Fungi, insects and abiotic factors associated with the death of *Euphorbia ingens* in South Africa

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Fungi, insects and abiotic factors associated with the death of *Euphorbia ingens* in South Africa

By

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Prof. Diana Six

Declaration

I, Johannes Alwyn van der Linde, declare that the thesis, which I hereby submit for the degree of Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Preface

Euphorbia ingens, iconic trees in the savanna landscape of South Africa, have been dying off in large numbers over the last 15-20 years, especially in the northern provinces of South Africa. Various pilot studies and a previous M.Sc. study led to the identification of several fungi and insects associated with *E. ingens* die-offs. Previous evidence suggested that climate change could be driving these die-offs, but the work was exploratory in nature and limited to a small number of sites (occurring in two provinces) and variables. The death of *E. ingens* appears to be caused by a combination of biotic and abiotic factors acting in concert.

The aim of this PhD study was to firstly investigate *E. ingens* health across South Africa in all of the localities where this tree occurs. Furthermore, the role of the various fungi and insects associated with *E. ingens* disease was investigated. The study set out to determine whether there could be a particular trigger for the die-offs. This was achieved by conducting in depth studies of land management practices and linking these to climate data for each site in the various provinces of South Africa where *E. ingens* occurs.

Chapter one of this thesis provides background and insight on the effect of anthropogenic climate change on insects and pathogens related to tree mortality. Globally, forests are affected by anthropogenic climate change resulting in unexpected tree die-offs. This chapter summarizes the effect of anthropogenic climate change on insects and pathogens and how this has become one of the most important triggers in tree die-offs. This chapter provides a foundation for studies in the thesis, specifically showing that tree die-offs are part of a very complex system. This includes numerous biotic and abiotic factors, which needed to be considered during the investigation of *E. ingens* die-offs in South Africa.

The study presented in chapter two describes and summarizes the biotic factors (fungi and insects) involved with *E. ingens* die-offs across South Africa. Freshly diseased and insect infested *E. ingens* plant material was collected over two years across South Africa where this tree occurs. The fungi and insects obtained were identified using both morphology and DNA sequence approaches.

Chapter three identified the ophiostomatalean fungi that occur in the tunnels of *Cyrtogenius africus* that infest *E. ingens*. Logs infested with this beetle were collected from the KwaZulu-Natal, Limpopo, Mpumalanga, and North West Provinces of South Africa. Ophiostomatalean fungi were identified using sequence data of multiple gene regions (β-tubulin, ITS1-5.8S-

ITS2 and LSU gene regions) as well as morphological characters. Their possible role in *E. ingens* die-offs was considered using artificial inoculation studies, on healthy trees in their natural environment, to assess pathogenicity to the host trees.

In chapter four, I investigated the seasonal flight patterns of the Cossoninae and Scolytinae that infest *E. ingens* in areas with different *E. ingens* die-off levels and climatic conditions. Lindgren traps were set up at three sites in two provinces in South Africa to gain an understanding of the seasonal activity of the beetles that attack *E. ingens*. Beetles were identified to species level by Dr Roger Beaver (Thailand). Data loggers were suspended from one trap at each site to measure temperature and relative humidity every hour from September 2013 to April 2015.

The study presented in chapter five summarizes the severity of *E. ingens* die-offs across South Africa and describes the triggers that apparently lead to the die-offs. The specific objectives were to 1) re-examine the role that climate plays in current patterns of *E. ingens* die-off, and 2) investigate whether tree mortality could also be associated with factors related to landscape degradation. Ten study sites were investigated across the Limpopo, North West, KwaZulu Natal and Mpumalanga Provinces. At each site transects were established to evaluate the symptoms associated with die-off, determine the percentage mortality in each transect and to score environmental variables (proxies) associated with savanna degradation. These proxies were dung counts (livestock), woody debris counts, plant and bare soil cover, soil nutrients, and density of *Dichrostachys cinerea* (Fabaceae), a savanna plant that dominates when disturbance is high. Minimum and maximum temperatures as well as precipitation were also compared among sites.

Findings presented in this thesis provide important new information related to the large-scale mortality of *E. ingens* trees in South Africa. Overall, the results provide new knowledge on the fungi and insects that attack *E. ingens* trees as well as a better understanding of the abiotic factors that trigger *E. ingens* die-offs. This new knowledge can be used by conservation and agricultural agencies to potentially reduce the number of dying trees and to prevent the loss of an iconic, native tree in South Africa.

Chapter 1

Literature review: Anthropogenic climate change effects on insects and pathogens affecting native and planted forests in the Northern Hemisphere and South Africa

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Abstract

Globally, over the last 30 years, there has been an increase in the number of reports of tree mortality related to anthropogenically driven climate change. Changes in climate not only directly affect plant and tree growth but also influence insects and microbes (pests and pathogens) that interact with plants. Increased temperatures have, for example, led to an explosion in mountain pine beetle (*Dendroctonus ponderosae*) populations resulting in the death of more than 10 million hectares of *Pinus contorta* in Canada and the United States of America. This review considers the known and predicted impact of anthropogenic climate change on insects and pathogens in forest environments where large scale tree die-offs have been experienced. Most of these reports are from the Northern Hemisphere, but there are also instances in the Southern Hemisphere, including South Africa where tree die-offs are occurring and where climate is believed to play a role. For example, *Euphorbia ingens* trees in South Africa have been reported to be dying-off in unprecedented numbers. In this case, it has been suggested that opportunistic pests and pathogens, driven by changes in climate, may be contributing to the death of these trees. Climate change associated tree die-offs are not only of concern in the natural forest environment but are also important in planted forests where commercial impacts are relevant. Overall, climate change has become an important issue relating to tree diseases and it must be taken into consideration when investigating the factors involved in unexpected tree die-offs.

Keywords: anthropogenic, *Euphorbia ingens*, forest diseases, opportunistic pests, tree mortality

1. Introduction

The onset of the industrial revolution and human population growth has been linked to unnatural global climate change (Crowley 2000, Karl and Trenberth 2003, Solomon *et al.* 2009). The industrial revolution significantly changed the impact of humans on the environment as a result of the increased combustion of fossil fuels (Mitchell 1989, Karl and Trenberth 2003, Garrett *et al.* 2006, Solomon *et al.* 2009). This era is also defined by an increase in land usage for mass industries and to accommodate for the dramatic rise in the human population (Weart 2003, IPCC 2014). These activities have been associated with an increase of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) in the earth's atmosphere (Mitchell 1989, IPCC 2014) (Fig. 1). These gasses form part of a complex known as "greenhouse gasses" which are known to trap heat in the atmosphere, keeping the Earth's surface warm (greenhouse effect) (Crowley 2000, Weart 2003, IPCC 2014). The increase in greenhouse gasses, brought on by the industrial revolution, has been implicated in the unnatural changes in global climate (Fig. 2), aptly known as anthropogenic climate change (ACC) (Crowley 2000, Weart 2003, Flannery 2006).

ACC has been characterized by increased temperature and CO_2 levels (IPCC 2014). The rate at which CO_2 levels and temperature are increasing is estimated to be 15-40 times faster than the natural fluctuations of climate experienced in the previous 100 000 years (Peters 1990, Walther *et al.* 2002, Schwartz *et al.* 2006, Beaubien and Hamann 2011). ACC is not only limited to increasing temperatures (Peters 1990, Houghton *et al.* 2001) but also involves highly variable regional and local changes in evaporation, precipitation and soil moisture (Donn and Shaw 1963, Landsburg 1970, Benton 1970, Mitchell 1989, Peters 1990). In this regard, it has been suggested (Houghton *et al.* 2001, Karl and Trenberth 2003) that the Earth's climate and associated CO_2 levels have changed beyond the limits of its natural variation (Fig. 3). The unnatural changes brought on by ACC will have a dramatic effect on natural ecosystems.

The health of a forest ecosystem is adversely affected by a multiplicity of natural disturbances, including insect and pathogen outbreaks, wildfires and climatic extremes (Dale *et al.* 2000, Dale *et al.* 2001). ACC has a direct effect on these natural disturbances, increasing the vulnerability of forests to regional and widespread mortality (Fig. 4) (Dale *et al.* 2000, Dale *et al.* 2001, Logan *et al.* 2003, Hogg *et al.* 2008, Allen *et al.* 2010). ACC indirectly affects tree physiological functions, tree vigour and defences (Konkin and Hopkins

2009, Allen *et al.* 2010, Anderegg *et al.* 2012). In recent years, there have been several reports of tree mortality associated with ACC (Sturrock *et al.* 2011, Pautasso *et al.* 2012, Sturrock 2012, Anderegg *et al.* 2015) and there is great concern that it will exacerbate the negative effect of natural disturbances on tree health (Dale *et al.* 2000, Dale *et al.* 2001, Adams *et al.* 2009). ACC has also been linked to the increased activity of forest pathogens and insects (Pautasso *et al.* 2012, Anderegg *et al.* 2015, Ramsfield *et al.* 2016) and increased stress on trees, making them more susceptible to pathogen and insect attack (Peters 1990, Chakraborty 2005, Garrett *et al.* 2006, Sturrock *et al.* 2011, Pautasso *et al.* 2012, Sturrock 2012).

Insects and pathogens, which have short lived generations with high reproductive rates, will respond more quickly to ACC compared to long-lived organisms such as higher plants (Menéndez 2007). The survival and reproductive rates of insects and pathogens are strongly influenced by temperature and precipitation (Pautasso *et al.* 2012, Anderegg *et al.* 2015, Ramsfield *et al.* 2016). The effects of ACC on insects and pathogens include changes in phenology (e.g. greater brood production for insects or greater spore release by fungal pathogens), range expansion and exposure to new susceptible tree species (Six and Bentz 2007, Bentz *et al.* 2010, Sturrock *et al.* 2011, Sturrock 2012, Pautasso *et al.* 2012). Severe cases include root rot caused by *Phytophthora cinnamomi* in the United Kingdom (UK) and Coastal Europe (Brasier 1996, Bergot *et al.* 2004, Sturrock *et al.* 2011) and mountain pine beetle outbreaks (*Dendroctonus ponderosae*) in western North America (Kurz *et al.* 2008, Konkin and Hopkins 2009, Bentz *et al.* 2010, Cudmore *et al.* 2010).

Insects and pathogens, affected by ACC, and linked to tree mortality have mostly been reported from the Northern Hemisphere (Sturrock *et al.* 2011, Pautasso *et al.* 2012, Sturrock 2012, Anderegg *et al.* 2015). It has been estimated that the Northern Hemisphere has had a higher rate (1.12°C/century) of land-based temperature increase compared to the Southern Hemisphere (0.84°C/century) for the period 1901 to 2010 (Jones *et al*. 2012). Even though the Southern Hemisphere has experienced a lower warming trend, the rate of warming over Africa has been comparable to land masses in the Northern Hemisphere (Engelbrecht *et al.* 2015). It has been estimated that the warming trend in southern Africa (more than 3.2°C/century) will be amongst the highest in the Southern Hemisphere (Engelbrecht *et al.* 2015). It is consequently likely that ACC will affect insect and pathogen outbreaks in the Southern Hemisphere, similar to the Northern Hemisphere.

This review presents examples of the effect of ACC (temperature and precipitation) on insects and fungal pathogens and their role in tree mortality. Various important examples, particularly from the Northern Hemisphere are discussed where insect pests and fungal pathogens have been or could possibly be affected by ACC. This is followed by a number of examples from South Africa, broadly attempting to outline the potential impact of ACC on tree health. The examples include not only natural but also planted forest environments.

2. The effect of anthropogenic climate change on tree insects and fungal pathogens

Survival and reproductive rates of insects and fungi are strongly influenced by temperature and precipitation (Dukes *et al.* 2009, Pautasso *et al.* 2012, Anderegg *et al.* 2015, Ramsfield *et al.* 2016). Insect metabolic rates are highly sensitive to changes in temperature; increasing drastically with increasing temperatures (Gillooly *et al.* 2001, Clarke and Fraser 2004). As a result, reproduction, herbivory and general activity of insects is likely to increase during periods of increased temperatures (Bale *et al.* 2002). Fungi are also strongly influenced by temperature and precipitation, for example, these organisms are capable of surviving and developing in a wide range of temperatures but epidemic growth is dependent on a narrow temperature range (Lonsdale and Gibbs 1994, Dukes *et al.* 2009). Epidemic growth of fungi is generally favoured by higher temperatures as well as high moisture conditions (Lonsdale and Gibbs 1994, Garrett *et al.* 2006). High moisture conditions (brought on by precipitation) will aid infection, spore development and dispersal of various fungi (Lonsdale and Gibbs 1994, Dukes *et al.* 2009).

ACC will likely affect both insects and fungal pathogens. It has already been shown that the length of insect life stages have been reduced by ACC (Harrington *et al.* 2001, Logan *et al.* 2003). Furthermore, various insects and pathogens have been extending their distribution northwards as a result of ACC (Parmesan 2006, Sturrock 2012, Pautasso *et al.* 2012). The increase in the minimum extreme temperatures have been shown to aid insect and fungal overwintering survival, leading to major outbreak epidemics (Trần *et al.* 2007, Dukes *et al.* 2009). These effects of ACC on insects and pathogens have already been linked to various severe tree die-offs and will be discussed below.

In the following sections, I have selected various insects and pathogens that have caused serious damage to forests in various parts of the world. Insects and pathogens have been selected based on the following criteria: there is extensive evidence supporting the affect of

ACC on their activity, ACC has supported/increased the activity of the insects and pathogens and, insects and pathogens selected have caused severe damage to forests as a result of ACC.

2.1 Insects

The insects selected in this section include bark beetles from North America and Europe, an ambrosia beetle from Asia as well as a moth from Europe. Bark beetles do not usually kill large numbers of their host trees, however, during the course of the last 20 years there have been increasing reports of bark beetles killing trees (in large numbers) where ACC has been implicated (Ramsfield *et al.* 2016). The bark beetles selected for discussion have been shown to be influenced by ACC, which has led to large epidemics and severe forest damage. Ambrosia beetles typically infest dead or severely stressed trees (Kühnholz *et al.* 2001, Hulcr and Dunn 2011). It is now becoming evident that some ambrosia beetles, together with their fungal symbionts, are infesting and killing healthy trees (Ramsfield *et al.* 2016). The example of *Platypus quercivora* chosen for discussion considers the effect of ACC on this ambrosia beetle that has led to severe oak die-back in Japan (Kamata *et al.* 2002). The effect of ACC on the pine processionary moth is also discussed because this example illustrates the profound impact of ACC on lepidopteran insects. ACC has had a substantial effect on the larvae and adults of this moth, which are now occurring in areas previously considered unfavourable for it and where it is infesting new hosts in southern Europe.

2.1.1 *Dendroctonus frontalis*

The southern pine beetle (*Dendroctonus frontalis*) is one of the greatest natural disturbances on pine forests in the south-eastern United States (Pennsylvania, Florida, Missouri and Texas) (Ungerer *et al.* 1999). This bark beetle feeds on the phloem of its host while living inside the inner bark (Thatcher and Conner 1985). *Dendroctonus frontalis* completes its lifecycle by killing mature pine trees weakened by fires, storms or other abiotic stressors, with populations reaching epidemic levels once 20 to 30 pines have been attacked (Turchin *et al.* 1991, Ylioja *et al.* 2005). Low lethal temperatures (during winters in the North) have until recently kept *D. frontalis* populations in the South, limiting the beetle's distribution to the North (Ungerer *et al.* 1999).

A warming trend occurred in the *D. frontalis* distributional area, from 1960 to 2004 with a 3.3°C increase in minimum winter temperatures (Trần *et al.* 2007). This warming trend allowed *D. frontalis* to extend its distribution northwards, as beetles experienced less severe winter temperatures (Ungerer *et al.* 1999, Trần *et al.* 2007). *Dendroctonus frontalis* caused

continued outbreaks at its most northern limit between 2001 and 2006 (New Jersey, Maryland, Ohio) (Trần *et al.* 2007). This distributional change was exacerbated by the beetles' short generation times, high dispersal abilities and wide distribution of suitable hosts (Ungerer *et al.* 1999, Trần *et al.* 2007). ACC will likely allow *D. frontalis* outbreaks and attacks to become more prevalent at its most northern limit in the future.

2.1.2 *Dendroctonus rufipennis*

Spruce beetles (*Dendroctonus rufipennis*) occur across North America as one of the most devastating pests of spruce trees (DeRose and Long 2007, Sherriff *et al.* 2011). *Dendroctonus rufipennis* colonizes and reproduces within the phloem of the host and upon heavy infestation interrupts the flow of nutrients and water throughout the tree (Hart *et al.* 2014). *Dendroctonus rufipennis* has a two year life cycle, overwintering at the base of the host during the 2nd winter of the lifecycle, to escape predation and cold temperatures (Massey and Wygant 1954). *Dendroctonus rufipennis* infestations and outbreaks usually occur in mature forests that have experienced a disturbance (fire, harvest or severe wind) leaving enough damaged and dying spruce trees for beetle reproduction and establishment (Berg *et al.* 2006).

Unexpected outbreaks of *D. rufipennis* were experienced in Alaska and the Yukon Territory (1989-2004) with no prior disturbance triggering these infestations (Berg *et al.* 2006). These unexpected outbreaks were attributed to higher summer temperatures which allowed for better overwinter survival and halving the beetles' maturation time from two to one year (Berg *et al.* 2006, Sherriff *et al.* 2011). The severity of the attacks were also directly influenced by the higher temperatures, as a drought occurred in the area further stressing the mature trees (Berg *et al.* 2006, Hart *et al.* 2014). *Dendroctonus rufipennis* outbreaks will likely be more frequent in the future, due to ACC, attacking trees without any prior disturbances.

2.1.3 *Dendroctonus ponderosae*

The mountain pine beetle (*Dendroctonus ponderosae*) is a bark beetle native to western North America (Kurz *et al.* 2008, Konkin and Hopkins 2009, Cudmore *et al.* 2010). This insect infests pine trees and has been known to have periodic outbreaks in its native environment (Kurz *et al.* 2008, Konkin and Hopkins 2009). Populations of MPB have always been regulated by the extreme cold temperatures that occur in western North America (Robertson *et al.* 2009). However, since the 1990's a severe MPB epidemic has been experienced in Canada and the United States of America (USA), occurring in British

Columbia, Alberta, Colorado, Idaho and Montana (Klutsch *et al.* 2009, Konkin and Hopkins 2009, Nordhaus 2009). This epidemic has led to over 10 million hectares of *Pinus contorta* (lodgepole pine) deaths in these regions (Kurz *et al.* 2008, Konkin and Hopkins 2009).

The reduced mortality and higher reproductive rates of MPB have been attributed to ACC (Bentz *et al.* 1991, Kurz *et al.* 2008, Nordhaus 2009). There has been an increase in the overall temperature in the affected regions, leading to warmer winters and summers, with reduced summer precipitation (Kurz *et al.* 2008, Konkin and Hopkins 2009). Increased temperatures associated with climate change have caused MPB populations to expand their geographic range with higher survival rates and increased brood production (Cudmore *et al.* 2010). The warmer temperatures have led to reduced mortality of MPB during winter and increased survival rates (Bentz *et al.* 1991, Nordhaus 2009). The increase in temperature has allowed MPB to reproduce more rapidly, producing two generations in a year (Kurz *et al.* 2008, Nordhaus 2009). A prolonged drought in western North America made host trees more vulnerable to MPB attack, because they were under severe moisture stress (Mattson and Haack 1987, Nordhaus 2009).

ACC has been proposed to alter the three way interaction between the MPB, their symbiotic fungi and their tree hosts (Hepting 1963, Krcmar-Novaic *et al.* 2000, Kühnholz *et al.* 2001, Six 2009, Six *et al.* 2011). The symbiotic fungi of the MPB are directly affected by ACC (Kurz *et al.* 2008, Konkin and Hopkins 2009). MPB have two symbiotic fungi; *Grosmannia clavigera* and *Ophiostoma montinum* (Bleiker and Six 2007). The warmer temperatures favour the growth of *G. clavigera*, outcompeting *O. montium* (Bleiker and Six 2007, Six and Bentz 2007, Bentz *et al.* 2010). *Grosmannia clavigera* has been shown to support faster and increased brood development of MPB than *O. montium* (Bleiker and Six 2007, Six and Bentz 2007, Bentz *et al.* 2010). The indirect influence of *G. clavigera*, reduced cold stress and increased geographical range has led to the largest epidemic for any bark beetle in recorded history (Kurz *et al.* 2008, Konkin and Hopkins 2009, Cudmore *et al.* 2010).

2.1.4 *Ips typographus*

The European spruce beetle (*Ips typographus*) is one of the most important biotic risks to European conifer trees (Müller *et al.* 2008, Linnakoski *et al.* 2016). *Ips typographus* mainly infests weakened and stressed trees, while living in the phloem of the tree, with attacks occurring on healthy trees if beetle populations reach suitable levels (Wermelinger 2004). Over the last two decades there has been an increase of *I. typographus* on European spruce

trees (Rouault *et al.* 2006, Marini *et al.* 2012), especially *Picea abies* (Norway spruce) (Müller *et al.* 2008, Linnakoski *et al.* 2016).

ACC will increase the swarming activity of *I. typographus* and allow for northwards expansion. The expected temperature warming in Europe is predicted to change *I. typographus* population dynamics linked to predicted hot spells and droughts for southern Sweden (Jönsson *et al.* 2007). The predicted increased temperatures (2°C to 3°C) will allow *I. typographus* to produce two generations per year, as opposed to one, and will also allow for increased swarming activity (Lange *et al.* 2006, Jönsson *et al.* 2007). It is predicted that *I. typographus* will have a northward expansion, linked to increased summer temperatures, applying increased pressure on alpine forests (Marini *et al.* 2012). Furthermore, the predicted droughts will place more stress on tree hosts, making the trees more susceptible to *I. typographus* attacks (Wermelinger 2004).

2.1.5 *Platypus quercivora*

Platypus quercivora is an ambrosia beetle native to Japan (Ito and Yamada 1998). *Platypus quercivora* and its fungal symbiont, *Raffaelea quercivora* (Kamata *et al.* 2002, Kubono and Ito 2002), are responsible for oak die-back in Japan and outbreaks have been reported since the 1930's (Ito and Yamada 1998, Kamata *et al.* 2002). The severity of the die-backs has increased in intensity with epidemics lasting more than 10 years (Ito and Yamada 1998, Kamata *et al.* 2002).

The increase in the oak die-back epidemics in Japan has been proposed to be triggered by an increase in temperature of 0.4°C, since the 1980's (Kamata *et al.* 2002). This is the highest increase in temperature in the last 100 years for that region (Kamata *et al.* 2002). The warmer climate has expanded *P. quercivora* to a more northerly range and higher altitudes where it encounters more *Quercus crispula* (Kamata *et al.* 2002). *Platypus quercivora* has a higher reproductive success on *Q. crispula*, compared to other hosts (Kamata *et al.* 2002). The increased infestation of *Q. crispula* by *P. quercivora* has played an important role in the rate of spread and intensity of oak die-back in Japan (Kamata *et al.* 2002). *Platypus quercivora* will likely experience greater reproductive success, due to ACC, leading to more severe tree die-back.

2.1.6 *Thaumetopoea pityocampa*

The pine processionary moth (*Thaumetopoea pityocampa*) is a defoliator moth that mainly occurs in the Mediterranean basin and feeds on *Pinus* species (Hódar *et al.* 2002, 2003). Eggs

are laid in the canopy of host trees during the summer (Devkota and Schmidt 1990) with larvae hatching after 45 days (Hódar *et al.* 2002). Larvae crawl around on its hosts and feed on the pine needles (Hódar *et al.* 2002). During winter the larvae aggregate into a silk nest for protection against the cold climatic conditions (Hódar *et al.* 2002, Battisti *et al.* 2006). During spring the larvae leave the nest and enter the ground at the base of the tree to pupate (Hódar *et al.* 2002, Battisti *et al.* 2006).

The distribution of *T. pityocampa* has been altered, with it now occurring closer to highly elevated *Pinus* plantations (Pennerstorfer *et al.* 2005, Battisti *et al.* 2005, 2006; Netherer and Schopf 2010). ACC has increased the activity of *T. pityocampa* larvae and has altered the distribution of the adult moths (Netherer and Schopf 2010). Warmer temperatures during winter have increased *T. pityocampa* larvae herbivory, which has caused severe defoliation of *Pinus* and *Cedrus* species (Battisti *et al.* 2005, Netherer and Schopf 2010). It has been found that *T. pityocampa* is starting to occur and attack trees in previously unfavourable areas within the Paris basin (Netherer and Schopf 2010), as well as at higher elevations in the Sierra Nevada Mountains, in Spain, attacking populations of *Pinus sylvestris* (Hódar and Zamora 2004, Menéndez 2007). Due to ACC, *T. pityocampa* will likely become a more prevalent pest of *Cedrus* and *Pinus* trees and expand into previously unfavourable areas and attacking alternative hosts.

2.2 Pathogens

The pathogens selected for discussion in this section include *Phytophthora* species, *Dothistroma septosporum* and species of the Botryosphaeriaceae. *Phytophthora* spp. are amongst the most destructive pathogens in the world (Kroon *et al.* 2012). The two examples discussed deal with the direct influence of ACC on *Phytophthora cinnamomi*,causing oak decline in Europe, and on the predicted influence of ACC on *Phytophthora ramorum*, which is a devastating pathogen in North America and the UK. *Dothistroma septosporum* that causes red band needle blight (Barnes *et al.* 2004) has been known to be a minor threat in its natural environment (Woods 2003, Bradshaw 2004, Woods *et al.* 2005). ACC has allowed this pathogen to establish a needle blight epidemic in its natural environment and it is likely to continue to become an increasingly important pathogen in North America (Woods *et al.* 2005, Woods *et al.* 2016). The Botryosphaeriaceae are opportunistic fungal pathogens, residing as asymptomatic endophytes in their hosts, causing disease only when the host trees are subjected to stressful conditions (Punithalingam 1980, Slippers and Wingfield 2007). ACC is known to increase stress in plants and forests systems, which will likely lead to an

increase in the pathogenic activity of fungi in the Botryosphaeriaceae (Desprez-Loustau *et al.* 2006, Sturrock *et al.* 2011).

2.2.1 *Phytophthora cinnamomi*

Phytophthora cinnamomi (Oomycetes, Stramenopiles) is a soil-borne plant pathogen with a global distribution and wide host range mainly affecting woody plants (Zentmyer 1988, Brasier 1996, de Sampaio e Paiva Camilo-Alves *et al.* 2013). It is primarily a pathogen of fine feeder roots, with moisture as the key factor in the survival and development of this pathogen (Zentmyer 1988, de Sampaio e Paiva Camilo-Alves *et al.* 2013). Disease development caused by this pathogen is restricted by cold winter temperatures, as it requires warm temperatures (25°C to 30°C) for development and infection as well as wet soil environments for zoospore distribution (Shearer and Tippett 1989). There is one example where ACC appears to be involved in disease caused by this pathogen, the disease known as oak decline in Europe.

Oak decline of *Quercus suber* and *Quercus ilex* occurs in the Mediterranean basin, specifically in France, Portugal and Spain (Brasier *et al.* 1993, Sánchez *et al.* 2002, de Sampaio e Paiva Camilo-Alves *et al.* 2013). It causes progressive decline of its hosts, associated with gradual loss of foliage and in some cases sudden death of the host tree (Gallego *et al.* 1999, Brasier *et al.* 1993). The roots of the declining trees usually have several dead fine roots especially in moister soils (Brasier *et al.* 1993, de Sampaio e Paiva Camilo-Alves *et al.* 2013). Oak decline in the Mediterranean basin has been occurring over the last 100 years with significant increased decline occurring over the last 40 years (de Sampaio e Paiva Camilo-Alves *et al.* 2013).

The predicted increased temperatures and extended wet periods in central Europe will aid *P. cinnamomi* establishment and longevity (Brasier 1996, Bergot *et al.* 2004). ACC will not only increase inoculum production and allow for higher winter survival but will also affect the distribution of *P. cinnamomi* (Brasier 1996, Bergot *et al.* 2004, Sturrock *et al.* 2011). Warmer winters will allow for higher annual survival of *P. cinnamomi* and potential range expansion into central Europe (Bergot *et al.* 2004). As a result, oak decline in central Europe of *Q. suber* and *Q. ilex* is likely to be exacerbated by ACC (Brasier 1996, Bergot *et al.* 2004).

2.2.2 *Phytophthora ramorum*

Phytophthora ramorum is a splash dispersed oomycete with its sporangia spread over long distances by wind or wind-driven rain (Davidson *et al.* 2005). Zoospores are released from

established sporangia onto a host, which will germinate and infect the host (Tooley *et al.* 2008). *Phytophthora ramorum* is adapted to cool temperatures (optimal growth at 20°C) and can survive adverse environmental conditions by producing chlamydospores in its infected hosts (Tooley *et al.* 2008, Grünwald *et al.* 2012). It infects the phloem of the host, girdling the tree, essentially cutting off the host's nutrient supply (Grünwald *et al.* 2008).

Phytophthora ramorum is a major pathogen of oak in North America and larch trees in the UK, as well as of ornamental plants across Europe (Werres *et al.* 2001, Brasier and Webber 2010, Grünwald *et al.* 2012, Pautasso 2013). *Phytophthora ramorum* is the cause of sudden oak death (SOD) in California and Oregon (Rizzo *et al.* 2002, Rizzo and Garbelotto 2003). This disease emerged in the mid-1990's and causes cankers and 'sudden' death of native coastal forest oaks (*Quercus agrifolia* and *Quercus kelloggi*) and tanbark-oaks (*Notholithocarpus densiflorus*) (Rizzo *et al.* 2002, Grünwald *et al.* 2008). SOD has affected hundreds of kilometres of coastal oak trees along the Californian coast (Davidson *et al.* 2002, Holdenrieder *et al.* 2004). In the UK the most severe epidemic of *P. ramorum* first occurred in England (Brasier and Webber 2010, Grünwald *et al.* 2012). This epidemic was associated with widespread decline and mortality of *Larix kaempferi* (Japanese larch) and is now widespread throughout the UK (Brasier and Webber 2010, Grünwald *et al.* 2012).

ACC will directly affect the development of *P. ramorum* as it has been shown that it is capable of producing larger lesions on host trees during warmer and wetter climatic conditions (Dodd *et al.* 2008, Donnelly *et al.* 2011). Indirectly, *P. ramorum* will also become more "pathogenic" to host trees as they will be under severe stress as a result of ACC, increasing their susceptibility to *P. ramorum* attacks (Pautasso *et al.* 2012). It is also predicted that *P. ramorum* will move into climatically suitable areas that are more favourable for its development, exposing itself to susceptible hosts (Venette 2009).

2.2.3 *Dothistroma septosporum*

Dothistroma needle blight (caused by *Dothistroma septosporum* and *Dothistroma pini*) is characterized by the occurrence of red bands on the needles of a wide range of *Pinus* species, as well as spruce, larch and Douglas fir (Barnes *et al.* 2004, Barnes *et al.* 2014, Barnes *et al.* 2016). This disease leads to premature needle drop, ultimately affecting wood quality, especially when younger trees are attacked, and in some cases can lead to tree mortality (Bradshaw 2004, Sturrock *et al.* 2011). The fungi that cause Dothistroma needle blight occur across various climatic environments in over 70 countries (Barnes *et al.* 2014, Drenkhan *et*

al. 2016). Spore production and severity of disease caused by *Dothistroma* species are mainly dependent on humidity and leaf wetness (Bradshaw 2004, Woods *et al.* 2016). Spores (produced by black fruiting bodies in the red bands on the needles) move to fresh needles via water droplets, with symptoms appearing from 32 to 114 days (Peterson 1973, Karadžič 1989, Bradshaw 2004). Temperature plays a less important role, compared to humidity, as infection can occur between 5°C and 26°C (Gilmour and Crockett 1972). Until recently, Dothistroma needle blight (specifically caused by *D. septosporum*) has been a minor threat in North America as the natural climate never allowed for optimal conditions for infection (Woods 2003, Bradshaw *et al.* 2004, Woods *et al.* 2005).

Severe changes in rainfall and temperature in British Columbia (BC) have led to a devastating needle blight epidemic, caused by *D. septosporum,* on *P. contorta* (Woods 2003, Bradshaw 2004, Woods *et al.* 2005). The epidemic in BC can be attributed to the higher occurrence of warm rain spells throughout the 1990's, leading to more favourable climatic conditions for *D. septosporum* to thrive (Woods *et al.* 2005, Woods *et al.* 2016). The warmer rain spells consists of a complex interaction between increased temperature and continuous precipitation, that allows for optimal infection rates of *D. septosporum* (Woods *et al.* 2005, Woods *et al.* 2016). The increased temperatures lead to a greater production of spores, acting together with prolonged moisture presence (continuous precipitation causes longer periods of needle wetness) and leading to rapid development and greater impact of this needle disease (Gadgil 1974, Woods *et al.* 2005, Sturrock *et al.* 2011, Woods *et al.* 2016). ACC will likely allow *D. septosporum* to continue to establish itself as a major needle blight disease in North America.

2.2.4 Botryosphaeriaceae

The Botryosphaeriaceae are known as opportunistic fungal pathogens that cause disease and death of numerous tree species (Punithalingam 1980, Slippers and Wingfield 2007, Slippers *et al.* 2017). Botryosphaeriaceae can enter the host through wounds, reproductive structures (seed) and natural openings (stomata), infecting stems, leaves and roots (Smith *et al.* 1996a,b, Mehl *et al.* 2013). They are capable of residing within a host without causing symptoms of disease (Sieber 2007). Fungi in the Botryosphaeriaceae are capable of switching from this endophytic cycle to a pathogenic lifecycle within the host (Smith *et al.* 1996a,b, Sakalidis *et al.* 2011). This pathogenic stage is triggered by the onset of stress on the host which includes environmental damage, climatic stress and water stress (Smith *et al.* 1996a,b; Sakalidis *et al.* 2011, Mehl *et al.* 2013).

ACC will likely affect the pathogenic activity of fungi in the Botryosphaeriaceae (Desprez-Loustau *et al.* 2006, Sturrock *et al.* 2011). ACC has been increasing stress on plants and forest systems with known tree mortalities, linked to the onset of stress, worldwide (Sturrock *et al.* 2011, Pautasso *et al.* 2012, Sturrock 2012). Several species within the Botryosphaeriaceae have already been associated with disease on hosts triggered by drought and water stress (Sturrock *et al.* 2011). *Botryosphaeria dothidea* has been associated with increased levels of disease, linked to drought and water stress, on apple trees in the Eastern USA (Brown and Hendrix 1981). *Diplodia sapinea* is consistently associated with shoot blight, linked to water stress, on pine trees in the USA (Bachi and Peterson 1985, Paoletti *et al.* 2001, Sturrock *et al.* 2011) and Europe (Adamson *et al.* 2015). The increase in the incidence of Botryosphaeriaceae related plant diseases will become a continuous trend worldwide and is predicted to be influenced by ACC (Desprez-Loustau *et al.* 2006, Sturrock *et al.* 2011, Zlatković *et al.* 2016).

3. Anthropogenic climate change in southern Africa and its potential impact on tree health

ACC is predicted to affect southern Africa to a greater degree than most other parts of the world (Engelbrecht *et al.* 2015). It is predicted that there will be an increase in winter and summer temperatures of 3°C to 7°C by the year 2100 (Boko *et al.* 2007, Houniet *et al.* 2009, Jury 2013), with an annual increase of 0.02°C per year (Du Plessis *et al.* 2003). Minimum temperatures will likely have a higher increase than maximum temperatures, leading to more significant changes in temperature during winter than in summer (Du Plessis *et al.* 2003). It is also predicted that rainfall will be highly variable, increasing or decreasing (depending on region) by 20% during the 21st century (Boko *et al.* 2007, Houniet *et al.* 2009). Specifically, South Africa has experienced a warming trend of more than 0.15°C per decade over the 20th century which will continue to increase in the 21st century (Boko *et al.* 2007, Houniet *et al.* 2009, Engelbrecht *et al.* 2015).

ACC will be of great concern to both natural and planted forests in South Africa (Van Staden *et al*. 2004). The commercial forestry industry is of great economic importance to the country and dependant on non-native *Acacia*, *Eucalyptus* and *Pinus* trees (Forestry South Africa 2017). The industry is affected by numerous important plant pathogens and insects, including *Fusarium circinatum* (causing pitch canker of pine trees), *Diplodia sapinea* (causing disease in stressed pine trees), *Sirex noctilio* (woodwasp that infests pine trees), *Gonipterus* snout

beetle and *Glycaspis brimblecombei* (both insects causing damage to *Eucalyptus*) (Garnas *et al.* 2016). Furthermore, the native plant environment in South Africa is under threat from, amongst others, root diseases (caused by *Armillaria mellea* and *P. cinnamomi*) and rust disease (*Austropuccinia psidii*) (Coetzee *et al*. 2003, Roux *et al.* 2015). ACC will not only place trees under severe stress but it will also increase the activity of pathogens and insects, increasing survival and distributional range, posing an extreme threat to South African flora.

The next sections discuss important fungal pathogens and insects, not only in the planted forest environment but also in the natural forest environment in South Africa. The examples selected for discussion are some of the most devastating pathogens and insects in South Africa and the predicted effect of ACC on these pathogens and insects is considered. Most of the information available, especially for the insects, pertains to the commercial forestry industry in South Africa. The activity of pathogens and insect pests in the natural tree environment in South Africa will likely be exacerbated by ACC, leading to unexpected die-offs. A lack of knowledge of these effects needs be addressed, as is the case of *Euphorbia ingens* die-offs (Roux *et al.* 2008; Roux *et al.* 2009, Van der Linde *et al.* 2012).

3.1 Pathogens

3.1.1 *Fusarium circinatum*

Fusarium circinatum (FC) is a known specialised pathogen of *Pinus* species worldwide, causing pitch canker disease within pine plantations (Wingfield *et al.* 2008, Gordon 2012). Pitch canker is a destructive disease of pine trees (especially *Pinus radiata* and *Pinus patula*) in commercial forests in South Africa (Wingfield *et al.* 2008). Pitch canker outbreaks have occurred in *P. patula*, *Pinus greggi* and *P. radiata* stands in South Africa with most outbreaks occurring in the coastal areas (Mitchell *et al.* 2011). It is predicted that *F. circinatum* will respond to the increase in minimum temperatures that will be brought on by ACC (Watt *et al.* 2011). *Fusarium circinatum* infection intensity is predicted to increase especially in coastal areas and northern parts of South Africa (Watt *et al.* 2011). This is of great concern since it is known that infection by *F. circinatum* is greater in coastal areas causing higher infection and mortality rates (Gordon *et al.* 2001, Coutinho *et al.* 2007, Mitchell *et al.* 2011).

3.1.2 Botryosphaeriaceae

Fungi in the Botryosphaeriaceae are known to occur on native as well as commercial forestry trees in South Africa (Smith *et al.* 1996a,b; Van der Linde 2011b, Jami *et al.* 2014, Jami *et al*. 2017). A well-known example of a South African fungal pathogen in the Botryosphaeriaceae

is *Diplodia sapinea* causing die-back of pine trees (particularly *P. patula* and *P. radiata*) that have been damaged after hail storms (Smith *et al.* 2002). Fungi in the Botryosphaeriaceae have also been found to cause die-back of stressed *Eucalyptus* trees in South Africa that have been exposed to strong wind, high temperatures, frost and hail (Smith *et al.* 1994, Slippers *et al.* 2009). In the native environment, *E. ingens* trees have been suffering from severe die-offs with fungi in the Botryosphaeriaceae occurring on diseased and dying *E. ingens* trees (Van der Linde *et al.* 2011b). It has also recently been found that fungi in the Botryosphaeriaceae are most dominant in tree hosts that occur in disturbed and urban environments (Pavlic-Zupanc *et al.* 2015, Pavlic-Zupanc *et al*. 2017).

Fungi in the Botryosphaeriaceae colonize their hosts naturally and in the absence of disease symptoms, resulting in disease only when their hosts have been subjected to stress (Punithalingam 1980, Sieber 2007, Slippers and Wingfield 2007, Mehl *et al.* 2013). ACC is known to lead to tree stress, which can potentially increase the incidence of tree diseases caused by fungi in this family (Desprez-Loustau *et al.* 2006, Sturrock *et al.* 2011). This should be of great concern in South Africa where various economic sectors (e.g. forestry, fruit and wine), native tree environments and urban tree environments are likely to be affected in the future.

3.1.3 *Phytophthora cinnamomi* and *Armillaria*

Root rot disease is threatening the native plant environment in the south Western Cape region of South Africa (Machingambi *et al.* 2015). This region is commonly known as the Cape Floristic Region (fynbos biome) and is dominated by plant species in the Proteaceae family (Palgrave *et al.* 2002). *Phytophthora cinnamomi* has been known to cause various native tree deaths across the Cape Floristic Region (Lübbe and Geldenhuys 1990). This pathogen is known to be affected by climate change and has caused severe mortality of native trees in Europe where ACC is of great concern (Brasier 1996, Bergot *et al.* 2004). It is predicted that ACC will cause increased activity of this pathogen, namely increased survival and increase in distributional range (Brasier 1996, Bergot *et al.* 2004), and can pose a great threat to the native plant environment in the Cape Floristic Region. Furthermore, *Armillaria* species are also known to cause disease in this region specifically affecting *Protea* species (Coetzee *et al.* 2003). *Armillaria* root disease is most prolific in drier Mediterranean and continental climates, with spread and infection of this pathogen affected by temperature and moisture (Shaw and Kile 1991). *Armillaria* species can grow at temperatures as high at 31°C and are known to cause disease in hosts that are drought stressed (Shaw and Kile 1991). *Armillaria*

root disease is likely to become more prolific with drier and warmer climatic conditions brought on by ACC in South Africa (Shaw and Kile 1991, Klopfenstein *et al.* 2009, Sturrock *et al.* 2011). This will be of great concern to *Protea* spp. in the Cape Floristic Region as climatic conditions are likely to become more optimal for this root pathogen, leading to increased disease incidence and spread.

3.1.4 *Austropuccinia psidii*

Austropuccinia psidii is a devastating rust pathogen that was first detected in the south-eastern coast of South Africa in 2013 (Roux *et al.* 2013). Recently it has been found that *A. psidii* has a wide distribution in South Africa with evidence of being present in the country longer than originally thought (Roux *et al.* 2016). This rust pathogen is native to South America where it affects various species within the Myrtaceae (Coutinho *et al.* 1998, Glen *et al.* 2007, Morin *et al.* 2012). *Austropuccinia psidii* is known to spread rapidly in its introduced environment especially on hosts in the Myrtaceae (Glen *et al.* 2007, Carnegie and Lidbetter 2012). In Australia this pathogen has become a major threat to the native Myrtaceae environment (Dayton and Higgins 2011). This rust pathogen is of great concern to South Africa as the forestry industry relies on non-native Myrtaceae (*Eucalyptus* spp.) with various native Myrtaceae present in the natural environment of South Africa (Palgrave *et al.* 2002, Roux *et al.* 2015, Forestry South Africa 2017).

Austropuccinia psidii will become an increasingly important threat to the native Myrtaceae on the south-eastern coastal region of South Africa. It is predicted that rust fungi, including *A. psidii*, will be affected by the unpredictable weather conditions (increased rainfall and windy conditions) caused by ACC, aiding in its dispersal (Kakishima *et al.* 2017). The development, survival and spread of this pathogen relies on wet conditions (humidity and leaf wetness) as well as wind and rainstorms (Glen *et al.* 2007). It has been shown that the south-eastern coastal region in South Africa is at highest risk for *A. psidii*, which relates to similar studies in Australia (Glen *et al.* 2007, Roux *et al.* 2015). This is of great concern as this pathogen is present in this region and will be aided greatly by the current and predicted climatic changes for South Africa (Glen *et al.* 2007, Roux *et al.* 2015).

3.2 Insects

Limited information is available regarding insects that will be affected by ACC in the native South African tree environment. However, *Euphorbia ingens* trees have been dying off unexpectedly in this country (Roux *et al.* 2008; 2009, Van der Linde *et al.* 2012). Various

insects (ambrosia beetles, weevils and a moth species) have been collected from dying *E. ingens* trees (Roux *et al.* 2008, Roux *et al.* 2009, Van der Linde *et al.* 2011a,b). Climate change (temperature and precipitation) has been implicated as the trigger for *E. ingens* die-offs (Van der Linde *et al.* 2012). The effect of these climatic changes on insects needs to be considered and remains only poorly understood.

Various foliar insects are of concern to the forestry industry in South Africa and are likely to be influenced by ACC (Garnas *et al.* 2016). Some of the main insects of concern include *Gonipterus* spp., *G. brimblecombei* and *Thaumastocoris peregrinus* (Garnas *et al.* 2016). These insects are introduced organisms to South Africa and attack the leaves of *Eucalyptus* trees, with severe infestations leading to tree death (Garnas *et al.* 2016). Introduced insects will be directly influenced by the climate in their new location, with ACC allowing invasive insects to establish in areas where it was previously not possible for them to survive (Logan *et al.* 2003, Sturrock *et al.* 2011, Anderegg *et al.* 2015, Ramsfield *et al.* 2016). ACC will also affect the establishment and activity of the insects indirectly, by causing stress in the host trees, resulting in them becoming more susceptible to insect attack (Anderegg *et al.* 2015, Ramsfield *et al.* 2016). The predicted climate change for South Africa could potentially increase the distribution and activity of the known insect pests. This is a major concern to the South African forestry industry as these insects are already established and any increase in distribution and activity will be devastating.

Sirex noctilio is an important insect pest to the pine industry in South Africa and is regarded as one of the most important insect pests of softwoods in the Southern Hemisphere (Hurley *et al.* 2007, Yousuf *et al.* 2014). This woodwasp is known to attack stressed and healthy trees (if population densities are high), eventually killing its host, together with its symbiotic fungus *Amylostereum areolatum* (Hurley *et al.* 2007). In South Africa the activity of *S. noctilio* has been reduced by implementing the biocontrol agent *Deladenus siricidicola* (parasitic nematode) (Hurley *et al.* 2008). ACC, specifically higher temperatures and reduced rainfall, could play a vital role in the control success of *S. noctilio* by *D. siricidicola*.

High temperatures and dry conditions are unfavourable for the development of the biocontrol of *S. noctilio* (Hurley *et al.* 2008, Yousuf *et al.* 2014). It has been shown that *D. siricidicola* prefers high moisture content within trees and high temperatures (above 24°C) reduces the parasitizing success of *S. noctilio* by this nematode (Hurley *et al.* 2008, Yousuf *et al.* 2014). Surprisingly, compared to most ACC predictions and impacts on insects, *S. noctilio*

development has been shown to be negatively affected by high temperatures (Yousuf *et al.* 2014). The experiments, involving *S. noctilio* development, were conducted in a laboratory, and are not a true representation of the actual changing climatic environment. If *S. noctilio* can adapt to the predicted ACC in South Africa, new strains of biocontrol, possibly even new species, might need to be considered.

4. Conclusions

The incidence of large scale tree mortality has increased globally during the course of the past two decades (Hogg *et al.* 2008, Raffa *et al.* 2008, Allen 2009, Allen *et al.* 2010). In some instances, a combination of ambrosia beetles, bark beetles and fungi have been associated with these deaths, and where there are known links to changes in climate, particularly increased temperatures (Kamata *et al*. 2002, Berg *et al.* 2006, Trần *et al.* 2007, Kurz *et al.* 2008, Six 2009, Six *et al.* 2011). Very little information is, however, available pertaining to ambrosia beetles, bark beetles and fungi, affected by potential ACC, linked to tree die-offs in Africa, especially native tree die-offs.

ACC is already having a significant impact on tree health, causing tree die-offs associated with new insect infestations and fungal infections (Sturrock *et al.* 2011, Pautasso *et al.* 2012, Sturrock 2012, Anderegg *et al.* 2015). ACC is predicted to play a major role in southern Africa with significant changes in temperature and precipitation (Engelbrecht *et al.* 2015). Various fungal pathogens and insects (e.g. Botryosphaeriaceae, *Phytophthora cinnamomi* and ambrosia beetles) are known to be associated with native tree health problems in South Africa (Van der Linde et al. 2011a,b, Jami *et al.* 2014, Machingambi *et al.* 2015). These fungal pathogens and insects are known to be influence by ACC in the Northern Hemisphere and are likely to become more of a threat to the natural fauna in the future in South Africa.

The paucity of information on fungi, ambrosia and bark beetles in South Africa, and especially how they are influenced by changes in climate, presents a significant challenge to our understanding of the mortality of *E. ingens* and other native tree species in the country. Improved knowledge of ACC and how this will influence the insects and pathogens will surely allow us to better understand native tree die-offs in the country.

5. Background and objectives of this thesis

Euphorbia ingens trees in the Limpopo province of South Africa are dying at an alarming rate. Analyses of climatic data revealed climate changes in the Limpopo province of 2°C

increase in temperature, which could have acted as a catalyst to the sudden dying of trees (Van der Linde *et al.* 2012). Comparisons with trees in healthier sites in the North West province revealed less severe changes in climate, with lower mean temperatures and higher precipitation levels compared to the more diseased sites in the Limpopo province (Van der Linde *et al.* 2012).

Investigations have found a high diversity of fungi and insects on diseased *E. ingens* (Roux *et al.* 2008; Roux *et al.* 2009, Van der Linde *et al.* 2011a,b). Ambrosia beetles (*Cyrtogenius africus*, *Cossonus* sp. and *Stenoscelis* sp.) and some fungal associates have also been found in association with disease symptoms on dying *E. ingens* (Roux *et al.* 2009, Van der Linde *et al.* 2011a,b). These fungi and insects, such as the Botryosphaeriaceae and ambrosia beetles, are mostly all known to be opportunistic pathogens, often associated with stressed hosts. The roles of these fungi and insects remain uncertain, but it seems that they are driven by an abiotic factor, stressing the trees and making them more susceptible to these fungi and insects.

The death of *E. ingens* appears to be caused by a combination of biotic and abiotic factors acting together. Although Van der Linde *et al.* (2012) provided some evidence to support suggestions of climate change driving these die-offs, their work was exploratory in nature and limited to a small number of sites and variables. Further studies are needed to consider the unexpected die-off of *E. ingens* in different provinces in South Africa and the role that the insects and their fungal associates play. These studies must also include more in depth analyses of climate data for each province in order to better understand the possible involvement of fungi, insects and climate in the decline of native trees in South Africa.

The research presented in the following chapters of this thesis aimed to investigate *E. ingens* die-offs in South Africa, considering the associated insects and fungi and investigating the potential triggers for the die-offs. This thesis will consider temperature and precipitation as potential climatic die-off triggers and the potential effect of ACC on insects that infest dying *E. ingens* trees.

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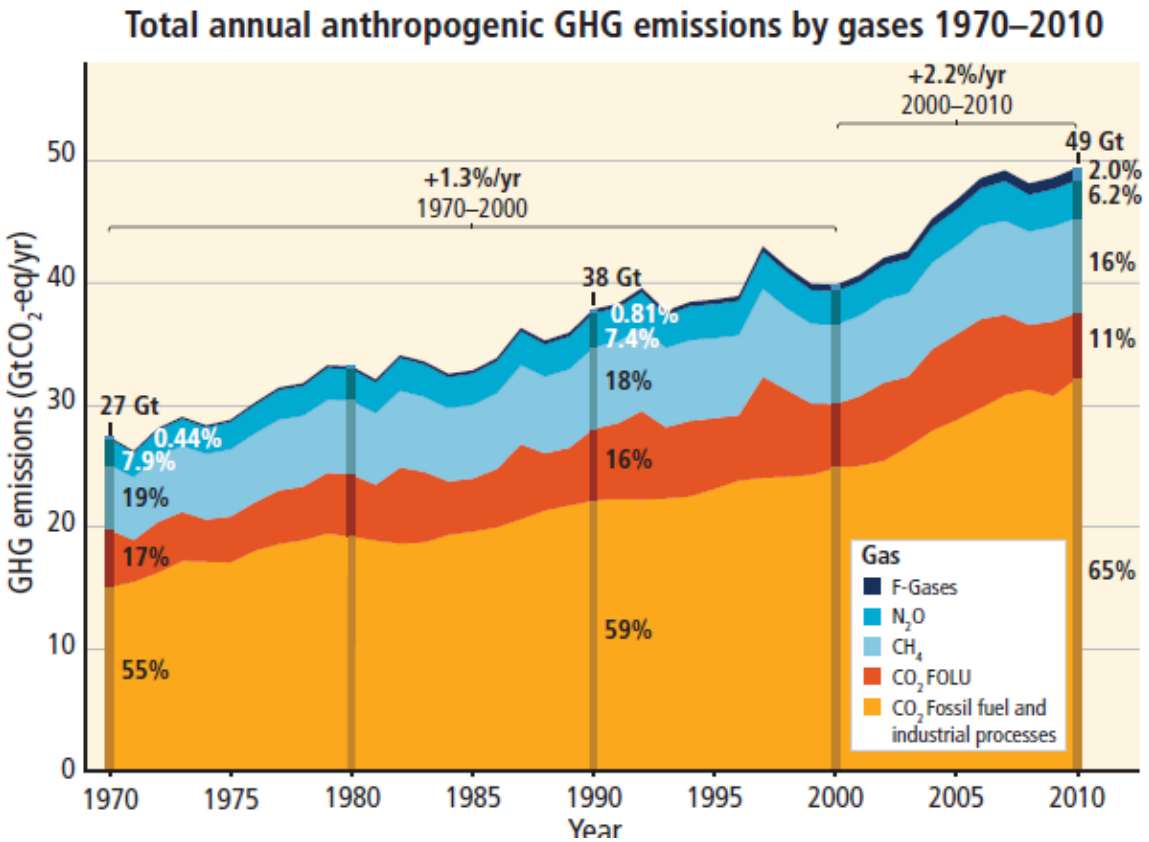

Figure 1: Total annual anthropogenic greenhouse gas (GHG) emissions (gigatonne of CO_2-equivalent per year, $GtCO_2$-eq/yr) for the period 1970 to 2010 by gases: CO_2 from fossil fuel combustion and industrial processes; CO_2 from Forestry and Other Land Use (FOLU); methane (CH_4); nitrous oxide (N_2O) (Figure source: IPPC 2014).

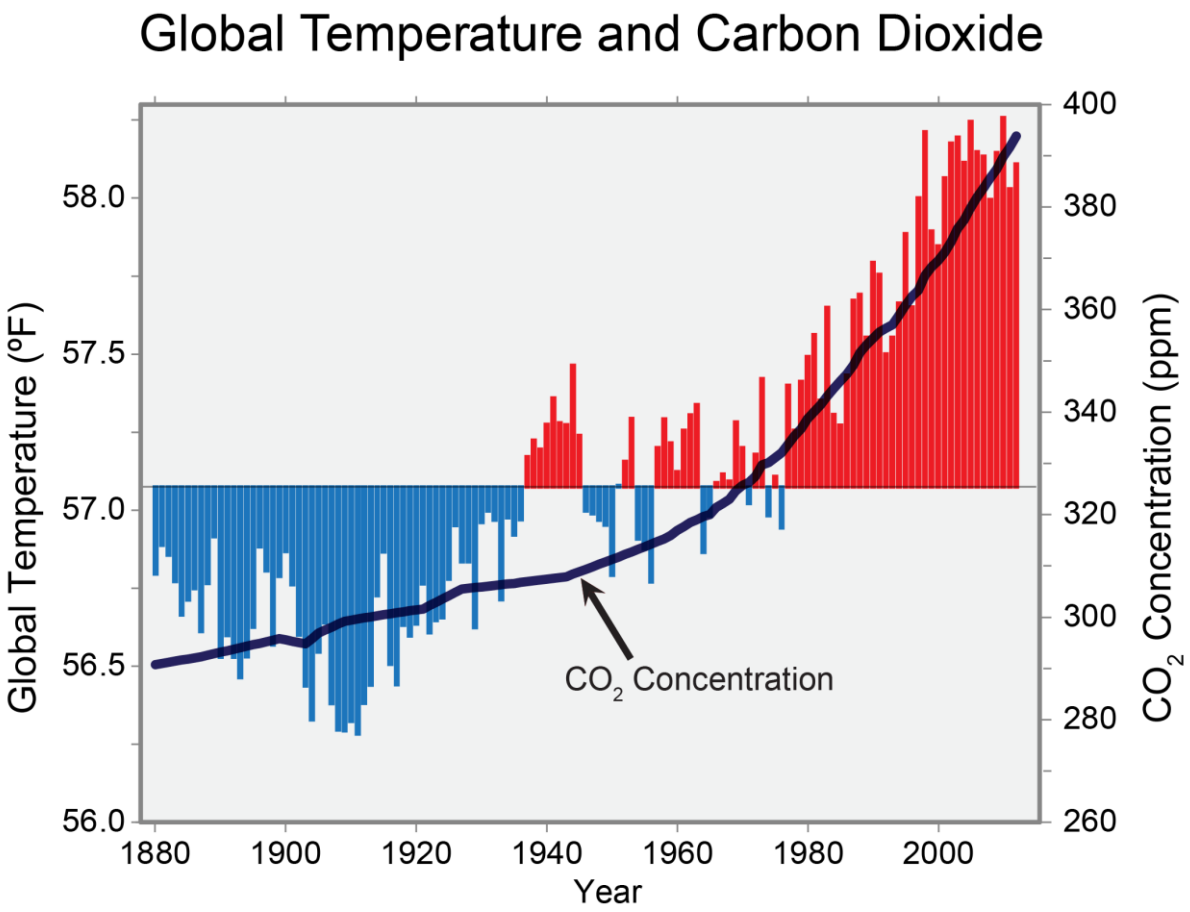

Figure 2: Global annual average temperature increase since 1880. (Figure source: updated from Karl *et al*. 2009).

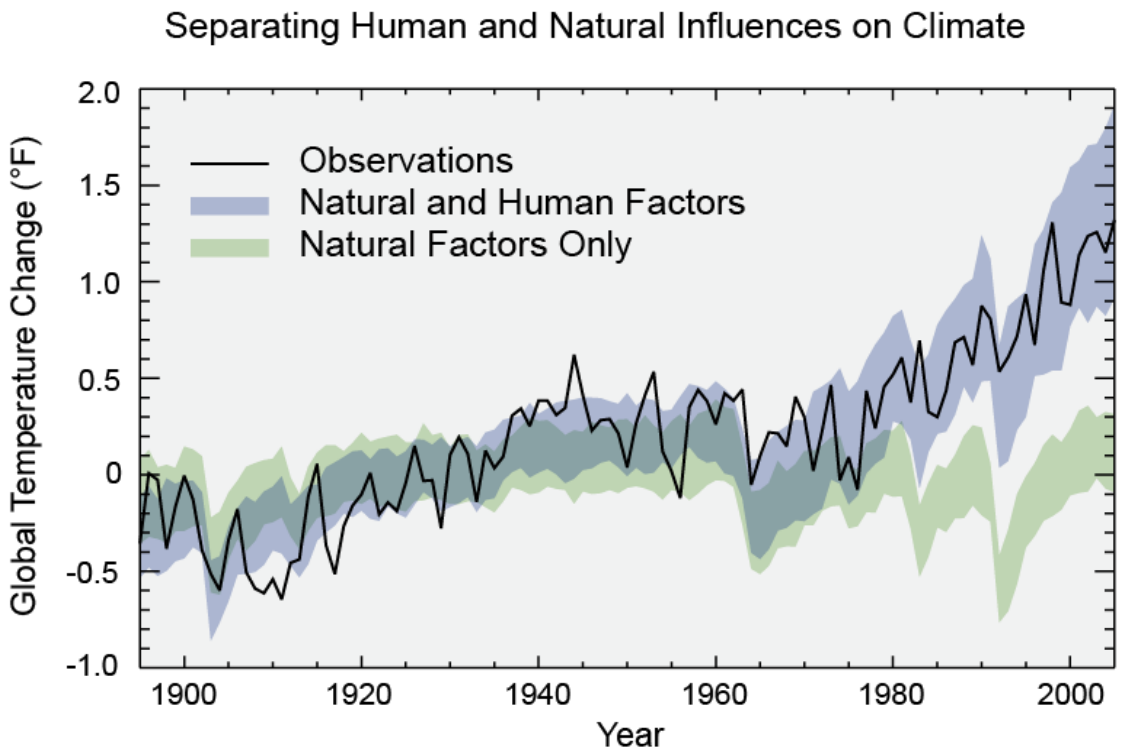

Figure 3: Observed global average changes (black line), model simulations using only changes in natural factors (solar and volcanic) in green, and model simulations with the addition of human-induced emissions (blue). (Figure source: adapted from Huber and Knutti 2011).

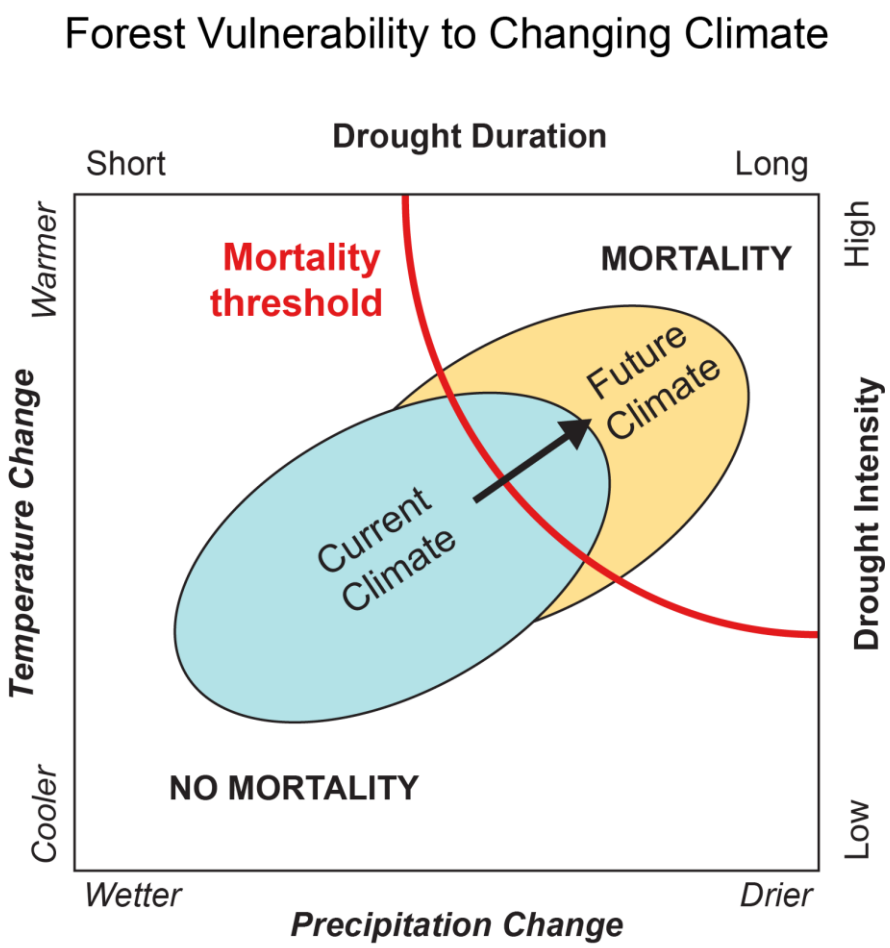

Figure 4: The figure shows a conceptual climate envelope analysis of forest vulnerability under current and projected future ranges of variability in climate parameters (temperature and precipitation, or alternatively drought duration and intensity) (Figure source: Allen *et al.* 2010).

Chapter 2

Fungi and insects associated with *Euphorbia ingens* die-off in South Africa

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Abstract

Euphorbia ingens, landmark succulent trees in savannas of South Africa, have been dying in large numbers over the last 10 to 15 years. Initial studies conducted in the Limpopo Province of South Africa revealed a diverse group of biotic agents including fungi, beetles, and moths associated with dying trees, but due to the limited geographic extent of these studies, it was not known if the same agents were associated with dying trees regionally. In this study, diseased and insect-infested trees were sampled for fungal pathogens and insects at six sites in four provinces located across South Africa. Fungi were identified based on morphology and DNA sequencing of the ITS, LSU, β-tubulin and TEF 1-α gene regions, and insects were identified based on morphology. Fungal isolates were identified as *Aureovirgo volantis*, *Fusarium solani*, *Lasiodiplodia×egyptiacae*, *Ophiostoma thermarum* and a *Readeriella* species. Five insects were identified, all in the family Curculionidae, including two ambrosia beetles, *Cyrtogenius africus* and a *Stenoscelis* species. All fungi and insects collected are known to be opportunistic and occur on stressed trees as secondary agents of mortality or disease. These results suggest that the die-off is not related to attack of the trees by aggressive insects or pathogens, but rather that *E. ingens* in this region is under stress from environmental factors that supports the ability of opportunistic insects and pathogens to establish.

Keywords: Ambrosia beetles, opportunistic pathogens, tree stress

1. Introduction

Euphorbia ingens (common names include giant euphorbia tree, candelabra tree and naboom) are dying at a rapid rate in some regions of South Africa. The first reports of large-scale *E. ingens* mortality were from the Limpopo Province. Causes of mortality were speculated to be from stress due to climate change or infestation by invasive insects or pathogens (Malan 2006, Roux *et al.* 2008, 2009). A subsequent study, comparing mortality at sites in the Limpopo and North West Provinces, indicated that die-off were most severe in the Limpopo region (Van der Linde *et al.* 2012) and were most likely related to changes in temperature and rainfall patterns that contributed to insect attack and disease development (Van der Linde *et al.* 2012). Additional studies implicated several fungal and insect agents as possible causes of tree mortality (Roux *et al.* 2008, 2009, Van der Linde *et al.* 2011a,b). These studies suggested that *E. ingens* die-off may be the result of environmental factors that create stress in the trees, leading to attack by opportunistic insects and pathogens (Van der Linde *et al.* 2011a,b; 2012). However, given the limited geographic extent of the initial studies, surveys made across a broader area were needed to know if this was indeed the case.

The aim of this study was to conduct surveys assessing symptoms associated with the die-off and associated insects and fungi across the range of *E. ingens* in South Africa. Furthermore, we wished to determine if any of these biotic agents were consistently associated with trees in areas experiencing die-off.

2. Materials and Methods

2.1 Estimation of mortality and disease symptoms associated with *E. ingens* die-off

In 2014, disease symptoms and mortality in declining *E. ingens* stands were scored at nine sites (Fig. 1) across South Africa, including five sites previously investigated by Van der Linde *et al.* (2012) in 2009 and 2010. Eight belt transects of 100m x 50m were established at each site and their location recorded using a Global Positioning System (GPS). Based on Van der Linde *et al.* (2012), two specific symptoms were evaluated for individual *E. ingens* trees: grey discoloration and rotting of succulent branches.

Grey discoloration and moth damage were scored, independently from one another, based on a ranking system of zero (no grey discoloration or moth damage) to four (1: 1-25% succulent branches grey and rotting from moth damage, 2: 26-50%, 3: 51-75%, 4: 76-100%). Grey discoloration and the rotting of succulent branches affect *E. ingens* trees differently, and even

with cases where the two symptoms occur on the same tree, they do not typically occur on the same branch. The moths attack succulent branches randomly with no clear pattern of rotting, hence the succulent crown was visualised as a quadrant with succulent branches scored accordingly. Grey discoloration was easier to score, occurring as a gradual progression starting at the lower ends of the succulent branches just above the main trunk.

Mortality was scored as a percentage of dead trees in each transect compared to living trees. The mean rank proportion of grey and moth-damaged trees, as well as percentage mortality, was calculated (data were tested for normality using Shapiro-Wilk's W) and compared among the nine sites using ANOVA. Mean separation analyses were conducted using Tukey-Kramer's test. Linear regression analysis, among all sites, was conducted to test if mortality was dependant on moth damage and/or grey discoloration. All statistical analyses were conducted using JMP Version 12.0.1 (SAS Institute Inc., Cary, North Carolina, 1989-2007) with $\alpha \leq 0.05$.

2.2 Fungus and insect collections

Surveys of diseased *E. ingens* were conducted in 2012 and 2013 at six sites with one inspection conducted at each site per year (Fig. 1; Sites 1 to 6). The same area, within which the belt transects were established for the symptom and mortality scoring survey, was used for the surveys at each site. At each site, one branch exhibiting each symptom type (grey discoloration of the succulent branches, rotting of the succulent branches surrounding moth damaged areas, or staining in the main woody stems associated with insect infestation) was collected from ten different trees for each symptom. Symptomatic tissue samples were placed in paper, and/or plastic bags and transported to the laboratory for further investigation.

Isolations for fungi were made by surface disinfesting plant tissue and cutting small segments from the leading edges of diseased areas and transferring the tissue to 2% malt extract agar (MEA; 15g agar and 20g malt extract l^{-1}; Biolab, Merck, Midrand, South Africa) amended with streptomycin (Streptomycin; 0.4g l^{-1}; Sigma-Aldrich, St Louis, USA). When fungal fruiting bodies were present on lesions or in insect tunnels, spore drops and/or hyphae were carefully removed from the plant material using a sterilized needle and placed on 2% MEA plates. The resultant colonies from tissues and fungal material were purified using single spore or hyphal tip transfers onto 2% MEA plates. After five days of growth, cultures were grouped according to each disease symptom and then further grouped based on the most commonly occurring pure cultures. Representatives from each morphological group were

sequenced (using the ITS, LSU, β-tubulin and TEF 1-α gene regions) and identified to genus, and where possible, species level. Representative isolates have been deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa.

Insects associated with diseased trees were obtained from freshly infested branches and stems by collecting 10 logs from 10 different trees from each site within the already established transect areas. Collections were made during March 2013 and 2014. For each site, four logs were placed in four emergence chambers which were monitored daily for insect emergence over a period of two weeks. Logs could not be kept for a longer period within the emergence chambers as *E. ingens* branches and stems rot and disintegrate very quickly due to their high moisture content. The remaining six logs, from each site, were dissected in the laboratory and insects collected pre-emergence. Insects collected from emergence chambers and dissected logs were grouped based on morphology using the keys in Wood (1986), and counts made for each group. Insects were identified by Dr. Roger Beaver (Thailand). Representative specimens of beetles were pinned and deposited with the National Collection of Insects, Plant Protection Research Institute, Agricultural Research Council, Roodeplaat, Pretoria, South Africa as well as the collection of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

3. Results

3.1 Estimation of mortality and disease symptoms associated with *E. ingens* die-off

Symptoms associated with die-off were present at all sites investigated and at varying levels of severity (Table 1). There was a significant difference in severity of the two main die-off symptoms (greying and rotting) as well as in the percentage mortality among the sites (Table 1). The sites with the highest proportion of rotting (moth damage) were Enzelsberg, Wolfaan, Ulundi followed by Bela-Bela, Euphorbia Drive, Last Post and Capricorn, Eshowe and Lydenburg. The sites with the highest proportion of grey discoloration were Euphorbia Drive, Last Post, and Ulundi followed by Capricorn, Eshowe, Lydenburg, Bela-Bela, Enzelsberg, and Wolfaan. Overall, the most severely affected sites (highest mortality) were Enzelsberg, Euphorbia Drive and Last Post with the least affected sites being Capricorn, Eshowe and Lydenburg.

Sites with the highest mean rank greying did not always have the highest percentage of mortality of *E. ingens* and does not seem to be correlated (R^2 = 0.01, P = 0.32). Euphorbia Drive, Last Post and Ulundi exhibited the highest mean rank greying with a correspondingly high percentage of mortality, while Capricorn had a high mean rank of grey discoloration with the lowest percentage mortality. Enzelsberg had the highest percentage mortality but the lowest mean rank of greying among all the sites. Moth damage was correlated (R^2 = 0.212, P < 0.001) with higher percentage mortality, with the sites with the highest degree of die-off having higher levels of moth-related damage.

3.2 Fungus and insect collections

Isolations from diseased tissue yielded a total of 351 isolates for the six sites, with most isolates being saprophytes such as *Penicillium* species. From the 351 isolates, 100 were identified as the most consistently associated with the observed disease symptoms (Table 2). The isolates were divided into three main groups based on morphology. Representative isolates (Table 3) from each morpho-group were further identified using DNA sequence analysis, from which five genera were identified (Table 3).

Based on DNA sequence data, isolates were identified as *Aureovirgo volantis* (TreeBase: 17782, 17783) described previously by Van der Linde *et al.* (2016a) from *E. ingens*, an undescribed *Fusarium* sp. (TreeBase: 17784, 17785) in the *Fusarium solani* species complex, *Lasiodiplodia×egyptiacae* (TreeBase: 17788, 17789) (recently identified as a hybrid of *L. theobromae* and possibly *L. parva* or *L. citricola*; Cruywagen *et al.* 2016), *Ophiostoma thermarum* (TreeBase: 17782, 17783) described previously by Van der Linde *et al.* (2016a) from *E. ingens*, and an apparently undescribed *Readeriella* sp. (TreeBase: 17786, 17787). *Lasiodiplodia×egyptiacae* and *F. solani* were isolated from stained areas of the main stems of trees heavily infested with weevils as well as rotted tissues associated with moth damage. *Readeriella* sp. was isolated from fruiting bodies in grey as well as green succulent areas on the outside of the branches. *Aureovirgo volantis* and *O. thermarum* were commonly found within the tunnels of the ambrosia beetles *Cyrtogenius africus* and *Stenoscelis* sp., in succulent branches and the sapwood of the main stems (Fig. 2).

Fungal isolations were successful from only 55 (out of the 180 collected) branches (each branch from a different tree, N = 55 trees). Most of the isolates obtained were associated with insect damage (rotting associated with moth attacks and staining associated with weevil attacks) with only six isolates from greyed areas. Isolates associated with insect damage were

obtained from all of the sites, while isolates from the grey discoloured tissue were obtained from only two sites (Table 2).

Five Curculionidae species (two ambrosia beetles and three other weevils) were collected from the emergence chambers (Table 2). The ambrosia beetles (Scolytinae), *Cyrtogenius africus* (AcP9546) and a *Stenoscelis* sp. (AcP9549), were reared from the main stems, while two weevils, *Mechistocerus* sp. (Molytinae) (AcP9551) and *Coleobothrus germeauxi* (Scolytinae) (AcP9544) were reared from the secondary phloem. The weevil *Cossonus* sp. (Cossoninae) (AcP9548) was reared from the vascular cambium (Fig. 3). Larvae of the moth *Megasis* sp. (Lepidoptera: Pyralidae) were identified at all sites and were associated with the rotting of the succulent branches.

4. Discussion

The results of this study expand on those of Roux *et al.* 2008, 2009 and Van der Linde *et al.* (2011a,b; 2016a) who reported a number of fungi and insects associated with the large-scale die-off of *E. ingens* in the Limpopo Province of South Africa. Sites for the present study were selected over a wider geographic distribution of *E. ingens* in the country, allowing a more comprehensive evaluation of the factors associated with the die-off. There were differences in severity of symptoms associated with die-off and mortality among the sites. Higher levels of moth damage were observed at sites with higher tree mortality. *Megasis* sp. occurred at all sites, suggesting a stronger correlation between tree death and infestation by this moth compared to grey discoloration. Isolations from grey discoloured branches yielded very few fungal isolates and the grey discoloration of the branches is not caused by fungal infections.

Relatively few fungal isolates were obtained from diseased material sampled in this study, despite the large number of samples collected. These results are similar to previous studies by Van der Linde *et al.* (2011a,b) and may either be a result of low isolation success and/or an indication of the secondary nature of fungal involvement in the death of *E. ingens*. Of the isolates obtained, only a few represented possible pathogens. Of these, *A. volantis*, *F. solani*, *L.* ×*egyptiacae*, and *O. thermarum* were most often isolated from *E. ingens* trees that were heavily infested by the moth *Megasis* sp. and ambrosia beetles (*C. africus* and *Stenoscelis* sp.). *Lasiodiplodia* species (Botryosphaeriaceae) are well known opportunistic fungal pathogens known to cause staining within wood, dieback and cankers of stressed trees and are associated with a wide variety of hosts (Damm *et al.* 2007, Slippers and Wingfield 2007, Phillips *et al.* 2008, Jami *et al.* 2015). Species in this genus, such as *L. theobromae* and *L.*

mahajangana, have been reported previously from dying *E. ingens* trees (Van der Linde *et al.* 2011b) and are also known from *Acacia*, *Eucalyptus*, *Pinus* and native *Syzygium* in South Africa (Crous *et al.* 2000, Burgess *et al.* 2003, Pavlic *et al.* 2004; 2007). *Lasiodiplodia×egyptiacae* was described from mango (*Mangifera indica*) plantations in Egypt (Ismail *et al.* 2012) and has been reported from Physic nut (*Jatropha curcas*) in Brazil as well as Baobabs (*Adansonia grandidiera*) in Madagascar (Machado *et al.* 2014, Cruywagen *et al.* 2016).

Aureovirgo volantis and *O. thermarum* are members of the *Ophiostoma sensu lato* complex (Ophiostomataceae). *Ophiostoma thermarum* resides in the *Sporothrix schenckii–Ophiostoma stenoceras* complex, a group of fungi known to be associated with soil and hardwoods as well as with conifer-infesting bark beetles (Zhou *et al.* 2001, De Beer *et al.* 2003, De Meyer *et al.* 2008, Roets *et al.* 2008). Even though fungi in this complex are known to cause staining in wood, their pathogenicity in host trees has been questioned, and they are considered as secondary agents to tree disease and mortality (De Beer *et al.* 2003, De Meyer *et al.* 2008). Species within this complex, in South Africa, have also been recorded from *Protea* species, *Eucalyptus grandis* and pine-infesting bark beetles (Wingfield *et al.* 1993, Zhou *et al.* 2001, Zhou *et al.* 2006, Roets *et al.* 2008). Pathogenicity trials, conducted by Van der Linde *et al.* (2016a) using *A. volantis* and *O. thermarum* on *E. ingens*, produced small lesions and internal rotting on succulent branches (Van der Linde *et al.* 2011b). Van der Linde *et al.* (2016a) did not find that *A. volantis* and *O. thermarum* are primary pathogens of *E. ingens*. Because they are not known to be virulent pathogens, the species isolated in this study are unlikely to be major drivers of *E. ingens* die-off.

The *Fusarium solani* species complex (FSSC) is comprised of at least 45 closely related species (Zhang *et al.* 2006). The FSSC fungi are known to be soil borne or to occur in decaying organic material (Zhang *et al.* 2006, Bogale *et al.* 2009). Species in this group have been isolated from soil and lesions on a wide variety of crops including potatoes, tomatoes, citrus plants, peas and soybeans (Roy *et al.* 1989, Cho *et al.* 2001, Romberg and Davis 2007, Zaccardelli *et al.* 2008, Rehman *et al.* 2012). In South Africa, *F. solani* has been reported to cause the die-back of English walnut (*Juglans regia*) and lisianthus (*Eustoma grandiflorum*) (Chen and Swart 2000, Truter and Wehner 2004). Species within FSSC are known to be associated with ambrosia beetles (Baker and Norris 1968, Windels *et al.* 1976, Beaver 1989, Rojas *et al.* 1999, Mendel *et al.* 2012) and in some cases occur as mutualists, e.g. of *Hypothenemus hampei (*Coffee borer beetle) (Rojas *et al.* 1999, Morales-Ramos *et al.* 2000).

It has been suggested that these fungi could be opportunist pathogens causing disease and death in stressed plants (Sherbakoff 1953, Kavroulakis *et al.* 2007, Bogale *et al.* 2009, Rehman *et al.* 2012). In the present study, isolates of *F. solani* were consistently isolated from disease margins of rotten succulent branches, which had been fed on by *Megasis* larvae and infested by weevils. It seems unlikely that this fungus would be the primary cause of tree die-off and is more likely a secondary agent of disease.

The fungal genus *Readeriella* belongs to the family Teratosphaeriaceae, a well-known group of fungi that causes diseases of the stems and leaves of *Eucalyptus* species (Crous 1998, Crous *et al.* 2004, Burgess *et al.* 2007, Carnegie 2007, Cheewangkoon *et al.* 2008; 2009, Hunter *et al.* 2011). This is the first report of a *Readeriella* species from *E. ingens* trees. The fungus was commonly isolated from black fruiting bodies on the outside of the grey discoloured and green succulent branches of *E. ingens*. The black fruiting bodies only occurred on the exterior of the branches in a very superficial manner, never extending into the tissue.

The insects reared from diseased and dying *E. ingens* trees have all previously been reported from Africa. *Coleobothrus germeauxi* is known to occur in dead branches of trees and has been recorded from *Euphorbia teke* Schweinf. ex Pax in Kenya and Uganda (Jordal and Hewitt 2004, Mandelshtam and Danielsson 2004), while *Mechistocerus* sp. has only one record from Africa, namely from Liberia (Briscoe 1947). *Stenoscelis* species are known to attack trees that are stressed, with recorded collections from Algeria, Kenya and South Africa (Konishi 1956). *Cossonus* species have been reported from decaying trees in Ethiopia and the KwaZulu-Natal Province in South Africa (Marshall 1905, Colonnelli 2014). *Cyrtogenius africus* was first recorded in 1988 from various *Euphorbia* species in Africa (Democratic Republic of Congo [formally known as Zaire from 1971-1997], Guinea, Kenya, Tanzania, Uganda) and again in 2009 from dead branches of *Euphorbia triangularis* Desf. in South Africa (Wood and Bright 1992, Jordal 2009). However, there is limited information on how and when these beetles infest trees, with the only records existing being for diseased trees or decaying wood (Marshall 1905, Briscoe 1947, Konishi 1956, Wood and Bright 1992, Jordal and Hewitt 2004, Mandelshtam and Danielsson 2004, Jordal 2009, Colonnelli 2014). The beetles appear to only occur as secondary insects, infesting stressed trees (Konishi 1956, Jordal 2009). Limited information is also available for host preferences and distribution of the moth, *Megasis* sp. Our specimens could only be identified to genus and are believed to be a

species native to South Africa (pers. Comm. Dr Martin Kruger, The Ditsong National Museum of Natural History, Pretoria, South Africa).

Van der Linde *et al.* (2012) found that *E. ingens* mortality was related to a specific province with temperature and rainfall having a significant effect on tree mortality. Temperature over the last 60 years has increased more significantly in the Limpopo Province compared to the North West Province and was identified as the main trigger for *E. ingens* die-off (Van der Linde *et al.* 2012). However, in this study, sites within a province had different levels of tree mortality. Euphorbia Drive and Last Post had higher tree mortality compared to Bela-Bela and Capricorn, with Last Post having significantly higher percentage mortality compared to Capricorn (two sites that are 18km from each other). Wolfaan had half the percentage mortality compared to Enzelsberg (North West), while Eshowe had lower percentage mortality compared to Ulundi (KwaZulu Natal). Percentage mortality, therefore, does not seem to be related to a specific province or area, as previously believed, and might be affected, not only by climate, but by more site specific conditions as suggested by Van der Linde *et al.* (2016b).

It is known that trees in disturbed environments can be under substantial stress leading to susceptibility by secondary fungi and insects that can lead to the death of the trees (Mueller-Dombois *et al.* 1983, Akashi and Mueller-Dombois 1995, Dale *et al.* 2000, Holdenrieder *et al.* 2004, Foden *et al.* 2007, Allen 2009, Allen *et al.* 2010). Our results lead us to believe that *E. ingens* die-off are likely driven by stressors in the environment that lead to attack by insects and infection by pathogens that then ultimately kill the tree.

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Table 1. Mean (SE) die-off factor and percent mortality in *Euphorbia ingens* at nine sites and one-way ANOVA statistics of comparisons among sites for each factor and mortality.

Site	Grey discoloration	Moth damage	% Mortality
Enzelsberg	0.148 (0.056)^{c}	0.689 (0.120)^{ab}	32.50 (3.694)^{a}
Euphorbia Drive	1.695 (0.073)^{a}	0.597 (0.054)^{ab}	25.52 (5.102)^{ab}
Last Post	1.407 (0.062)^{a}	0.584 (0.047)^{ab}	20.90 (4.454)^{abc}
Ulundi	1.659 (0.083)^{a}	0.775 (0.047)^{a}	17.47 (5.889)^{abcd}
Wolfaan	0.351 (0.044)^{bc}	0.774 (0.080)^{a}	16.40 (4.061)^{abcd}
Bela-Bela	0.560 (0.071)^{b}	0.504 (0.092)^{ab}	14.90 (2.039)^{bcd}
Eshowe	0.657 (0.118)^{b}	0.406 (0.068)^{bc}	10.62 (3.196)^{bcd}
Lydenburg	0.594 (0.141)^{b}	0.166 (0.048)^{c}	7.00 (2.479)^{cd}
Capricorn	0.739 (0.082)^{b}	0.170 (0.026)^{c}	2.50 (1.732)^{d}
ANOVA Statistics	F = 43.847, df = 8, P < 0.001	F = 10.863, df = 8, P < 0.001	F = 5.702, df = 8, P < 0.001

Same letters within a column indicate that means are not significantly different

Table 2. Number of isolates obtained from isolations from dying *Euphorbia ingens* trees exhibiting the three main symptoms of disease at six sites in South Africa.

Fungal species	Disease symptom[a]	Enzelsberg	Wolfaan	Lydenburg	Bela-Bela	Ulundi	Eshowe	Total isolates
Fusarium solani f. sp. nov.	**rotting of succulent branch**	3 [2][b]	*	27 [7]	5 [2]	2 [1]	2 [1]	39
Lasiodiplodia×egyptiacae	**rotting of succulent branch**	*	12 [4]	*	*	3 [3]	*	15
Readeriella sp. nov.	**grey discoloration**	*	*	2 [2]	*	*	4 [4]	6
Ophiostoma thermarum	**stain/galleries in main woody stem**	*	*	*	16 [7]	*	*	16
Aureovirgo volantis	**stain/galleries in main woody stem**	6 [6]	4 [4]	7 [7]	4 [3]	*	3 [3]	24
Total isolates		**9**	**16**	**36**	**25**	**5**	**9**	100
Total branches/Trees [c]		**8**	**8**	**16**	**12**	**3**	**8**	55
Insect presence								
Megasis sp.[d]		+	+	+	+	+	+	
Cossonus sp.		-	+ (32)	-	-	+ (22)	-	
Mechistocerus sp.		+ (5) [e]	+(15)	-	-	-	-	
Stenoscelis sp.		+ (105)	+ (53)	+ (23)	+ (45)	-	-	
C. germeauxi		+ (163)	+ (180)	-	-	-	-	
C. africus		+(90)	+ (145)	+ (41)	+ (28)	-	+ (38)	

*** Indicates no isolations or collections**

[a] 10 branches from 10 trees for each disease symptom

[b] Number of isolates [no of branches]

[c] Out of a possible 30 at each site (i.e. 180 branches in total)

[d] Presence/absence based on field observation

[e] Number of beetles that emerged from rearing containers

Table 3. Genbank accession numbers and locality of collection of representative isolates sequenced and identified in this study.

Species	**CMW**	**Locality**	**ITS**	**TEF 1-α**	**LSU**	**β-tubulin**
Fusarium solani	–	Enzelsberg	KU519629	KU519634	–	–
F. solani	–	Lydenburg	KU519630	KU519635	–	–
F. solani	–	Bela-Bela	KU519631	KU519636	–	–
F. solani	–	Ulundi	KU519632	KU519637	–	–
F. solani	–	Eshowe	KU519633	KU519638	–	–
Lasiodiplodia×egyptiacae	38914	Wolfaan	KU519639	KU519643	–	–
L.×egyptiacae	38915	Wolfaan	KU519640	KU519644	–	–
L.×egyptiacae	38916	Wolfaan	KU519641	KU519645	–	–
L.×egyptiacae	38917	Ulundi	KU519642	KU519646	–	–
Ophiostoma thermarum	38929	Bela-Bela	KR051114	–	KR051126	KR51102
O. thermarum	38930	Bela-Bela	KR051115	–	KR051127	KR51103
O. thermarum	38931	Bela-Bela	KR051116	–	KR051128	KR51104
Aureovirgo volantis	42282	Eshowe	KR051123	–	KR051133	KR51109
A. volantis	42285	Lydenburg	KR051121	–	KR051134	KR51110
A. volantis	42287	Bela-Bela	KR051124	–	KR051135	KR51111
A. volantis	42290	Enzelsberg	KR051122	–	KR051136	KR51112
A. volantis	42292	Wolfaan	KR051125	–	KR051137	KR51113
Readeriella sp.	44675	Eshowe	KU519647	KU519649	–	–
Readeriella sp.	44676	Lydenburg	KU519648	KU519650	–	–

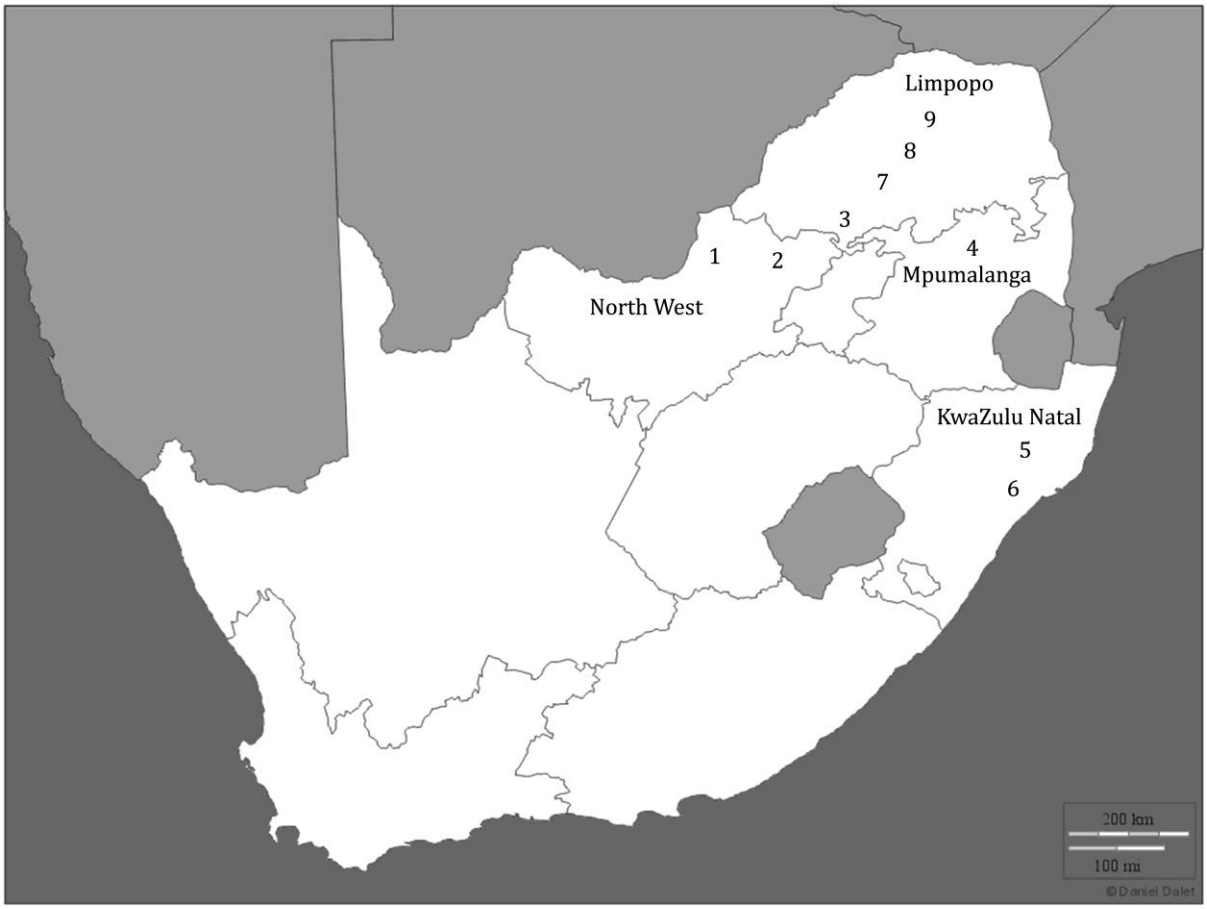

Figure 1: Sites at which insect and fungal surveys and die-off symptom scoring were conducted. 1 = Enzelsberg, 2 = Wolfaan, 3 = Bela-Bela, 4 = Lydenburg; 5 = Ulundi, 6 = Eshowe, 7 = Euphorbia Drive, 8 = Capricorn, 9 = Last Post.

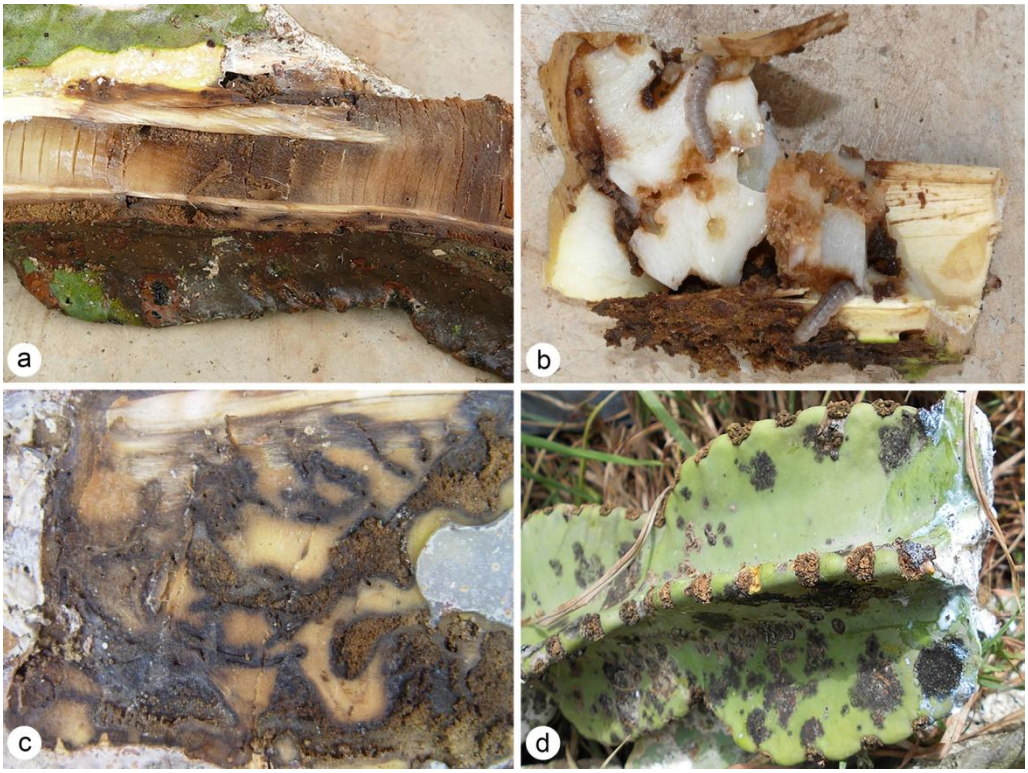

Figure 2: Symptoms of disease and insect infestation associated with *Euphorbia ingens* die-off. (a) Moth-attacked branch from which *L.* × *egyptiacae* and *F. solani* were isolated, (b) Larvae and damage caused by *Megasis* sp., (c) Staining associated with beetle tunnelling from which *A. volantis* and *O. thermarum* were isolated, (d) Black fruiting bodies of *Readeriella* sp. on succulent branches.

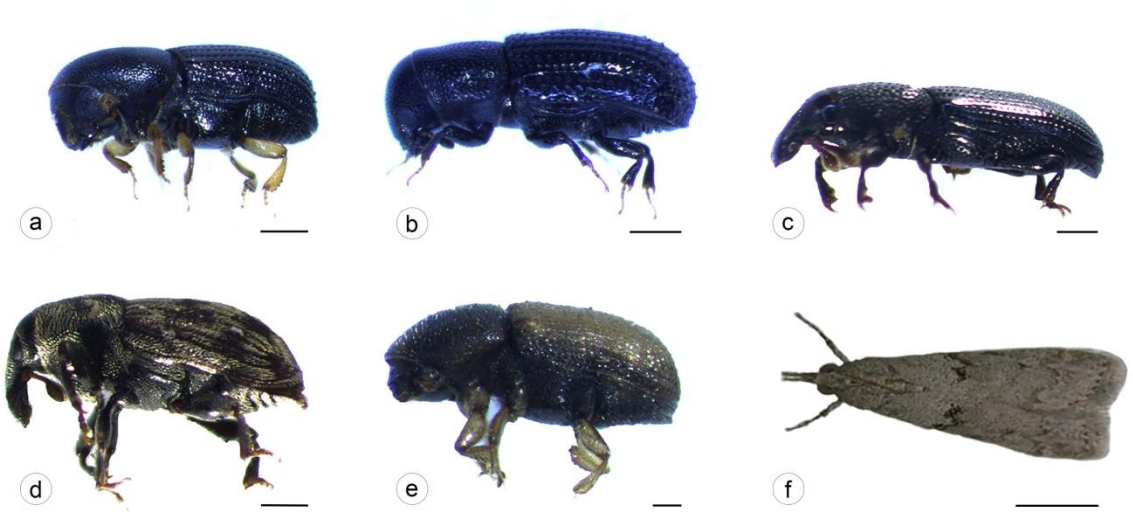

Figure 3: Insects identified from diseased *Euphorbia ingens* trees. (a) *C. africus* (sapwood), (b) *Stenoscelis* sp. (sapwood), (c) *Cossonus* sp. (vascular cambium), (d) *Mechistocerus* sp. (secondary phloem), (e) *C. germeauxi* (secondary phloem), (f) Adult *Megasis* sp. (secondary phloem). Scale bars a to d = 500µm, e = 200µm and f = 5mm.

Chapter 3

Novel ophiostomatalean fungi from galleries of *Cyrtogenius africus* (Scolytinae) infesting dying *Euphorbia ingens*

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Abstract

Euphorbia ingens trees have been dying in large numbers in the Limpopo Province of South Africa for approximately 15 years. The ambrosia beetle *Cyrtogenius africus* is often found infesting diseased and dying trees. The aim of this study was to identify the ophiostomatoid fungi occurring in the galleries of *C. africus*. Logs infested with this beetle were collected from the KwaZulu-Natal, Limpopo, Mpumalanga, and North West Provinces of South Africa. Fungi belonging to the Ophiostomatales were identified based on morphology and comparison of sequence data for the β-tubulin, ITS1-5.8S-ITS2 and LSU gene regions. A novel species of *Ophiostoma* and a novel genus in the Ophiostomatales were identified. Inoculation studies with these fungi produced lesions in the branches of healthy *E. ingens* trees.

Keywords: *Ophiostoma*, Ophiostomataceae, ophiostomatalean fungi, Ophiostomatales, Scolytinae

1. Introduction

The ophiostomatoid fungi were originally classified, based on morphology, as a group of fungi with similar sexual structures that had evolved in close association with insects (Wingfield *et al*. 1993, De Beer *et al*. 2013). These structures include flask-shaped ascomata with long necks raised above the substrate and exuding sticky spore drops (containing ascospores) that aid in dispersal via insects (Dowding 1984, Malloch and Blackwell 1993). Three ascomycete genera, *Ceratocystis*, *Ceratocystiopsis,* and *Ophiostoma*, were originally included in the group referred to as ophiostomatoid fungi (Wingfield *et al*. 1993). Phylogenetic inference based on DNA-sequence analyses later revealed that the ophiostomatoid fungi represent a polyphyletic assemblage, comprising two distinct orders, the Ophiostomatales and the Microascales (Hausner *et al*. 1993, Spatafora and Blackwell 1994, Zipfel *et al*. 2006). The Ophiostomatales contains only one family (Ophiostomataceae) with six genera, *Ceratocystiopsis*, *Fragosphaeria*, *Leptographium sensu lato*, *Ophiostoma sensu lato*, *Raffaelea sensu stricto* and *Graphilbum* (De Beer *et al*. 2013), while the Microascales is comprised of five families, two of which, the Ceratocystidaceae and Graphiaceae, accommodate the ophiostomatoid genera (Réblová *et al*. 2011, De Beer *et al*. 2013, De Beer *et al*. 2014).

Ophiostomatalean fungi have various ecological associations with ambrosia and bark beetles (Paine *et al*. 1997, Harrington 2005). While bark beetles feed in the phloem and sometimes have dependent associations on the fungi they vector, ambrosia beetles bore into the xylem of host trees where they are completely dependent upon their fungal associates for food (Beaver 1989, Hulcr and Dunn 2011, Six 2012). Ambrosia beetles are known to infest dead or severely stressed trees (Batra 1967, Wood 1982). However, studies have shown that increasing numbers of these beetles, together with their fungal partners, can infest and kill healthy trees leading to substantial tree mortality (Hulcr and Dunn 2011, Ploetz *et al*. 2013, Ranger *et al*. 2015). For example, in Japan increasing levels of oak die-back caused by *Platypus quercivorus* Murayama and its fungal symbiont *Raffaelea quercivora* Kubono & Shin. Ito, have been reported (Kamata *et al*. 2002, Kubono and Ito 2002). Likewise, in the United States of America (USA) laurel wilt disease is caused by *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva that is vectored by the invasive ambrosia beetle *Xyloborus glabratus* Eichhoff (Fraedrich *et al*. 2008, Harrington *et al*. 2008).

Euphorbia ingens E. Meyer: Boissier trees, native in the savanna landscape of South Africa, have been dying in large numbers for several years (Roux *et al*. 2008, 2009, Van der Linde *et al*. 2011a,b). The first reports of die-offs were from the Limpopo Province of South Africa (RSA) and were associated with various biotic factors (Malan 2006, Roux *et al*. 2008, 2009). Pilot studies showed the presence of several ophiostomatalean fungi in the tunnels of beetles infesting dying *E. ingens* (Roux *et al*. 2008, 2009). Two weevils [*Cossonus* sp. Claireville and *Stenoscelis* sp. Wollaston (Cossoninae)] as well as the ambrosia beetle *Cyrtogenius africus* Wood (Scolytinae) were identified from diseased and dying *E. ingens* trees (Van der Linde *et al*. 2011a,b). Van der Linde *et al*. (2011b), however, identified only a single species of ophiostomatalean fungi, *Knoxdaviesia serotectus* (J.A. van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf. (Ceratocystidaceae), from the secondary phloem of dying trees associated with a *Cossonus* species.

The aim of this study was to identify ophiostomatalean fungi collected in the tunnels of *C. africus* on *E. ingens* in all provinces of South Africa where the tree occurs. Ophiostomatalean fungi were identified using sequence data of multiple gene regions and their potential role in tree die-offs was considered using artificial inoculation studies to assess pathogenicity to the host tree.

2. Materials and Methods

2.1 Collection of samples and isolations

Sections of *E. ingens* stems were cut from trees showing signs of beetle infestation at six sites in South Africa over a period of three years (2012-2014), from early autumn (March) to early spring (August) during each year. One log was sampled from each of 10 trees at each site. The sites were located in KwaZulu-Natal (Eshowe - March 2013, Coordinates: 28°48'42.64"S 31°30'30.10"E), Limpopo (Bela-Bela - August 2012/July 2013, 24°51'48.30"S 28°20'5.90"E; Last Post - June 2014, 23°17'21.39"S 29°55'27.93"E), Mpumalanga (Lydenburg - July 2012, 24°55'53.87"S 30°19'7.09"E) and North West Provinces (Wolfaan - July 2013, 25°42'59.27"S 27°42'9.24"E; Enzelsberg - July 2013, 25°22'58.05"S 26°16'4.21"E).

Logs were carefully dissected in the laboratory to expose galleries of *C. africus*. Isolations were made directly from fungal structures, typical of ophiostomatalean fungi, in the galleries of *C. africus* as well as from stained tissue surrounding the galleries. Spore drops from ascomata were placed, using a sterilised needle, on 2% MEA (MEA; 15g agar and 20g malt extract per 1000mL distilled water) containing streptomycin sulphate (0.4g/L). MEA plates

were incubated at 25°C for up to five days and single hyphal tips from germinating spores transferred to fresh MEA plates. Isolates were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and representative isolates were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Herbarium specimens representing novel species were deposited with the National Fungal Collection (PREM), Pretoria, South Africa.

2.2 Fungal Morphology and Growth

Fruiting structures of the ophiostomatalean fungi, obtained from both plant material and MEA plates, were examined by mounting structures in distilled water on glass microscope slides and examining these using a Zeiss microscope. Images of relevant, representative structures were captured with an Axiocam digital camera (Axiovision 3.1). Informative characteristics for the ophiostomatalean fungi were measured (50 measurements each) and presented as (min–) avg. ± std. dev. (–max) for the length and width of the structures (l/w).

Optimal temperatures for growth of the fungi were determined by placing 5 mm agar discs, obtained from five-day-old cultures, with the mycelium facing down, at the centers of 90 mm MEA plates. Five replicates were used for each isolate at each temperature. Plates were incubated at temperatures ranging from 10 °C to 35 °C at 5 °C intervals. Incubation was conducted in the dark for 10 days with measurements taken every 24 hours. Two measurements of the total diameter were made perpendicular to one another. Averages of the diameters were calculated and one-way analysis of variance (ANOVA) was conducted ($P \leq 0.05$ as significant, JMP Version 12.0.1, SAS Institute Inc., Cary, North Carolina, 1989-2007) to determine at which temperature each of the species grew best. Data were tested for normality (Shapiro-Wilk's W, Shapiro and Wilk 1965) with non-normal data analysed using a Kruskal-Wallis one way ANOVA if data were not normal after transformation.

2.3 DNA extraction, PCR, Sequencing and Phylogenetic analyses

DNA was extracted from the mycelium of five-day-old isolates (with ophiostomatalean culture morphology) using PrepMan® Ultra (Applied Biosystems, Foster City, USA). DNA concentrations were determined using a Nanodrop ND-1000 Spectrophotometer (Thermo-Fisher Scientific, Wilmington, USA). The polymerase chain reaction (PCR), using an Applied Biosystems Veriti thermocycler, was used to amplify the β-tubulin (BT) gene region using the primers T10 (O'Donnell and Cigelnik 1997) or Bt2A together with Bt2B (Glass and Donaldson 1995), the internal transcribed spacer (ITS) regions 1 and 2 including the 5.8S

gene using the primers ITS1 and ITS4 (White *et al*. 1990), and the ribosomal large subunit (LSU) gene region using the primers LR5 and LROR (Vilgalys and Hester 1990). PCRs were conducted using the protocol described by Van der Linde *et al*. (2011b). PCR products were confirmed using an agarose gel (2%; Whitehead Scientific, Cape Town, South Africa) loaded with GelRed (Anatech, USA), visualised under UV illumination. A 100 bp DNA molecular marker (O'RangeRuler™ 100 bp DNA ladder, Fermentas Life Sciences, Vilnius, Lithuania) was used to estimate the sizes of the PCR products. Amplification products were purified using DNA Clean & Concentrator™-5 (ZYMO Research, Irvine, USA).

Purified PCR products were sequenced using an ABI 3700 DNA analyser (Applied Biosystems) following the instructions provided by the manufacturer. Mega 5.0 (Tamura *et al.* 2007) was used to construct contigs based on forward and reverse sequences. Sequences were submitted to searches in the BLASTn database to establish closest matches. Sequences of the most closely related fungi for all three gene regions were downloaded from Genbank and included in phylogenetic analyses. Sequences obtained from this study and Genbank were aligned using MAFFT 5.851 (Katoh *et al*. 2002).

Aligned sequences were analyzed with Maximum Parsimony (heuristic searches, with random stepwise addition and tree bisection as branch swapping algorithms) in PAUP* 4.0b10 (Swofford 2002). Bootstrap analyses, with 1000 replicates (Felsenstein 1985), were determined for all datasets. Posterior probabilities were determined using Bayesian inference (MrBayes 3.1.2, Huelsenbeck and Ronquist 2001) using the Monte Carlo Markov chain (MCMC) method (parameters set at four chains producing 5,000,000 generations, recording trees every 100 generations). The appropriate nucleotide substitution model was determined using jModelTest 0.1.1 (Posada 2008) with burn-in values determined using graphical analysis (Tracer 1.5) at the point where values converged.

2.4 Pathogenicity study

Three isolates of each of the fungi identified in this study were selected for pathogenicity trials conducted on *E. ingens* trees growing under field conditions at Bela-Bela (24°51'48.30"S 28°20'5.90"E) in the Limpopo Province. Sterilized wooden tooth picks were autoclaved in malt extract broth and placed onto the surfaces of 2% MEA plates. These plates were left for three days to ensure sterility before inoculation with the test fungi (Van der Linde *et al*. 2011b). Five plates were prepared for each of the isolates as well as the control and grown for five days prior to inoculation. The control consisted of sterile toothpicks on the

surface of MEA that had not been inoculated with fungi. Toothpicks for each isolate (seven in total), including the control, were inserted into five separate branches on seven trees. Toothpicks were inserted to a depth of 3 mm in secondary tier healthy succulent branches. Branches ranged between 25 and 30 cm in circumference.

Six weeks after inoculation, all inoculated branches were removed for evaluation. External lesions extending from the entry point of the toothpicks were measured (parallel with branch length). The depth and width of internal rotting was also measured after cutting open the branches at the point of inoculation. Isolations were made from any visible lesions to confirm fungal identity and to comply with Koch's postulates. The entire experiment was repeated once. Data from both experiments were analysed separately and then combined for each isolate to determine variation in external lesion length and internal rotting (depth and width). The mean of the external lesion length and area of internal rotting (depth and width) was compared among all the isolates tested using one-way analysis of variance (ANOVA) with $P \leq 0.05$ set as significant (JMP 12.0.1). Normality of data were tested with Shapiro-Wilk's W with non-normal data transformed. If normality was not achieved after transformation the data were analysed with a Kruskal-Wallis one way ANOVA. Significant F-tests were subjected to mean separation tests (Tukey-Kramer's, HSD). No variance was found with the control (replicates showed zero lesion length) and consequently each isolate was compared against zero using independent, one sided t-tests, Bonferroni-corrected for multiple comparisons ($\alpha \leq 0.05$, JMP 12.0.1).

3. Results

3.1 Fungi isolated

Cyrtogenius africus was obtained from logs at all sites. Fifty-five isolates resembling species of ophiostomatalean fungi were obtained from beetle galleries and surrounding stained tissues from 40 of the 60 logs. Of the 55 isolates obtained in this study two different species were identified. Thirty-nine of the isolates belonged to one species, with 13 directly isolated from sporocarps in *C. africus* tunnels and 26 obtained from fungal stain surrounding the tunnels. Of the 39 isolates, four were obtained from Bela-Bela (three trees), four from Wolfaan (four trees), three from Eshowe (three trees), six from Enzelsberg (six trees), 15 from Last Post (10 trees) and seven from Lydenburg (seven trees). Sixteen of the 55 isolates were identified as another species with six obtained directly from sporocarps in *C. africus* tunnels and 10 obtained from fungal stain surrounding the tunnels. All sixteen isolates of the

second species were obtained from Bela-Bela (7 trees), with none from the other sites investigated. The two fungal species identified were not found in the same *C. africus* tunnels in Bela-Bela.

3.2 Fungal Morphology and Growth

Sexual and asexual structures were observed in the galleries of *C. africus*, with the two states not observed to occur within the same tunnels. The one state was characterized by ascomata with spore droplets and the other state had hyaline sporothrix-like conidiophores in the tunnels. All cultures obtained were white with two distinct culture morphologies; one with radiate-to chrysanthemum-like (in reference to the flower morphology of this plant genus, usually used to describe *Phytophthora* and *Pythium* culture morphology, Mrázková *et al*. 2011) aerial mycelial growth, with the other culture morphology type having no aerial mycelia and an inconspicuous, near translucent, shiny appearance.

The six isolates (three each of the two species, Table 1) selected for further study had significant differences in growth rates at the different temperatures. The optimal growth rate was 30 °C for species 1 ($F = 327.906$, $df = 5$, $P < 0.001$) and species 2 ($F = 1394.727$, $df = 5$, $P < 0.001$). The temperature range supporting growth was similar for both species.

3.3 DNA sequence analyses

Analyses of the ITS data (TL = 717, CI = 0.517, RI = 0.856, 45.7% of characters parsimony informative, TreeBase: TB17782) (Fig. 1) revealed that the isolates obtained from *E. ingens* in the present study formed two groups, representing taxa distinct from all known species in the Ophiostomatales. The first taxon was most closely related to *Ophiostoma bragantinum*, and grouped peripheral to the *S. schenckii–O. stenoceras* complex within *Ophiostoma sensu lato*. The second taxon grouped with a lineage closest to, but clearly distinct from the *Sporothrix lignivora* complex and the genus *Graphilbum*. The two taxa exhibited similar relationships with other ophiostomatalean lineages in the LSU tree (TL = 283, CI = 0.525, RI = 0.832, 24.8% of characters parsimony informative, TreeBase: TB17783) (Fig. 2). The ITS and LSU datasets were supported by high bootstrap values as well as posterior probabilities obtained from Bayesian analysis (ITS model: GTR+G, LSU model: TIM3+I+G, burn-in values for both datasets were 3000). BT sequences are not available for all taxa included in the ITS and LSU trees and so a similar tree comparison could not be made for these gene sequences. However, based on BLAST searches (data not shown) the sequences for both taxa were distinct from all available BT sequences for the Ophiostomatales.

Taxonomy

Analyses of DNA sequences of the ITS, LSU and BT gene regions of isolates obtained in this study confirmed that these isolates represented two novel taxa distinct from previously described species. One of these clearly represents a new genus in the Ophiostomatales, while the other fungus is described as a new species of *Ophiostoma sensu lato*.

Aureovirgo J.A. van der Linde, Z.W. de Beer & Jol. Roux gen. nov.

Mycobank MB813870

Etymology: Genus name refers to the golden appearance of the immature ascomata and the pure white color of the cultures ("Aureovirgo" refers to a golden maiden with an unstated overtone of virginal whiteness).

Type species: ***Aureovirgo volantis*** J.A. van der Linde, Z.W. de Beer & Jol. Roux

Ascomatal bases honey colored (19") when immature to fuscous (13""k) when mature, necks dark, ostiolar hyphae hyaline, ascospores allantoid with ellipsoidal sheaths. Leptographium-like asexual state: Conidiophores mononematous, hyaline, stipe cylindrical and simple. Conidia oblong-elliptical and oval ovate.

Aureovirgo volantis J.A. van der Linde, Z.W. de Beer & Jol. Roux sp. nov. (Fig. 3)

Mycobank MB813872

Etymology: the species name is derived from the Latin word "volanti" for flying. This describes the distribution of the fungus with the insect *C. africus*.

Mycelium on MEA produces pure white radiate to chrysanthemum aerial growth. Sexual state found only in insect galleries, not observed in culture. *Ascomatal bases* honey coloured (19") when immature, to fuscous (13""k) when mature (77.5–) 78.7 – 94.9 (–97.3) x (90.61–) 92.1 – 106.9 (–110.6) µm (86.8 x 99.5 µm, l/w 0.9), *necks* dark brown (164.5–) 202.2 – 345.2 (–359.3) x (16.0–) 15.9 – 21.3 (–23.9) µm (273.7 x 18.6 µm, l/w 14.7), *ostiolar hyphae* hyaline (33.6–) 34.1 – 67.1 (–72.9) x (3.6–) 3.7 – 4.5 (–4.6) µm (50.6 x 4.1 µm, l/w 12.3), *ascospores* allantoid with ellipsoidal sheaths (6.9–) 7.2 – 8.0 (–8.3) x (1.5–) 1.7 – 2.1 (–2.2) µm (7.6 x 1.9 µm, l/w 4.0). *Asexual state* leptographium-like. *Conidiophores* mononematous, hyaline (22.4–) 25.2 – 46.4 (–52.0) x (1.0–) 0.9 – 1.7 (–1.9) µm (35.8 x 1.3 µm, l/w 27.5), *conidia* oblong-elliptical and oval ovate (3.5–) 3.7 – 4.8 (–5.9) x (1.6–) 1.9 – 2.9 (–3.8) µm

(4.3 x 2.4 µm, l/w 1.8). Optimum temperature for growth temperature 30 °C, growing at 9.7 mm/day, with minimum growth at 15 °C and maximum growth at 35 °C.

HOLOTYPE. SOUTH AFRICA, LIMPOPO PROVINCE: Last Post, isolated directly from *Cyrtogenius africus* galleries on diseased *Euphorbia ingens* trees, June 2014, Van der Linde JA, holotype PREM 61236 dry culture on MEA, ex-holotype culture CMW41238 = CBS139648; paratype PREM 61236, ex-paratype culture CMW41250 = CBS139649. Ascomata not observed in culture.

Additional specimens examined: Limpopo Province (Bela-Bela; July 2013, Van der Linde JA; CMW42287), KwaZulu-Natal (Eshowe; March 2013, Van der Linde JA; CMW42282), Mpumalanga (Lydenburg; July 2012, Van der Linde JA; CMW42285), North West (Wolfaan; July 2013, Van der Linde JA; CMW42292 = CBS139645 and Enzelsberg; July 2013 Van der Linde JA; CMW42290) isolated from *C. africus* galleries on diseased *E. ingens* trees.

Ophiostoma thermarum J.A. van der Linde, Z.W. de Beer & Jol. Roux sp. nov. (Fig. 4)

Mycobank MB813873

Etymology: The species name refers to the locality from where this species was collected, Bela-Bela, which is surrounded by geothermal springs ("thermarum" meaning place of warm baths).

Growth on MEA barely visible, shiny white yeast-like growth with no aerial mycelium. Sexual state not observed on plant material or culture. *Asexual state* sporothrix-like. *Conidiophores* (56.0–) 62.3 – 101.3 (–107.4) x (1.00–) 1.1 – 2.1 (–2.4) µm (81.8 x 1.6 µm, l/w 51.1), *conidia* oblong-elliptical and obovate (4.1–) 4.4 – 5.6 (–6.5) x (1.4–) 2.0 – 2.8 (–3.3) µm (5.0 x 2.4 µm, l/w 2.1). Optimum temperature for growth 30 °C, growing at 9.4 mm/day, with minimum growth at 15 °C and maximum growth at 35 °C.

HOLOTYPE. SOUTH AFRICA, LIMPOPO PROVINCE: Bela-Bela, isolated from *Cyrtogenius africus* galleries on diseased *Euphorbia ingens* trees, August 2012, Van der Linde JA, holotype PREM 61238 dry culture on MEA, ex-holotype culture CMW38930 = CBS139747; paratype PREM 61240, ex-paratype culture CMW38940 = CBS140082.

Additional specimens examined: Bela-Bela, isolated from *C. africus* galleries on diseased *E. ingens* trees, August 2012, Van der Linde JA; CMW38929 = CBS140081; CMW38932; CMW38931.

3.4 Pathogenicity

Isolates of *O. thermarum* (CMW38940, CMW38930, CMW38929) and *A. volantis* (CMW41238, CMW41250, CMW42292) produced internal rotting and external lesions at the points of inoculation on healthy *E. ingens* trees. The external lesions at the points of inoculation surrounding the toothpicks were brown in color with dark brown rotted areas leading into the internal succulent tissue (Fig. 5a, b, c). Control inoculations produced no external lesions or internal rotting of the succulent branches (Fig. 5d). Both experiments produced similar results with significant differences in the length of the external tissue lesions, between the isolates extending from the points of inoculation (F test statistic = 13.250, df = 5, $P < 0.001$). There was no significant difference among depths (F test statistic = 1.6907, df = 5, P = 0.17) and widths, between the isolates, (F test statistic = 1.0913, df = 5, P = 0.39) of the internal lesions (Fig. 6). Both species produced lesions that were significantly larger (Values from the Bonferroni procedure were all significant with $P < 0.05$) than those of the controls. The two fungi used in the inoculations were consistently re-isolated and identified based on morphology, while no growth was produced from isolations from the control inoculations.

4. Discussion

Fungi in the Ophiostomatales were identified from dying *E. ingens* trees at all sites investigated in this study. Previously, fungi in the Microascales were identified from dying *E. ingens* trees infested by *Cossonus* species in the Limpopo Province (Roux *et al*. 2009, Van der Linde *et al*. 2011b), but these fungi were not found here. This is the first report of fungi in the Ophiostomatales from dying *E. ingens* trees in South Africa. A species in a novel genus and a new *Ophiostoma* species were identified and described from the fungi isolated from the galleries of the ambrosia beetle *C. africus* infesting dying *E. ingens* trees.

The newly described genus, *Aureovirgo*, can be distinguished from other genera in the Ophiostomatales based on DNA sequence data and distinct morphological features. *Aureovirgo* is phylogenetically most closely related to *Graphilbum* H.P. Upadhyay & W.B. Kendr. and species in the *Sporothrix lignivora* complex. It can, however, be distinguished from those genera based on its conidiophore morphology, as well as its conidia and

ascospores. *Aureovirgo* has a leptographium-like asexual state as opposed to the sporothrix-like state in *S. lignivora* and pesotum-and hyalorhinocladiella-like states in *Graphilbum* (De Beer and Wingfield 2013). The ascospores are the most distinctive feature of *Aureovirgo. Graphilbum* species have rod-shaped ascospores with ossiform sheaths (De Beer and Wingfield 2013), while those of *Aureovirgo* are uniquely allantoid with ellipsoidal sheaths. A single species of *Aureovirgo* was identified in this study and described as *A. volantis* sp. nov.

A new species in the genus *Ophiostoma* Syd. & P. Syd was identified and described as *Ophiostoma thermarum* sp. nov. *Ophiostoma thermarum* grouped peripheral to the *Sporothrix schenckii–Ophiostoma stenoceras* complex (De Beer *et al*. 2003), a group of fungi known to be associated with soil, hardwoods, *Protea* infructescences and mites (De Beer *et al*. 2003; Roets *et al*. 2008). Its closest phylogenetic neighbour is *O. bragantinum* Pfenning & Oberw., described in Brazil from soil samples (Pfenning and Oberwinkler 1993). *Ophiostoma bragantinum* has shorter conidiophores (20 to 40µm) and different conidial morphology (guttuliform or fusiform) compared to *O. thermarum* (Pfenning and Oberwinkler 1993).

Previous studies on dying *Euphorbia* trees in South Africa identified two ophiostomatalean fungi from these trees, namely *Knoxdaviesia serotectus* (J.A. van der Linde, Jol. Roux) Z.W. de Beer & M.J. Wingf. (on *E. ingens*) and *K. ubusi* (J.A. van der Linde, Jol. Roux) Z.W. de Beer & M.J. Wingf. (on *E. tetragona* Haw.) (Van der Linde *et al*. 2011b). These species reside in the Microascales and were associated with rotting branches and a weevil in the genus *Cossonus*. Neither of these species were obtained from *C. africus* tunnels in diseased *E. ingens* trees in the current study. The weevil *Cossonus* infests only succulent parts of diseased *E. ingens* branches which is very different from *C. africus*, an ambrosia beetle that infests the woody xylem of diseased trees. The absence of *Knoxdaviesia* spp. in this study is thus not surprising.

Aureovirgo volantis and *O. thermarum* both produced external lesions and internal rotting in the inoculated *E. ingens* branches in the pathogenicity tests. Previously Van der Linde *et al*. (2011b) inoculated healthy *E. ingens* branches with two *Knoxdaviesia* species, which produced results similar to those found in the present study. Relatively small lesions and areas of internal rot were found associated with fungal inoculations in both studies although the results were variable (possibly due to genetic variation of *E. ingens* in their natural environment). This is in contrast to inoculations with *Lasiodiplodia* spp., also isolated from diseased *E. ingens* and capable of producing larger lesions (Van der Linde *et al*. 2011a). The

results of the present study failed to show that either of the two ophiostomatalean fungi are primary pathogens. However, in the case of mass infestations, they could possibly contribute to tree death.

This study expands the base of knowledge regarding the diversity of ophiostomatalean fungi that occur in diseased *E. ingens* trees. It is now known that this group of fungi occurs on diseased *E. ingens* trees across South Africa (where *E. ingens* populations occur). It was surprising that only two fungal species were obtained from the tunnels of *C. africus*. Interestingly, *A. volantis* and *O. thermarum* were never isolated from the same tunnel. Many ambrosia beetles carry more than one fungal partner and these fungi are often isolated together (Carrillo *et al*. 2014, Kostovcik *et al*. 2015). The association of ophiostomatalean fungi with *C. africus* and *E. ingens* die-offs deserves further study including additional collections from trees and beetles at different times of the year. Mass inoculations may also be used to simulate the effect of fungi on trees after mass attacks of host beetles.

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Table 1. Locality and Genbank accession numbers of representative isolates sequenced and used in phylogenetic analyses.

					Genbank accession no.		
Species	**CMW no.[a]**	**CBS no.[b]**	**PREM no.[c]**	**Locality**	**ITS**	**LSU**	**β-tubulin**
Ophiostoma thermarum	38929[d]	140081	61239	Bela-Bela	KR051114	KR051126	KR51102
O. thermarum	38930[d,e]	139747	61238	Bela-Bela	KR051115	KR051127	KR51103
O. thermarum	38931	–	–	Bela-Bela	KR051116	KR051128	KR51104
O. thermarum	38932	–	–	Bela-Bela	KR051117	KR051129	KR51105
O. thermarum	38940[d]	140082	61240	Bela-Bela	KR051118	KR051130	KR51106
Aureovirgo volantis	41238[d,e]	139648	61235	Last Post	KR051119	KR051131	KR51107
A. volantis	41250[d]	139649	61236	Last Post	KR051120	KR051132	KR51108
A. volantis	42282	–	–	Eshowe	KR051123	KR051133	KR51109
A. volantis	42285	–	–	Lydenburg	KR051121	KR051134	KR51110
A. volantis	42287	–	–	Bela-Bela	KR051124	KR051135	KR51111
A. volantis	42290	–	–	Enzelsberg	KR051122	KR051136	KR51112
A. volantis	42292[d]	139645	61237	Wolfaan	KR051125	KR051137	KR51113

[a] CMW, Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), South Africa
[b] CBS, Centraalbureau voor Schimmelcultures, Netherlands
[c] PREM, South African National Collection of Fungi, South Africa
[d] Isolates used in pathogenicity and growth study
[e] Type strains

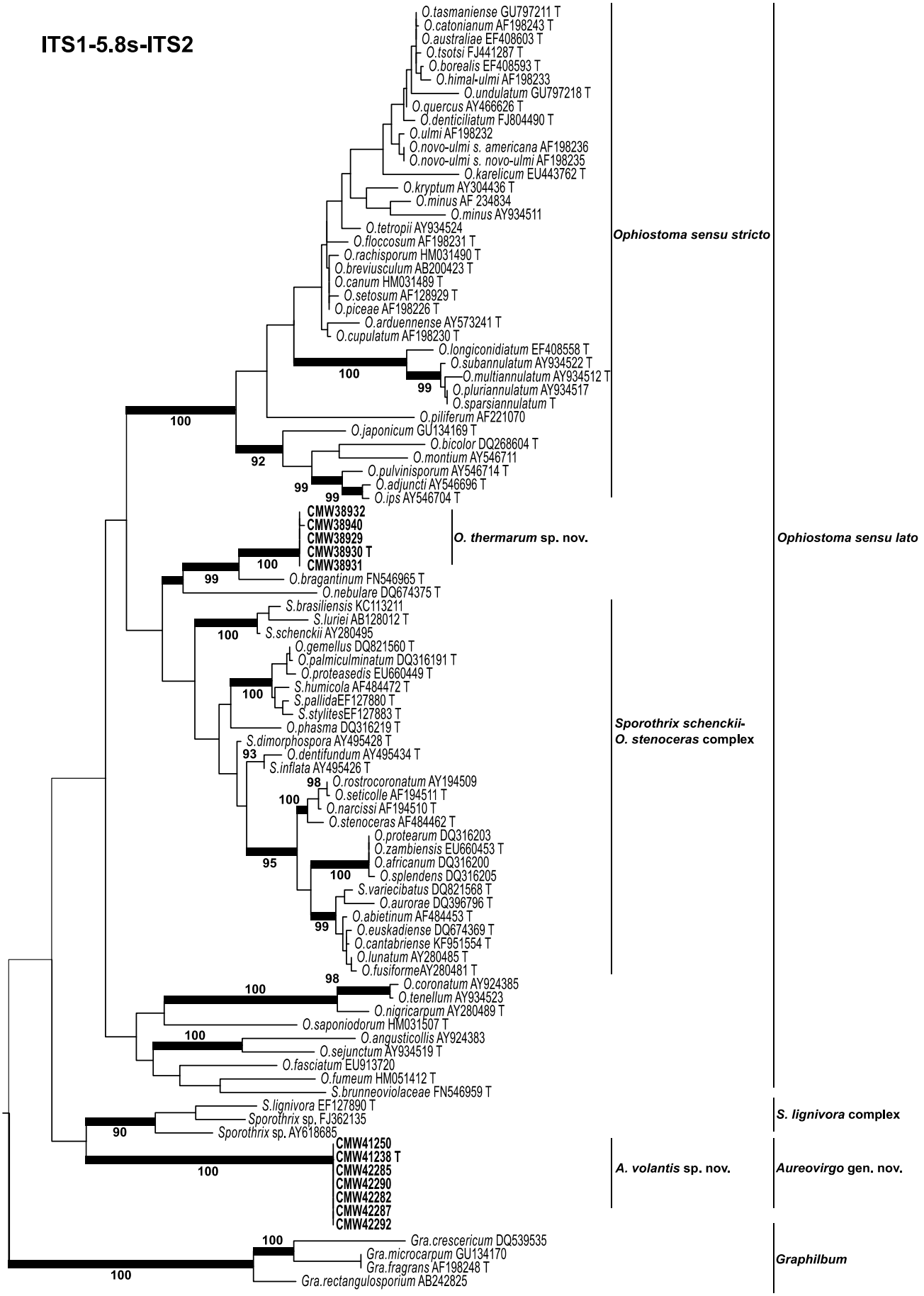

Figure 1: The most parsimonious tree obtained from maximum parsimony analyses of ITS1-5.8s-ITS2 sequence data. Nodes in bold indicate MCMC posterior probabilities values ≥ 0.95 and values at the nodes are maximum parsimony bootstrap values. T indicates ex-type strains.

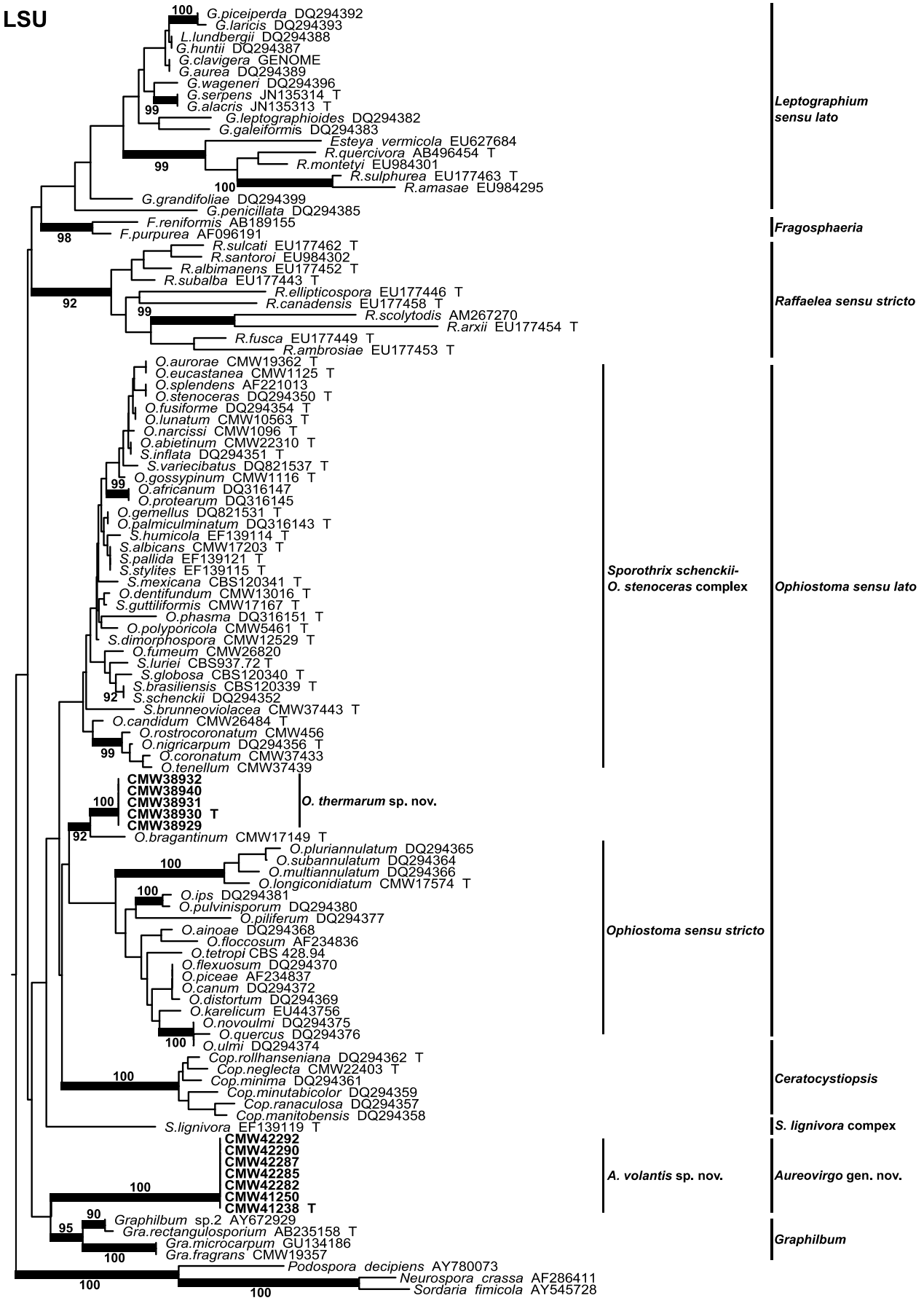

Figure 2: The most parsimonious tree obtained from maximum parsimony analyses of LSU sequence data. Nodes in bold indicate MCMC posterior probabilities values ≥ 0.95 and values at the nodes are maximum parsimony bootstrap values. T indicates ex-type strains.

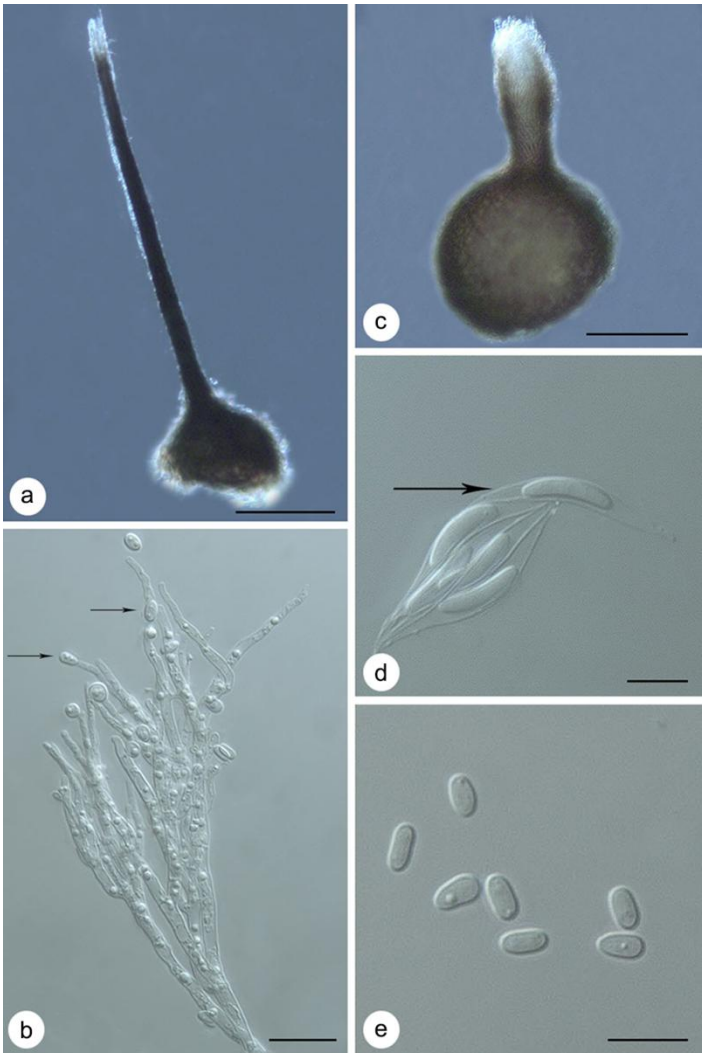

Figure 3: *Aureovirgo volantis*. (a) Mature ascocarp with ostiolar hyphae. (b) Leptographium-like conidiophore with conidiogenous cells. (c) Immature ascocarp with typical honey coloured base. (d) Allantoid ascospores with ellipsoidal sheath. (e) Oblong-elliptical and oval ovate conidia. Scale bars a & c = 100µm, b = 10µm, d & e = 5µm.

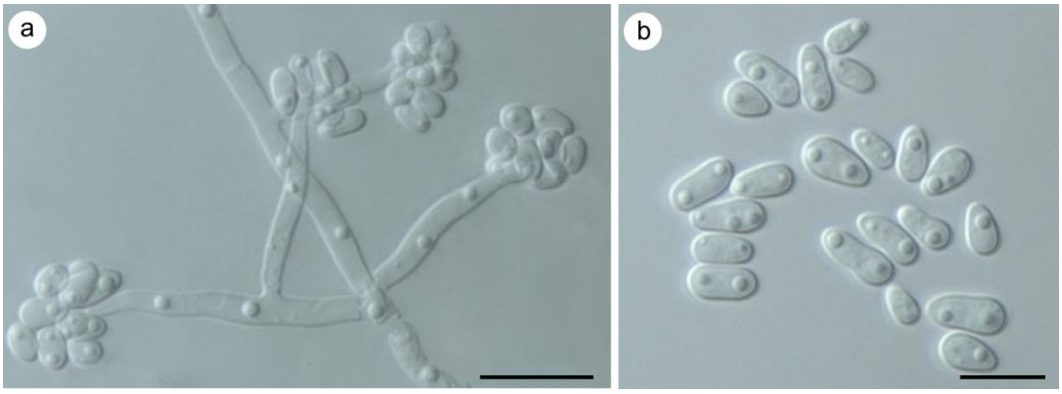

Figure 4: *Ophiostoma thermarum*. (a) Sporothrix-like condiophore with condiogenous cells. (b) Oblong-elliptical and obovate conidia. Scale bars a = 10µm, b = 5µm.

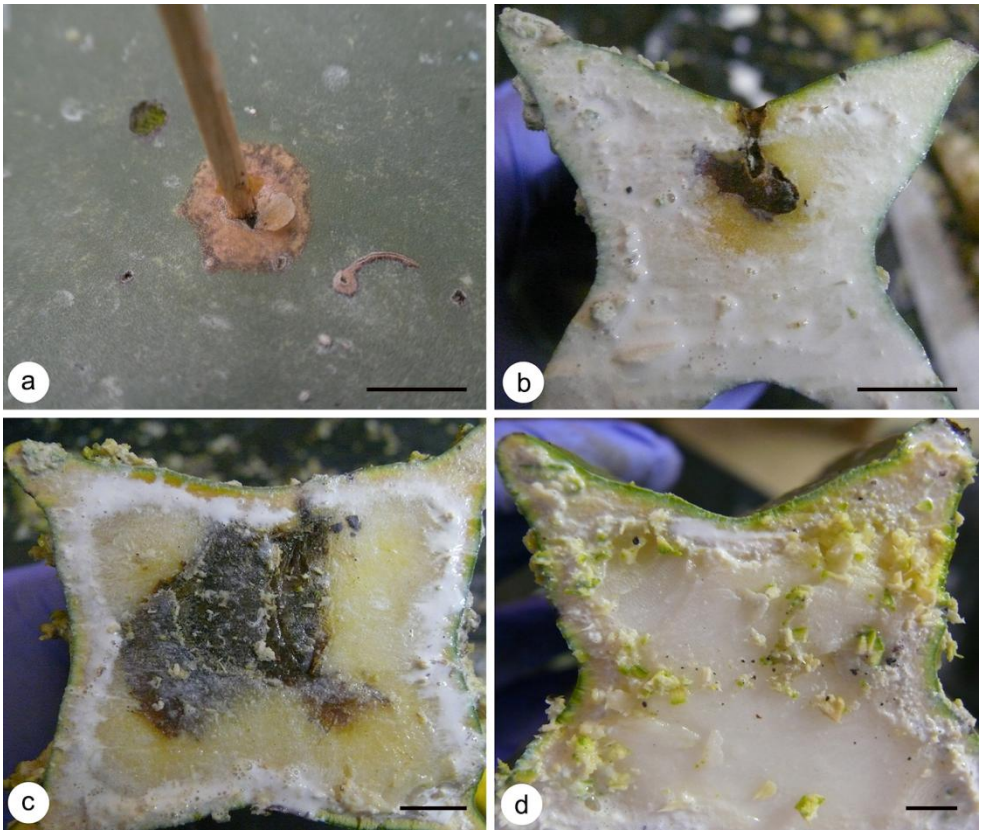

Figure 5: Sections through *Euphorbia ingens* branches inoculated with *Aureovirgo volantis*, *Ophiostoma thermarum* and control inoculation. (a) Lesion produced by *A. volantis* (CMW42292) on the exterior of the succulent branches. (b) Internal rotting of succulent tissue produced by *O. thermarum* (CMW38929). (c) Extensive rotting found in some cases, in this case produced by *A. volantis* (CMW41250). (d) Control inoculation showing no infection. Scale bars a to d = 10mm.

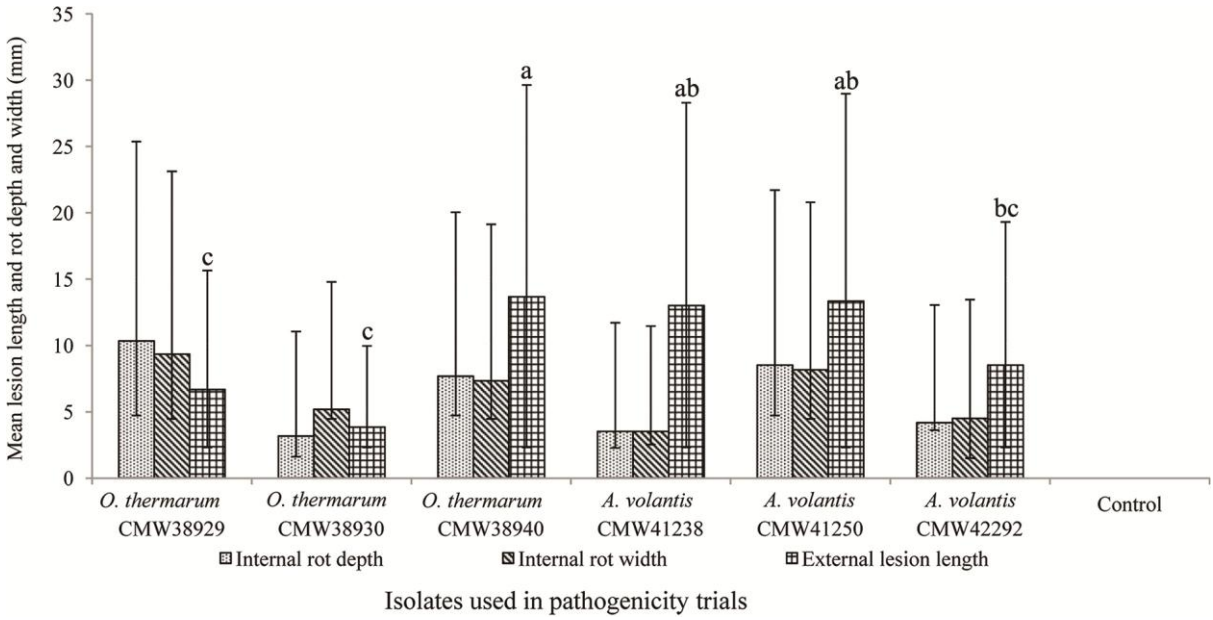

Figure 6: Mean external lesion and internal rotting depth and width measurements obtained in inoculations of *Euphorbia ingens* branches with *Ophiostoma thermarum* (CMW38929, CMW38930, CMW38940) and *Aureovirgo volantis* (CMW41238, CMW41250, CMW42292). Error bars indicate 95% confidence limits for each isolate. Similar letters, for external lesion length, do not indicate significant differences.

Chapter 4

Seasonal flight patterns of Curculionidae (Cossoninae and Scolytinae) infesting dying *Euphorbia ingens* in South Africa

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Abstract

There is limited knowledge regarding the biology of beetles in the Cossoninae and Scolytinae (Curculionidae) in South Africa. It has recently been found that beetles in these two weevil subfamilies are associated with *Euphorbia ingens* die-offs in the country. Lindgren traps baited with 95% ethanol were set at three sites in two provinces of South Africa to gain an understanding of the seasonal activity of the beetles that infest *E. ingens*. Temperature and relative humidity were monitored at each site to correlate environmental conditions with beetle flight patterns. Seven beetle species, of which six were in the Scolytinae and one in the Cossoninae, were captured in the traps over a period of 20 months. *Eccoptopterus spinosus*, *Premnobius cavipennis* and *Xyleborinus spinifer* were the most commonly caught beetles. *Ambrosiodmus natalensis*, *E. spinosus*, *P. cavipennis* and *X. spinifer* are reported from South Africa for the first time. Of the seven beetle species, two, *Cyrtogenius africus* and a *Stenoscelis* sp., are known to colonize diseased and dying *E. ingens* trees, but these were trapped in low numbers, possibly due to the choice of bait used. The number of *C. africus* and *Stenoscelis* sp. caught varied with temperature and humidity, but only temperature had a significant effect on numbers captured. The number of *C. africus* and *Stenoscelis* sp. caught appeared to be a function of site and climatic conditions as opposed to *E. ingens* mortality levels.

Keywords: Ambrosia beetles, *Cyrtogenius africus*, giant *Euphorbia* tree, Lindgren funnel trap, weevils

1. Introduction

The Cossoninae and Scolytinae are subfamilies of the Curculionidae (weevils) (Wood 1973). The Scolytinae are known as bark and ambrosia beetles, infesting woody plants (Bright 1976, Wood 1982, Marvaldi 1997). The incidence of infestation on seemingly healthy trees by Cossoninae and Scolytinae has increased over the last 20 years and has been attributed to their increased introductions into novel environments (primarily due to a rise in global trade) and climate change (Liebhold *et al.* 1995, Pautasso *et al.* 2012, Pawson *et al.* 2013). Well-known examples include the exotic ambrosia beetle *Xyleborus glabratus* Eichhoff, an invasive, and its fungal symbiont *Raffaelea lauricola* Harrington & Fraedrich (Fraedrich *et al.* 2008), which causes laurel wilt disease in native Lauraceae in the United States of America (USA) and the extensive infestation of *Pinus contorta* Douglas (lodgepole pine) in British Columbia by the native bark beetle *Dendroctonus ponderosae* Hopkins which is attributed to chronic warming and drought (Konkin and Hopkins 2009, Bentz *et al.* 2010, Cudmore *et al.* 2010).

Beetles in the Cossoninae and Scolytinae have recently been collected from diseased *Euphorbia ingens* E. Meyer: Boissier in the KwaZulu Natal, Limpopo, Mpumalanga and North West Provinces of South Africa (Roux *et al.* 2009, Van der Linde *et al.* 2011a,b,c; 2016a,b). *Euphorbia ingens* is a succulent tree native to Africa occurring from South Africa to Kenya and Uganda, as well as Botswana, Malawi, Mozambique, Zambia and Zimbabwe (Van Wyk and Van Wyk 1997, Palgrave *et al.* 2002, Schmidt *et al.* 2002, Gildenhuys 2006). During the course of the last fifteen years, large-scale die-offs of *E. ingens* have been observed in South Africa (Malan 2006, Roux *et al.* 2008, 2009). Initial investigations of dying trees in the Limpopo Province reported the presence of *Cyrtogenius africus* Wood and *Euwallacea piceus* Motsch on *E. ingens* (Roux *et al.* 2009). Later studies also identified *C. africus* (Scolytinae), a *Cossonus* sp. (Cossoninae) and a *Stenoscelis* sp. (Cossoninae) from dying *E. ingens* (Van der Linde *et al.* 2011a,b,c; 2016a,b).

Significant differences in climatic conditions (temperature and precipitation) between areas with different levels of *E. ingens* mortality were observed in the Limpopo and North West Province of South Africa (Van der Linde *et al.* 2012). Such differences in environmental conditions could affect beetle activity and the timing and degree of attack on *E. ingens*. However, nothing is known regarding the flight activity of the Cossoninae and Scolytinae in these two provinces. It is also not known whether differences in climatic conditions between

the two provinces affect beetle flight timing and activity. The objectives of this study were, therefore, to monitor the seasonal flight patterns of the Cossoninae and Scolytinae that infest *E. ingens* in areas with different *E. ingens* die-off levels and climatic conditions.

2. Materials and Methods

2.1 Study sites and collection of beetles

Cossoninae and Scolytinae were collected at three sites; two in the Limpopo (Last Post Game Ranch: GPS 23°17'21.39"S 29°55'27.93"E, Capricorn: GPS 23°21'50.67"S 29°44'40.27"E) and one in the North West Province (Enzelsberg: GPS 25°22'58.05"S 26°16'4.21"E) of South Africa. Last Post and Enzelsberg are two privately owned game farms while Capricorn is a privately owned production cattle farm. Sites in the Limpopo and North West Province had different levels of *E. ingens* die-off as well as different climatic conditions (Van der Linde *et al.* 2012). Selection of trapping sites was based on previous studies by Van der Linde *et al.* (2012) who described different levels of *E. ingens* die-off and infestation at the sites. The two sites in the Limpopo had the most significant climatic changes (increased temperature and variable rainfall patterns) over the last 50 years (Van der Linde *et al.* 2012), with Enzelsberg being the most severely affected site followed by Last Post and Capricorn (Van der Linde *et al.* 2016a).

The traps used in this study were eleven-unit black Lindgren funnel traps (Lindgren 1983), typically used for Scolytinae, suspended at a height of 1.5m above ground level from a metal frame. Traps were baited with 95% ethanol (Reding *et al.* 2010) using a 300ml squeeze bottle containing 285ml 100% ethanol and 15ml distilled water with a 6.0mm rope wick. Ethanol was used as a lure since stressed trees are known to release this volatile organic compound which is used as a cue by many ambrosia beetles to locate suitable hosts (Kimmerer and Kozlowski 1982, Ranger *et al.* 2010). The wick was suspended into the liquid in the bottle, secured with a cable tie at the top with 5cm of one end exposed to the air. Each lure was attached, halfway along the length of the Lindgren trap using cable ties. Ethanol was topped up biweekly during visits to collect beetles from traps. Ethylene glycol (Wynn's antifreeze/coolant, Wynn's Oil Company, Irwindale, California) was placed in the collection cups, which was also refilled on collection days, with the collected beetles placed into 70% ethanol and transported to the laboratory for identification and quantification. Three traps were placed along a linear transect at each site within *E. ingens* stands, with a minimum of 50m between each of the traps. The traps were placed in the field at the beginning of

September 2013 (early spring) and removed at the end of April 2015 (mid-autumn). Beetles were divided into morphological groups (morphogroups) based on distinguishable characters (antennae, shape of the eye, elytral declivity, scutellum and protibia) and then identified to species by Dr. Roger Beaver (Thailand).

2.2 Temperature and relative humidity

Data loggers (Maxim iButton DS1923, Fairbridge Technologies, Johannesburg, South Africa) were suspended from one trap at each site to measure temperature and relative humidity every hour from 1 September 2013 through 30 April 2015. The data were downloaded with ColdChain ThermoDynamics software version 4.9 (Fairbridge Technologies) and exported to Microsoft Excel for data analyses.

2.3 Statistical Analyses

The mean monthly numbers captured for each beetle morphogroup was compared among each of the sites investigated. Normality of the data was tested using Shapiro-Wilk's W. Data that failed to meet the assumptions of normality were transformed (ln+1). If normality was not achieved after transformation, the data were analyzed with Kruskal-Wallis one-way ANOVA (analysis of variance). ANOVA was applied to raw data that met the assumptions of normality and t-tests were used when only two beetle morphogroups were compared. The mean temperature and relative humidity for each of the 20 months during which collections were conducted were compared among the three sites using ANOVA. Significant F and H tests were subjected to mean separation tests using Tukey-Kramer's HSD. In all tests, α was set at ≤ 0.05. Linear regression analyses were conducted to test whether the number of beetles caught was correlated with humidity and/or temperature. All statistical analyses were run using JMP Version 12.0.1 (SAS Institute Inc., Cary, North Carolina).

3. Results

3.1 Study sites and collection of beetles

Beetles caught at the three sites (Capricorn, Enzelsberg, Last Post) could be divided into seven morphogroups. Six were Scolytinae and one in Cossoninae. The Scolytinae were identified as *Ambrosiodmus natalensis* Schedl, *C. africus*, *Eccoptopterus spinosus* Olivier, *Premnobius cavipennis* Eichhoff, *Xyleborinus spinifer* Eggers and *Xyloctonus latus* Eggers and the one Cossoninae as *Stenoscelis* sp. (Table 1). Two of the beetle species, *C. africus* and a *Stenoscelis* sp., are known to infest diseased *E. ingens* trees (Roux *et al.* 2009; Van der Linde *et al.* 2011a,b; 2016a,b).

There was a significant difference in the total number of beetle species caught among the sites (H = 84.42, df = 6, P < 0.001). The greatest number of total beetles caught was at Last Post (Table 1). The most commonly trapped beetle was *E. spinosus* (Table 1) although it was collected only at sites in the Limpopo Province and not at Enzelsberg in the North West. Very low numbers of *A. natalensis*, *C. africus*, *Stenoscelis* sp. and *X. latus* (*X. latus* was not caught at Capricorn) were collected. The most commonly caught beetle at Enzelsberg and Capricorn was *P. cavipennis*, while at Last Post, *E. spinosus* was the most commonly collected (Table 1). Except for *X. latus* there was a significant difference in numbers of *A. natalensis*, *C. africus*, *E. spinosus*, *P. cavipennis*, *X. spinifer* and *X. latus* caught among the sites (Table 1).

Cyrtogenius africus was caught in the highest numbers at the two sites in the Limpopo Province while *Stenoscelis* sp. was caught in highest numbers at Enzelsberg (Table 1). There was a significant difference in the number of *C. africus* beetles caught among sites (F = 7.48, df = 2, P = 0.01). Mean separation analysis revealed no significant difference in the number of *C. africus* caught between the two sites in Limpopo (P = 0.89) while there was a significant difference in the number of *C. africus* caught at Enzelsberg and Last Post (P = 0.01) and Enzelsberg and Capricorn (P = 0.01). Similarly, there was a significant difference in the number of *Stenoscelis* sp. caught among all sites (F = 5.04, df = 2, P = 0.01). Mean separation analysis revealed no significant difference in the number of *Stenoscelis* sp. caught between the two sites in the Limpopo (P = 0.94) while there was a significant difference in numbers of *Stenoscelis* sp. caught between Enzelsberg and Last Post (P = 0.01) as well as between Enzelsberg and Capricorn (P = 0.03).

Seasonal flight patterns varied among the beetle species caught and among sites for certain species. *Ambrosiodmus natalensis* was caught much earlier in the year at Last Post (October to April peaking in November; mid-spring to mid-autumn) compared to Capricorn and Enzelsberg (January to April peaking in February; mid-summer to mid-autumn). *Eccoptopterus spinosus* was caught during the same time of the year at Capricorn and Last Post (July to January peaking in November; mid-winter to mid-summer) with *X. spinifer* caught from July to February, peaking in November, (mid-winter to end-summer) at all three sites. *Premnobius cavipennis* was caught throughout the year at all three sites with peak flight from February to June (end-summer to start-winter peaking in March) at Capricorn, July to September at Enzelsberg (mid-winter to start-spring peaking in September) and July to

November (mid-winter to start-summer peaking in September) at Last Post. *Xyloctonus latus* was caught from November to January (start-summer to mid-summer) at all three sites.

The seasonal flight pattern of *C. africus* was very similar in Capricorn and Last Post (early summer to mid-autumn), with the beetles flying earlier in the year at Last Post. The flight pattern of *Stenoscelis* sp. in the North West was much earlier in the year (early spring to end of summer), compared to *C. africus* in the Limpopo Province (Fig. 1, only *C. africus* and *Stenoscelis* sp. shown as they are known to infest *E. ingens*). *Cyrtogenius africus* was mostly caught from November to April (peaking in March), while *Stenoscelis* sp. was mostly caught from September to February (peaking in December).

3.2 Temperature and relative humidity factors

There was a significant difference in the monthly mean temperatures (Sept 2013 to April 2015; compared among the three sites over the 20 month period) at the Limpopo and North West sites ($F = 3.32$, $df = 2$, $P = 0.04$), with Capricorn being significantly different from Enzelsberg. Monthly relative humidity compared over the 20 months, for which data were collected (Sept 2013 to April 2015), was significantly different between the sites in the Limpopo and North West Province ($F = 15.98$, $df = 2$, $P < 0.001$). The number of beetles caught fluctuated over the 20 months investigated. However, only the effect of temperature was significant ($R^2 = 0.17$, $P = 0.001$). The effect of humidity was not significant ($R^2 = 0.01$, $P = 0.07$). The peak flight times for most of the beetle species coincided with the time of the year with the highest mean temperature (Fig. 2, 3 & 4; only *C. africus* and *Stenoscelis* shown as they infest *E. ingens*).

4. Discussion

Seven beetle species were trapped in stands of *E. ingens* trees in this study. Of these, only two, *C. africus* and a *Stenoscelis* sp., have previously been collected from the stems and branches of dying *E. ingens* (Van der Linde *et al.* 2011a,c; 2016a,b). Of the other beetles, one, *E. spinosus*, represents a new report for the African continent, while the remaining four are new reports for South Africa.

Cyrtogenius africus and *Stenoscelis* species are known to infest stressed trees (Konishi 1956, Jordal 2009). Beetles in the genus *Stenoscelis* are known to infest trees that are nearly dead, or initially infested with other bark beetles, with collections recorded from Algeria, Kenya and the Western Cape of South Africa (Konishi 1956). *Cyrtogenius africus* was first recorded

in 1988 from various *Euphorbia* species in Africa (Democratic Republic of Congo [formally known as Zaire], Guinea, Kenya, Tanzania and Uganda) and again in 2009 from dead branches of *Euphorbia triangularis* Desf. in the Western Cape (Wood and Bright 1992, Jordal 2009). In studies of dying *E. ingens* trees, these beetles were obtained from branches of severely diseased trees (Van der Linde *et al.* 2011a,c; 2016a,b).

Van der Linde *et al.* (2016a) reported different levels of *E. ingens* mortality for the sites used in this study. In that study, Enzelsberg had the highest percentage mortality (32.5%) followed by Last Post (20.9%) and Capricorn (2.5%) (Van der Linde *et al.* 2016a). In the current study, there was no significant difference in the number of *C. africus* and *Stenoscelis* sp. (species that infest *E. ingens*), between the two sites in the Limpopo Province (Last Post and Capricorn) despite differences in *E. ingens* mortality. Furthermore, there was a significant difference in the numbers of *C. africus* and *Stenoscelis* sp. caught at Last Post and Enzelsberg with no significant difference in *E. ingens* mortality between the two sites (Van der Linde *et al.* 2016a). A significantly greater number of *C. africus* were caught at Capricorn compared to Enzelsberg although Capricorn had significantly lower *E. ingens* mortality.

The seasonal flight patterns and the effect of temperature and humidity on flight activity of Scolytinae and Cossoninae had not been previously investigated in relation to *E. ingens* mortality. In this study, there was a significant difference in the number of *C. africus* and *Stenoscelis* sp. captured between the two provinces that had significant differences in monthly temperature and humidity. From our results it appears that flight activity and numbers of beetles at a site may be more related to weather and climatic conditions than levels of *E. ingens* mortality. Effects of temperature on insect flight activity is well known (Tanaka *et al.* 1987, Dowdy 1994, Fadamiro and Wyatt 1995, Stock *et al.* 2014) however, the lack of an effect by host tree mortality levels on insect activity is surprising. *Euphorbia ingens* trees rot and decompose rapidly after they have died off (due to their high moisture content) becoming an unsuitable host for the weevils to infest. The weevils (*C. africus* and *Stenoscelis* sp.) infest highly stressed *E. ingens* trees at the final stages of mortality (Van der Linde *et al.* 2016a,b), and so the number of trees actually producing beetles at any one time may be similar among sites since trees that have already died are not suitable for beetle production.

Although the majority of beetles trapped in this study have not been found to infest diseased or dying *E. ingens* trees, their discovery is significant because they all represent new reports

for South Africa. The ambrosia beetle, *E. spinosus,* is known to infest small stems and branches of coffee, cocoa and mango, but it is not known as a primary pest, occurring throughout the Pacific islands and most of the oriental and tropical regions (Beaver 1987, 1988, 2005, Hulcr and Cognato 2010). *Premnobius cavipennis* is a polyphagous beetle native to Africa (Wood 1977, Zanuncio *et al.* 2005, Rabaglia *et al.* 2006). This beetle has been introduced into South and North America and is known to damage stressed *Eucalyptus* L'Hér trees in Brazil (Flechtmann *et al.* 2001, Zanuncio *et al.* 2005). There is very limited information available for the other beetle species collected, although all of the genera (*Ambrosiodmus* Hopkins, *Cyrtogenius* Strohmeyer, *Stenoscelis* Wollaston, *Xyleborinus* Reitter, *Xyloctonus* Eichhoff) have previously been recorded in Africa (Konishi 1956, Browne 1963, Wood 1982, Wood and Bright 1992, Rabaglia *et al.* 2006, Faccoli *et al.* 2009). This study reports the first records of *A. natalensis*, *E. spinosus*, *P. cavipennis*, *X. latus* and *X. spinifer* in South Africa. Most of these beetles are known to occur on a wide variety of hosts and they could potentially represent a threat to native and commercial trees in South Africa.

The number of *C. africus* and *Stenoscelis* sp. caught in this study was low compared to most of the other beetle species caught. Since *C. africus* and *Stenoscelis* sp. are known to infest *E. ingens*, a more specific bait for these species may need to be developed. Ambrosia beetles are commonly attracted to alcohols and volatiles released from the wood of dying and recently dead trees (Ranger *et al.* 2010). Stressed plants also release a variety of volatiles and some ambrosia beetles are attracted to specific host plant compounds (e.g. *Xyleborus glabratus* Eichhoff) as opposed to ethanol which is often released by decomposing plant material (Hanula and Sullivan 2008, Harrington *et al.* 2011). Studies of the volatiles released by stressed *E. ingens*, and the response of beetles to these substances, could lead to the development of more appropriate lures for these insects. This, in turn, would allow for more specific trapping and a more comprehensive knowledge of the beetles that infest and contribute to the death of *E. ingens* trees in South Africa.

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Table 1. Mean (SE) number of beetles and total number of beetle, caught (Sept 2013 to April 2015) at three sites in South Africa, and ANOVA and t-test statistics for comparisons among sites

Site/Total beetles	**Total beetles caught**	***Ambrosiodmus natalensis***	***Cyrtogenius africus*[1]**	***Eccoptopterus spinosus***	***Premnobius cavipennis***	***Stenoscelis* sp.[1]**	***Xyleborinus spinifer***	***Xyloctonus latus***
Capricorn	2597	1.75 (1.02)[a]	2.50 (1.19)[a]	46.90 (15.60)	60.4 (9.00)[ab]	0.45 (0.23)[b]	17.9 (5.86)[ab]	─
Enzelsberg	562	1.20 (1.01)[a]	0.25 (0.55)[b]	─	21.5 (4.91)[b]	3.10 (1.24)[a]	0.50 (0.22)[b]	0.10 (0.10)
Last Post	9173	10.3 (4.80)[a]	2.90 (4.52)[a]	309.8 (127.1)	77.60 (25.60)[a]	0.10 (0.07)[b]	57.8 (20.4)[a]	0.30 (0.21)
Total beetles		294	113	7134	3187	73.00	1523	8
ANOVA Statistics		**F = 3.08, df = 2, P = 0.05**	**F = 7.48, df = 2, P = 0.01**	**t = 2.05, df = 1, P = 0.05**	**F = 3.26, df = 2, P = 0.05**	**F = 5.04, df = 2, P = 0.01**	**F = 5.71, df = 2, P = 0.01**	**t = 0.87, df = 1, P = 0.39**

[1]Beetle species known to infest *E. ingens*

Same letters within a column indicate no significant difference

─ Not collected

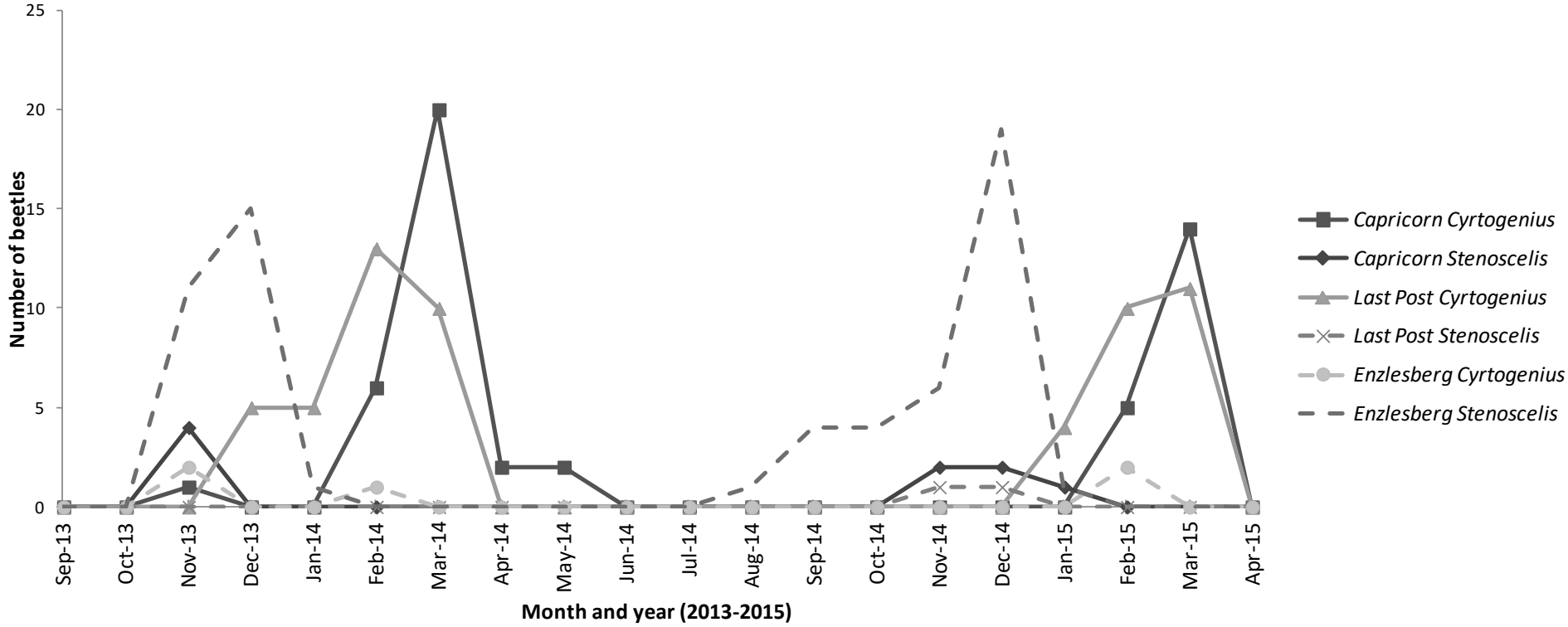

Figure 1: Seasonal flight pattern of *Cyrtogenius africus* and *Stenoscelis* sp. caught at Capricorn, Enzelsberg and Last Post from September 2013 to April 2015.

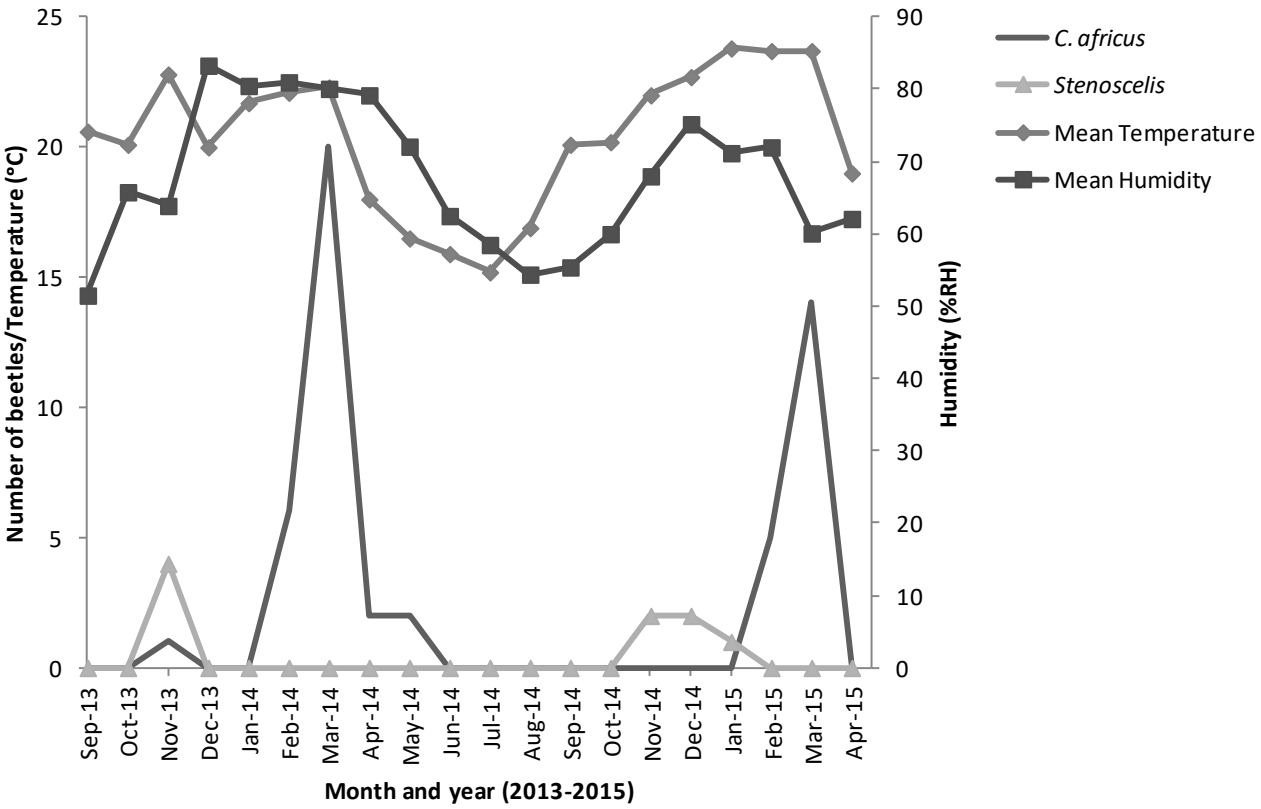

Figure 2: Mean temperature and mean relative humidity associated with *Cyrtogenius africus* and *Stenoscelis* sp. trap captures at Capricorn from September 2013 to April 2015.

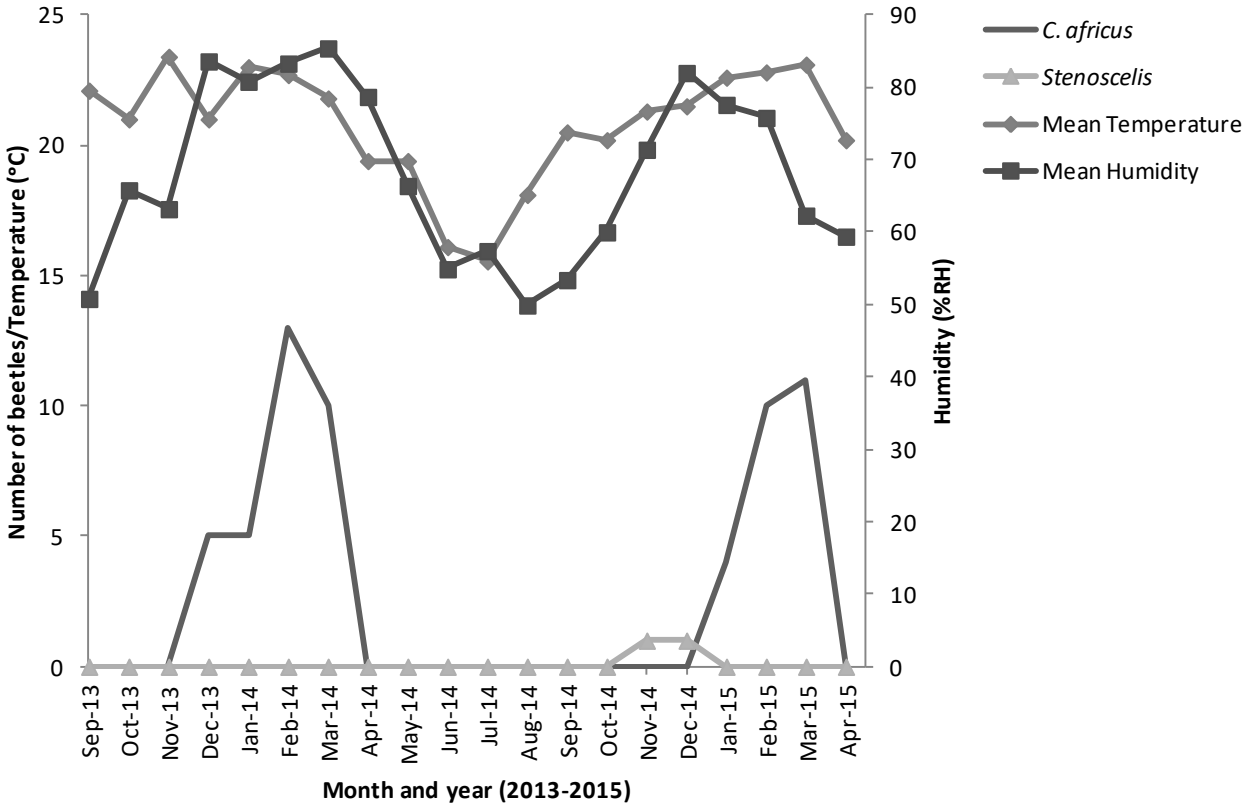

Figure 3: Mean temperature and mean relative humidity associated with *Cyrtogenius africus* and *Stenoscelis* sp. captures at Last Post from September 2013 to April 2015.

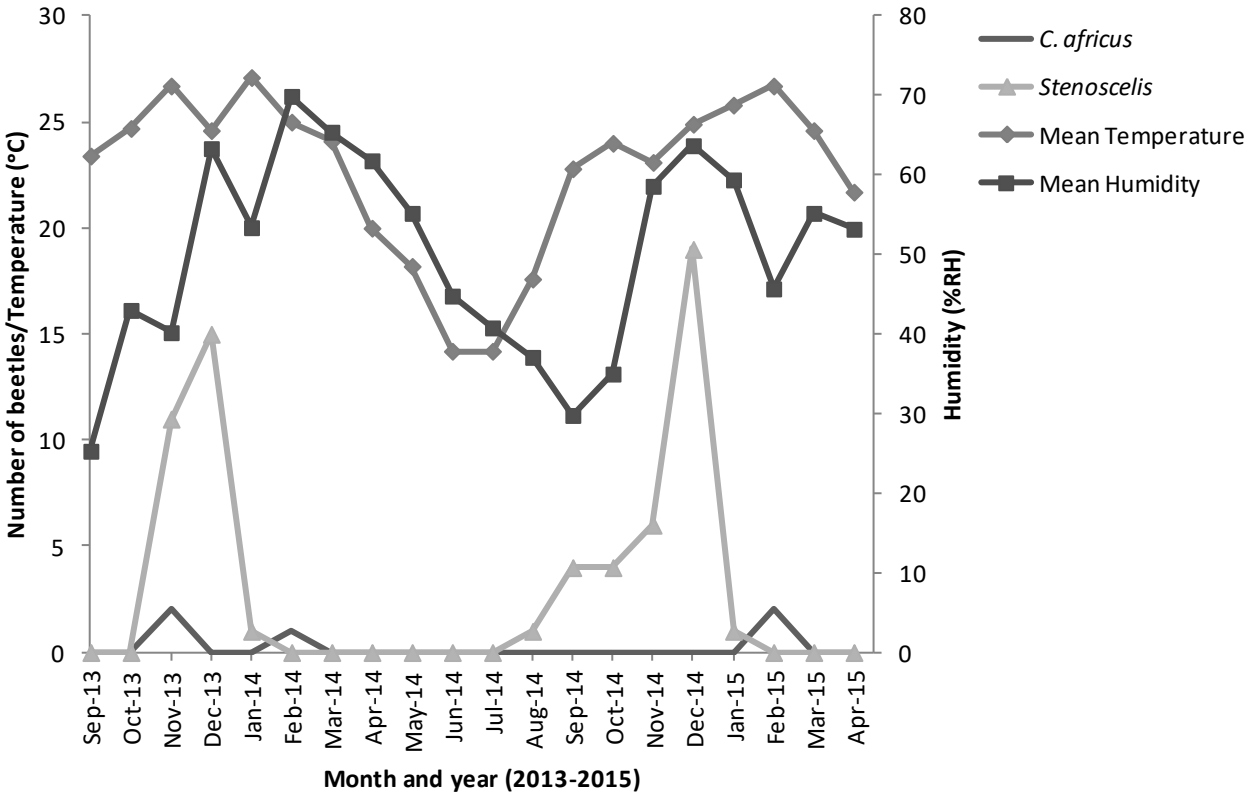

Figure 4: Mean temperature and mean relative humidity associated with *Cyrtogenius africus* and *Stenoscelis* sp. captures at Enzelsberg from September 2013 to April 2015.

Chapter 5

Landscape degradation may contribute to large-scale die-offs of *Euphorbia ingens* in South Africa

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Abstract

Euphorbia ingens, a large succulent tree species native to southern African savanna ecosystems, have died in large numbers in recent years in some areas of South Africa. A previous study found that changes in climate (higher temperatures and lower or more variable rainfall) likely play an important role in causing mortality. However, anecdotal evidence suggests that stress due to habitat degradation may also contribute to *E. ingens* die-offs. In this study, we evaluated *E. ingens* die-offs in South Africa at 10 sites. Specifically, we aimed to examine the roles of both climate and landscape degradation in causing the die-offs. We used a combination of climate data, estimates of tree mortality and ratings of die-off symptoms (categories of grey discoloration and rotting associated with moth attacks), and proxies for landscape degradation associated with livestock grazing. We assessed which sites exhibited greater mortality and die-off associated symptoms, and whether they exhibited spatial auto-correlation (did distance between sites correlate with severity of *E. ingens* die-off). We also used correlation analysis to compare tree mortality to proxies of savanna ecosystem degradation. These proxies were dung counts (livestock), woody debris counts, plant and bare soil cover, soil nutrients, and density of *Dichrostachys cinerea* (Fabaceae), a savanna plant that dominates when disturbance is high. Minimum and maximum temperatures as well as precipitation were compared among sites. There was no spatial autocorrelation between distance and die-off severity among sites, and sites with greater levels of tree mortality were associated with proxies indicating degradation. This suggests that die-offs of *E. ingens* are likely due to a complex of stressors, including both changes in climate and poor land-use practices. Our results indicate that sustainable rangeland practising of South African savannas may aid in conserving *E. ingens* and retaining this iconic tree on the landscape.

Key words: Anthropogenic disturbance, biodiversity conservation, climate change, habitat degradation, savanna

1. Introduction

Euphorbia ingens E. Meyer: Boissier is native to southern Africa where it is found primarily in savanna ecosystems (Van Wyk and Van Wyk 1997, Palgrave *et al.* 2002). It is a large succulent tree with a branching crown and a main woody stem supported by a shallow, widely-spread root system (Van Wyk and Van Wyk 1997, Palgrave *et al.* 2002, Gildenhuys 2006). Several animals rely on this tree for moisture and nutrients (most notably nitrogen), especially during periods of drought (Brown *et al.* 2003, Dudley 1997, Heilmann *et al.* 2006). Culturally, the tree is important to local human communities that use the chemically complex latex it produces to stun or kill fish allowing easy capture and to produce an array of traditional medicines (Dudley 1997, Brown *et al.* 2003, Gildenhuys 2006, Heilmann *et al.* 2006). The tree is also one of the most iconic examples of convergent evolution in the world (Bennici 2002, Horn *et al.* 2012).

Unfortunately, rapid localized die-offs of *E. ingens* are increasingly reported. The first reports of high levels of mortality of the tree were from the Limpopo Province of South Africa (Malan 2006, Roux *et al.* 2008, 2009). The main symptoms exhibited by dying trees were a grey discoloration of the succulent branches and the rotting of branches associated with feeding by the larvae of a moth in the genus *Megasis* Guenée (Lepidoptera: Pyralidae) (Malan 2006, Roux *et al.* 2008, 2009, Van der Linde *et al.* 2011a). Subsequent studies revealed that various beetles and fungi were associated with diseased and dying *E. ingens*, but none were clear primary causal agents of mortality (Van der Linde *et al.* 2011b,c; 2016).

Van der Linde *et al.* (2012) found evidence that changes in climatic conditions (higher temperatures and lower or more variable rainfall) were involved in *E. ingens* die-offs. Changes in local climate could result in stress to *E. ingens*, allowing insects and pathogens, that are otherwise relatively benign, to contribute to tree mortality. However, anecdotal observations also suggested that die-offs may be triggered by locally-induced stressors such as poor land-use practices, including intensive grazing. However, the hypothesis that land degradation plays a role had not been investigated.

The savanna ecosystem in which *E. ingens* occurs covers approximately 35% of South Africa (Scholes 1997, Scholes and Archer 1997). Savanna ecosystems are maintained by interactions among fire, herbivory and precipitation (Backéus 1992, Scholes 1997, Scholes and Archer 1997, Van Langevelde *et al.* 2003, Archibald *et al.* 2005, Van Wilgen 2009). Two types of savanna systems occur in South Africa; mesic savanna and xeric savanna which are

differentiated by mean annual precipitation (Scholes 1997). Mesic savanna landscapes generally have a higher woody component compared to xeric savannas due to higher rainfall (Scholes 1997, Scholes and Archer 1997). Xeric savannas are drier with a lower woody plant to grass ratio (Scholes 1997). Historic fire regimes maintain both grasses and trees in savannas (Bond *et al.* 2003). A lack of fire or reduction in mean fire intervals, due to fire suppression, can lead to increased woody vegetation and a loss of grass cover especially in mesic savanna (Bond *et al.* 2003, Sankaran *et al*. 2005, Van Wilgen *et al.* 2008, Van Wilgen 2009; Parr *et al.* 2012). In contrast, higher mean fire return intervals due to human-caused fires lead to reduced woody and grass cover in mesic and xeric savannas respectively (Bond *et al.* 2003, Van Wilgen 2009, Parr *et al.* 2012). Herbivory also plays an important role in maintaining the ratio of grass to woody plants typical of savannas (Scholes and Archer 1997, Van Langevelde *et al.* 2003, Wakeling and Bond 2007). In particular, grazers are most important in xeric savannas while browsers are more important in mesic savannas (Scholes and Archer 1997, Van Langevelde *et al.* 2003, Wakeling and Bond 2007).

Apart from the direct effect on grass to tree ratios, herbivores indirectly affect savanna ecosystems by their physical activities. In the context of human land use effects, livestock can have major negative effects on plant communities through high levels of grazing, trampling, and compaction (Scholes and Archer 1997, Van Langevelde *et al.* 2003). Overgrazing, especially in environments with clay soils, can lead to soil degradation, soil compaction (reducing water infiltration and thus water availability), higher surface run-off (that can wash away important water soluble nutrients needed by plants, e.g. nitrate) and crust formation. All of these can reduce the ability of grasses and trees to grow (Kelly and Walker 1976, Rietkerk *et al.* 1997; 2000, Van Langevelde *et al*. 2003, Savadogo *et al.* 2007).

Savanna ecosystems in South Africa are under increasing pressure to support livestock production (Scholes 1997, Wakeling and Bond 2007, Van Wilgen 2009). Overstocking is common and fire suppression is often practiced to protect the animals (Van Langevelde *et al.* 2003, Van Wilgen 2009). Overgrazing and fire suppression in savanna ecosystems not only leads to a loss of grass cover and erosion (Scholes 1997, Bond *et al.* 2003, Van Langevelde *et al.* 2003), but also encroachment by woody pioneer species (Roques *et al.* 2001, Wakeling and Bond 2007).

In South Africa, *Dichrostachys cinerea* Wight and Arn., a native woody plant, is a common encroacher in response to overgrazing (Hoffman *et al.* 1999, Roques *et al.* 2001, Wakeling

and Bond 2007, Orwa *et al.* 2009). Herbivores feed on the seed capsules of the plant and play a major role in its dispersal, while locally the plant can spread as a clone through lateral roots (Hoffman *et al*. 1999; Wakeling and Bond 2007). This allows *D. cinerea* to establish very quickly in a poorly managed system such as one with high levels of grazing, a high percentage bare soil and reduced fires. Once established, it becomes very difficult to control (Wakeling and Bond 2007).

The overall objective of this study was to elucidate the factors leading to the massive, rapid die-offs of *E. ingens* in South Africa. We revisited sites previously studied by Van der Linde *et al.* (2012) and included a number of new sites to increase sampling frequency and geographic distribution. The specific objectives were to 1) re-examine the role that climate plays in current patterns of *E. ingens* die-off, and 2) investigate whether tree mortality could also be associated with factors related to landscape degradation.

2. Materials and Methods

2.1 Study sites

Study sites included five previously sampled by Van der Linde *et al.* (2012) in 2010, as well as five new sites. Of the previously sampled sites, three were located in the Limpopo Province [Euphorbia Drive (Coordinates: 24°10'14.02"S 29°3'4.86"E, Elevation: 1180m), Last Post (23°17'21.39"S 29°55'27.93"E, 940m) and Capricorn (23°21'50.67"S 29°44'40.27"E, 1110m)], and two in the North West Province [Enzelsberg (25°22'58.05"S 26°16'4.21"E, 1170m) and Wolfaan (25°42'59.27"S 27°42'9.24"E, 1236m)] of South Africa. Of the new sites, two were located in the province of KwaZulu Natal [Eshowe (28°48'42.64"S 31°30'30.10"E, 450m) and Ulundi (28°26'8.47"S 31°18'25.70"E, 735m)], two in Limpopo [Bela-Bela (24°51'48.30"S 28°20'5.90"E, 1200m) and Modimolle (24°44'53.75"S 28°21'55.43"E, 1216m)] and one in Mpumalanga [Lydenburg (24°55'53.87"S 30°19'7.09"E, 1155m)] (Fig. 1). The sites were chosen from accessible *E. ingens* populations, where we had permission to conduct field studies, from each province in South Africa where this tree occurs.

2.2 Assessment of *E. ingens* mortality, degree of die-off, and the relationship of mortality and symptoms to climate and landscape variables

At each site, eight 100m x 50m transects were established. Measurements were conducted in November 2014, coinciding with the timeframes used for previous sampling in 2010 and 2012. Within each transect, symptoms associated with die-off (grey discoloration and rotting

associated with *Megasis* sp., hereafter referred to as moth damage) were scored for each living tree within each transect (mature and juveniles). Dead trees were also counted and percentage mortality was calculated relative to total trees in each transect. Grey discoloration and moth damage were scored, independently of one another, based on a ranking system of zero to four [1: (1-25% succulent branches grey discoloured and rotten from moth damage), 2: (26-50%), 3: (51-75%), 4: (76-100 %)]. Grey discoloration and moth damage have different patterns of disease progress on *E. ingens* trees, hence they were scored using different systems. Grey discoloration starts at the bottom end of the tree just above the trunk and gradually moves upwards to the crown while moth damage generally affects the succulent branches more or less randomly (Fig. 2 and 3). Not all sites were monitored for the same period of time, therefore, percentage mortality and estimations of disease severity were compared using data from a four-year period for Enzelsberg, Wolfaan, Euphorbia Drive, Capricorn and Last Post (2010-2014) and a two-year period for Bela-Bela, Modimolle, Lydenburg, Ulundi and Eshowe (2012-2014).

To score environmental variables (proxies) associated with savanna degradation, a linear 100m belt transect was established within each of the 100m x 50m transects at each site. Quadrants (1x1m) were located every 2m within each transect (50 quadrants x 8 transects per site = 400 quadrants per site). Within each quadrant, the percentage area covered by living plants and bare soil was estimated. Coverage of dung and dead wood within each quadrant was estimated using a ranking system of low (wood or dung clumps did not occur or only occurred in one quarter of the quadrant), medium (wood or dung clumps occurred in half of the quadrant area) and high (wood or dung clumps occurred in three quarters of the quadrant area). This classification was used rather than percentage cover because these variables often consisted of scattered faecal pellets, which made a discrete estimate of percentage cover difficult.

Dichrostachys cinerea encroachment within each transect at each site was scored based on a binary rank (low or high). *Dichrostachys cinerea* was scored around each *E. ingens* tree within a transect, with low being less than 50% of around each *E. ingens* tree surrounded by *D. cinerea* and high being more than 50% of the *E. ingens* tree encroached by *D. cinerea*. The area scored around each *E. ingens* tree for *D. cinerea* encroachment was determined using the crown size of the *E. ingens* tree in question. This binary system used to score *D. cinerea* was deemed more appropriate than using the quadrant data since *D. cinerea* trees

typically occurred around *E. ingens* individuals and in most cases there were either very dense stands of *D. cinerea*, or hardly any plants present.

For soil analyses, the first 954cm^3 (at a depth of 15cm) of top soil was taken every 20m on each 100m belt transect. Since we only wished to compare sites (and not individual transects within a site), soil samples from all eight transects within a site were pooled. Samples were transported to the laboratory and dried. Rocks, twigs and insects were removed before analyses. Soil samples were analyzed by the Department of Plant Production and Soil Science, at the University of Pretoria, for texture, pH, percentage carbon, mineral nitrogen, phosphorus and cation exchange capacity (CEC). The soil pH was determined using a 1:2,5 soil:water ratio suspension (Schofield and Taylor 1955), CEC was determined using a pH drop with ammonium acetate (1M dm^{-3}) solution buffered at pH 7 (Schollenberg and Simon 1945) with the hydrometer method used to determine particle size (Bouyoucos 1962). Organic carbon content was determined using the Walkley-Black method (Walkley 1935), ammonium and nitrate were extracted with 2M Potassium Chloride using a 1:10 soil:extractant ratio and a 1 hour end-over-end shake followed by filtration (Magill and Aber 2000; Shahandeh *et al.* 2005) while phosphorus was determined by the extraction method of Bray and Kurtz (1945).

Precipitation and temperature data for each site were obtained from the South African Weather Service (www.weathersa.co.za). Weather stations used were Lydenburg (station code: 0554816A7, distance from site: 27.8km) for the Lydenburg site, Warmbad Towoomba (05895941, 4km and 17km) for Bela-Bela and Modimolle, Babanango (03373825, 11km) for Ulundi and Mtunzini (03043576, 25km) for Eshowe. The same weather stations that were used by Van der Linde *et al.* (2012) for Enzelsberg (Tuscany, distance from site: 16km [precipitation] and Marico, 17km [temperature]), Wolfaan (Brits Hartbeespoortdam, 19km [precipitation] and Buffelspoort II AGR, 18km [temperature]), Euphorbia Drive (Palmer estate, 6km [precipitation] and Mokopane, 18km [temperature]), Capricorn (Mara-Pol, 30km [precipitation] and Mara, 49km [temperature]) and Last Post (Mara, 40km [precipitation and temperature]) were used. Daily (minimum and maximum) temperatures and precipitation data were used to calculate monthly means and these were compared to determine if there were any changes over time that might account for increased mortality. Precipitation and temperature data were available for all but three sites from 1960 to 2014. The exceptions were Euphorbia Drive, Eshowe and Ulundi for which data were available only from 1996 to 2014.

2.3 Data Analyses

Analysis of variance (ANOVA) was used to determine if there were significant differences in die-off symptoms (moth damage and grey discoloration) and mortality among sites. ANOVA was conducted using the mean percentage values of mortality and mean rank of the proportion of trees with grey discolouration or moth damage (from data acquired in 2014) in each transect for each site. In order to determine if there were changes in symptom severity and mortality over time; data from 2010 (Van der Linde *et al.* 2012) and 2012 were compared with data acquired in 2014. The mean rank of each die-off symptom (grey discoloration and moth damage), as well as mean percentage mortality of *E. ingens* for each transect at each site was calculated and compared using a t-test.

To determine whether there had been any significant changes in temperature and precipitation over time at the study sites, ANOVA was used to compare monthly means (minimum and maximum temperatures and precipitation) by decade (1965-1974, 1975-1984, 1985-1994, 1995-2004 and 2005-2014) for all sites except Euphorbia Drive, Eshowe and Ulundi. Temperature data for Euphorbia Drive and precipitation and temperature data for Eshowe and Ulundi were compared for 1996-2005 and 2006-2014 using a student's t-test.

Data were tested for normality using Shapiro-Wilk's W. Kruskal-Wallis one-way ANOVA was used if data were not normal after transformation (ln+1; to account for zeros in the data). Mean separation tests (Tukey-Kramer's, HSD) were conducted on significant F-tests and H-tests for all interactions. Linear regression analyses, among all sites, were conducted to test if mortality was correlated to any of the soil properties tested in this study. For all tests, α was set at $P \leq 0.05$. All statistical analyses were conducted using JMP Version 12.0.1 (SAS Institute Inc., Cary, North Carolina, 1989-2007).

To test for possible spatial auto-correlation between tree die-off symptoms and distance between sites (if closer sites were more alike in symptom severity), we used the RELATE function in PRIMER 6 (PRIMER-E, Lutton, Plymouth, UK), a Mantel-type test that correlates two similarity matrices with one another. For the RELATE test, we used Spearman's Rho with 9999 permutations. The number of trees within each disease category effectively functioned as the 'abundance' of individual trees expressing a symptom at each site. Using the same disease symptom data, we also executed a redundancy analysis (RDA) with the forward selection command in CANOCO 5 (ter Braak and Šmilauer 2012) to test whether those sites that had higher incidences of moth damage, greying or both also had

higher incidences of dead trees in the landscape. Another RDA was performed in CANOCO 5 to observe the principle environmental components that constitute the study sites, after which we used the forward selection function in the CANOCO program to correlate percentage tree mortality with these site conditions. For both RDA analyses, we used 9999 permutations to calculate correlation coefficients.

3. Results

3.1 Levels and changes over time of *Euphorbia ingens* die-off symptoms and mortality

Die-off symptoms and mortality were present at all the sites, but occurred at different levels of severity. There was a significant difference in grey discoloration, moth damage and mortality among all the sites investigated (Table 1). Greater tree mortality was significantly correlated with those landscapes where moth damage and grey discoloration were more severe (pseudo-F = 4.4; P = 0.03; Fig. 4). There was no spatial autocorrelation between the symptoms of *E. ingens* die-off and distance between sites (Spearman Rho = -0.05, P = 0.41).

There were significant changes in mortality and die-off factors across both the 4 and 2 year observation periods (Table 2). Only one site (Lydenburg) had no significant change in die-off factors and mortality. Bela-Bela, Enzelsberg, Eshowe, Last Post, and Ulundi all exhibited a marked increase in mortality over the last two to four years. Capricorn, Modimolle and Ulundi showed a significant increase in grey discoloration while Ulundi and Wolfaan showed a significant increase in moth damage. Capricorn and Euphorbia Drive showed a significant decrease in moth damage over the last four years.

3.2 Precipitation and temperature

Significant site-specific changes were observed for minimum temperature, maximum temperature and precipitation (Table 3). Lydenburg changed the most with a significant increase in minimum and maximum temperatures and a significant decrease in precipitation. There was a significant increase in minimum temperature at Bela-Bela, Modimolle and Wolfaan, while at Euphorbia Drive, minimum temperature decreased significantly. Maximum temperature increased significantly across all sites except Euphorbia Drive, Eshowe and Ulundi. Except for Lydenburg, there was no significant change in precipitation. Increased percentage tree mortality was generally associated with sites that had overall higher temperatures and less rainfall.

3.3 *Euphorbia ingens* mortality and landscape degradation

Higher levels of *E. ingens* mortality were associated with landscape parameters that are indicative of land degradation. Sites with the highest levels of *E. ingens* mortality displayed clear characteristics of degradation (high levels of bare soil, high levels of dead wood, higher dung counts and high bush encroachment by *D. cinerea*) (pseudo-F = 2.7; P = 0.003; Figure 5). These severely diseased sites (Enzelsberg, Euphorbia Drive, Last Post and Ulundi) were overgrazed with low levels of living grass cover and *E. ingens* trees were severely encroached with *D. cinerea*. Sites with the lowest levels of mortality (Capricorn, Eshowe, Lydenburg and Modimolle) were associated with high levels of living plant cover (specifically grass cover) with low levels of dead wood and low levels of *D. cinerea* encroachment (Fig. 6). Sites with higher levels of *E. ingens* die-offs were correlated with high levels of nitrates (P = 0.04, R^2 = 0.419) and CEC (P = 0.02, R^2 = 0.492). The other soil characteristics tested were not significantly correlated to *E. ingens* mortality; ammonium (P = 0.66, R^2 = 0.025), carbon (P = 0.20, R^2 = 0.194), clay (P = 0.55, R^2 = 0.046), loam (P = 0.51, R^2 = 0.057), pH (P = 0.15, R^2 = 0.242), phosphorus (P = 0.68, R^2 = 0.022) and sand (P = 0.45, R^2 = 0.074) (Table 4).

4. Discussion

Previous studies have described the insects and pathogens that are associated with mortality of *E. ingens* in areas experiencing die-off. But none of these agents were considered primary pathogens or pests (Van der Linde *et al.* 2011a,b,c). This suggests that some factor is predisposing *E. ingens* to attacks by these otherwise secondary insects and pathogens. Although evidence exists linking climate change to the die-off (Van der Linde *et al.* 2012), we show here that disease symptoms and mortality could also be explained by savanna degradation. While there were significant changes in temperature and precipitation at most of the sites in this study, these changes did not necessarily relate to symptom and die-off severity. This challenges the role of climate as the only trigger that has led to *E. ingens* die-offs. For example, Ulundi, which exhibited a significant increase in symptoms and mortality, experienced no significant change in temperature and precipitation over the last 20 years. Likewise, Lydenburg, which had a significant increase in minimum temperature, maximum temperature and a decrease in precipitation, showed no significant increase in symptoms or mortality. Capricorn, which had a significant increase in maximum temperature, was one of the healthiest sites included in this study with a mean percentage mortality of only 2.5%.

Landscape parameters that indicate savanna disturbance were highly correlated with *E. ingens* mortality across sites. Since livestock farming (subsistence and commercial) is an established activity in these landscapes, the observed rates of mortality suggest that *E. ingens* trees are negatively affected by this activity. The stress from intensive livestock grazing could predispose *E. ingens* populations to insect and pathogen damage and could eventually lead to local extinction. While climate change may play a role in the die-off, our results indicate that ecosystem degradation very likely exacerbates and may even trigger *E. ingens* die-offs.

Euphorbia ingens has a shallow root system typical of succulent-type plants in a xeric environment (Van Wyk and Van Wyk 1997, Palgrave *et al.* 2002). This type of root system allows the plants to rapidly access moisture in rare wet events. High livestock stocking rates would lead to compaction of the soil that could damage roots, restricting moisture and nutrient uptake and it could place *E. ingens* under further stress (Rietkerk *et al.* 1997; 2000, Van Langevelde *et al*. 2003). Apart from physical damage to the roots by the grazers, crust formation occurs (causing soil erosion and run-off) leading to the possibility of less water availability at the root level (Kelly and Walker 1976, Rietkerk *et al.* 1997; 2000, Van Langevelde *et al*. 2003, Savadogo *et al.* 2007). Results of this study showed that sites with high levels of *E. ingens* mortality had the highest concentrations of NO_3^- and CEC in the soil, contradicting the fact that the soil might have lowered water retention and nutrient availability at these specific sites. CEC measures the soils ability to hold exchangeable cations and the availability of important nutrients to plants, with higher CEC values linked to fertile soils with good water retention (Bot and Benites 2005, Saidi 2012). Water in the soil is needed to convert organic nitrogen to ammonium (NH_4^+) which is eventually converted to nitrates (NO_3^-), which provides plants with their most usable form of nitrogen (Anthonisen *et al.* 1976). Therefore, low levels of NO_3^- usually indicate low levels of water in the soil (Anthonisen *et al.* 1976). Nutrient uptake ability, due to damaged roots and compaction, might more likely be the stress factor. Furthermore, water and nutrient availability to *E. ingens* trees can also be reduced due to uptake and competition by *D. cinerea* in areas where it has encroached on *E. ingens* habitat (Rietkerk *et al.* 1997; 2000, Van Langevelde *et al*. 2003, Savadogo *et al.* 2007).

The knowledge that habitat degradation is likely involved in *E. ingens* die-offs is important because it indicates that appropriate rangeland management of xeric savannas could aid in conserving *E. ingens* in the landscape. This is especially relevant because other indicated causes of *E. ingens* die-off, such as climatic variability, are far less practical to mitigate. An

attempt to sustainably manage the studied savanna landscapes may not only help to conserve this iconic tree species, but it will also aid biodiversity conservation ideals in general, since many more species are inevitably affected by such landscape degradation.

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Table 1. Mean (SE) die-off factor and percentage mortality of *Euphorbia ingens* among all sites investigated in 2014.

Site	Grey discoloration	Moth damage	% Mortality
Bela-Bela	0.560 (0.071) [bcd]	0.504 (0.092) [ab]	14.90 (2.04) [bcd]
Capricorn	0.739 (0.082) [b]	0.170 (0.026) [cd]	2.50 (1.73) [d]
Euphorbia Drive	1.695 (0.073) [a]	0.597 (0.055) [ab]	25.50 (5.10) [ab]
Enzelsberg	0.148 (0.056) [e]	0.689 (0.120) [ab]	32.50 (3.70) [a]
Eshowe	0.657 (0.118) [bc]	0.406 (0.068) [bc]	10.62 (3.12) [bcd]
Last Post	1.407 (0.062) [a]	0.584 (0.048) [ab]	20.90 (4.45) [abc]
Lydenburg	0.594 (0.041) [bcd]	0.166 (0.048) [cd]	7.00 (2.48) [cd]
Modimolle	0.215 (0.054) [de]	0.054 (0.017) [d]	4.10 (1.60) [cd]
Ulundi	1.659 (0.083) [a]	0.775 (0.047) [a]	17.40 (5.89) [abcd]
Wolfaan	0.351 (0.044) [cde]	0.774 (0.080) [a]	16.40 (4.06) [abcd]
ANOVA statistics	F = 47.612, df = 9, P < 0.001,	F = 15.489, df = 9, P < 0.001	F = 6.629, df = 9, P < 0.001

Same letters within a column indicate that means are not significantly different

Table 2. Mean (SE) die-off factor and percentage mortality increase of *Euphorbia ingens* from 2010 to 2014 (Capricorn, Euphorbia Drive, Enzelsberg, Last Post and Wolfaan) and 2012 to 2014 (Bela-Bela, Eshowe, Lydenburg, Modimolle and Ulundi). Significant comparisons in bold.

Site	Year Scored	Grey discoloration	t-test result	Moth damage	t-test result	% Mortality	t-test result
Bela-Bela	2012	0.724 (0.046)	t = 1.923, df = 14, P = 0.07	0.461 (0.107)	t = 0.018, df = 14, P = 0.98	6.50 (1.823)	**t = 3.062, df = 14, P = 0.008**
	2014	0.560 (0.072)		0.504 (0.082)		14.90 (2.039)	
Capricorn	2010	0.427 (0.104)	**t = -2.346, df = 14, P = 0.03**	0.431 (0.063)	**t = 3.581, df = 14, P = 0.003**	0.630 (0.625)	t = -1.018, df = 14, P = 0.33
	2014	0.739 (0.082)		0.170(0.028)		2.50 (1.732)	
Euphorbia Drive	2010	1.877 (0.064)	t = 1.878, df = 14, P = 0.08	0.889 (0.051)	**t = -2.261, df = 14, P = 0.04**	22.13 (3.476)	t = -0.547, df = 14, P = 0.59
	2014	1.695 (0.073)		0.597 (0.055)		25.50 (5.103)	
Enzelsberg	2010	0.036 (0.014)	t = 1.919, df = 14, P = 0.08	0.652 (0.071)	t = 0.263, df = 14, P = 0.79	14.00 (4.310)	**t = -3.259, df = 14, P = 0.006**
	2014	0.148 (0.056)		0.689 (0.085)		32.50 (3.694)	
Eshowe	2012	0.537 (0.092)	t = -0.800, df = 14, P = 0.44	0.384 (0.054)	t = 0.087, df = 14, P = 0.93	2.25 (1.521)	**t = -2.366, df = 14, P = 0.03**
	2014	0.657 (0.118)		0.406 (0.067)		10.62 (3.196)	
Last Post	2010	1.311 (0.093)	t = -0.862, df = 14, P = 0.40	0.616 (0.037)	t = 1.066, df = 14, P = 0.31	2.00 (1.614)	**t = -3.984, df = 14, P = 0.001**
	2014	1.407 (0.062)		0.584 (0.054)		20.90 (4.453)	
Lydenburg	2012	0.529 (0.128)	t = -0.341, df = 14, P = 0.74	0.374 (0.133)	t = 1.470, df = 14, P = 0.16	4.10 (1.604)	t = -0.986, df = 14, P = 0.34
	2014	0.594 (0.141)		0.166 (0.048)		7.00 (2.479)	
Modimolle	2012	0.086 (0.024)	**t = -2.189, df = 14, P = 0.05**	0.040 (0.015)	t = -0.434, df = 14, P = 0.67	3.40 (1.603)	t = -0.331, df = 14, P = 0.74
	2014	0.215 (0.054)		0.054 (0.016)		4.10 (1.597)	
Ulundi	2012	1.284 (0.079)	**t = -3.279, df = 14, P = 0.005**	0.556 (0.036)	**t = -3.366, df = 14, P = 0.005**	3.40 (2.442)	**t = -2.196, df = 14, P = 0.04**
	2014	1.659 (0.083)		0.775 (0.050)		17.40 (5.889)	
Wolfaan	2010	0.205 (0.096)	t = -1.381, df = 14, P = 0.19	0.122 (0.067)	**t = -5.756, df = 14, P < 0.001**	6.30 (2.833)	t = -2.045, df = 14, P = 0.06
	2014	0.351 (0.044)		0.774 (0.075)		16.40 (4.062)	

Table 3. Results of analyses (ANOVA, Kruskal-Wallis one-way ANOVA and T-Test) of monthly mean minimum temperature, maximum temperature and precipitation (per decade) from 1965 to 2014 (Bela-Bela, Capricorn, Euphorbia Drive, Enzelsberg, Last Post, Lydenburg, Modimolle and Wolfaan) and from 1996 to 2014 (Euphorbia Drive, Eshowe and Ulundi).

Site	Decade	Mean minTemp (°C) (SD)	Result	Mean maxTemp (°C) (SD)	Result	Precipitation (mm) (SD)	Result
			H = 11.871, df =		**H = 21.415, df =**		H = 2.839, df =
Bela-Bela	1965-1974	11.22 [a]	**4, P = 0.02**	26.52 [b]	**4, P < 0.001**	603.77	4, P = 0.59
	1975-1984	10.72 [b]		26.55 [b]		692.39	
	1985-1994	11.50 [a]		27.06 [b]		612.33	
	1995-2004	11.34 [a]		27.09 [b]		631.31	
	2005-2014	11.59 [a]		28.8 [a]		572.93	
SE		**0.162**		**0.238**		**48.98**	
			H = 5.102, df =		**H = 22.159, df =**		H = 3.824, df =
Capricorn	1965-1974	12.45	4, P = 0.28	26.8 [c]	**4, P < 0.001**	429.71	4, P = 0.43
	1975-1984	12.00		27.03 [bc]		513.43	
	1985-1994	12.34		27.70 [ab]		416.81	
	1995-2004	12.50		27.48 [bc]		510.86	
	2005-2014	12.35		28.25 [a]		414.83	
SE		**0.174**		**0.182**		**47.98**	
Euphorbia			**t = 2.641, df =**		t = -1.950, df = 17,		H = 4.539, df =
Drive	1996-2005	13.74 (0.499)	**17, P = 0.02**	27.60 (0.872)	P = 0.07		4, P = 0.34
	2006-2014	13.12 (0.527)		28.22 (0.425)			
	1965-1974					553.37	
	1975-1984					567.89	
	1985-1994					478.78	
	1995-2004					545.35	
	2005-2014					530.44	
SE						**35.70**	
			F = 2.727, df =		**F = 7.624, df = 4,**		H = 3.135, df =
Enzelsberg	1965-1974	11.72	4, P = 0.08	27.27 [b]	**P < 0.001**	602.14	4, P = 0.54
	1975-1984	12.21		27.31 [b]		591.3	
	1985-1994	11.50		29.86 [a]		569.52	

	1995-2004	11.97		26.98 [b]		665.04	
	2005-2014	11.83		28.35 [a]		517.08	
SE		**0.177**		**0.429**		**56.948**	
Eshowe	1996-2005	16.66 (0.503)	t = 1.999, df = 17, P = 0.06	27.43 (0.521)	t = -0.168, df = 17, P = 0.87	1203.44 (258.19)	t = -0.010, df = 17, P = 0.99
	2006-2014	15.94 (1.011)		27.48 (0.654)		1204.87 (388.20)	
Last post	1965-1974	12.45	H = 5.102, df = 4, P = 0.28	26.8 [c]	**H = 22.159, df = 4, P < 0.001**	429.71	H = 3.824, df = 4, P = 0.43
	1975-1984	12.00		27.03 [bc]		513.43	
	1985-1994	12.34		27.70 [ab]		416.81	
	1995-2004	12.50		27.48 [bc]		510.86	
	2005-2014	12.35		28.25 [a]		414.83	
SE		**0.174**		**0.182**		**47.98**	
Lydenburg	1965-1974	9.28 [b]	**F = 29.888, df = 4, P < 0.001**	22.73 [c]	**H = 20.405, df = 4, P < 0.001**	680.57 [a]	**H = 25.381, df = 4, P < 0.001**
	1975-1984	9.26 [b]		23.10 [bc]		740.04 [a]	
	1985-1994	10.24 [a]		23.06 [bc]		745.28 [a]	
	1995-2004	10.38 [a]		23.34 [ab]		350.26 [b]	
	2005-2014	10.36 [a]		23.81 [a]		424.00 [b]	
SE		**0.109**		**0.145**		**48.89**	
Capricorn	1965-1974	12.45	H = 5.102, df = 4, P = 0.28	26.8 [c]	**H = 22.159, df = 4, P < 0.001**	429.71	H = 3.824, df = 4, P = 0.43
	1975-1984	12.00		27.03 [bc]		513.43	
	1985-1994	12.34		27.70 [ab]		416.81	
	1995-2004	12.50		27.48 [bc]		510.86	
	2005-2014	12.35		28.25 [a]		414.83	
SE		**0.174**		**0.182**		**47.98**	
Ulundi	1996-2005	14.33 (0.340)	t = 1.416, df = 17, P = 0.18	26.61 (0.650)	t = 1.125, df = 17, P = 0.28	641.77 (136.58)	t = 0121, df = 17, P = 0.91
	2006-2014	13.71 (1.345)		26.22 (0.851)		630.82 (248.70)	
Wolfaan	1965-1974	10.94 [b]	**F = 12.719, df = 4, P < 0.001**	26.42 [ab]	**H = 13.572, df = 4, P = 0.009**	704.17	F = 0.767, df = 4, P = 0.55

	1975-1984	11.25 [b]	25.88 [b]	678.55
	1985-1994	11.21 [b]	26.29 [ab]	549.95
	1995-2004	11.82 [b]	26.30 [ab]	611.17
	2005-2014	13.94 [a]	26.96 [a]	574.62
SE		**0.343**	**0.196**	**75.52**

SD = standard deviation
***SE* = Standard error**
Same letters within a column, specific to a site, indicate that means are not significantly different

Table 4. Soil characteristics tested and results from each site investigated in the current study.

Site	pH	P (mg/kg)	NO_3^- mg/kg	NH_4^+ mg/kg	CEC cmol(+)/kg	% Carbon	% Sand	% Loam	% Clay
Bela-Bela	6.19	11.91	8.72	6.97	19.1	2.28	69	11	20
Capricorn	6.4	4.05	4.94	6.9	22.1	0.63	81	7	12
Euphorbia Drive	7.47	4.05	9.59	5.22	23.06	1.4	62	19	19
Enzelsberg	6.82	7.88	11.17	6.55	24.44	1.61	68	17	15
Eshowe	5.89	11.91	7.42	5.08	16.64	1.47	67	18	15
Last post	6.4	17.78	8.58	14.28	25.31	1.59	75	15	10
Lydenburg	6.57	7.25	8.93	5.64	17.28	1.4	55	29	16
Modimolle	6.23	4.49	8.61	7.42	14.55	0.68	81	6	13
Ulundi	5.85	12.16	12.57	11.03	19.79	1.19	78	10	12
Wolfaan	6.31	39.97	9.8	6.09	20.58	2.56	70	14	16

P = Phosphorus, NO_3^- = Nitrate, NH_4^+ = Ammonium CEC = Cation Exchange Capacity

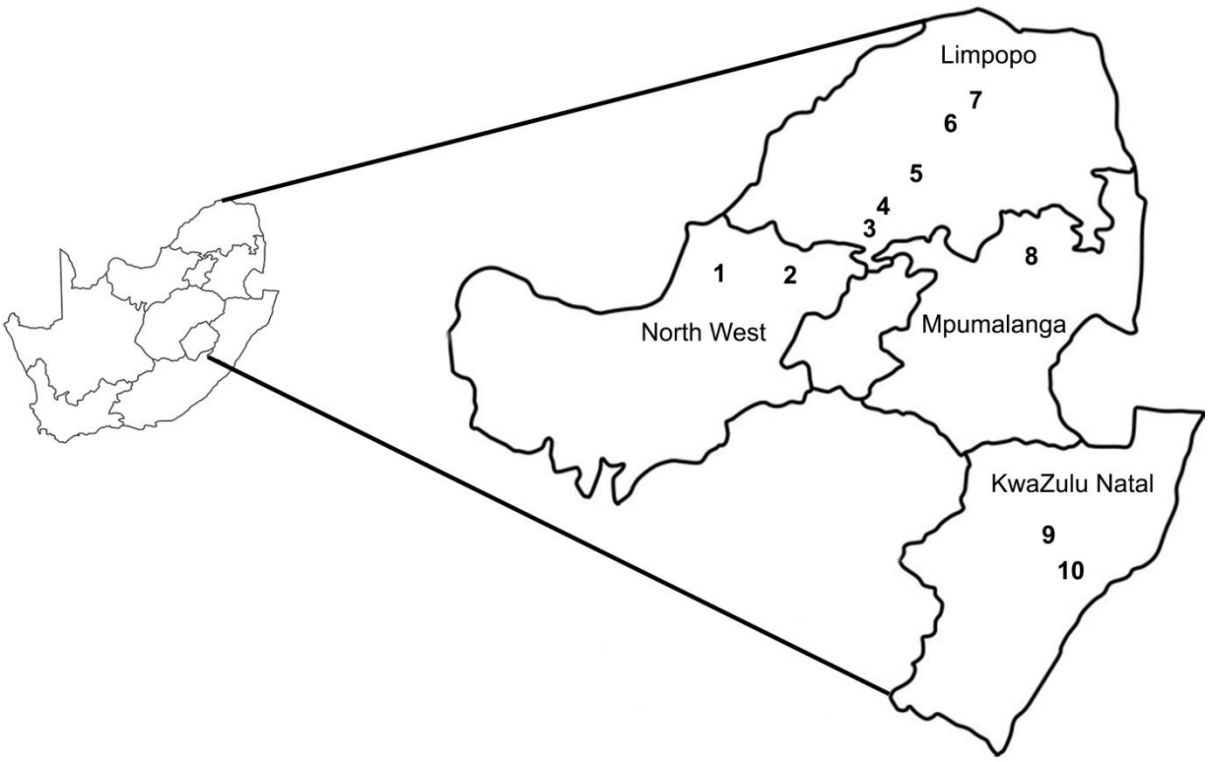

Figure 1: Sites at which *Euphorbia ingens* die-offs were investigated. 1 = Enzelsberg, 2 = Wolfaan, 3 = Bela-Bela, 4 = Modimolle, 5 = Euphorbia Drive, 6 = Capricorn, 7 = Last Post, 8 = Lydenburg, 9 = Ulundi and 10 = Eshowe.

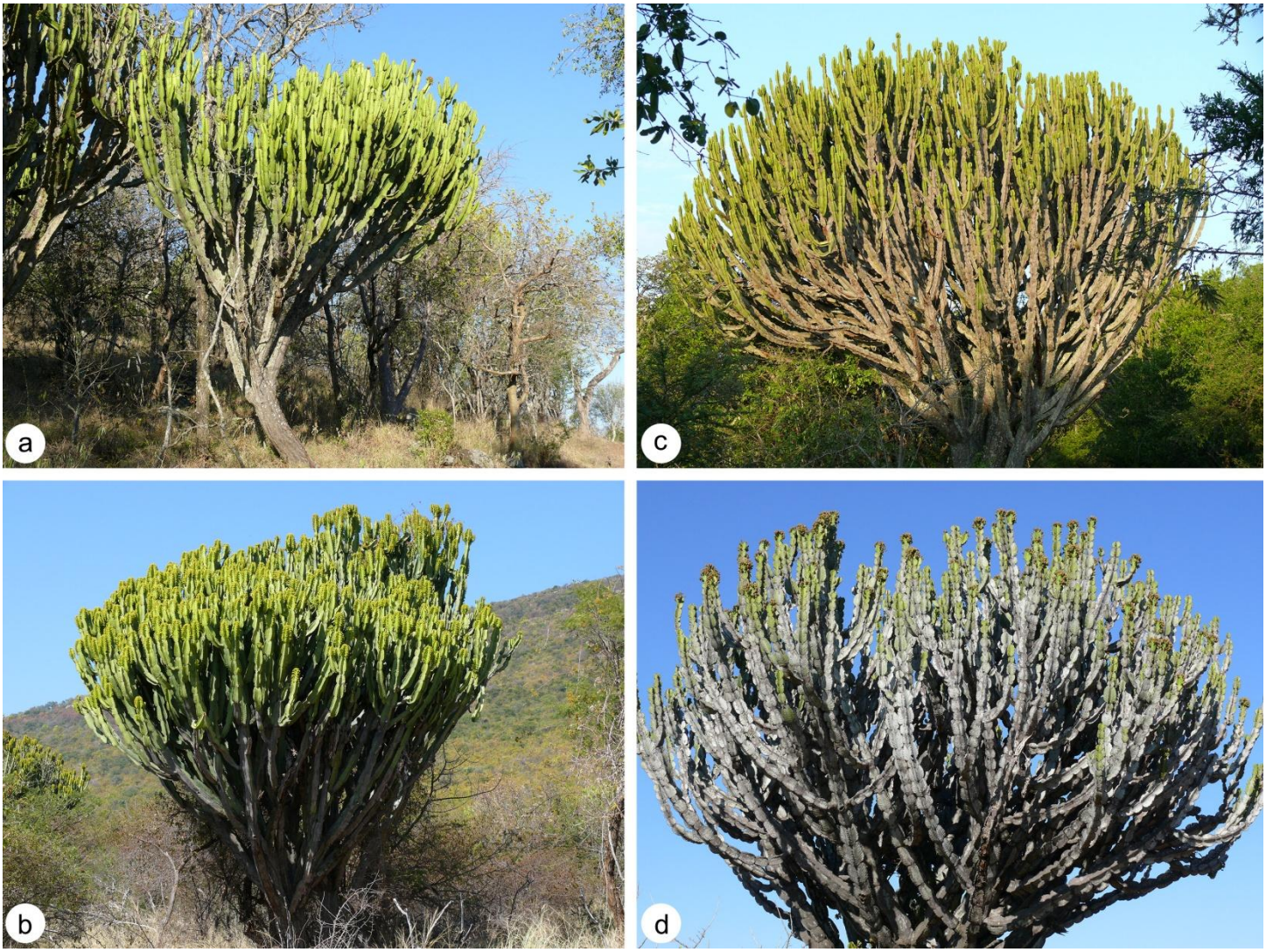

Figure 2: Ranking of grey discoloration on *Euphorbia ingens* trees. (a) G1 (0-25% affected). (b) G2 (26-50% affected). (c) G3 (51-75% affected). (d) G4 (76-100% affected).

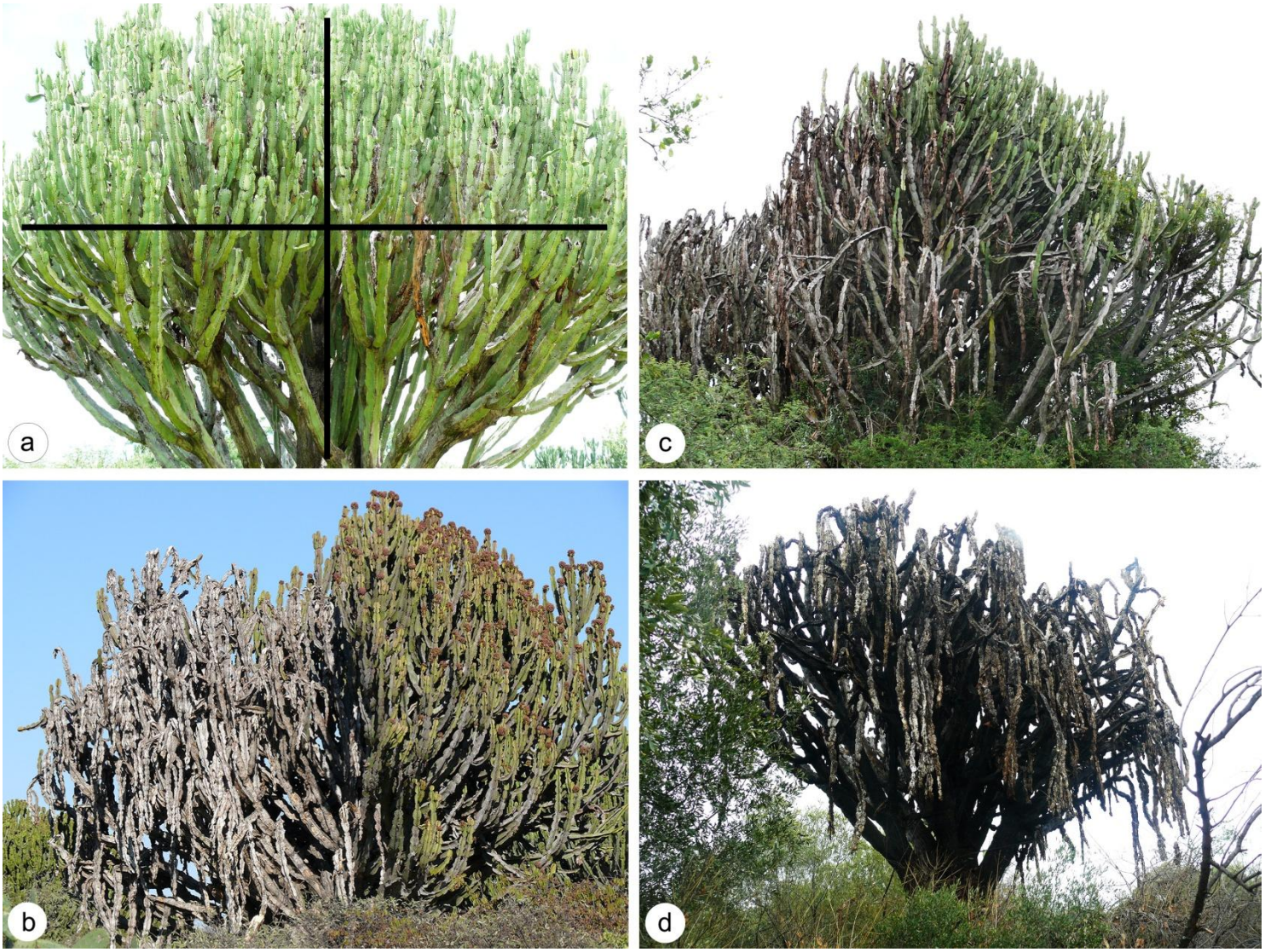

Figure 3: Ranking of rotting associated with *Megasis* attacks on *Euphorbia ingens*. The crown of an *Euphorbia ingens* tree is divided into four quadrants and the percentage damage was calculated according to the proportion of quadrants containing moth-damaged branches. (a) M1 (0-25% affected). (b) M2 (26-50% affected). (c) M3 (51-75% affected). (d) M4 (76-100% affected).

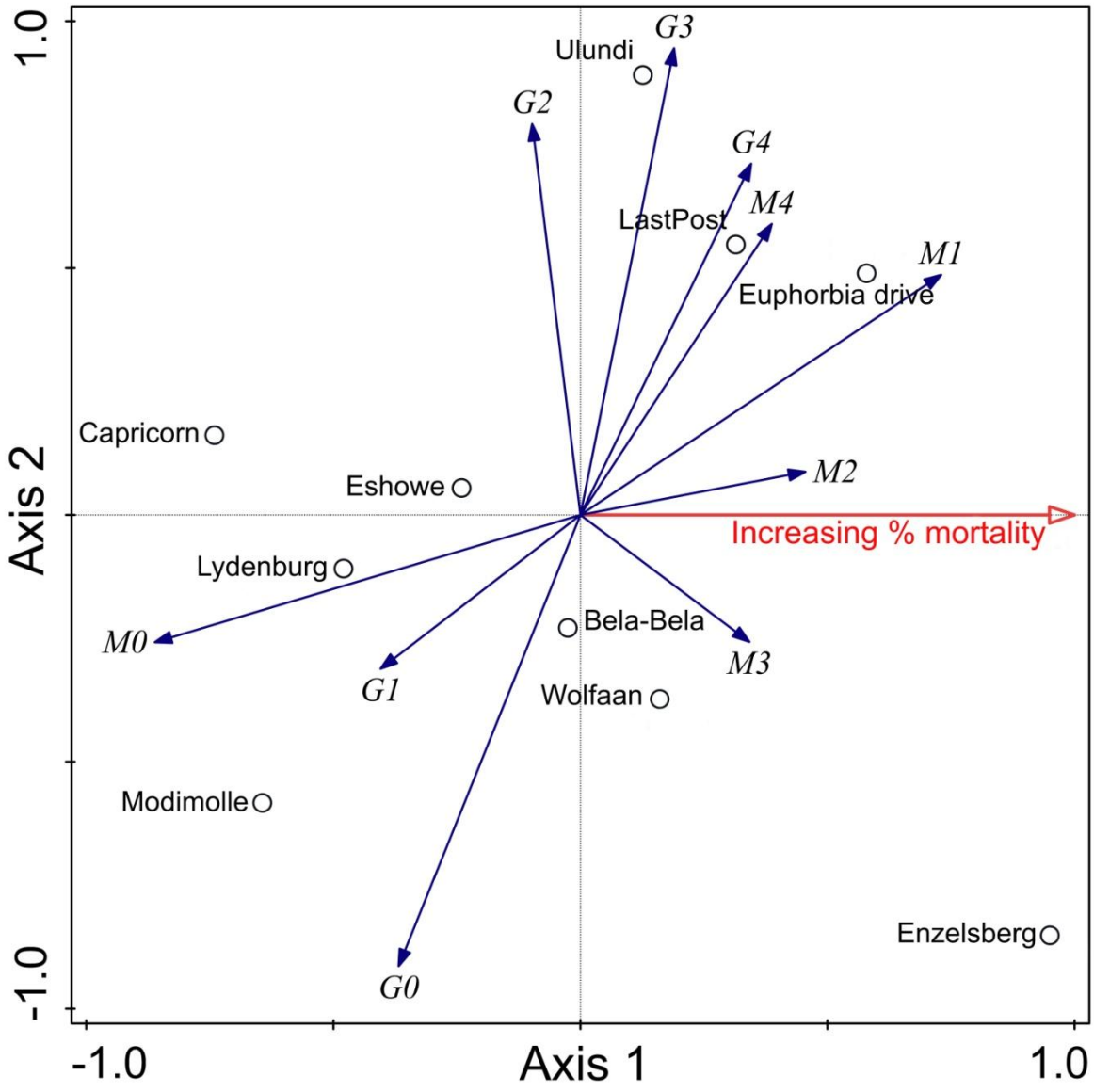

Figure 4: Redundancy analysis (RDA; pseudo-F = 4.4; P = 0.03) showing relationship among symptoms associated with *Euphorbia ingens* die-off and increasing mortality. G0-G4 indicate scores of greying of *E. ingens* branches with zero being absent to low and 4 being very high. M0-M4 indicate scores of moth damage with zero being absent or low and 4 being very high.

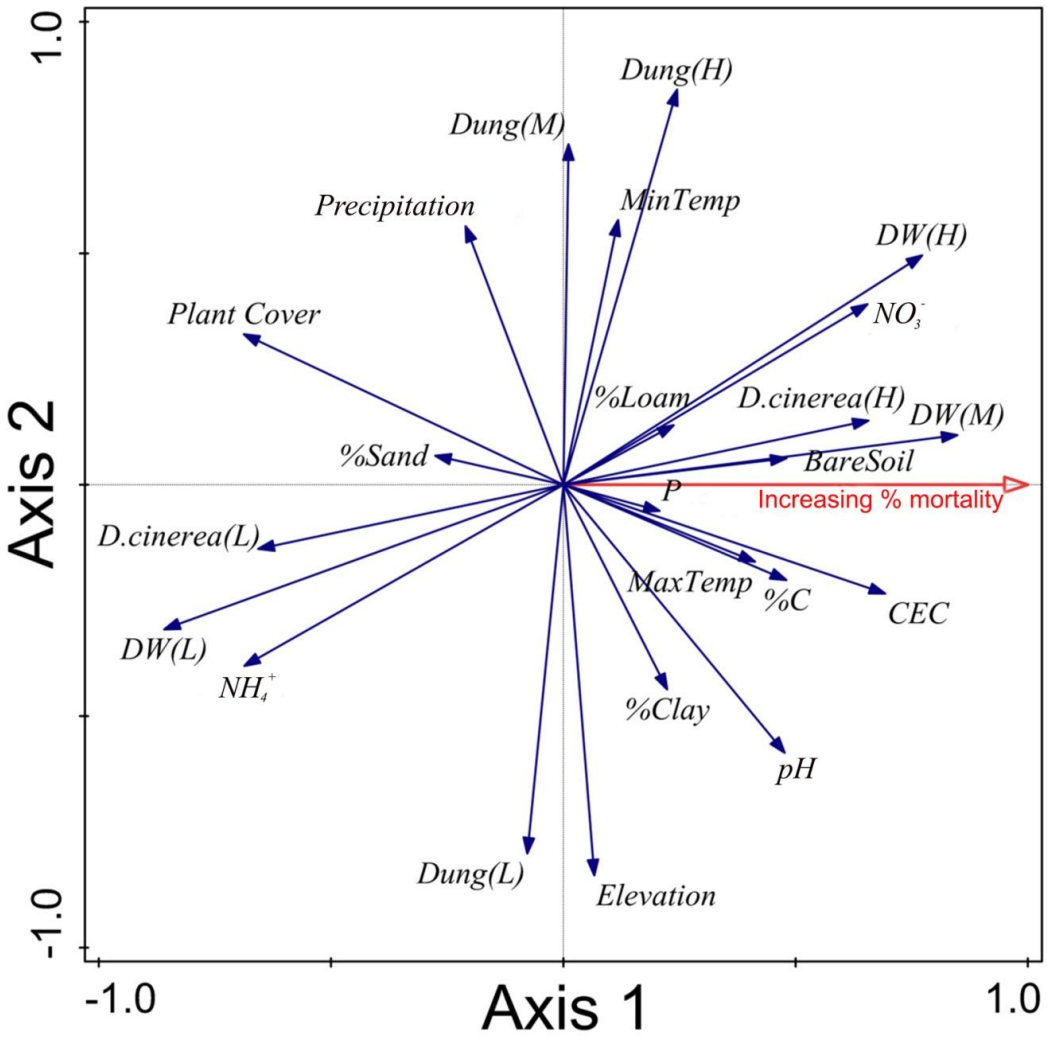

Figure 5: Redundancy analysis (RDA; pseudo-F = 2.7; P = 0.003) showing increasing mortality of *Euphorbia ingens* in relation to landscape variables scored in this study. DW = dead woody debris, *D. cinerea* = *Dichrostachys cinerea*, CEC = soil cation-exchange capacity, %C = % soil carbon, Precipitation = monthly mean precipitation, MinTemp = monthly mean minimum temperature and MaxTemp = monthly mean maximum temperature. H, M and L in parentheses are High, Medium or Low. Bare soil, higher levels of dead wood and high levels of *D. cinerea* encroachment were associated with increased mortality (more diseased sites) while higher grass cover, lower levels of dead wood and lower levels of *D. cinerea* encroachment were associated with healthier sites (low levels of *E. ingens* mortality).

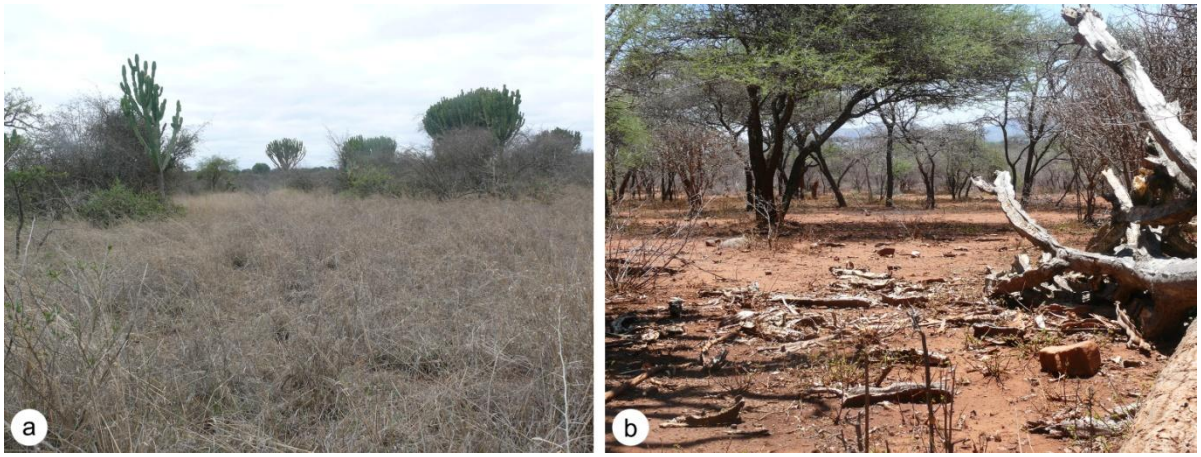

Figure 6: Comparison of a typical site where *Euphorbia ingens* mortality is low and high. (a) Capricorn (*E. ingens* mortality 2.5%); high level of live ground plant cover and hardly any *Dichrostachys cinerea* encroachment. (b) Euphorbia Drive (*E. ingens* mortality 25.50%); high levels of bare soil and *D. cinerea* encroachment.

Summary

Euphorbia ingens trees are under severe environmental stress in South Africa. Initially thought only to be of concern in the Limpopo Province of South Africa, results of this study have shown that these iconic trees are suffering from disease and are dying across South Africa. The incidence of disease symptoms and levels of mortality are closely associated with landscape management (health of the ecosystem) and climatic changes (temperature and precipitation) driven further by fungal infections and insect infestation.

A diverse group of insects and fungi were associated with disease symptoms of *E. ingens*, including ambrosia beetles, weevils, a moth species, fungi in the Botryosphaeriaceae, and previously undescribed fungi in the Ophiostomataceae, Nectriaceae and Teratosphaeriaceae. No specific fungal species was consistently isolated or associated with specific disease symptoms or mortality of *E. ingens* trees. Ambrosia beetles and weevils infested *E. ingens* trees that were already stressed and dying off. The moth, *Megasis* sp., was more aggressive and was found to infest succulent branches of healthy looking trees. Grey discoloration of the succulent branches revealed no clear association with fungi or insects and is likely a stress-related symptom. Flight activity of beetles that infest *E. ingens* trees were significantly associated with climatic conditions (temperature and humidity) and did not seem to be dependent on *E. ingens* tree health at any particular site. There was a significant difference in the incidence of disease symptoms and mortality levels at the different sites surveyed. These differences were observed among sites within a province (where climatic conditions were similar) and among sites in different provinces. Furthermore, field studies showed a correlation between poor land management and landscape degradation (indicative of over grazing and intrusion of *Dichrostachys cinerea*) and *E. ingens* die-offs, with die-offs triggered by site specific conditions. Previously, *E. ingens* die-offs were thought to be exclusively triggered by climate change (temperature and precipitation) in a specific region. Results of this study has led to the view that this problem arises from a complex interaction of abiotic, site specific conditions (ecosystem health and climatic changes specific for a site) with die-offs further driven by a complex diversity of biotic (fungi and insects) factors.