediately transported to the University of KwaZulu-Natal at Pietermaritzburg.

The mistletoe and host seedlings were planted on separate sand-filled 2 L pots with perforated bases to facilitate drainage. Prior to use, the sand was acid-washed and oven-dried to ensure that it was free of any nutrients and microbial organisms which would affect the growth and conditions of the experiment. Based on pilot studies, a quarter strength Hoagland’s solution was used as a nutrient source, with 10 mL applied three times per week. In addition, plants were watered daily to keep the substrate moist. Seedlings were kept in the greenhouse for 6 mo before commencement of the experiment.

Leaf sampling

After 6 mo, mistletoe seedlings had sufficient leaves for use in experiments. From the established plants, we selected 14 individuals of each species from which we collected leaves for use in the experiment (14 plants × 4 species = 56 plants). During the morning, we sampled one pair of leaves (one for measuring the dorsal surface of the leaf and the other one for the ventral) from each of seven plants of the four species (morning batch: 2 leaves × 7 plants × 4 species = 56 leaves). Similarly, at midday we sampled another batch of 56 leaves from the same plants that we used for the morning batch. In the ABA treatments, seven randomly selected plants of each species were watered with 10 mL of ABA (50 µM). This was done in the morning when we expected the stomata to be open so that these leaves could be compared to the untreated morning and midday leaf responses of the plant species used in the study. Based on our own pilot studies and following the protocol of Hussain *et al*. (2012) for exogenous application of ABA, we excised leaves after 2.5 h. For each sample, leaves of similar size and position on the plant were excised using a sharp razor blade.

Scanning Electron Microscopy

Preparing biological samples for observation with a scanning electron microscope requires a standard procedure of chemical fixation, preservation by dehydration/drying, mounting on a stub and coating with a metal (e.g., chromium, gold and platinum) (Pathan *et al*. 2008). In our experiment, the excised leaf samples were immediately immersed in 3% glutaraldehyde and left overnight at room temperature (Pathan *et al*. 2008). Fixed leaves were washed two times with 0.05 M cacodylate buffer pH 7.2 for 30 min each. The samples were dehydrated through a graded series of ethanol washes (30%, 40%, 60%, 80%, 90%) once for 10 min consecutively at each step, ending with three 100% ethanol washes each for 10 min. The specimens in 100% ethanol were transferred to a critical point dryer (CPD) (HCP-2, Hitachi. Japan) and flashed with liquefied carbon dioxide for 30 min. During this process, the water content of the leaf tissue was first replaced by ethanol, which was in turn replaced by CO_2. After CPD, the specimens were mounted on aluminium stubs and gold-coated using a polaron ES100 Sputter Coater (Ibaraki, Japan). The specimens were then loaded and viewed in the SEM (ZEISS EVO®, Carl Zeiss SMT, Germany).

In our pilot study, we observed that the stomata of the plants were concentrated at the centre of the blade. For each leaf surface, we randomly focussed on one spot from five different fields of view which were all at the middle of the leaf blade. We captured the micrograph images of *A. karroo–A. natalitius* pairs at ×600 magnification. At ×600 magnification the field of view was approximately 25,000 μm^2. We doubled the magnification to ×1.2k for the *V. obovata–E. dregei* pairs because the host leaves had smaller and fewer stomata. At ×1.2k the area of field of view was approximately 44,000 μm^2, which is almost double the area used for *A. karroo–A. natalitius* pairs.

Stomatal measurements

After the images were captured by SEM, size measurements were carried out using image processing software, analySIS v. 3.2. In this software the measurements were calibrated to measure the length of the stomata. Stomatal size (μm) was defined as the length between the junctions of the guard cells at each end of the stoma. Parlange and Waggoner (1970) showed that in the passage of gases through stomata, it is the linear dimensions rather than the area of the stomata that is important. We therefore measured length as an indication of stomata size.

Statistical analysis

The means of water potentials of mistletoes and their host trees at predawn and midday were compared using a paired t-test. We also conducted regression analysis to investigate if mistletoe water potential is related to that of their host trees. We applied a multi-level nested Generalised Linear Model (GLIM) with a Poisson distribution (logarithm link function) to test the variation of number of stomata between the mistletoe–host pairs. At the same time we investigated whether there is any variation in the distribution of stomata on the dorsal and ventral surfaces of leaves within each species and between the mistletoe–host pairs. We also applied a multi-level nested GLIM to investigate the variation in stomatal length between mistletoe–host pairs (normal distribution with identity link function). Length was treated as a continuous variable, while species and side of the leaf surface (dorsal or ventral) were treated as independent factors. Similarly, we applied a multi-level nested analysis of GLIM to the proportion of the closed stomata (binomial distribution with logit link function). SPSS version 21 was used for all statistical analyses.

Results

In the field we found significant differences in water potentials of mistletoes and their host trees at midday and predawn (range of t = 2.88-7.69, range of P = <0.001-0.018, error df = 9), with mistletoe water potentials being more negative than those of their host trees (Table 1). However, for *V. obovata* and *E. dregei*, predawn water potentials of mistletoes and their host trees were not significantly different (t = -1.44, P = 0.521, error df = 9). We also found that the mistletoes were water stressed when the host trees were stressed (Fig. 1). At midday, both mistletoes and their hosts had a significantly positive relationship (*A. karroo*–*A. natalitius*: r = 0.92, $F_{1,9}$ = 42.28, P = 0.001

and *V.obovata–E. dregei*: $r = 0.86$, $F_{1,9} = 23.61$, $P = 0.001$). At predawn, the relationship was siginificantly positive between *A. karroo* and *A. natalitius* ($r = 0.66$, $F_{1,9} = 6.10$, $P = 0.039$), but not between *V. obovata* and *E. dregei* ($r = 0.26$, $F_{1,9} = 0.59$, $P = 0.464$). This indicates that mistletoes are able to coordinate and maintain more negative water potentials than their host trees but the mistletoes do respond to the conditions of their host trees.

The micrograph images (Fig. 2) revealed that the number of stomata was significantly different between mistletoes and their host trees, with differences also between the type of leaf surface (dorsal or ventral) (Table 2). Mistletoes had a significantly higher number of stomata than their host trees (Fig. 3). For both mistletoes and their hosts, the ventral surface of the leaf had a significantly higher number of stomata than the dorsal surface (Fig. 3). Host trees had few stomata on the dorsal surface; in the case of *V. obovata,* there were almost no dorsal stomata (Fig. 3).

Mistletoes had significantly longer stomata than their host trees (Table 3, Fig. 4). Stomatal length was similar between the dorsal and ventral surfaces of the same leaf in both mistletoes and their hosts. The proportion of closed stomata was siginificantly affected by the species, sampling time and ABA treatments (Table 4). Leaves sampled at midday had more closed stomata than during the morning and when plants were treated with abscisic acid (Fig. 5), but the proportion of closed stomata in mistletoes was lower than for their host trees. Mistletoes and host trees respond the same way in closing of their stomata, but to a different degree for the high midday temperatures and ABA.

Discussion

The opening and closing of stomata is homeostatically controlled by leaf-xylem water potential (Brodribb and Holbrook 2003). When more water is lost than supplied by the host's xylem, water potential decreases, which triggers stomatal closure (Brodribb and Holbrook 2003; Brodribb *et al*. 2003). However, mistletoes are able to maintain a flux gradient, avoid stomatal closure and wilting and tolerate a more negative water potential than their host trees (Glatzel and Geils 2009).

Mistletoes passively uptake nutrients and use unidirectional flow, which usually enables them to concentrate mineral nutrients in the leaf (Lamont and Shouthall 1982; Pate *et al*. 1991). Several studies have shown that mistletoes exhibit higher transpiration rates than their host trees (Fisher 1983; Glatzel 1983; Schulze *et al*. 1984; Ullmann *et al*. 1985; Urban *et al*. 2012). Thus mistletoes can bypass the homeostatic control of water by the host trees. Several studies have proposed that mistletoes have little control of their stomata and that guard cells remain turgid or open all the time (Hellmuth 1971; Fisher 1983; Escher *et al*. 2008; Ziegler *et al*. 2009; Zweifel *et al*. 2012). Our finding shows that mistletoes have more negative water potentials than their host trees.

From our results it is clear that the abundance of stomata on mistletoe leaves was higher than on their host trees. The length of the mistletoe stomata was also much greater than their host trees. In addition, we showed that the stomata of mistletoes stay open during the morning but are closed during midday, which is similar to the pattern shown by their host trees. Thus, as the day progresses, changes in the microclimate signal the stomata to close so that the mistletoe plants conserve water. It is also clear that mistletoe stomata are distributed in a similar pattern for both ventral and dorsal surfaces of the leaf. This strengthens the ability of mistletoes to maintain more negative water potentials than their host trees by opening the stomata of both surfaces of the leaf.

Abscisic acid was also found to initiate the closing of stomata of mistletoes and their host trees. A number of studies have shown that an exogenous application of ABA has a similar effect to endogenous ABA of plants (Franks and Farquhar 2001; Hussain *et al*. 2012). For exogenous application of ABA, the quantities applied range from 5-100 µm, with response times of 0.5-4 h (Hussain *et al*. 2012). For example, exogenous application of ABA to soybean reduces the yield of the crop (Liu *et al*. 2004). It seems that the mistletoes of our study also shut their stomata in response to a similar range of ABA. The results of our study are consistent with findings that mistletoes can transpire more than their host trees (Schulze *et al*. 1984; Ullmann *et al*. 1985; Goldstein 1989; Bowie and Ward 2004; Okubamichael *et al*. 2011) but rejects the hypothesis of water loss of mistletoes in an uncontrolled fashion. Glatzel (1983) illustrates differences in stomatal closure and wilting for the *Loranthus europaeus* Jacq. and *Quercus robur* L. system. By tolerating more negative water potentials, the hemiparasite *L. europaeus* is capable of extracting water from the host and delaying stomatal closure to a much lower xylem water potential compared to the host (Glatzel and Geils 2009). This is consistent with our finding that the proportion of closed stomata in mistletoes was never as high as those of their host trees. Zweifel *et al*. (2012) showed that there was a slight deviation of measured transpiration from potential transpiration under very dry conditions in their study of *Viscum album* parasitising Scots pine (*Pinus sylvestris*). They interpreted stomatal closure to be likely when there is failure of the water-conducting system. Strong and Bannister (2002) also showed that transpiration by mistletoes was more coordinated when the host was water-stressed. Davidson *et al*. (1989) proposed that there is coordinated stomatal behaviour in *Amyema linophyllum* parasitising *Casuarina obesa.* These findings are consistent with and supported by direct evidence from our study.

It is commonly assumed that mistletoes can stress their host tree through excessive water loss and that host trees cannot control the water lost by the mistletoe (Garkoti *et al*. 2012). Zweifel *et al*. (2012) showed that infections by the mistletoe *Viscum album* disrupts the stomatal control system in Scots pine (*Pinus sylvestris*), causing early and oscillating closure of host stomata, thereby diminishing host photosynthetic carbon assimilation. In addition, mistletoes take up nutrients from their hosts, which could eventually result in a reduction of host productivity and growth (Tennakoon and Pate 1996; Rigling *et al*. 2010). In the long run, survival of host trees could be compromised by mistletoe infection. Our findings, however, show that mistletoes can control their water loss. Over-exploitation and drying of host trees would outweigh the short-term benefits to mistletoes and disadvantage them in the long term (Ward *et al*. 2006).

Mistletoes evolved in dry and nutrient-poor environments (Atsatt 1973). Thus, it is unlikely that mistletoes would have lost their ability to regulate loss of water. Goldstein et al. (1989) explained the ability of mistletoes to synchronise their water potential to that of their host trees resulting in their coexistence. Similarly, Urban *et al*. (2012) also indicated that mistletoes could have greater adaptation to water stress than their host trees and hence could potentially control water loss, which is consistent with and supported by direct evidence from our findings. We conclude that mistletoes coexist with their host trees by assimilating a similar pattern of stomatal response to diurnal microclimates and ABA. A higher density of larger stomata on both leaf surfaces than host leaves enables mistletoes to transpire more than their host trees once they are open.

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Table 1. The water potential (MPa) (mean ± SE) of mistletoes and their host trees at midday and predawn for *Acacia karroo–Agelanthus natalitius* and *Vitex obovata–Erianthemum dregei*.

	Water potential (MPa) (Mean ± SE)	
Time	***A. karroo–A. natalitius***	***V. obovata–E. dregei***
Midday	-1.85 ± 0.14/-2.28 ± 0.12*	-2.13 ± 0.18/-2.49 ± 0.15*
Predawn	-0.44 ± 0.06/-0.76 ± 0.12*	-1.44 ± 0.21/-1.33 ± 0.14

*Denotes statistical significance at a level of $p < 0.05$.

Table 2. Nested generalised linear model (GLIM) test results for number of stomata on the leaves of host trees and their mistletoes (*Acacia karroo–Agelanthus natalitius* and *Vitex obovata–Erianthemum dregei*). Leaf side = dorsal or ventral leaf surfaces. Statistical significance at a level of p < 0.05 is indicated in bold. The residual (error) d.f. = 85 for the analysis of each mistletoe–host pair.

Source of variation	**df**	***A. karroo–A. natalitius***		***V. obovata–E. dregei***	
		Wald Chi Square	***P***	**Wald Chi Square**	***P***
Species	1	115.2	**< 0.001**	27.9	**< 0.001**
Tree(species)	12	14.2	0.056	9.9	0.702
Leaf side(tree(species))	14	59.8	**< 0.001**	83.1	**< 0.001**
Error	85				

Table 3. Nested generalised linear model (GLIM) test results for length of stomata of host trees and mistletoes on both surfaces of the leaf (side = dorsal or ventral) for two pairs of host trees with their mistletoes (*Acacia karroo–Agelanthus natalitius* and *Vitex obovata–Erianthemum dregei*). Statistical significance at a level of $p < 0.05$ is indicated in bold. The residual (error) d.f. = 85 for the analysis of each mistletoe–host pair.

Source of variation		***A. karroo–A. natalitius***		***V. obovata–E. dregei***	
	df	**Wald Chi Square**	***P***	**Wald Chi Square**	***P***
Species	1	95.9	**< 0.001**	250.8	**< 0.001**
Tree(species)	12	16.6	0.081	14.4	0.278
Leaf side(tree(species))	14	228.5	**< 0.001**	25.7	**0.004**

Table 4. Nested generalised linear model (GLIM) test results for proportion of closed stomata at a given time on both surfaces (side = dorsal or ventral) for two pairs of host trees and their mistletoes (*Acacia karroo–Agelanthus natalitius* and *Vitex obovata–Erianthemum dregei*). ABA = abscisic acid application. Time = pre-dawn or midday. Statistical significance at a level of p < 0.05 is indicated in bold. The residual (error) d.f. = 55 for the analysis of each mistletoe–host pair.

Source of variation	**Df**	***A. karroo*–*A. natalitius***		***V. obovata*–*E. dregei***	
		Wald Chi Square	***P***	**Wald Chi Square**	***P***
Time/ABA	2	16.25	**< 0.001**	29.365	**< 0.001**
Species	1	0.041	0.839	0.274	0.601
Tree(species)	12	47.003	**< 0.001**	131.930	**< 0.001**
Leaf side(tree(species))	14	58.695	**< 0.001**	526.410	**< 0.001**
Time × leaf side(tree(species))	28	43.59	**0.030**	481.669	**< 0.001**

Figure captions

Fig. 1. The water potentials of mistletoes and their host trees were positively related at midday for *Acacia karroo–Agelanthus natalitius* (**B**) and *Vitex obovata–Erianthemum dregei* (**D**) but this relationship was only marginally significant at predawn for *A. karroo–A. natalitius* (**A)**. There was no significant relationship for the water potential of *V. obovata–E. dregei* (**C)** at predawn.

Fig. 2. Scanning electron micrograph of the ventral view of leaves of two mistletoe–host pairs: *Acacia karroo* (**A**) and *Agelanthus natalitius* (**B**), *Vitex obovata* (**C**) and *Erianthemum dregei* (**D**). Bars at the bottom right indicate a scale of 20 µm.

Fig. 3. (**A**) Number of stomata on leaves of *Acacia karroo* and *Agelanthus natalitius* (per 25,000 μm^2) and (**B**) Number of stomata on leaves of *Vitex obovata* and *Erianthemum dregei* (per 44,000 μm^2).

Fig. 4. Stomatal length of the mistletoes and their host trees for *Acacia karroo–Agelanthus natalitius* (**A**) and *Vitex obovata–Erianthemum dregei* (**B**). Mistletoe stomatal length was much longer than that of their hosts. The length of stomata did not vary much between the dorsal and ventral surface of the leaf.

Fig. 5. Proportion of closed stomata of a leaf during morning (Mor), after abscisic acid treatment (ABA) and midday (Mid) for the mistletoe–host pairs *Acacia karroo–Agelanthus natalitius* (**A**) and *Vitex obovata–Erianthemum dregei* (**B**). A higher proportion of stomata remained open during the morning but were closed at midday. Similarly, when plants were treated with ABA in the morning when they are expected to be open, they shut their stomata.

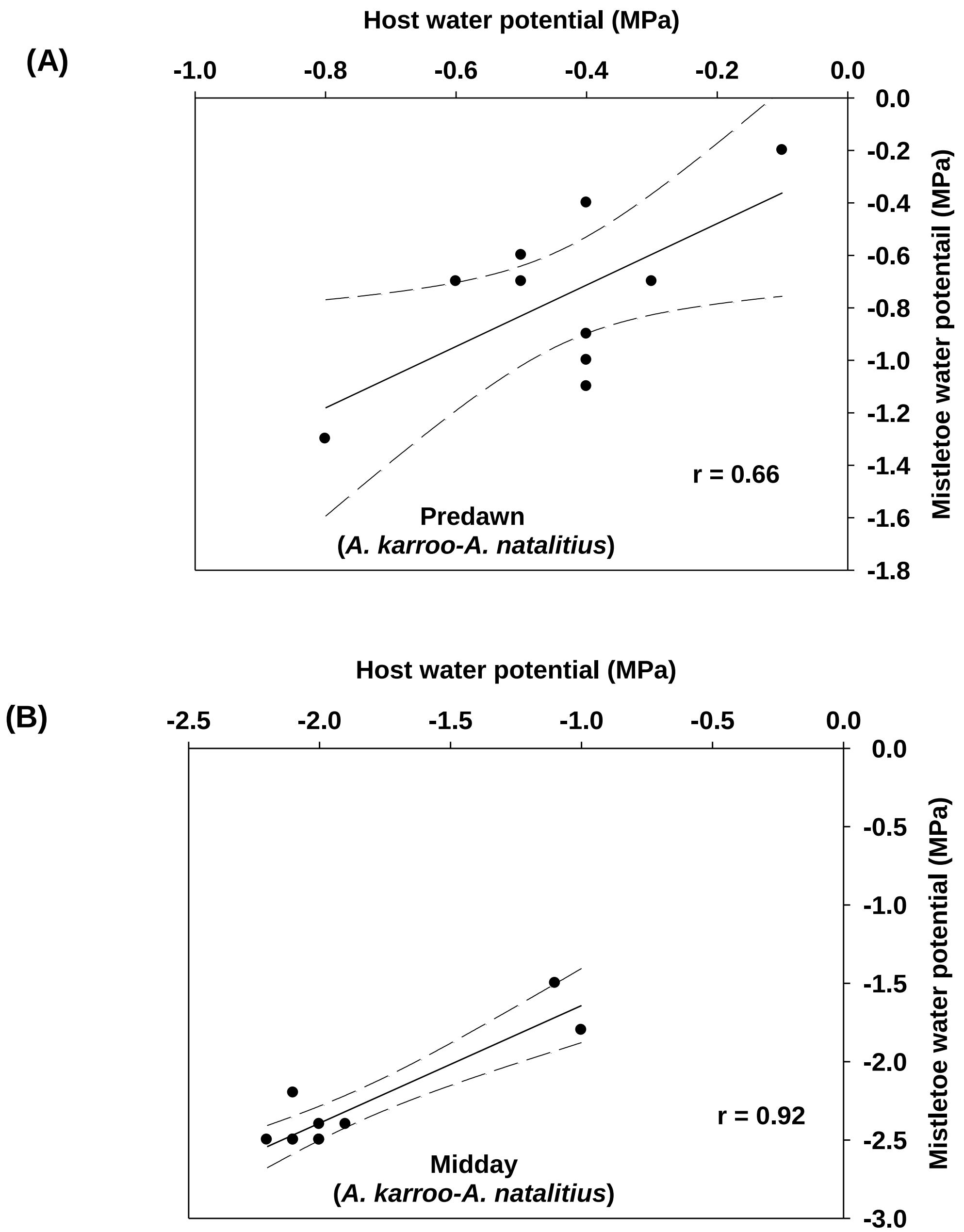

(A)
Host water potential (MPa)
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
Mistletoe water potentail (MPa)
r = 0.66
Predawn
(A. karroo-A. natalitius)
(B)
Host water potential (MPa)
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Mistletoe water potential (MPa)
r = 0.92
Midday
(A. karroo-A. natalitius)

Fig. 1.

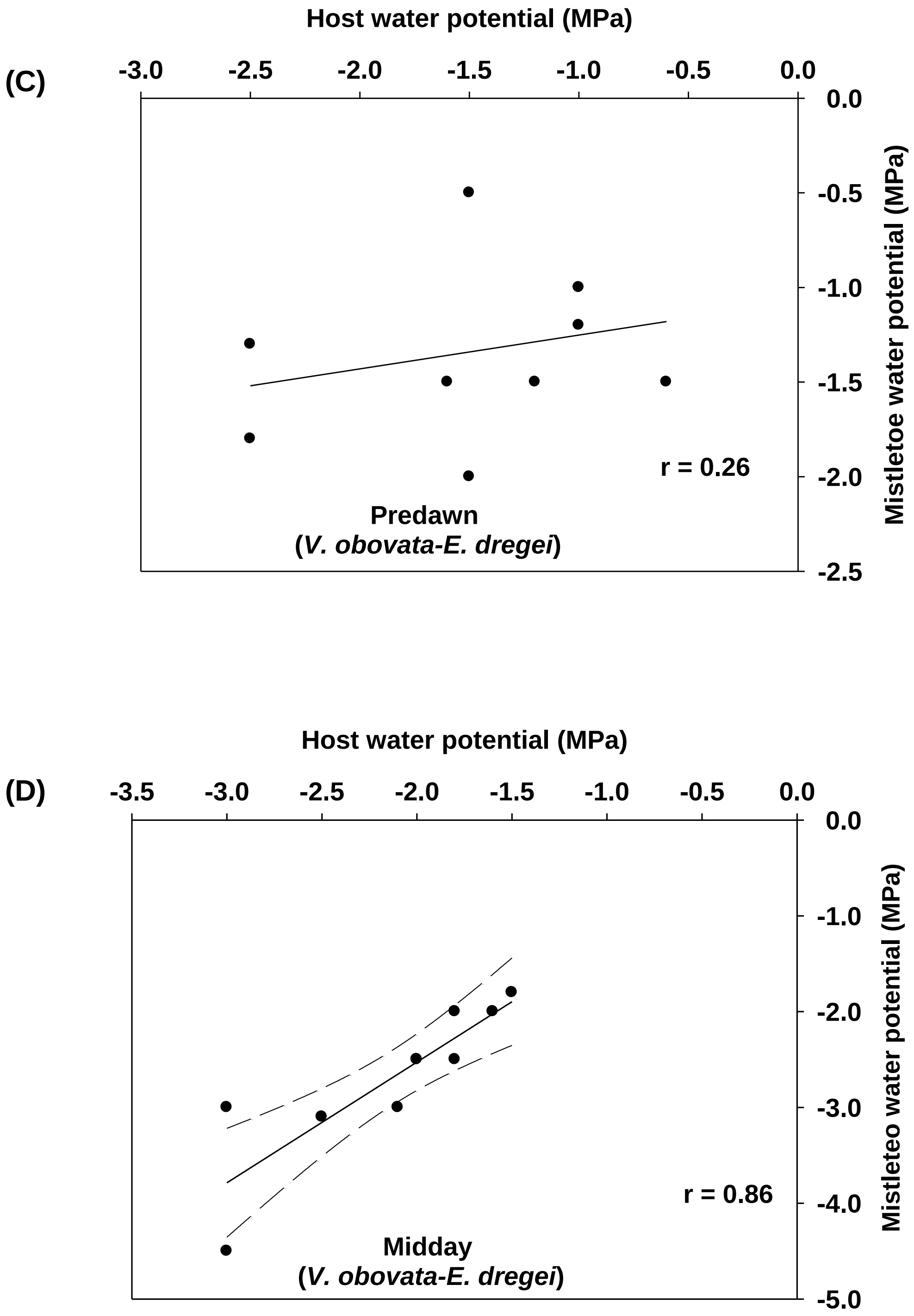
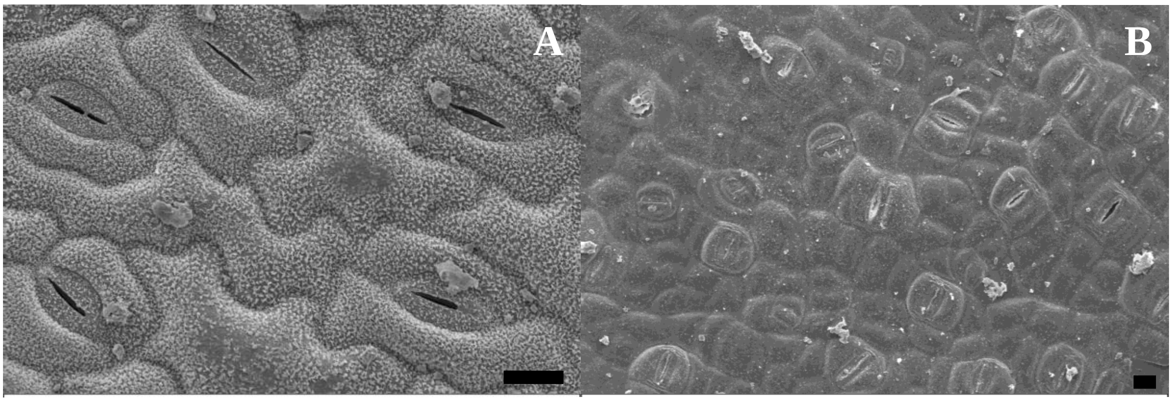
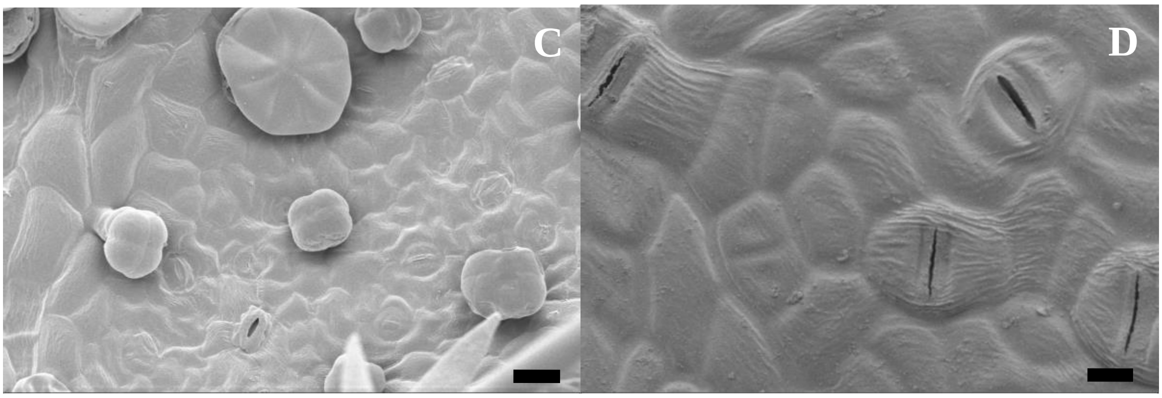

(C)
Host water potential (MPa)
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
Mistletoe water potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
r = 0.26
Predawn
(*V. obovata-E. dregei*)
(D)
Host water potential (MPa)
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
Mistleteo water potential (MPa)
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
r = 0.86
Midday
(*V. obovata-E. dregei*)

Fig. 1.



Acacia karroo

Agelanthus natalitius



Vitex obovata

Erianthemum dregei

Fig. 2.

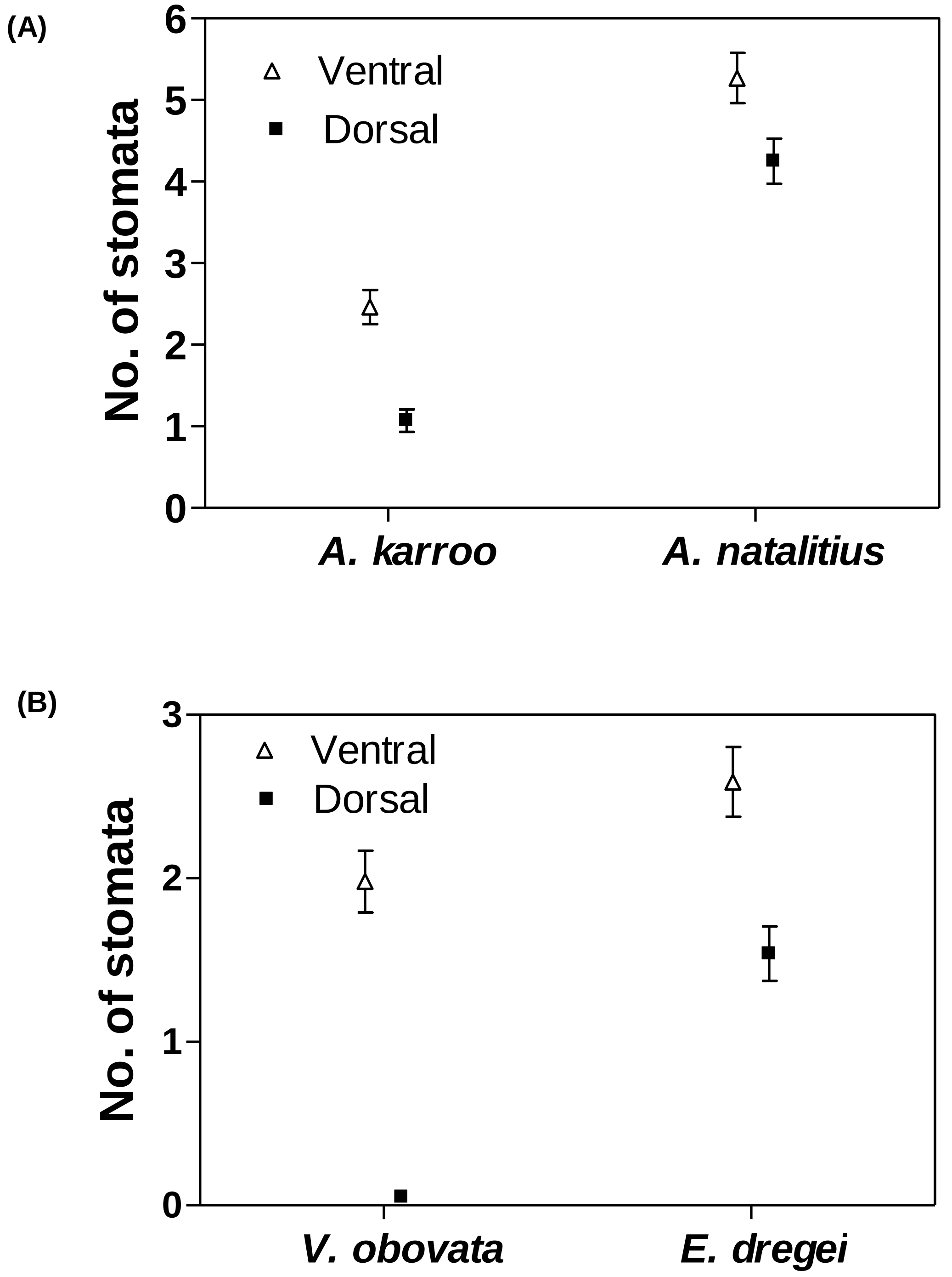
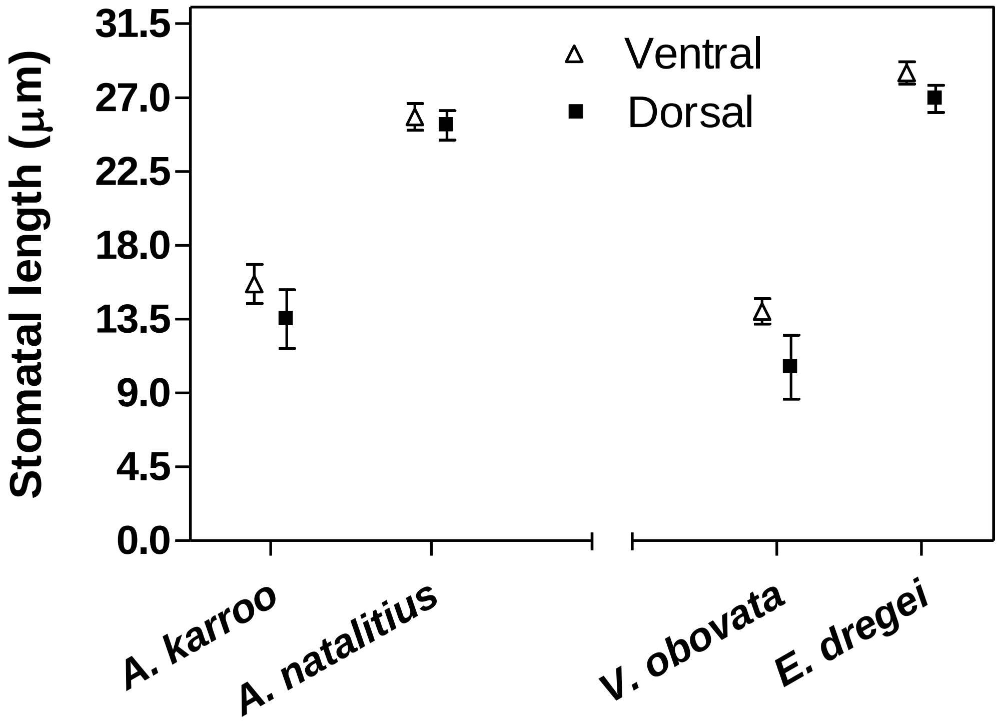

(A)
No. of stomata
6
5
4
3
2
1
0
Ventral
Dorsal
A. karroo
A. natalitius
(B)
No. of stomata
3
2
1
0
Ventral
Dorsal
V. obovata
E. dregei

Fig. 3.



Stomatal length (µm)
31.5
27.0
22.5
18.0
13.5
9.0
4.5
0.0
Ventral
Dorsal
A. karroo
A. natalitius
V. obovata
E. dregei

Fig. 4.

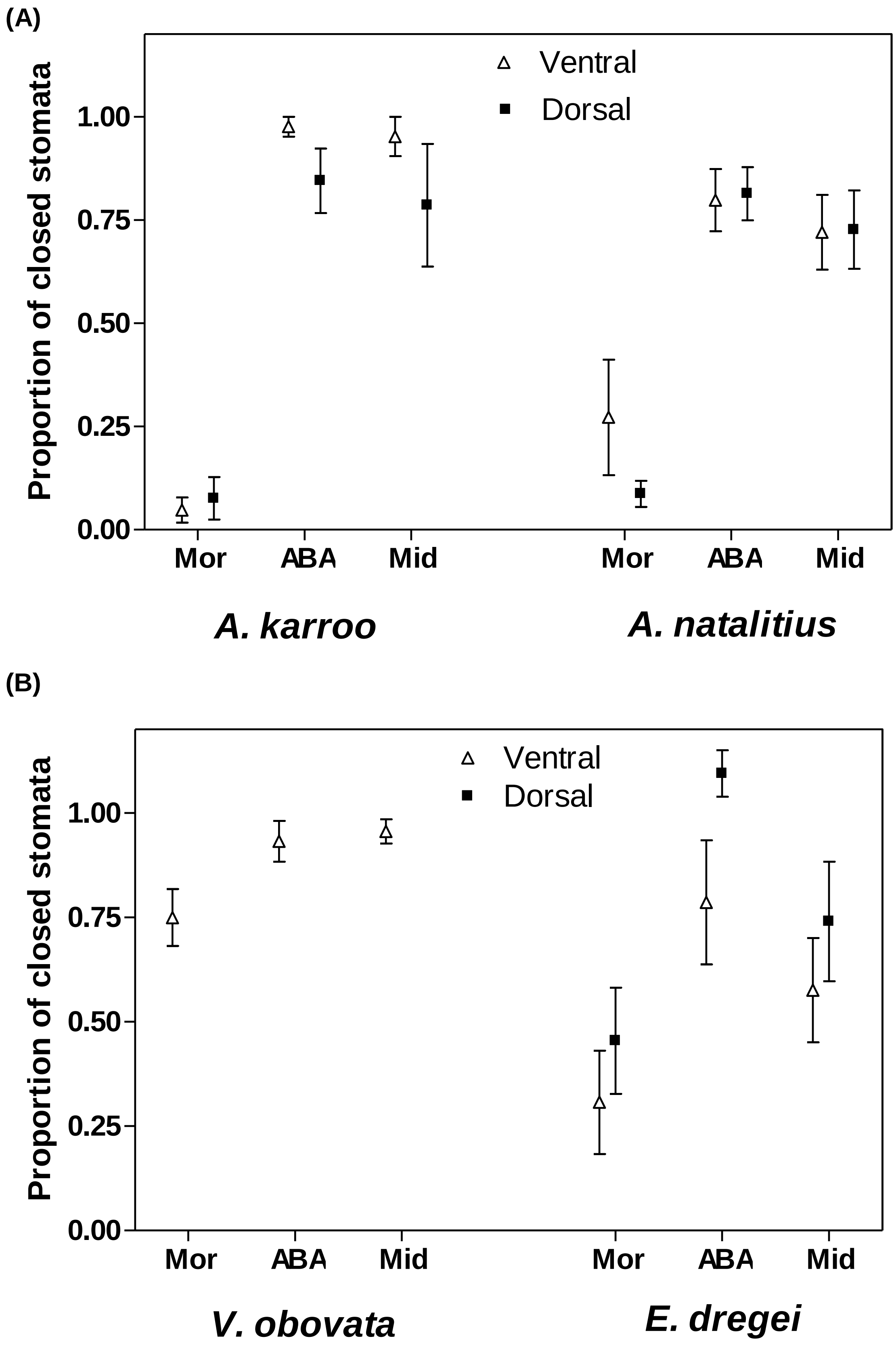

(A)
Ventral
Dorsal
Proportion of closed stomata
1.00
0.75
0.50
0.25
0.00
Mor
ABA
Mid
Mor
ABA
Mid
A. karroo
A. natalitius
(B)
Ventral
Dorsal
Proportion of closed stomata
1.00
0.75
0.50
0.25
0.00
Mor
ABA
Mid
Mor
ABA
Mid
V. obovata
E. dregei

Fig. 5.

CHAPTER 7

Summary, Conclusions and Future Directions

Host specificity has been a major focus in the study of mistletoes in recent years, with many important advances. However, most of these studies are localised and lack a holistic approach in understanding host specificity. Here I synthesise my findings on the mechanisms of host specificity and compare it with other findings. I also suggest where research should be focused in future studies. My aim is to promote discussion, set priorities and focus on developing a more comprehensive understanding of host specificity in parasitic plants.

The distribution of mistletoes within a plant community depends on the availability of suitable host trees for colonisation (Dean et al. 1994, Overton 1994, Downey 2004, Kavanagh and Burns 2012). A few mistletoes, such as the genus *Misodendrum*, are constrained to specific host species and only interact with these hosts over a very narrow range (Vidal-Russell and Nickrent 2007). However, most mistletoe species are generalists that infect multiple host species that widely differ morphologically and genetically (Norton and Carpenter 1998, Norton and de Lange 1999, Okubamichael et al. 2011a). Mistletoes that are host generalists at a larger spatial scale may infect a subset of available host species at a local scale (Norton and Carpenter 1998). I consider this to be an example of local host specificity (a measure of coevolution of mistletoes and their host trees), which is defined as the preferential parasitism of mistletoes of certain host species within a locality. Studies using inoculation experiments (Clay et al. 1985, Rödl and Ward 2002), allozymes (Glazner et al. 1988, Nickrent and Stell 1990), amplified fragment length polymorphisms (Jerome and Ford 2002) and molecular phylogenies (Amico et al. 2007, Amico and Nickrent 2009) have identified host races in mistletoes. Given the importance of host race formation in revealing sympatric speciation, more extensive and robust studies of host races are still warranted, as recommended by Amico et al. (2007).

In this thesis I synthesised information on host specificity from available literature on mistletoes, giving particular focus to mistletoe distribution patterns in southern Africa (Chapter 2). Mistletoe parasitism is not random in plant genera and species. The geographic mosaic model (Thompson 1994) can explain mistletoe–host interactions, host switches and help us to understand patterns of host specificity in mistletoes across their ranges. My synthesis showed that host compatibility (Hoffmann et al 1986, Roxburgh and Nicolson 2005, Fadini 2011) and host abundance (Norton and Carpenter 1998, Norton and de Lange 1999, Press and Phoenix 2005) are the most important factors determining host specificity of mistletoes (Chapter 2). It is known that volatile compounds direct the growth of dodder plants (*Cuscuta*) to the right host, leading to differentiation of the haustorium (Runyon et al. 2006). A similar mechanism was reported recently for mistletoes by Cannon et al. (2011), who demonstrated that *Phoradendron serotinum* (now *P. leucarpum*) seedlings respond to host volatile compounds in much the same manner. This initial finding will likely invite further investigation in more mistletoe species, which will enhance our understanding of the interactions that determine host specificity.

The findings of my research (Chapter 3) confirm that the mistletoe *V. rotundifolium* is specific on the host species *Ziziphus mucronata* in the Kimberley area although it occasionally parasitises *Ehretia rigida* (Chapter 3). The evolution of host specificity is usually related to the development of ecological dominance in plant communities (Barlow and Wiens 1977). Mistletoes tend to perform better on the host species that are most frequently encountered (Norton and Carpenter 1998, Norton and de Lange 1999, Press and Phoenix 2005). However, in this study the preferred host of *V. rotundifolium* was less abundant than the other potential host trees (Chapter 3). Thus, host compatibility may have played a more important role in directing the host specificity of *V. rotundifolium.* There is a high potential for birds to disperse seeds of *V. rotundifolium* to *Z. mucronata* because both have fruits that are consumed by frugivorous birds. Another factor to consider is the larger scale distribution of *Z. mucronata*. It is reported that *Z. mucronata* is quite common in adjacent properties (Bezuidenhout 1994, 1995, 2009), especially in Rooipoort Nature

Reserve. Thus, *V. rotundifolium* may have frequent interactions with the host species *Z. mucronata* across a wider geographic range than was included in this study.

Autoinfection is the most frequent form of mistletoe parasitism, through which a parasitised tree receives more mistletoe seeds each time mistletoe fruiting occurs (Aukema and Martínez del Rio 2002a,b, Ward 2005, Ward and Paton 2007, Okubamichael et al. 2011b). The interaction of generalist mistletoes (such as *V. rotundifolium*) and their host trees increases the likelihood of compatibility. In my research, I found that compatibility was manifested at an early stage of mistletoe establishment, survival and growth in *V. rotundifolium* (Chapter 3).

The results of my study on *Agelanthus natalitius* suggest that this mistletoe is adapted to the most common host trees in KwaZulu-Natal (Chapter 4). Roxburgh and Nicolson (2005) also showed that *Plicosepalus kalachariensis* was host specific on abundant host trees in southern Africa. These findings are also well supported in the New World; López de Buen and Ornelas (2002) found that *Psittacanthus schiedeanus* is most compatible with the abundant host tree, *Liquidambar styraciflua,* in central Veracruz, Mexico. I found that mistletoes placed on their preferred host trees had better growth and survival than those placed on other potential host trees (Chapter 4). Similarly, other studies showed that mistletoe seeds transferred to other potential host trees are not compatible with the new host substrate and may not survive (Clay et al. 1985, Yan 1993, Rödl and Ward 2002). In addition, these studies showed that mistletoes perform better on source host than non-source trees. I suggest that the ability of mistletoes to be successful on source host species promotes host specificity in mistletoes, albeit incompletely, which may reinforce and promote selective parasitism.

Within a host species, the degree of mistletoe infection is positively correlated with tree height (Chapters 3 and 4). This can be explained to some degree by the behaviour of birds that perch preferentially on tall trees (Aukema and Martínez del Rio 2002a,b). Tall trees are usually older and can thus accumulate mistletoes over time (Overton 1994, Ward et al. 2006). Roxburgh and Nicolson (2007) also showed that big trees provide higher nutrient concentrations than small trees, which would promote greater survival of mistletoes dispersed to those hosts. Mistletoes on tall trees may have greater access to light, which also contributes to the success of mistletoes (Ward and Paton 2007). In the field, the mistletoe species studied here tended to be common on exposed parts of trees, although this was not quantified. Similarly, other studies reported that mistletoes are abundant on the upper canopy (Ward 2005, Robinson and Geils 2006). This was supported by the findings of my mistletoe germination experiment, which showed that light availability and moisture were critical for the survival of mistletoe seedlings (Chapter 5).

Surprisingly there are not many studies on the effect of host quality on host specificity, although it is commonly accepted that mistletoes evolved a parasitic life history strategy to acquire nutrients (see a review on parasitic plant host choice by Pennings and Callaway 2002). Nitrogen is commonly the most limiting macronutrient in plants (Atsatt 1973, Pennings and Callaway 2002, Agren et al. 2012). It has long been thought that mistletoes selectively parasitise host species that are high in nitrogen (Schulze and Ehleringer 1985, Schulze et al. 1991, Dean et al. 1994, Pennings and Callaway 2002). However, host quality in terms of nutrients and water status had little effect in determining host specificity in our studies. I found that neither *V. rotundifolium* nor *A. natalitius* parasitised host trees that were high in water and nutrients, specifically nitrogen. Four *Acacia* species at Pniel potentially fix nitrogen (Kambatuku et al. 2012), but these species are not parasitised by *V. rotundifolium* (Chapter 3). My germination experiment also showed that moisture supply improved the growth of mistletoe seedlings in the growth chamber. My results are consistent with a recent re-analysis of data from a study by Dean et al. (1994), which has found that mistletoe species richness is not related to the nitrogen content of host genera (Griffiths et al., submitted). Taken together, these findings suggest that host quality—in terms of nutrients and water—may not determine host specificity in the mistletoes used in this study.

I found that the mistletoes used in this series of studies had more negative water potentials than their hosts, which allows the mistletoes to access nutrients passively (Chapters 3, 4 and 6). This finding was consistent with other studies that reported that mistletoes tend to have a lower water potential than their host trees (e.g., Bowie and Ward 2004, Escher et al. 2008, Glatzel and Geils 2009). Mistletoes may also use active uptake to access nutrients from the phloem of host trees, in which case water potential and nutrient uptake may not be strongly coupled (Chapters 3 and 4). Mistletoes that are generalist at a large scale parasitise host trees of different nutrient quality. Thus, it is expected that host choice based on nutrient quality would limit the ability of mistletoes to infect a wide range of tree species. In this study the mistletoe *V. rotundifolium* was found to potentially access nutrients both passively and actively. I suggest that mistletoes maintain plasticity in this uptake mechanism to use predominantly one or the other depending on the environment and host tree (e.g., nutrient quality and compatibility).

Passive uptake is widely accepted to be the mechanism for nutrient uptake in mistletoes because it is less costly for the parasite to maintain. The hypothesis for the underlying mechanism is that mistletoes keep their stomata open and have little control of water loss. However, I found this to be inaccurate. My direct evidence showed that mistletoes do close their stomata and control their water loss to a certain degree. My findings for two pairs of mistletoes and their hosts (*A. natalitius*–*A. karroo* and *Erianthemum dregei*–*Vitex obovata*) showed that mistletoes do close their stomata (Chapter 6). However, when the stomata are open, mistletoes transpire more rapidly than their hosts because they have a higher density of larger stomata (Chapter 6, Whittington and Sinclair 1988, Davidson et al. 1989). These studies show that mistletoes synchronise with the conditions of the host trees and do not simply lose water in an uncontrolled fashion. This contradicts findings of other researchers in different genera of mistletoes (Ehleringer et al. 1985, 1986, Escher et al. 2008).

Future directions

Reviews on parasitism

Several reviews exist on mistletoe–bird–host interactions (e.g., Stewart and Press 1990, Norton and Carpenter 1998, Watson 2001, Aukema 2003, Mathiasen et al. 2008, Glatzel and Geils 2009, Nickrent 2011). Research on the evolution and adaptation of animal parasites to hosts has been extensively reviewed (e.g., Poulin et al. 2006; Johnson et al. 2009; Johnson et al. 2011) because the parasites negatively impact their hosts. For similar reasons, studies on root parasites and mistletoes that parasitise conifers (*Arceuthobium*) are more advanced than those on other mistletoes because they affect agriculturally important plants. However, it is possible to draw connections between research on animal and root parasites with aerial parasites. For example, Nickrent and García (2009) compared root parasitic Orobanchaceae with aerial parasites Santalales for loss of photosynthesis and found that *Arceuthobium* maintains photosynthetic function despite significant alteration and truncation of its plastome. It would be interesting to synthesise this larger body of research to help identify gaps in our knowledge and to develop a more holistic understanding of parasite–vector–host interactions.

The role of plant community composition in determining mistletoe host specificity

Many reports on differences in mistletoe–host infection usually do not describe patterns of host preference, occasional parasitism, host composition and frequency of each host species in an area. Such reports usually result from surveys that do not reflect the infection patterns of mistletoes and do not report whether these mistletoes are specialists or generalists in their whole range (Norton and Carpenter 1998). For example, in my study, I found that: (a) *Viscum rotundifolium* is host specific on *Ziziphus mucronata* in the Kimberley area (Chapter 3); (b) *Agelanthus natalitius* had a higher intensity of infection on *A. karroo* at Mtontwane and Highover (Chapter 4); (c) *Erianthemum dregei* is host specific on *Vitex obovata* at Mtontwane (but reported to parasitise at least 25 host genera; Dzerefos et al. 2003) (Chapter 1) and (d) *Viscum combreticola* is host specific on *Combretum erythrophyllum* (but reported to parasitise 19 host genera, Visser 1981) while *Tapinanthus rubromarginatus* is host specific on *Protea caffra* (but reported to parasitise 14 host genera, Visser 1981) at Walter Sisulu National Botanical Garden (Chapter 2). Host specificity may change over the geographic range based on local adaptations of the mistletoes to available hosts. Thus, it would be interesting to determine host specificity of mistletoes where major and minor hosts vary among sites.

Multiple interactions determine the adaptation of mistletoe species to their hosts and the selection pressures on mistletoes vary throughout their geographic range to create an ever-shifting geographic mosaic (Chapters 2, 3 and 4). It would be interesting to study the geographic mosaic model using mistletoe–host combinations at a larger geographic scale and to test if the patterns of mistletoe infection persist or differ using several mistletoe–host combinations. The Global Biodiversity Information Facility (GBIF), published records on the distribution of mistletoes of Africa (e.g., Polhill and Wiens 1998) and herbarium collections may be used as a baseline to conduct such surveys.

Host availability is an important parameter that influences the infection patterns of mistletoes and thereby affects the mistletoe composition in an area (Norton and Carpenter 1998, Barlow and Wiens 1977, Kavanagh and Burns 2012). In a plant community where there is no single dominant host species—such as in tropical rainforest—mistletoes tend to be generalists (Norton and Carpenter 1998, Barlow and Wiens 1977, Kavanagh and Burns 2012). In a plant community dominated by a few species—such as temperate forests—mistletoes tend to be highly specialised or host specific (Barlow and Wiens 1977). It would be important to test how and why the specificity or generality of mistletoes varies with changes in host community composition in savannas (for a similar approach in Australia, see Kavanagh and Burns 2012). This could potentially allow us to trace patterns of host switches across a wide range of mistletoes in southern Africa.

Reciprocal transplant experiments and molecular genetic studies

Once host specificity or variation of a single host species across a geographic gradient has been established, reciprocal transplant germination experiments in multiple sites can be considered. A mistletoe species can act as a ring species that is gradually differentiated morphologically and genetically across the species range (*sensu* Ward 2011), with occasional switching of host species.

Populations of a mistletoe species at the extreme ends of the range may be sufficiently differentiated to infect completely different host trees, resulting in substantial limitation of gene flow. Adaptation to a preferred host at a given locality may ultimately lead to the development of host races. I propose that hypocotyl growth and haustorium formation are good traits to use in future studies evaluating adaptive phenotypic plasticity in mistletoes because their performance on different host trees can be measured and quantified easily.

Molecular genetic analyses should be conducted to screen mistletoe populations parasitising different host species for variation (Glazner et al. 1988). Host trees may also be genetically differentiated, which may be matched by distinct populations of mistletoes. An analysis of the genetic variation within and between mistletoe races may indicate incipient speciation. This could also be used to estimate how far the mistletoe–host populations have diverged along the path to speciation. In addition, biogeography should be taken into consideration, to determine whether mistletoes parasitise phylogenetically or biogeographically similar hosts.

A phylogenetic comparison of mistletoes and their hosts can reveal the relative importance of cospeciation and host-switching events in mistletoe speciation (see e.g., Norton and Carpenter 1998, Nickrent 2011). Amico et al. (2007) and Amico and Nickrent (2009) reconstructed the speciation of *Tristerix corymbosus* to cacti-specific *T. aphyllus*. They incorporated sequence variation with other ecological factors (host, seed dispersal and environmental factors) to reveal the processes of speciation. *Tristerix corymbosus* exhibits geographic variation in fruit colour. Temperate forest populations of *T. corymbosus* were originally dispersed by marsupials that fed on their ripe green fruits. However, environmental conditions in the Chilean matorral favoured mistletoes that produce yellow fruits, are dispersed by birds and utilise cacti as hosts. *Tristerix corymbosus* became reproductively isolated and speciated to *T. aphyllus* in sympatry (Amico et al. 1997).

Nutrient access and water potential strategies of mistletoes

Mistletoes are hemiparasitic plants able to photosynthesise independently but they face several physiological challenges in infecting host trees (Glatzel and Geils 2009). A single species of mistletoe interacts with many host individuals and species that have marked physiological differences. There is likely to be a complex physiological response of the different mistletoe–host combinations in terms of light, temperature, water, nutrients and other chemicals (Lüttge et al. 1998). However, research on the physiological compatibility of mistletoes to their respective hosts is scant. Studies have shown that mistletoes not only depend on the extraction of mineral nutrients from their host trees, but are also dependent on photosynthates of the host (Schulze and Ehleringer 1985, Schulze et al. 1991, Wang et al. 2008) (Chapters 3 and 4). Research on several mistletoe–host combinations across sites with different environmental conditions would resolve some of the relationships of mistletoes with their respective hosts and environments.

It is possible to create different conditions of host physiology, chemical and nutrient supply in greenhouse or laboratory studies. Examining how host specificity could be affected by mistletoe physiology rather than ecology, *per se,* may be of interest in future studies. Most importantly, it would be important to understand how mistletoes overcome the physiological challenges imposed by their various host trees. For example, the parasitic plant dodder (*Cuscuta pentagona*), can simultaneously parsitise many different hosts that have different stem anatomies and use different photosynthetic pathways (C3, C4). The hastoria are able to adapt to all of the hosts individually, even when connected to the same stem (Dawson et al. 1994, Baráth and Csiky 2012). Dodder is likely to be a good model to compare with mistletoe species that are able to infect host trees of different physiology.

Mistletoes maintain more negative water potentials than their host trees so that they can access water from the xylem stream. Future studies on the effects of different types of stresses to mistletoe–host pairs would help us understand their comparative tolerance in terms of water potential. It would be interesting to follow diurnal stomatal behaviour and responses to abscisic acid for different mistletoe–host combinations. The stomatal response of mistletoes could be affected by different concentrations of nitrogen. When mistletoes receive low nitrogen supply they would be expected to maintain very negative water potentials to concentrate sufficient nitrogen for photosynthesis (see Ehleringer et al. 1985).

Some studies have shown that mistletoes have lower photosynthetic efficiency than their host trees because the mistletoes can access the carbon of the host trees (Johnson and Choinski 1993, Strong et al. 2000). Other studies have shown that mistletoes did not differ from their hosts in terms of light-capturing ability (Lüttge et al. 1998, Bannister and Strong 2001). Given these conflicting results, I did some preliminary investigations that are not reported in this thesis. In this study I grew two species of mistletoes (Loranthaceae) in pots of sand with a supply of quarter strength Hoagland’s solution in a greenhouse. The preliminary investigation showed that the host plant *Acacia karroo* had a significantly higher relative electron transfer rate (ETR) than the mistletoe *Agelanthus natalitius* (i.e., the host photosynthesises at a faster rate than the mistletoe, consistent with Maxwell and Johnson 2000). However, the host *Vitex obovata* had a lower ETR than the mistletoe *Erianthemum dregei*. It is still not clear what drives this mechanism or what photoprotective measures are used by the mistletoes in this study, as they both commonly appear in the upper canopy of savanna trees. Mistletoes typically do not use sunlight effectively and they therefore must have an efficient mechanism for leaf photoprotection (Johnson and Choinski 1993, Strong et al. 2000).

Concluding remarks

The mistletoes used in my studies are known to be host generalists at a large spatial scale. *Viscum rotundifolium* and *Agelanthus natalitius* parasitise 39 and 11 genera, respectively (Wiens and Tolken 1979, Visser 1981, Polhill and Wiens 1998). However, I found that they were host specific at a local scale. At my study sites, *Viscum rotundifolium* parasitised *Ziziphus mucronata* and *Ehretia rigida*. These host trees were not the most abundant in the study site, thus host compatibility played a more important role in directing the infection of *V. rotundifolium*. On the other hand, *Agelanthus natalitius* seems to parasitise the most locally abundant host, *Acacia karroo*, followed by *A. caffra*. The germination experiment confirmed that mistletoe seeds from the two mistletoe species (*V. rotundifolium* and *A. natalitius*) deposited on their preferred hosts have a greater chance of attachment and subsequent survival than those seeds deposited on other co-occurring potential host species. However, host nutrient and water status had little effect in determining host specificity at a local scale. I conclude that the interactions between generalist mistletoes and their hosts vary across the geographic range of the mistletoes and that the mistletoes may alternate among several hosts. This creates multiple locally host-specific mistletoe populations and produces a complex geographic mosaic of mistletoe–host combinations across space and time. I suggest that mistletoe populations may comprise numerous lineages incapable of parasitising the full range of host species, which could potentially lead to the formation of distinct host races over time.

Some mistletoe species can negatively affect host trees by reducing fecundity in the host, inducing premature mortality and reducing the quality and quantity of wood. Dwarf mistletoes *Arceuthobium* spp. from North America (Hawksworth and Wiens 1996) and *Amyema* spp. from Australia (Reid et al. 1994) are destructive to their host trees. However, I argue that mistletoes cannot be considered as merely destructive pests. Only quite recently have we begun to understand the function of mistletoes and their role in an ecosystem (Norton and Carpenter 1998). Watson (2001) and Watson and Herring (2012) suggested that mistletoes are keystone species that directly or indirectly influence several species in an ecosystem. Mistletoes are consumed by many animals that range from insects, birds and large mammalian browsers such as giraffes and elephants (Midgley and Joubert 1991). Their fruit is available to birds during the winter season when few other fruits are available in the ecosystem. Thus, there will be many situations where the ecosystem functions of mistletoes outweigh any negative influence on their host trees. There is a lot to learn from mistletoes and they offer an opportunity to understand parasite–vector–host interactions.

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