Factors associated with the decline of *Euphorbia ingens* in the Limpopo Province of South Africa

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Factors associated with the decline of *Euphorbia ingens* in the Limpopo Province of 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 Magister Scientiae 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.

Johannes Alwyn van der Linde

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PREFACE

Euphorbia ingens is the largest of the succulent *Euphorbia* species in Southern Africa. This species occurs mostly in the Northern Provinces of South Africa, growing in different habitats including areas of open veldt, ridges, rocky outcrops and steep rock. Dramatic deaths of this tree have been occurring in the Limpopo Province over the last 10 years. Initial disease symptoms and death of trees were noticed in the mid - to late nineties, leading to various pilot studies to identify the possible cause of the disease and deaths. Pilot studies revealed a high diversity of insects and fungi occurring on diseased and dying trees, but no clear answer to the cause of tree deaths. This provided the justification for a more in depth study to investigate the cause of *E. ingens* deaths in the Limpopo Province of South Africa.

The aim of the work described in this dissertation was to expand on the pilot studies conducted previously and to identify the major fungal and insect pests associated with *E. ingens* in the Limpopo Province. This included an evaluation of possible abiotic factors that might contribute to the decline and death of *E. ingens* in the Limpopo Province. Abiotic factors were also considered at sites where *E. ingens* appeared healthy. The possible role of climate change in the death of these trees was also evaluated.

Chapter one of this dissertation provides a background on the genus *Euphorbia* with specific reference and focus on *E. ingens*. This chapter also summarizes available information on pests and diseases on *E. ingens.* Finally, a discussion is provided on how climate change might be affecting *E. ingens* in light of international examples where climate change has had a severe impact on tree health.

Chapters two and three characterize two groups of fungi associated with dying *E. ingens* trees. Chapter two described two previously unknown *Gondwanamyces* spp., while chapter

three identifies species of Botryosphaeriaceae. Fungi were identified based on morphology and multi-gene phylogenies. Additionally, these fungi were used in pathogenicity trials to assess their possible roles in the disease on *E. ingens*.

Chapter four of the dissertation considered possible climatic change over the last 40 years in the Limpopo and North West Provinces. Temperature and rainfall data were obtained for four sites where *E. ingens* is dying (Limpopo Province) compared to sites of apparently unaffected *E. ingens* trees (North West Province). Water balance and evapo-transpiration levels were also calculated and together, this information was used to evaluate whether climate change is a driving factor in the death of *E. ingens*.

Chapter five provides an overview of the whole study, summarizing the results described in the individual chapters and putting this into context with hypotheses raised by other researchers regarding the possible cause of *E. ingens* deaths. This chapter also includes information from a two year monitoring study of permanent sampling plots and a summary of all insects and fungi obtained from diseased trees over the past four years.

This is the first study to investigate fungi and insects occurring on native *E. ingens* trees. It also provides the first attempt to link the possible involvement of climatic changes in *E. ingens* decline, which I hope will provide a valuable foundation for future studies on this problem.

Chapter 1

Literature Review: The genus *Euphorbia*, with a focus on *Euphorbia ingens* and possible factors associated with its decline

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ABSTRACT

The genus *Euphorbia* consists of a group of plants with a diverse morphology occurring all over the world. In South Africa species of the succulent *Euphorbia* trees are mostly found in the Northern provinces with *Euphorbia ingens* being the largest. In the last 10 years, symptoms of the disease have been reported on *E. ingens* trees in the Limpopo Province, which led to mass mortality. Pilot studies did not reveal a specific abiotic or biotic factor as the primary cause of the decline and the problem seems to result from a combination of these factors. The genus *Euphorbia* and specifically *E. ingens* is reviewed revealing the limited information known of insects and pathogens and the associated death of these trees. Climate change is also addressed in this review with examples from other countries as a possible factors leading to the deaths of *E. ingens*. To gain a better understanding into the deaths of *E. ingens* all factors will be investigated by expanding on the previous pilot studies done in the Limpopo Province and conducting comparative studies in areas where the trees are not as severely affected.

1. INTRODUCTION

The genus *Euphorbia* (Euphorbiaceae), comprised of approximately 2100 species, is one of the most diverse groups of flowering plants on the planet. Species of *Euphorbia* produce a white, often toxic, milky latex. They have a diverse morphology, including both succulent and herbaceous forms. They occur in Africa, tropical Asia and the Americas (Palgrave 2002, PBI Euphorbia project, www.euphorbiaceae.org).

Numerous succulent, herbaceous and woody species of *Euphorbia* occur in South Africa. (Palgrave 2002, Van Wyk & Van Wyk 1997). The highest diversity of *Euphorbia* trees are found in four of the Northern Provinces of South Africa, namely Gauteng, the Limpopo Province, Mpumalanga and the North West Province (41 species) (Gildenhuys 2006, Palgrave 2002). The three most abundant woody to succulent species in the Northern provinces are *Euphorbia ingens* E. Meyer: Boissier, *E. cooperi* N.E. Brown: Berger var. *cooperi* and *E. tirucalli* L., with *E. ingens* being the largest of the three species (Gildenhuys 2006).

In the last 10 years, it has been observed that *E. ingens* trees in the Limpopo Province are becoming diseased and dying. A survey conducted in 2006 at the National Zoological Gardens Biodiversity Conservation Centre (NZG) in Mokopane (Potgietersrus) revealed that all *E. ingens* trees in this reserve were diseased and many had died over a short period of time (personal communication, Rentia Malan). In addition, there was no recruitment of new trees and the decline was linked to several possible factors. These included damage due to baboons (*Papio ursinus* Kerr) and vervet monkeys (*Cercopithecus aethiops* L.) that eat the branch terminals and fruit of these trees, preventing the development and release of seed into

the environment (Malan 2006). Malan (2006), suggested that an insect pest or microbial disease may have been the cause of the deaths of the trees.

A pilot study conducted in 2007, aimed at characterizing disease on *E. ingens* at the Game Breeding Centre in Mokopane, identified several fungi and insects associated with dying trees (Roux *et al*. 2008, 2009). A gray discoloration of tree stems and branches, as reported previously by Malan (2006), was also found. Additionally, rotting and browning of the succulent branches, white and yellow spots on succulent branches, blue stain of the wood in the main stems and numerous insects infesting the stems and branches were found (Roux *et al*. 2008, 2009). Various genera of fungi were isolated from diseased material including *Cibiessii* spp., *Fusarium* spp., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., undescribed *Gondwanamyces* and *Graphium* species and an undescribed fungal genus residing in the Microascales. Insects found were primarily those in the Curculionidae including ambrosia beetles and *Cossonus* weevils (Roux *et al*. 2009).

No specific pathogens or insect affecting *E. ingens* appeared to be the primary cause of the rapid death of the trees. Therefore, the cause of the decline and death of *E. ingens* in the Limpopo Province remains uncertain. The decline of this species appears to involve a number of interacting biotic and abiotic factors. Stress resulting from a changing climate may also influence the susceptibility of the trees to insect invasion and pathogen infection in South Africa. The aims of this review are to provide background information on the genus *Euphorbia,* with special reference to *E. ingens,* and to summarize available information on pests and diseases of these trees. The review also considers the impact of climate change and other abiotic factors that might contribute to the decline of *E. ingens* in South Africa.

2. THE GENUS *EUPHORBIA*

2.1. Distribution

Euphorbia spp. occur in various habitats and soil types worldwide. The succulent species are the most diverse and can be found in the Americas, Madagascar, tropical Asia and Africa (Angola, Botswana, Cameroon, Ethiopia, Kenya, Lesotho, Madagascar, Malawi, Mozambique, Namibia, Nigeria, South Africa, Swaziland, Somalia, Tanzania, Uganda, Zambia and Zimbabwe) (Palgrave 2002, PBI Euphorbia project, www.euphorbiaceae.org, Desert Tropicals, www.desert-tropicals.com). The herbaceous euphorbias are mostly restricted to temperate zones (cold winters and mild summers) (FIG. 1) (PBI Euphorbia project, www.euphorbiaceae.org).

2.2. Morphology

Euphorbia spp. are distinct from other members of the Plant Kingdom in having a unique structure known as the cyathium. The cyathium is composed of reduced flowers grouped together in distinct clusters or inflorescences (FIG. 2). This characteristic has various modifications and can be used to distinguish between species of *Euphorbia* (PBI Euphorbia project, www.euphorbiaceae.org).

Four different modifications have evolved in the cyathia of *Euphorbia* spp. The associated bracts, or cyathophylls, vary in colour or may be absent (FIG. 3). Likewise, the number and the shape of the involucral glands, gland appendages and aggregated inflorescent units can vary and the cyathia can form with many planes of symmetry (PBI Euphorbia project, www.euphorbiaceae.org).

The fruits of *Euphorbia* spp. are encapsulated and typically contain three seeds that can vary in surface features, shape and size (FIG. 3). Once ripe, the fruit splits open and explosively

releases the seeds, some of which have a fleshy appendage (caruncle) (PBI Euphorbia project, www.euphorbiaceae.org).

Two prominent features of the genus *Euphorbia* are the variety of life forms (Fig. 4) and the different habitats that these plants occupy. This genus includes annual herbs, as well as plants with succulent photosynthetic stems. The largest number of *Euphorbia* plants are the succulent types, which have limited leaf cover, thorns and often well-developed underground tubers (PBI Euphorbia project, www.euphorbiaceae.org).

2.3. Use by humans and wildlife

Euphorbia spp. have considerable social, economic and ecological value. For example, there are hundreds of cultivars of Poinsettia (*Euphorbia pulcherrima* Willd. : Klotzsch), which is the most popular ornamental pot plant during the Christmas season in the United States, Europe and other countries (PBI Euphorbia project, www.euphorbiaceae.org).

Parts of *Euphorbia* plants have been used for their medicinal value. For example, *Euphorbia hirta* L. extracts are widely used in East and West Africa as well as Australia (Johnson *et al*. 1999). This medicinal extract is obtained by boiling the plant material and the resulting product is used for treating asthma, chronic bronchitis, as well as relieving hay fever and catarrh (Johnson *et al*. 1999). Similarly, the roots and leaves, as well as the stems of *Euphorbia fusiformis* Buch. Ham. : D. Don are used to treat various ailments including gout, arthritis and to relieve fever and inflammation (Natarajan *et al*. 2005). In Africa, the latex of certain *Euphorbia* spp., such as *E. cooperi* and *E. ingens*, is used as a glazing agent, lip balm and even to heal wounds (PBI Euphorbia project, www.euphorbiaceae.org).

The toxic properties of *Euphorbia* latex are used in various parts of the world as a poison for fishing and as an aid to hunting. The latex of *E. tirucalli* is preferred due to its high level of

potency and its ease of collection. The latex is either dropped into streams in small containers or made into poisonous mud balls using soil that are then thrown into the water. In some cases the succulent branches are submerged into the water using rocks as weights (Getahun 1976, Palgrave 2002, Neuwinger 2004). In West Africa, *Euphorbia poisonii* Pax. is used as a fishing poison by applying the latex on the tips of arrows which is then used to shoot the fish (Evans & Schmidt 1975). In Central Africa *Euphorbia* juices, for example those of *Euphorbia cereiformis* L., are mixed with other poisons, for example snake poison, to create a complex mixture that is applied to arrow heads used to hunt larger animals (Hall & Whitehead 1927).

The milky sap (latex) of *Euphorbia* spp. contains compounds known as terpene esters that are severe irritants with caustic properties. When the latex comes into direct contact with skin or even through aerosol exposure, this latex can lead to inflammation. It can also cause a temporary blindness when it comes into contact with the eyes. Certain species of *Euphorbia* produce latex that is potentially carcinogenic (PBI Euphorbia project, www.euphorbiaceae.org, Palgrave 2002).

The black rhinoceros (*Diceros bicornis* L.) and baboons are known to feed on certain *Euphorbia* spp. The *Euphorbia* spp. preferred by these mammals include *E. ingens*, *Euphorbia tetragona* Haw, *Euphorbia bothae* Lotsy & Goddijn and *Euphorbia triangularis* Desf. : A. Berger. Damage to the woody bases of these succulent euphorbias can also be caused by porcupines (*Hystrix cristata* L.). These animals apparently prefer to feed on *Euphorbia* spp. because of their high moisture and nitrogen content, especially during the dry season (Brown *et al*. 2003, Dudley 1997, Heilmann *et al*. 2006).

3. *EUPHORBIA INGENS*

3.1. Distribution and Habitat

Euphorbia ingens, first described by E. Meyer in 1843, is known only from Africa where it occupies a variety of habitats ranging from tropical to drier savanna and bushveld areas. These trees occur on numerous soil types as well as open veldt, ridges, rocky outcrops and even steep rock faces. This species is widely distributed across Southern Africa, occurring in Botswana, Mozambique, South Africa, Swaziland and Zimbabwe (FIG. 5) (Gildenhuys 2006, Palgrave 2002, Van Wyk & Van Wyk 1997). In South Africa, it is most abundant in the Limpopo Province but also occurs in the Gauteng Province, KwaZulu-Natal and the North West Province (Gildenhuys 2006).

3.2. Morphology

E. ingens trees have succulent crowns with sturdy wooden main stems, and when mature, they can reach heights of up to 10 meters. This massive size is reflected in the species name, "ingens", which is Latin for huge, vast or monstrous. This combination of woody bases and succulent branches is also reflected in the common Afrikaans name of the trees, "naboom" (close to/like a tree), which reflects the fact that its growth form is similar to a tree (FIG. 6). The branches consist of four to five angled segments with spines. Juvenile *E. ingens* (FIG. 6) differ from mature trees (FIG. 6) by being a darker green with a light green discoloration on the succulent stems. Before the woody trunk and branches develop, a juvenile will grow as a single stem plant (Gildenhuys 2006, PBI Euphorbia project, www.euphorbiaceae.org, Van Wyk & Van Wyk 1997).

The flowers of the genus *Euphorbia* are arranged in a bundle or inflorescence. In the case of *E. ingens*, the inflorescence consists of three upright liable cymes, each with three cyathia, one being male surrounded by two bisexual cyathia maturing after the male cyathium. After

pollination, the ovaries ripen to form a globose fruit (August) with a fleshy outer layer approximately one centimeter in diameter (FIG. 6) (Gildenhuys 2006, Malan 2006, Palgrave 2002).

3.3. Use by humans and wildlife

Baboons, black rhinoceros, giraffes (*Giraffa camelopardalis* L.) and vervet monkeys are particularly prone to feed on *E. ingens* trees. These animals target the highly nutritious succulent branches and the fruit (Brown *et al*. 2003, Dudley 1997, Heilmann *et al*. 2006, Mark Howitt, Pers. Comm.).

The latex of *E. ingens* is caustic and toxic. Regardless, this milky sap is still utilized by local cultures in traditional healing medicines and for fishing (Gildenhuys 2006). There have been many cases where overdoses have occurred that have resulted in severe abdominal pain and vomiting, and in extreme cases, death (Palgrave 2002).

4. DEATH OF *E. INGENS*

During the course of the last 15 years, *E. ingens* trees in the Limpopo Province of South Africa have begun to exhibit extreme symptoms of disease, which is followed rapidly by death. A survey of one area in this province, the Biodiversity Conservation Centre (NZG) of the National Zoological Gardens in Mokopane (Potgietersrus), in 2006 was unable to detect even a single healthy *E. ingens* tree (Malan 2006). As part of this survey, Malan (2006) also conducted a detailed evaluation of the health status of *E. ingens* and assessed several factors that may have affected the health of the trees. These included herbivory, fire damage, temperature, and rainfall. The author also conducted an evaluation of overall tree condition and recruitment. A transect, 900 m by 50 m, including a total of 225 *E. ingens* trees, was evaluated. The trees were placed into four disease categories (dead, sickly 1, sickly 2 and

vigorous). Trees in early stages of decline exhibited a general greying of the succulent branches. Trees in the most advanced stage of decline had died and collapsed completely. All trees in the transect were found to be in some stage of decline with 25% already dead at the time of the evaluation (Malan 2006). Additionally, Malan (2006) found that all but one tree, in the transect, was a juvenile.

5. POTENTIAL FACTORS INFLUENCING THE *E. INGENS* DEATH

5.1. Insects and pathogens

Limited information is available regarding insects and diseases of *Euphorbia* spp. This is especially true for species with a succulent growth form and for those occurring in Africa. The few existing reports of insect and disease problems have not been published in the scientific literature and identifications of causal agents have not been confirmed by experts. Cork-like growths on the epidermis of plants have been ascribed to natural aging or to exposure to sunshine of previously shaded plants. Recorded insect problems include mealy bugs (Pseudococcidae), red spider mites (Tetranychidae) and *Sciara* flies. Microbial problems reported from *Euphorbia* include root rot ascribed to *Fusarium*, mildew, black mould associated with feeding of moths (Pyralidae), and the tobacco mosaic virus (IES, www.euphorbia-international.org).

Roux *et al*. (2008, 2009), conducted pilot studies at the same site used by Malan (2006) to further test some of the conclusions made by Malan (2006), but with a specific focus on the potential involvement of insects or pathogens. Samples were collected from diseased and dying *E. ingens* trees over three periods. The authors described a similar greyish discoloration (FIG. 7) as reported by Malan (2006). However, they also noted internal symptoms such as rotting and browning of the succulent branches, white and yellow spots on

the exterior of succulent branches, blue stain of the stem tissue and insect infestation (Roux *et al*. 2008, 2009).

Various genera of fungi were isolated from dying *E. ingens* trees at Mokopane by Roux *et al.* (2008, 2009). These included species of *Cibiessii* (white and yellow spots), *Fusarium* spp. and *L. theobromae*, which was associated with blue stain in the main trunks and brown lesions on succulent branches. Undescribed Ophiostomatoid fungi were also isolated including undescribed species of *Gondwanamyces* and *Graphium,* and a fungus thought to represent an undescribed genus (Roux *et al.* 2008, 2009).

Insects found by Roux *et al.* (2008, 2009) included members of the Curculionidae (mainly ambrosia beetles), that attack weak or recently killed trees. These beetles excavate tunnels in the main stems of trees and are mutualistically associated with fungi upon which they feed as a sole food source (Paine *et al.* 1997, Farrell *et al.* 2001). The ophiostomatoid fungi detected in this study were isolated from these beetles. The other main insect group found on *E. ingens* were moths. The insect responsible for feeding damage on the succulent branches was identified as a moth in the genus *Megasis* Guenee (Pyralidae, Lepidoptera).

Very limited information is available on the genus *Megasis*. Personal communication with Dr Martin Kruger (Ditsong National Museum of Natural History, Pretoria, South Africa) supplied the following information:

(i) Two species in this genus were described in the late 1800's and early 1900's, however, no recent revision of the genus has been done and there is uncertainty as to whether these two species still reside within *Megasis*.

(ii) There is no indication that this moth has been introduced to South Africa and it is likely to be native.

(iii) All records of plant hosts for species in *Megasis* are from Euphorbiaceae. Damage to *E. ingens* was observed in the 1990's by L.E.O. Braack, associated with *Megasis*, in the Kruger National Park in South Africa.

A parasitoid in the genus *Syzeuctus* (Banchinae, Ichneumonidae) was found associated with the *Megasis* samples collected by Roux *et al*. (2008, 2009). This wasp is likely to be an unidentified species (pers. Comm. Dr. Simon van Noort, Iziko Museums, Cape Town, South Africa). Other members of *Syzeuctus* are important in the control of African cereal stem borers (Pyralidae) (Muli *et al*. 2009).

5.2. Wildlife impacts

The lack of regeneration and decline of adult *E. ingens* in Mokopane was suggested to possibly be due to the impact of wildlife in the reserve (Malan 2006). Baboons, giraffes and vervet monkeys eat the fruit of these trees, preventing the development and release of seed into the environment (Heilmann *et al*. 2006, Malan 2006). Heavy feeding on *E. ingens* by these animals might explain the lack of juvenile trees.

5.3. Disturbance

Malan (2006), excluded fire damage, erosion and drought as causes of the *E. ingens* death at Mokopane (Malan 2006). No fire had occurred in the study area since 2001 and soil erosion does not occur extensively enough in this area to have an effect on these trees. The rainfall between 2000 and 2005 exhibited an overall decline. However, rainfall during this period was highly variable; during 2004 rainfall was double that experienced in 2003, but extremely low in 2005 (FIG. 8) (Malan 2006). The rainfall data on its own does not indicate whether any change in precipitation was correlated with the incidence of decline and mortality at the site.

Longer term data are required to more conclusively associate these factors with the decline of *E. ingens* at Mokopane.

Studies conducted by Roux *et al*. (2008, 2009) and Malan (2006) respectively, did not provide concrete answers regarding the cause of decline and death of *E. ingens*. The prevalence of fungi, such as those in the Botryosphaeriaceae, as well as ambrosia beetles, both of which are known to respond to stress in host trees, suggests an abiotic environmental trigger to the decline. In the next section of this review, the impact of climate change on trees is discussed in more detail, with the aim of providing a foundation for more detailed future studies into its potential role in the decline and death of *E. ingens* in South Africa.

6. CLIMATE CHANGE

Climate change is affecting southern Africa to a greater degree than most other parts of the planet (Du Plessis *et al.* 2003, Boko *et al.* 2007) and is expected to increase greatly in its impacts over the next few decades. Given the lack of conclusive evidence that pathogens and insects are playing primary roles in the die-off of *E. ingens*, climate change is a likely driving factor. It is predicted that there will be an increase in winter and summer temperatures by three to seven degrees Celsius by the year 2100 (Boko *et al.* 2007, Houniet *et al.* 2009). Minimum temperatures will more likely have a higher increase than maximum temperatures and this will lead to more significant changes in temperature during winter than summer (Du Plessis *et al.* 2003). It is also predicted that rainfall will increase or decrease, depending on region, by 20% during the 21st century (Boko *et al.* 2007, Houniet *et al.* 2009). With climate change leading to higher temperatures and unpredictable rainfall, the range of occurrence of some pathogens in South Africa will increase. This is of great concern to not only the forestry industry in South Africa but also the native environment (van Staden *et al.* 2004).

6.1. Possible effects of a changing climate on *E. ingens*

Forest ecosystems are influenced by disturbances that play an integral role in their structure and function. Natural disturbances maintain the structure and function of forests over time. However, when disturbances are unnatural or when natural disturbances are altered in their severity or occurrence, they may act to disrupt and alter communities and ecological processes and services resulting in negative cascading effects that ultimately may result in permanent alterations (Bergeron & Leduc 1998, Dale *et al*. 2001, Logan 2003). Climate change can alter disturbance regimes in such a way that they occur outside of their natural range of variation. These disturbances include fire, and insect and pathogen outbreaks (Dale *et al.* 2000, Dale *et al*. 2001, Logan 2003, Williamson *et al*. 2009, Sturrock *et al.* 2011).

Rainfall and temperature are two major components of climate. Increased rain followed by warm dry periods can have severe impacts on forest ecosystems. It can serve to act in favour of certain pathogens, and may result in increased stress to trees, making them more susceptible to fungal infection/impact (La Porta *et al*. 2008, Dukes *et al*. 2009, Tubby *et al*. 2010). Various organisms and plant roots rapidly deplete the gaseous oxygen in waterlogged soils. This creates an anoxic environment where the carbon dioxide levels are very high and various compounds become soluble in the soil in their toxic forms. Within the plant roots respiration occurs without oxygen, retarding energy utilization and leading to reduced mineral and water uptake, resulting in severe stress to plants and eventual death (Sinclair and Lyon 2005). During this period plants are also increasingly susceptible to disease (Sinclair and Lyon 2005). High levels of free moisture in the soil is favourable for the germination of, for example *Phytophthora* spp., affecting their dispersal and infection of plant roots due to the water acting as a medium for the dispersal of the pathogen's zoospores (Sinclair and Lyon

2005). Infection by *Phytophthora* spp. results in root death that becomes especially pronounced under conditions of high temperatures and dry conditions (Frankel 2007, 2008).

Higher temperatures during winter pose a threat to tree health. Reports of tree decline, as a result of higher winter temperatures have already been noted in Europe and North America (Brasier 1996, Breshears *et al.* 2005). These changes in climatic conditions favour pathogens and pests, posing a great risk to forest ecosystems (Dale *et al.* 2001), due to the additional physiological stress on the plants as a result of more water being lost by transpiration than absorbed by roots from the dry soil (Sinclair & Lyon 2005, Stone *et al.* 2007, Sturrock *et al.* 2011).

6.2. Examples of climate change impact from the Northern Hemisphere

A number of examples of climate change driven increases in tree mortality have been observed in Europe, Canada and the USA. Changes in climate has as yet not been explored in terms of its impact on the symbiosis between tree hosts, insects and pathogens, but is most probably the biggest threat to ecosystems globally (Brasier 1996, Dale *et al.* 2001, Breshears *et al.* 2005, Allen 2009, Six 2009). These examples from the Northern Hemisphere may provide important clues for the identification of the factors driving the die-off of *E. ingens* in South Africa.

Global warming predictions suggest that between the mid 1990s and 2050, the mean temperature in Europe will increase by 1.5 - 4.5˚C (Pearman & Jäger 1988, Brasier 1996). The increase in temperature will also be accompanied by erratic rainfall periods. This type of climatic change is of great concern in Europe as it favors the activity of plant pathogens such as *P. cinnamomi* Rands (Brasier & Scott 1994, Brasier 1996, Bergot *et al.* 2004, Davidson *et al.* 2005). Higher temperatures will increase inoculum production, while higher rainfall

during warm periods will lead to prolonged wet soils which will alter and increase the distribution of *P. cinnamomi* (Brasier 1996).

Unusually wet seasons in Europe have been blamed for the decline and death of European beech (*Fagus sykvatica* L.). An unusually wet season was followed by a dry season, in this case triggering disease caused by various species of *Phytophthora* (Jung 2009). In 2002, Bavaria experienced a very wet season that was followed by a dry period in 2003. Between 2003 and 2007, 86.6% of beech trees were affected by *Phytophthora*- type disease symptoms in a selection of 134 beech stands. It was found that approximately 80% of the trees in the affected stands were infected with *Phytophthora* spp. In total, 11 species of *Phytophthora* were found, including *P. cambivora* (Petri) Buisman and *P. citricola* Sawada (Jung 2009). All evidence from the decline of oaks in Europe due to infection by *Phytophthora* spp. suggest that this epidemic is driven by changes in climate, acting as a catalyst (Duniway 1977, Jung *et al.* 2003). Jung (2009), hypothesized that during the wet season, the fine root hairs of the trees became infected by the pathogen and due to the waterlogged soils and widespread occurrence of high numbers of zoospores even larger roots and the bark of mature trees become infected. The dry spell that followed placed severe pressure on the trees which were unable to regenerate their fine root hair systems, leading to leaf fall and eventual tree death (Jung 2009).

It is not only pathogens that have been affected by increased temperatures. The continued trend of increasing temperatures throughout the Mediterranean basin has affected the defoliator moth, *Thaumetopoea pityocampa* Schiff. This moth attacks *Pinus* and *Cedrus* spp. and is known to occur as far as the Northern Italian Alps (Netherer & Schopf 2010). Warmer temperatures during winter eliminate lethal survival temperature ranges and have improved the performance of the moth larvae (Netherer & Schopf 2010). The distribution of *T. pityocampa* has also been altered and it now occurs closer to highly elevated *Pinus*

plantations (Pennerstorfer *et al*. 2005, Battisti *et al*. 2005, Netherer & Schopf 2010). It has been found that *T. pityocampa* is starting to occur and attack trees in previously unfavorable areas within the Paris basin (Netherer & Schopf 2010). *T. pityocampa* is also infesting new hosts to which it had not previously been exposed and that do not have natural resistance to it (Netherer & Schopf 2010).

Severe changes in rainfall and temperature in British Columbia (BC) has led to a devastating needle blight epidemic on *Pinus contorta* Douglas ex Loudon (Woods 2003, Bradshaw 2004, Woods *et al.* 2005). Needle blight is caused by *Dothistroma septosporum* (Dorog.) M. Morelet and is a very important disease of Pine trees in temperate forests worldwide (Harrington & Wingfield 1998, Bradshaw 2004, Woods *et al.* 2005, Barnes *et al.* 2007). The current epidemic in BC can be attributed to the occurrence of warm rain spells throughout the 1990's, leading to more favorable climatic conditions for *D. septosporum* to thrive in its native habitat (Woods *et al.* 2005).

Warmer winters in Canada have led to increased pressures on forests, especially from their natural insect pests. *Pinus contorta* is the preferred host of the native mountain pine beetle (*Dendroctonus ponderosae* Hopkins) in western Canada. In the past century, climate change has played a vital role in British Columbia (BC) in terms of influencing this insect-host interaction. There has been an increase in the overall temperature in BC leading to warmer winters (Konkin & Hopkins 2009). This has led to the mountain pine beetle having an increased survival rate as well as to it expanding its natural geographic range, giving rise to the biggest epidemic for any bark beetle in the recorded history of the world. The mountain pine beetle in Canada has already affected an estimated half a billion cubic meters of timber and it is predicted that by 2015 the epidemic will have killed more than three quarters of the pine volume of BC (900 million cubic meters of timber) (Konkin & Hopkins 2009, Kurz *et al.* 2008). Certain areas in western USA have been experiencing increased temperatures and

droughts combined with outbreaks of *D. ponderosae*, which is thought to have led to severe decline of *Pinus albicaulis* Engelm (Logan & Powell 2001).

In northern California in 2006, heavy rain fell over a period of 25 days. This was followed by the longest continuous period of hot dry weather in the history of the state. This resulted in severe sudden oak death outbreaks caused by *Phytophthora ramorum* Werres, De Cock & Man in't Veld (Frankel 2007, 2008). *P. ramorum* is known to have deciduous sporangia that can be dispersed with rain splashing and windblown rain. During the rainy spell, the oxygen deprived stressed trees become more susceptible to *P. ramorum* attack and the increased dispersal of this pathogen leads to higher infection rates. This pathogen will attack the roots, the root collar and the vascular system of the trees. During the following dry season, the trees will struggle with the high demand of water need/conductance due to the tissue death caused by *P. ramorum*, resulting in high levels of mortality (Sinclair and Lyon 2005, Frankel 2007, 2008).

The impact of climate change on mutualistic symbiotic interactions, including those between trees, insects and micro-organisms is complex and difficult to define (Six 2009). Changes in this intricate interaction can, however, have significant impact on species distribution and forest health globally (Dale *et al.* 2001, Six 2009). *Dendroctonus ponderosae*, for example, carries two known symbiotic fungi, *Ophiostoma montium* (Rumbold) Arx and *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M. J. Wingf. (Six & Bentz 2007). It has been shown that these symbionts have different optimal growth temperatures, with *O. montium* flourishing at higher temperatures while *G. clavigera* prefers lower temperatures. The presence of two different symbionts will allow *D. ponderosae* to occur in various environments, thus surviving temperature alterations leading to more severe epidemics (Six & Bentz 2007).

Die-back of deciduous oak trees, *Quercus crispula* Blume, has been recorded in Japan as early as the 1930's. The occurrence has never been considered a serious problem up until the 1980's (Ito *et al.* 1998, Ito & Yamada 1998, Kamata *et al.* 2002). It has been found that in the last 30 years the epidemics have occurred for longer periods of time and in previously unrecorded areas (Ito & Yamada 1998, Kamata *et al.* 2002). Climate change is most likely the major driver in leading to an increase in oak death, resulting in considerable concern for the survival of these trees in Japan (Ito & Yamada 1998, Kamata *et al.* 2002).

Oak decline in Japan, linked to climate change, has been attributed to *Raffalea quercivora* Kubono et Shin and the ambrosia beetle, *Platypus quercivorus* Murayama (Kamata *et al*. 2002). The distribution of *P. quercivorus* has altered due to a 0.4 ˚C increase in temperature, in Japan, since the 1980's (Kamata *et al.* 2002). Not only has the distribution of this beetle become more widespread but it has also started to attack hosts of greater susceptibility, for example *Q. crispula*. The high susceptibility of *Q. crispula* to this fungal – beetle interaction can be due to their lack in co-evolution because of previous differences in their distribution before the drastic changes in climate (Kamata *et al.* 2002).

7. CONCLUSIONS

Euphorbia ingens trees are dying dramatically in the Limpopo Province of South Africa. However, the cause of the mortality is not known. It has been suggested that the trees are dying due to climate change or due to fungal or bacterial pathogens. No data, however, exist to substantiate any of the suggestions, or on which to build possible management plans. The lack of knowledge on *E. ingens* decline is exacerbated by the fact that very limited information is available on the fungi and insects of native trees in Africa (Crous *et al*. 2006). This is especially true for species in the genus *Euphorbia*, for which there is very limited information regarding their pests and diseases**.**

The aim of the research conducted in this dissertation will be to provide information to promote a clearer understanding of the decline and death of *E. ingens* in the Limpopo Province of South Africa. This will be done by expanding on the pilot studies conducted by Malan (2006) and by Roux *et al.* (2007, 2008). Information will be gathered regarding symptom/disease progress and the major fungi and insect pests associated with *E. ingens* in the Limpopo Province. Secondly, the abiotic factors that might contribute to the decline and death of *E. ingens* in the Limpopo Province will be investigated. The same abiotic factors will also be considered at sites where *E. ingens* seem to be healthy. It is hoped that this information will provide a foundation for further studies to understand the death of *E. ingens*, and if appropriate, the development of management strategies to reduce the incidence of the decline.

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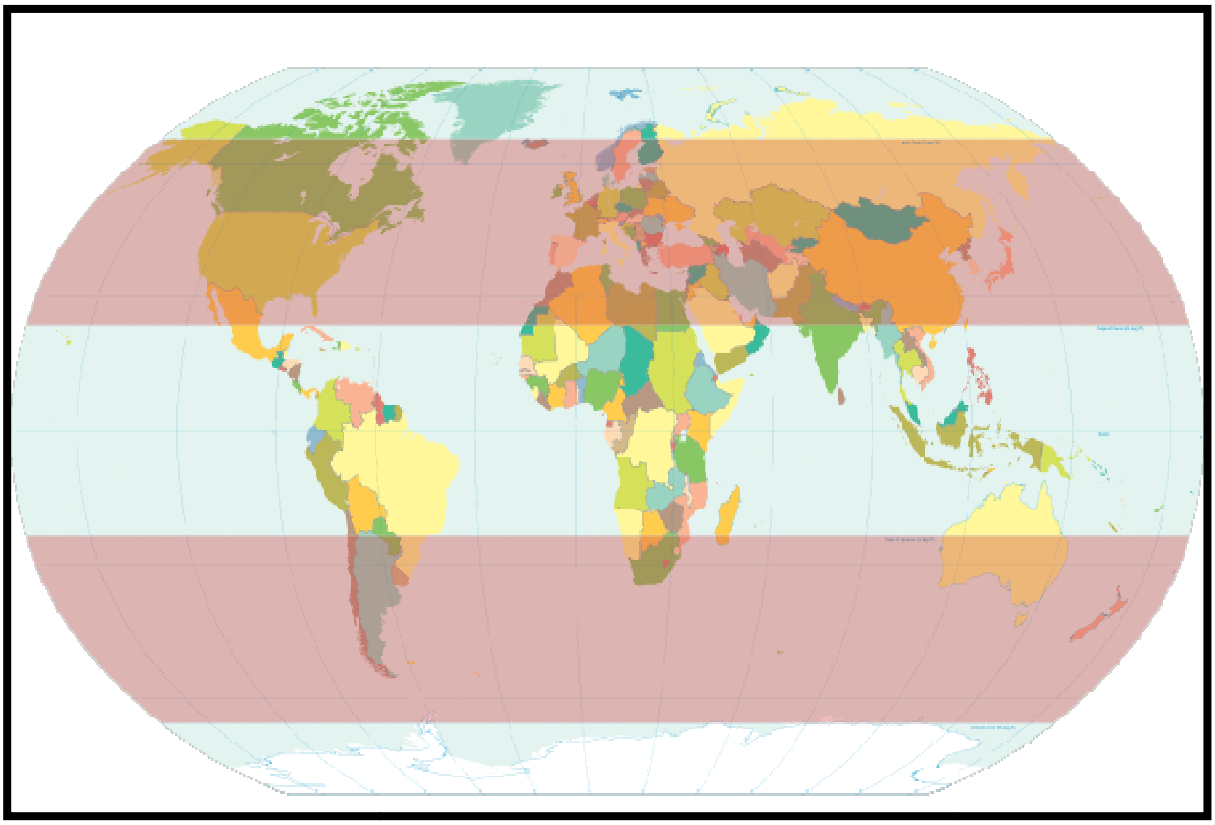

FIG. 1. World map indicating the temperate zones shown by the red bands. (www.uwsp.edu)

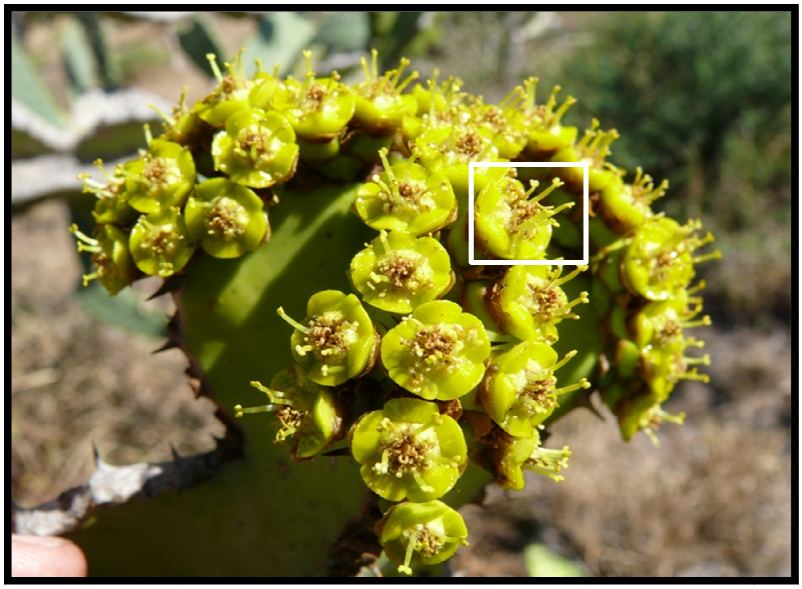

FIG. 2. The cyathium of *Euphorbia cooperi*, indicated by the white block.

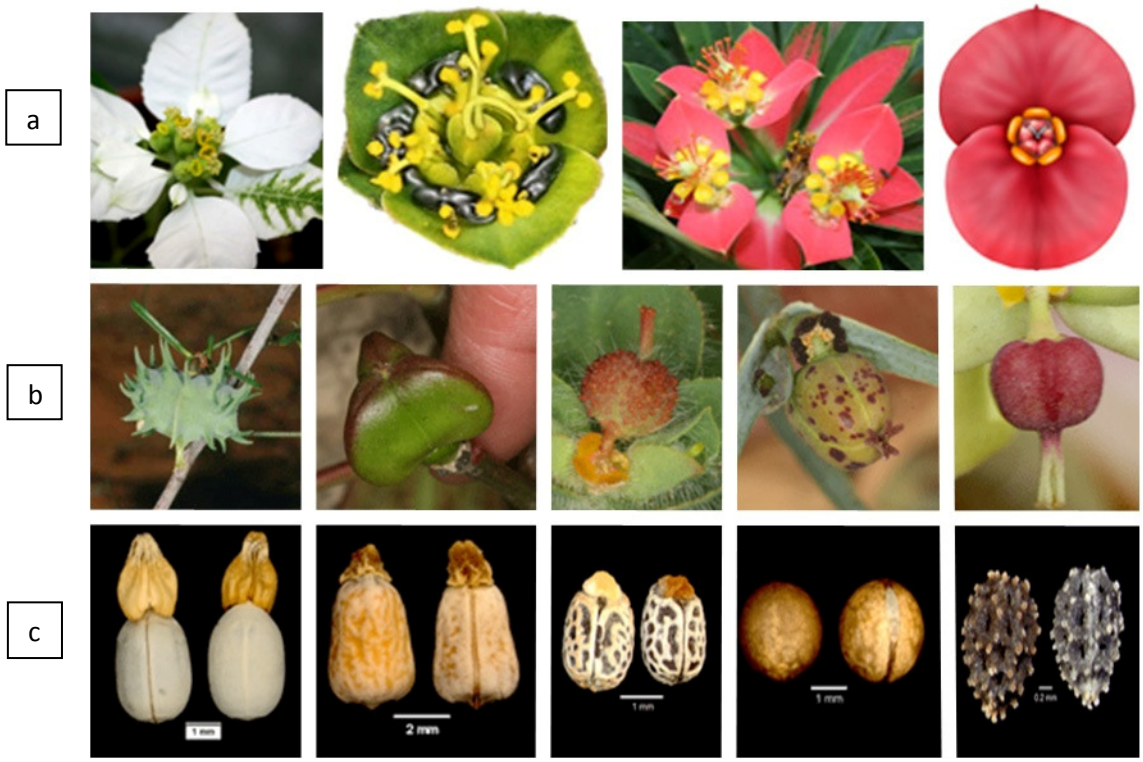

FIG. 3. Variation of the flowers, fruit and seed of *Euphorbia*. (a) Variation of the associated bracts or cyathophylls (PBI Euphorbia project). (b), (c) Variation of the fruit and seeds within the *Euphorbia* genus (PBI Euphorbia project).

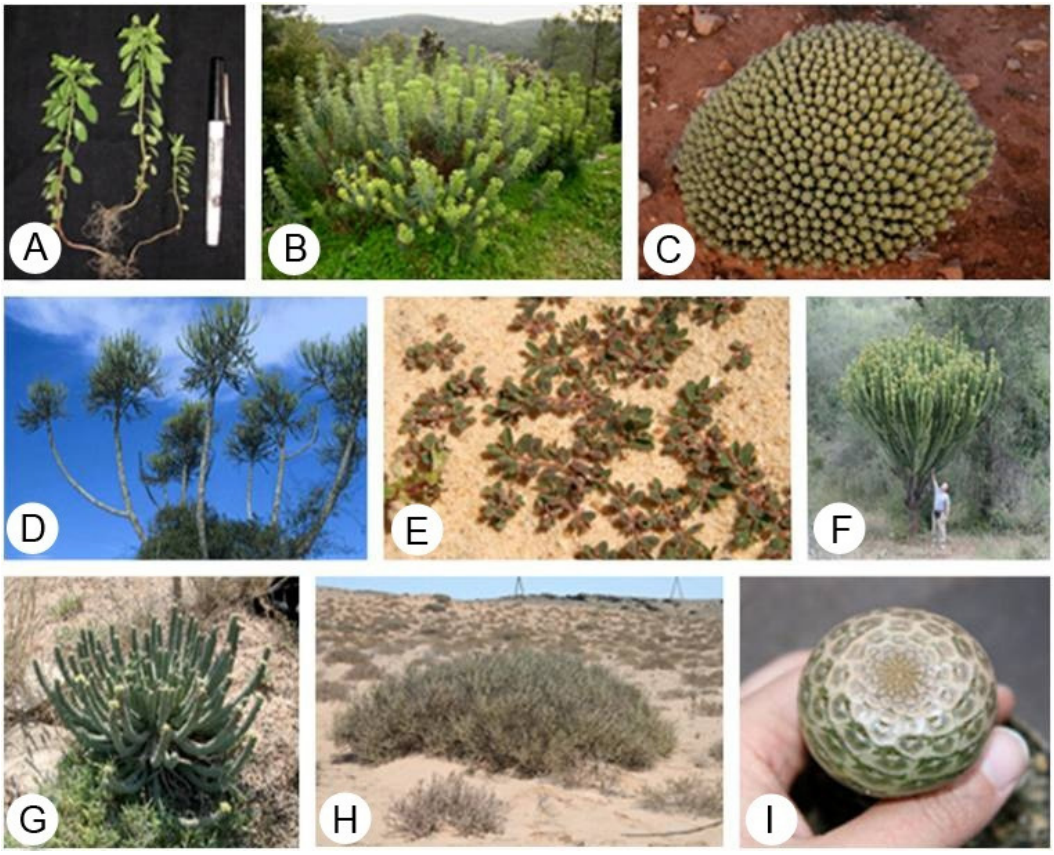

FIG. 4. Variety of life forms within the *Euphorbia* genus, ranging from herbs (a, b, e), succulent shrubs (c, g, h), succulent tree like plants (d, f) and bulbous succulents (i) (PBI Euphorbia project).

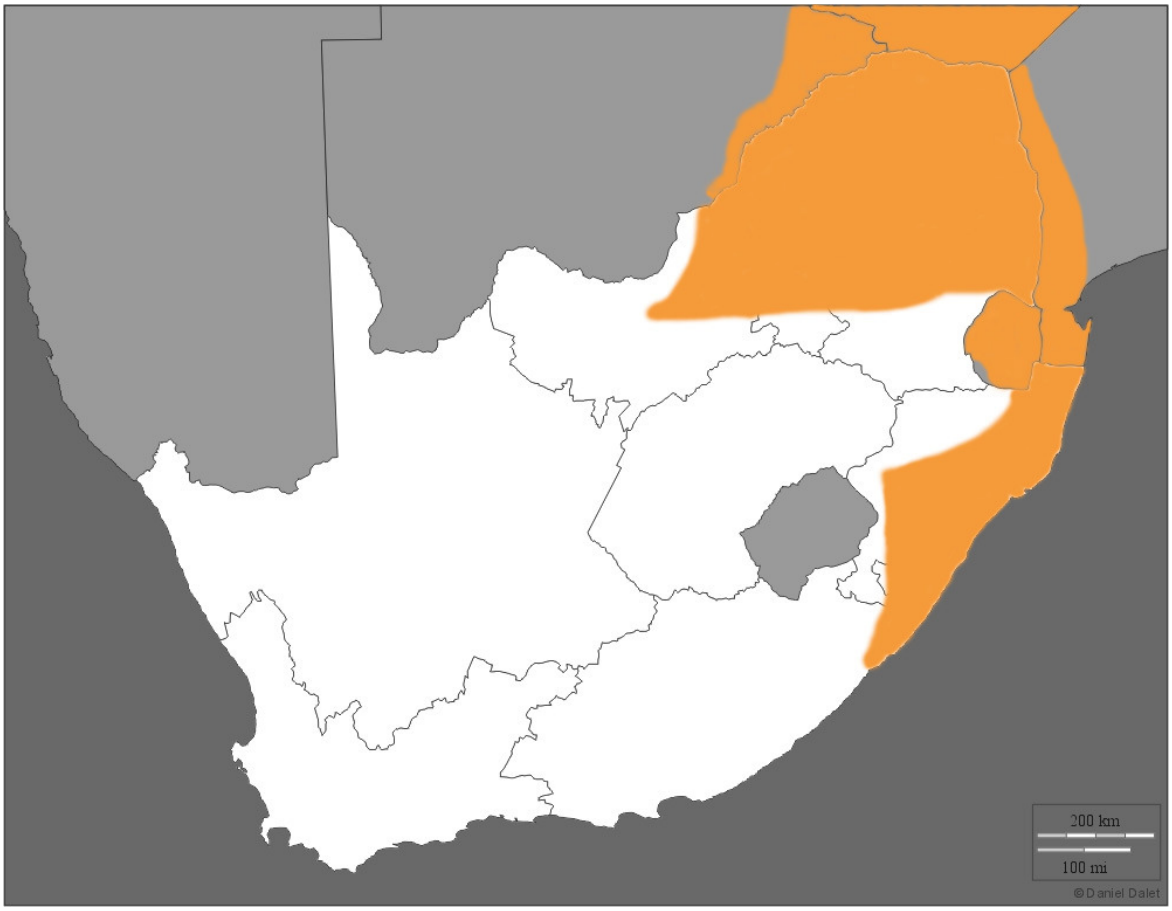

FIG. 5. Map of Southern Africa indicating the areas where *Euphorbia ingens* occur, illustrated by the orange shading (Palgrave 2002, Van Wyk & Wyk 1997).

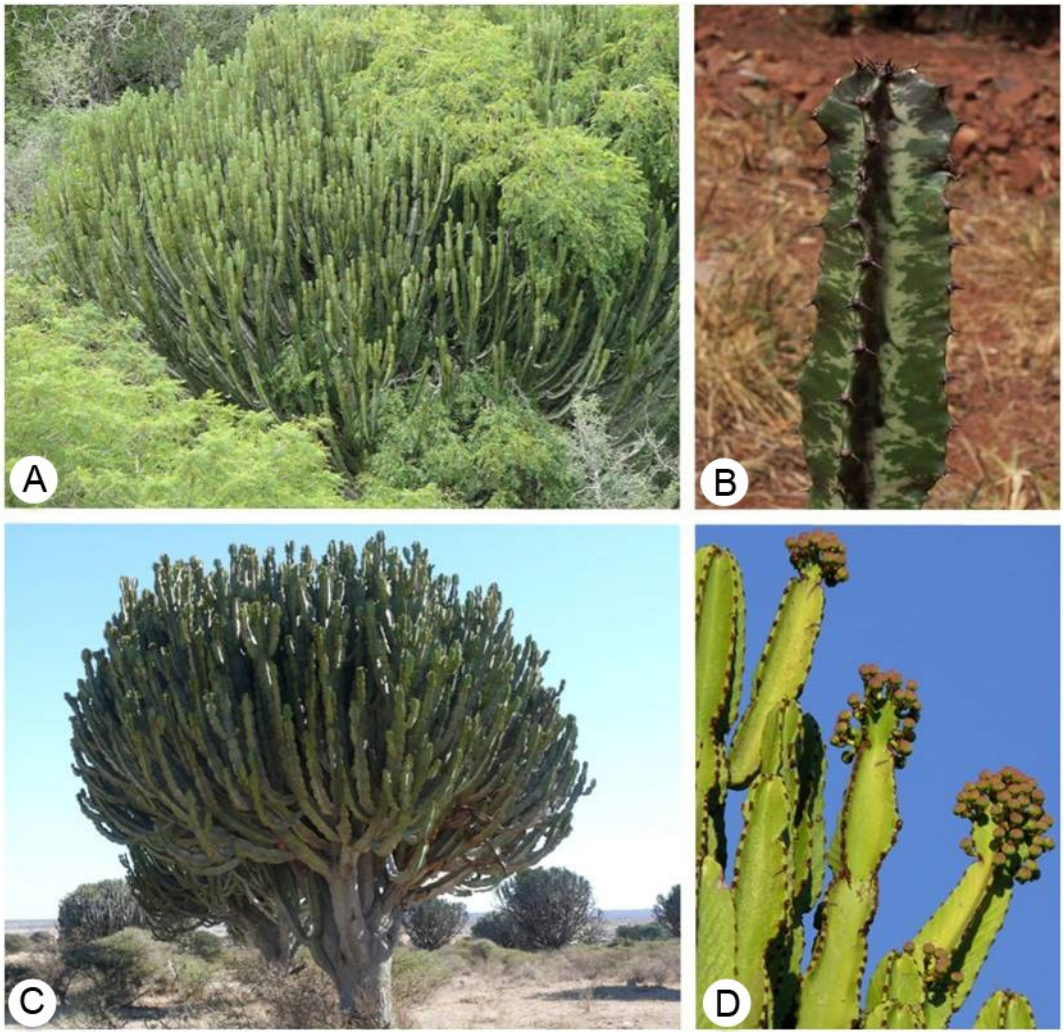

FIG. 6. Morphology of mature and juvenile *E. ingens* trees. (a) A healthy mature *E. ingens* tree. (b) A juvenile *E. ingens* with characteristic light green discoloration and four segments (Gildenhuys 2006). (c) A mature *E.ingens* tree with main woody stem supporting succulent branches. (d) Fruit of mature *E. ingens* tree.

FIG. 7. *E. ingens* tree showing grayish discoloration.

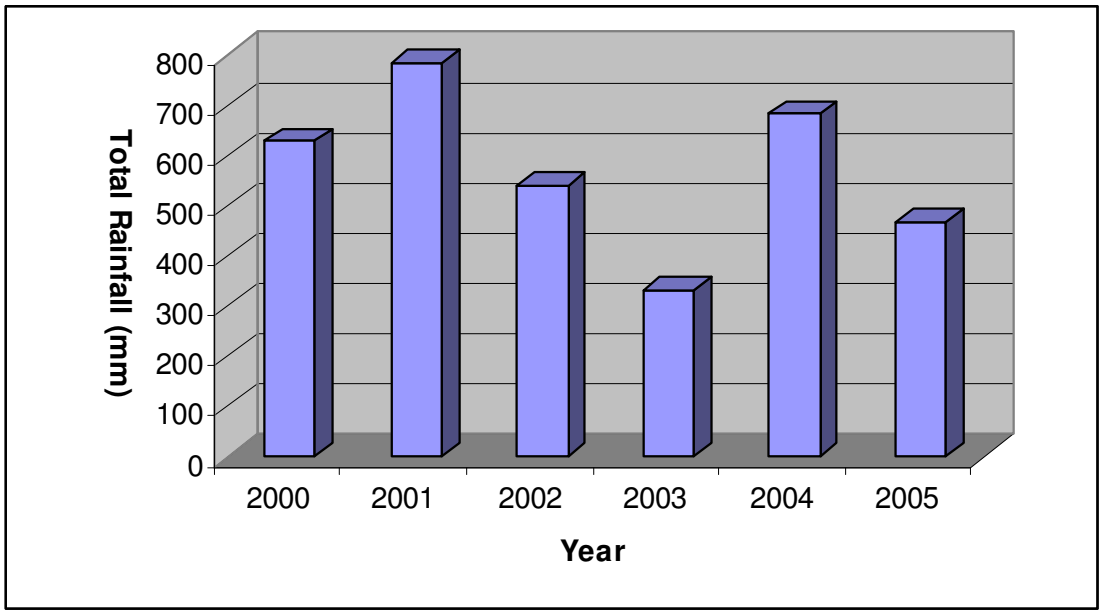

FIG. 8. Variation in total Rainfall for Mokopane from 2000 to 2005 (Malan 2006).

Chapter 2

New species of *Gondwanamyces* from dying *Euphorbia* trees in South Africa

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This chapter has been submitted for publication as: Van der Linde JA, Six DL, Wingfield MJ, Roux J. New species of *Gondwanamyces* from dying *Euphorbia* trees in South Africa. (Accepted by *Mycologia*, in press)

ABSTRACT

Gondwanamyces and its *Custingophora* anamorphs were first described from *Protea* infructescences in South Africa. Subsequently, these unusual fungi were also found on *Cecropia* in Central America. During the course of an investigation into the decline and death of native *Euphorbia* trees in South Africa, several fungal isolates resembling the anamorph state of *Gondwanamyces* were obtained from diseased tissues. In this study, these isolates are identified based on morphology and comparisons of DNA sequences. Two previously unknown *Gondwanamyces* species were identified, one of which was associated with damage caused by a beetle (*Cossonus* sp.). Inoculation studies showed that the new species of *Gondwanamyces* are pathogenic on *Euphorbia ingens* and may contribute to the decline of these trees.

Keywords: Tree diseases, Cossonus, Custingophora, Euphorbia ingens, Euphorbia tetragona, Insect-fungus interactions, Knoxdavesia

1. INTRODUCTION

The genus *Gondwanamyces* Marais & M.J. Wingf was established in 1998 for two species of fungi collected from the infructescences of native *Protea* plants in the Cape Floristic Region (CFR) of South Africa (Marais *et al.* 1998). These fungi were described as having anamorphs in a new genus, *Knoxdaviesia* (Wingfield *et al.* 1988, Wingfield & Van Wyk 1993), but DNA sequence data later showed that they could be accommodated in *Custingophora* (Kolařík & Hulcr 2009). *Custingophora* species have mononematous conidiophores that terminate in obovoid conidiogenous cells with distinct collarettes and conidia. The *Gondwanamyces* teleomorphs are characterized by ascomata similar to those of species of *Ceratocystis* and *Ophiostoma*, with globose ascomatal bases and long necks bearing ascospores in slimy masses (Marais *et al.* 1998). Phylogenetic studies based on DNA sequences have shown that these fungi reside in the Microascales and are closely related to but distinct from species of *Ceratocystis* (Marais *et al.* 1998, Zhang *et al.* 2006).

Until recently, *Gondwanamyces* spp. was known only from Southern Africa, however, Kolařík **& Hulcr (2009) described two more species, *Custingophora cecropiae* M.** Kolařík (no sexual state found) **and *G. scolytodis* M.** Kolařík **from** *Cecropia angustifolia* Trécul in Costa Rica. The discovery of *Gondwanamyces* on native trees in the **Neotropics in Central America calls to question a previous hypothesis that these fungi are specific to the southern hemisphere (Roets *et al.* 2009a).**

Species of *Gondwanamyces* are the dominant fungi in the infructescences of many *Protea* spp. in the CFR. One species, *Gondwanamyces proteae* Marais & M.J. Wingf., **is specific to the infructescences of *Protea repens* L. (Wingfield *et al.* 1988). In contrast, *Gondwanamyces capensis*** Marais & M.J. Wingf **is found in the floral parts of many *Protea* spp.** (Wingfield & Van Wyk 1993)**. Recently, it has been shown that *Ophiostoma* spp.**

occurring on *Protea* are vectored by mites and that *Gondwanamyces* might also be dispersed by the same vectors in a complex association with *Protea* spp. in the CFR (Roets *et al.* 2007, 2009b, 2011). *C. cecropiae* from Costa Rica is associated with a scolytine beetle, *Scolytus unipunctatus* Blandford, but whether it is also associated with mites is unknown.

Diseased and dying *Euphorbia ingens* E. Meyer: Boissier trees were observed in the Limpopo Province in the 1990s (Malan 2006, Roux *et al*. 2008, 2009). These trees are native to Africa and represent, one of the largest succulent *Euphorbia* species. The species consists of a main woody stem supporting cactus-like succulent branches (Van Wyk & Van Wyk 1997, Palgrave 2002, Gildenhuys 2006). The succulent branches can reach heights of up to 10 m and contain a milky latex that has caustic and toxic properties (Van Wyk & Van Wyk 1997, Palgrave 2002, Gildenhuys 2006). During investigations to determine the cause of death of these trees, fungal fruiting bodies resembling those of species of *Gondwanamyces* were observed on diseased plant material, as well as in the tunnels of weevils (Scolytinae: Curculionidae) infesting these trees (Roux *et al.* 2009). The aim of this study was to identify these fungi using DNA sequence comparisons and morphology. Inoculation experiments on *E. ingens* were also undertaken to consider the possible role of these fungi in the decline and death of these trees in South Africa. We also included isolates from diseased *Euphorbia tetragona* Haw trees in the Eastern Cape Province, which were collected from insect tunnels in diseased plant material.

2. MATERIALS AND METHODS

2.1. Collection of samples and isolations

Isolates used in this study were obtained from two different *Euphorbia* spp. growing in climatically different areas of South Africa. Isolates from *E. ingens* trees were collected

during the summer and winter of 2009 and the summer of 2010 at four different sites in the Limpopo Province. Isolates from *E. tetragona* were collected in March 2010 in the Great Fish River Nature Reserve near Grahamstown in the Eastern Cape Province. All isolates were obtained from diseased plant material, insect tunnels and directly from insects associated with the dying trees.

Isolations were made directly from fungal fruiting bodies observed on wood placed in moist chambers. Freshly produced spore drops were removed from conidiophores and ascomata using a sharp dissection needle and placed on 2 % malt extract agar (MEA; 15 g agar and 20 g malt extract per 1000 mL distilled water; Biolab, Merck, Midrand, RSA) containing streptomycin sulphate (0.4 g/L; Sigma-Aldrich, St. Louis, USA). For isolations directly from insects, the insects were crushed in 100 µL sterile distilled water using a plastic pestle, after which a series of dilutions were made (10^{-2} and 10^{-3}) and streaked onto water agar (WA; 15 g per 1000 mL distilled water; Biolab). The Petri plates were incubated at 20 C for up to six d and single hyphal tips or spore drops of resultant fungi were transferred to MEA. Isolates were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and representative isolates were also deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS) based in Utrecht, Netherlands. Herbarium material of the new species was deposited with the National Fungal Collection (PREM) Pretoria, South Africa.

2.2. Morphological Characteristics

Pure cultures of *Gondwanamyces*-like isolates were incubated on MEA at 20 C under near-ultra violet light to stimulate growth and fruiting body production. After 10 d, cultures were examined and fruiting bodies mounted on glass microscope slides in 75 % lactic acid. A Zeiss microscope (Carl Zeiss Ltd., Germany) was used to examine and study the fungal structures

and digital images of structures were taken with an Axiocam digital camera, with Axiovision 3.1 software, mounted on the microscope. Colors of colonies and morphological characters were determined using the color notations of Rayner (1970). Fifty measurements were made of each fungal character and are presented as (min -) ave. ± std. dev. (- max) for the length and width of the structures (l × w) representing the minimum, maximum, standard deviation (SD), mean values and the length/width (l/w) ratios.

2.3. Growth studies

Growth studies were conducted to determine optimum growth temperatures. The study was conducted at temperatures ranging from 10 C to 35 C at 5 C intervals. Five mm disks from six-day-old colonies on MEA were placed at the centres of 90 cm Petri plates containing MEA, with the mycelium side of the disks placed flat on the agar. The plates were incubated in the dark for 10 d with growth measured at 24 h intervals. For each plate, two diameter measurements perpendicular to each other were made resulting in a total of 10 measurements for each isolate at each temperature. The experiment was repeated once, under the same conditions. A student's t test was conducted, $P < 0.05$ designated as significant, to determine significant differences in growth rates between the different fungi at each of the selected temperatures.

2.4. DNA extraction and PCR

Cultures were grown for 14 d on MEA to obtain material for DNA extraction using the protocol described by Möller *et al.* (1992). Conidiophores were removed from the surfaces of MEA plates using a sterile scalpel and placed in 2 mL Eppendorf tubes and freeze-dried for 24 h. DNA was then extracted and concentrations measured using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

The internal transcribed spacer (ITS) regions (ITS1, ITS2) and the 5.8S gene were amplified using the primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) with an Applied Biosystems Veriti thermocycler (Applied Biosystems, Foster City, USA). The 25 µL PCR reactions consisted of 0.2 µL Super-therm polymerase (5 u/µL) (Hoffmann-La Roche, Nutley, USA), 2.5 µL (2.5 mM) dNTPs (Fermentas, Vilnius, LIT), 0.5 µL (25 mM) $MgCl_2$ (Roche Diagnostics, Mannheim, DE), 2.5 µL 10 × PCR buffer with $MgCl_2$ (Roche Diagnostics), 0.5 µL (10 mM) of each primer (InqabaBiotec, Pretoria, RSA), 16.3 µL sterile distilled water (Adcock Ingram, Bryanston, RSA) and 2.0 µL of DNA at a concentration of 10 ng/µL. Confirmation of amplified products was made on 2 % agarose gels (Whitehead Scientific, Cape Town, RSA) loaded with GelRed (Anatech, USA) under UV illumination. To estimate the sizes of the PCR products, a 100 bp DNA molecular marker ladder was used (O'RangeRuler™ 100 bp DNA ladder, Fermentas Life Sciences). Amplified products were purified for sequencing using Sephadex G-50 columns (1 g in 15 mL distilled water; SIGMA, Steinheim, DE) following the manufacturer's instructions.

2.5. Sequencing and phylogenetic analyses

Amplification products were sequenced using a Big Dye Cycle Sequencing kit Version 1.1 (Applied Biosystems) and an ABI3700 DNA analyzer (Applied Biosystems) following the instructions provided by the manufacturer. Forward and reverse sequences were obtained and compared using MEGA (Molecular Evolutionary Genetics Analysis) Version 4.0 (Tamura *et al.* 2007). Blastn (www.ncbi.nih.nlm.gov) was used to confirm gene identity and to obtain related sequences from GenBank for phylogenetic analyses. Sequences generated in this study, and those of closely related species obtained from GenBank were aligned using the online platform of MAFFT (Method for rapid sequence Alignment based on Fast Fourier Transform) version 5.851 (Katoh *et al.* 2002). Aligned sequences were compared using the

programme PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2002). Heuristic searches, using random stepwise addition and tree bisection and reconstruction as branch swapping algorithms, were used to produce most parsimonious trees. Maximum parsimony and bootstrap analysis, using 1000 replicates (Felsenstein 1985) were determined for all data sets. *Ceratocystis fimbriata* Ellis & Halsted *sensu stricto* was used as the outgroup taxon for the analyses and was treated as a monophyletic sister group to the ingroup.

Bayesian analyses, using the Monte Carlo Markov Chain (MCMC) method (parameters set at four chains producing 5 000 000 generations recording trees every 100 generations) was used to determine the posterior probabilities. MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) was used to run the Bayesian analysis with the appropriate nucleotide substitution model that was determined using the programme jModelTest 0.1.1 (Posada 2008). The model used for ITS, according to Akaike Information Criterion (AICc), was determined as the TPM2uf model. Graphical analysis (Tracer Version 1.5) was used to determine the burn-in value at the point where values converged. Posterior probabilities were determined from the trees produced in MEGA Version 4.0 (Tamura *et al.* 2007).

2.6. Pathogenicity studies

Two isolates (CMW36767, CMW36768, CMW36769, CMW36770) of each of the species identified in this study were grown for 10 d on MEA. Material for inoculation was prepared by soaking wooden toothpicks in sterile MEA and allowing the test fungi to colonize them for two wk. Colonized toothpicks as well as control toothpicks that had been soaked in MEA only, were inserted into succulent branches of healthy *E. ingens* trees, showing no symptoms of disease, to a standard depth of 3 mm for each treatment. Each species was inoculated into branches of five trees that were located in their natural environment in the North West

Province. After six wk inoculated branches were examined for lesion development and lesion lengths were measured. Where lesions were observed, branch material was incubated in moist chambers and examined for the presence of fruiting bodies resembling species of *Gondwanamyces*. In order to complete Koch's postulates, pieces from the diseased tissue were placed on MEA in order to re-isolate the fungi. Data were analysed by first determining the distribution and normality of the data, after which variation in lesion lengths were compared using a one-way analysis of variance (ANOVA), with $P < 0.05$ as significant. Since there was no variance in the control (all replicates showed zero lesion length) we compared each isolate against zero using independent, one sided t-tests, Bonferroni-corrected for multiple comparisons ($\alpha = 0.05$). All tests were conducted using JMP Version 9.0.2 (SAS institute 2011).

3. RESULTS

3.1. Collection of samples and isolations

Ten isolates of *Gondwanamyces* were obtained from diseased *E. ingens* in the Limpopo Province (FIG. 1A) and two isolates were obtained from diseased *E. tetragona* in the Eastern Cape Province. The isolates from the Limpopo Province were collected at three localities, the Capricorn Toll Plaza region, Last Post Private Game Reserve (Bandelierskop) and Euphorbia drive at the National Game Breeding Centre in Mokopane. These isolates were obtained from *Cossonus* Clairville (Coleoptera: Curculionidae, Cossoninae) beetles as well as from discolored plant material found in the brown internal parts of succulent branches on diseased trees (FIGS. 1B, C, D). The isolates from the Eastern Cape Province were obtained from insect tunnels in dying stems of one tree occurring in the Great Fish River Nature Reserve (Grahamstown).

3.2. Morphological characteristics

Only the asexual states of *Gondwanamyces* were observed. These were characterized on MEA by light colored, abundant conidiophores with typical *Custingophora* morphology producing spore drops at the tops of erect, brown mononematous conidiophores. The cultures turned olivaceous buff (21'''d) with age and had no aerial mycelium. Isolates from the Limpopo Province had longer conidiophores with a greater number of conidiogenous cells, producing shorter but wider conidia compared to those from the Eastern Cape.

3.3. Growth studies

Statistical analysis revealed highly significant differences between growth rates of the fungi at 10 C ($P = 0.022$, $df = 6.22$), 15 C ($P < 0.001$, $df = 5.06$), 20 C ($P < 0.001$, $df = 8.00$), 25 C ($P < 0.001$, $df = 4.32$) and 35 C ($P < 0.001$, $df = 4.79$). The fungi from the Limpopo Province grew faster than those from the Eastern Cape Province at 10 C to 25 C and 35 C. Optimal growth rates were similar for the two groups, with the optimum temperature for growth of both species being 30 C.

3.4. DNA sequence analyses

Blast searches in GenBank supported initial identifications based on morphology, with sequences showing a high similarity to those of species of *Gondwanamyces*. The dataset consisted of 18 sequences of which four represented isolates from *E. ingens*, two from *E. tetragona* and 12 obtained from GenBank representing the previously described species of *Gondwanamyces* in the Microascales (TABLE I). Phylogenetic analysis revealed that these isolates clustered in two distinct groups, Group I including isolates from the Limpopo and Group II including isolates from the Eastern Cape Province (FIG. 2, TABLE II). These groups were also distinct from any previously described species of *Gondwanamyces*, suggesting that

they represent novel species (Table III). The MP analysis (652 characters, 29 % of characters parsimony informative) generated one tree (TL = 645, CI = 0.859, RI = 0.885, RC = 0.760). In congruence with high bootstrap (BS) values, the dataset was strongly supported statistically with values obtained from the Bayesian analysis (burn-in value: ITS 104) (TreeBase: http://purl.org/phylo/treebase/phylows/study/TB2:S11524).

3.5. Pathogenicity tests

Isolates of both groups produced lesions on healthy *E. ingens* branches. Lesions were found on the exterior of the succulent branches, in the cambium, and more significantly in the internal core of the succulent branches (Fig. 1E). Lesions were dark brown and circular on the exterior of the branch and extended upwards and downwards in the cambium from the points of inoculation. Lesions in the internal core represented an amorphous mass of rotten tissue. Control inoculations (Fig. 1F) produced only small external lesions (Fig. 5). Statistical analysis did not show significant differences in pathogenicity between groups with P values of 0.6380 (F ratio: 0.5707), 0.6627 (F ratio: 0.5329) and 0.4167 (F ratio: 0.9718) for the cambium lesions, internal lesion depths and internal lesion width data, respectively (Degrees of freedom = 3). The calculated P-values from the Bonferroni procedure were all significant with $P < 0.05$. Isolates of the same organisms used to inoculate the trees were re-isolated from the lesions on the inoculated trees. No fungi were isolated from the controls.

3.6. Taxonomy

Based on differences in morphological characters and DNA sequence comparisons, the isolates collected in this study clearly represent two undescribed species that phylogenetically belong in the genus *Gondwanamyces*. Although only the asexual states of these fungi were seen, the trend to provide a single name for fungi (Hawksworth 2005, McNeill *et al.* 2005) is followed here and we describe these fungi as species of *Gondwanamyces*.

Gondwanamyces serotectus van der Linde, Jol. Roux, sp. nov. MB = 561550 (FIG. 3)

Etymology: name refers to the ability of the fungus to resist the poisonous latex of *E. ingens*.

Conidiophorae mononematae, macronematae in collo superiori cum usque ad 6 anfractibus, in superficie MEA pervulgatae, e rhizoideis 1–5 exorientes, cellulis conidiogenis 4–10 terminantes; etiam cum conidiophoris secondariis cellulas conidiogenas ferentibus. *Cellulae conidiogenae* cum colliculis distinctis, interdum cum cellulis conidiogenis secondariis inter sese colligatis, obovoideae olivaceo-brunneae. *Conidia* apice basique rotundata, oblonga hyalina non septata 8.4 × 4.2 µm.

Colonies olivaceous buff (21'''d) with age on MEA. *Conidiophores* sepia (17''m), mononematous, macronematous, apically sinuate up to six constrictions in the upper neck, abundant on the surface of MEA, arising from rhizoids, one to five, (7.0–) 11.4–23.4 (–31.4) × (2.7–) 5.0–8.1 (–10.2) µm (average of 50 rhizoids 17.4 × 6.6 µm, l/w 2.6) and terminating in four to ten conidiogenous cells, secondary conidiophores bearing conidiogenous cells, (49.2–) 74.0–208.0 (–345.0) × (5.6–) 6.4–9.2 (–11.8) µm (average of 50 conidiophores 141.0 × 7.8 µm, l/w 18.0) (FIG. 3A). *Conidiogenous cells* fawn (13''') with distinct collarettes, occasionally giving rise to secondary conidiogenous cells, obovoid, (5.5–) 7.2–10.6 (–14.1) × (2.6–) 3.0–4.0 (–4.5) µm (average of 50 conidiogenous cells 8.9 × 3.5 µm, l/w 2.6) (FIGS. 3D, E). *Conidia* vinaceous buff (17'''d), rounded at base and apex, oblong, hyaline, aspetate, (5.9–) 7.4–9.5 (–10.6) × (2.9–) 3.7–4.7 (–5.3) µm (average of 50 conidia 8.4 × 4.2 µm, l/w 2.0) (FIGS. 3B, C). *Optimum temperature for growth* 30 C growing at 9.5 mm/d, minimum growth temperature at 10 C and maximum growth temperature at 35 C.

HOLOTYPE. SOUTH AFRICA, LIMPOPO PROVINCE: Last Post (S23 17.738 E29 55.467, 900–1000 m) isolated from *Cossonus* on diseased *Euphorbia ingens*, May 2009, van der

Linde JA and Roux J, holotype (PREM 60566), dry culture on MEA CMW 36767 = CBS 129738; ex-type culture CMW 36768 = CBS 129739 (PREM 60567). Teleomorph not observed.

Additional specimens examined. SOUTH AFRICA, LIMPOPO PROVINCE: Last Post and Capricorn (S23 21.910 E29 44.621, 1000–1100 m) isolated from *Cossonus* occurring on diseased *E. ingens*, May 2009, van der Linde JA and Roux J, CMW 34100 (CBS = 129740), CMW 34101 (CBS = 129741). Teleomorph not observed.

Gondwanamyces ubusi van der Linde, Jol. Roux, sp. nov. MB = 561551 (FIG. 4)

Etymology: name derived from the Xhosa word for honey reflecting the fact that the host tree is commonly known as the honey tree.

Conidiophorae mononematae, macronematae in collo superiori cum usque ad 7 anfractibus, in superficie MEA pervulgatae, e rhizoideis 1–3 exorientes, cellulis conidiogenis 3–7 terminantes; etiam cum conidiophoris secondariis cellulas conidiogenas ferentibus. *Cellulae conidiogenae* cum colliculis distinctis, interdum cum cellulis conidiogenis secondariis inter sese colligatis, obovoideae olivaceo-brunneae. *Conidia* apice basique rotundata, oblonga hyalina non septata 10.0 × 3.5 µm.

Colonies olivaceous buff (21'''d) with age on MEA. *Conidiophores* sepia (17''m), mononematous, macronematous, sinuous at apex with up to seven constrictions (sinuae) in the upper part, abundant on the surface of agar, one to three rhizoids, (10.3–) 13.7–28.1 (–38.6) × (4.0–) 5.2–8.2 (–11.0) µm (average of 50 rhizoids 20.9 × 6.7 µm, l/w 3.1) at base and terminating in three to seven conidiogenous cells, (11.0–) 69.8–146.0 (–181.0) × (4.5–) 5.6–8.1 (–9.5) µm (average of 50 conidiophores 108.0 × 6.8 µm, l/w 15.9) (FIG. 4A). *Conidiogenous cells* fawn (13'''), terminating in distinct collarettes, occasionally with secondary conidiogenous cells, obovoid, (8.4–) 10.1–12.5 (–13.4) × (2.3–) 3.2–4.5 (–5.1)

µm (average of 50 conidiogenous cells 11.3 × 3.9 µm, l/w 2.9) (FIGS. 4D, E). *Conidia* vinaceous buff (17'''d), rounded at the base and apex, oblong, hyaline and aspetate, (5.6–) 8.5–11.5 (–13.6) × (1.7–) 2.9–4.0 (–4.7) µm (average of 50 conidia 10.0 × 3.5 µm, l/w 2.9) (FIGS. 4B, C). *Optimum temperature for growth* 30 C growing at 9.5 mm/d, minimum growth at 10 C and maximum growth at 35 C.

HOLOTYPE. SOUTH AFRICA, EASTERN CAPE PROVINCE: Great Fish River Nature Reserve (S33 1.764 E26 48.702), isolated from insect tunnels on diseased *Euphorbia tetragona*, Mar 2010, Roux J, holotype (PREM 60568), a dry culture on MEA CMW 36769 = CBS 129742; ex-type culture CMW 36770 = CBS 129743 (PREM 60569). Teleomorph not observed.

G. serotectus and *G. ubusi* were identified based on a combination of DNA sequence data and morphology. Previous studies of *Gondwanamyces* using the ITS region of the nuclear DNA and this, together with differences in morphology (TABLE IV), provided robust evidence for the unique nature of the fungi from *Euphorbia* species. *G. ubusi* and *G. serotectus* are quite different morphologically and relatively easy to distinguish from each other. The latter species has longer conidiophores with a greater number of rhizoids, which are shorter than those of *G. ubusi. G. serotectus* also produces shorter conidia with a greater number of conidiogenous cells than those of *G. ubusi*. *G. serotectus* has conidiophores that proliferate, giving rise to new conidiophores at a higher level, a characteristic never seen in *G. ubusi*.

Nomenclatural changes

Based on DNA sequence data from previous studies and the results of this study, most species of *Custingophora* and the previously described species of *Knoxdavesia* represent anamorph states of *Gondwanamyces* in the order Microascales. A single nomenclature for this group of fungi is presented giving precedence to the teleomorph name. For that reason *Custingophora*

cecropiae is transferred herein to *Gondwanamyces*. Even though *Custingophora olivaceae* shares similar morphological characters with *Gondwanamyces* anamorphs, this fungus appears to represent a distinct genus based on DNA sequence phylogeny and it is not placed in *Gondwanamyces***.**

Gondwanamyces **Marais & M.J. Wingf., Mycologia 90, 1998**

Globose to subglobose black ascomatal bases with long necks that tapers towards the apexes, ending in ostiolar hyphae. Asci evanescent, ascospores hyaline, aseptate, with or without sheaths (Réblová *et al.* 2011).

Gondwanamyces cecropiae (M. Kolařík) van der Linde, Jol. Roux & M.J. Wingf., **comb. nov.** PREM 858087. Basionym: *Custingophora cecropiae* M. Kolařík, Fungal Biology 1: 113. 2009.

4. DISCUSSION

This study provides new insights into the distribution, species diversity and host range of the unusual and ecologically intriguing (Roets *et al.* 2009a, 2009b) genus *Gondwanamyces*. Previously, only two species of *Gondwanamyces* were known from South Africa, *G. proteae* and *G. capensis* (Wingfield *et al.* 1988, Wingfield & Van Wyk 1993), both from the western Cape Province. These species were restricted to insect-colonized infructescences of *Proteae* species (Wingfield *et al.* 1988, Wingfield & Van Wyk 1993, Roets *et al.* 2009a). The discovery of *G. cecropiae* (previously *C. cecropiae*) and *G. scolytodis* associated with *C. angustifolia* in Costa Rica (Kolařík & Hulcr 2009), showed for the first time that these fungi are not restricted to South Africa, although they remain restricted to the Gondwana region. In this study, two additional *Gondwanamyces* species were found on *E. ingens* and *E. tetragona*

in South Africa. These results suggest that *Gondwanamyces* is more widespread, at least in the Gondwana region, than originally believed.

G. serotectus was isolated from discolored plant material as well as from the bodies of the weevil *Cossonus*. This insect has not previously been reported from *E. ingens* and was described in 1798 by the Swiss entomologist Joseph Philippe de Clairville. There is no clear understanding of its origin or global distribution (www.itis.gov). The association of a *Gondwanamyces* sp. with insects is not unexpected given that *G. proteae* and *G. capensis* occur in insect-infested *Protea* infructescences and were initially believed to be vectored by one or more of the insects in this niche (Wingfield *et al.* 1988). Likewise, *G. cecropiae* was isolated from the body of *S. unipunctatus* and *G. scolytodis* from galleries in the sapwood of *C. angustifolia* (Kolařík & Hulcr 2009).

Various ophiostomatoid fungi have been shown to be closely associated with mites, phoretic on the beetles that were originally thought to be the primary vectors of the fungi (Moser 1985, Klepzig *et al.* 2001). Intriguingly, the *Ophiostoma* species that are found in *Protea* infructecenses (Wingfield & Van Wyk 1993, Marais *et al.* 2001, Roets *et al.* 2009a) have been shown to be primarily vectored by mites (Roets *et al.* 2007, 2009b) and recently the same has been shown for *G. proteae* (Roets *et al.* 2011). It is thus likely that the *Gondwanamyces* species described in this study are vectored by mites that are associated with insects that infest *Euphorbia* species. Further studies will be required to resolve this interesting question.

This study presents the first evaluation of the pathogenicity of *Gondwanamyces* species on any plant. The *E. ingens* trees inoculated were growing in the North West Province where these trees seem to be more healthy compared to the Limpopo Province. Both of the newly described *Gondwanamyces* species produced lesions on the healthy succulent branches in

contrast to the control inoculation in which internal lesion development and discoloration was absent. This suggests that these fungi and the insects that carry them could play a role in the decline of *E. ingens* in the Limpopo Province. Great variation in lesion length was found for the two species described in this study, which could possibly be attributed to genotypic differences between the individual *E. ingens* trees. Additional inoculations on both *E. tetragona* and *E. ingens* as well as other *Euphorbia* spp. in other regions of the country should be carried out to understand this relationship more clearly. Furthermore, it appears that this fungus-insect-host interaction might be linked to an environmental and/or an anthropogenic trigger that has initiated the sudden and severe decline of these trees.

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TABLE 1: Isolates used in Phylogenetic Analyses.

Species[a]	Culture Number[c]	Host	Location	Genbank Accession Number
				ITS
Ceratocytis fimbriata	CMW7765	*Eucalyptus* sp.	South Africa	DQ520635
Custingophora olivaceae	CBS335.68	Compost	Germany	AM267269
Gondwanamyces capensis	CMW1150	*Protea magnifica*	South Africa	EU660444
G. capensis	CMW1145	*P. coronata*	South Africa	EU660441
G. capensis	CMW978	*P. neriifolia*	South Africa	EU660443
G. capensis	CMW974	*P. coronata*	South Africa	EU660442
G. cecropiae	CCF3568	*Cecropia angustifolia*	Costa Rica	AM267266
G. cecropiae	CCF3565	*C. angustifolia*	Costa Rica	AM267267
G. proteae	CMW1042	*Protea repens*	South Africa	EU660436
G. proteae	CMW3757	*P. repens*	South Africa	EU660435
G. proteae	CMW1043	*P. repens*	South Africa	EU660434
G. scolytodis	CCF3569	*Cecropia angustifolia*	Costa Rica	AM267268
[a]*G. serotectus*	CMW36767	*Euphorbia ingens*	South Africa	JF947182
[a]*G. serotectus*	CMW34100	*E. ingens*	South Africa	JF947183
[ab]*G. serotectus*	CMW36768	*E. ingens*	South Africa	JF947184
[a]*G. serotectus*	CMW34101	*E. ingens*	South Africa	JF947185
[a]*G. ubusi*	CMW36769	*E. tetragona*	South Africa	JF947186
[ab]*G. ubusi*	CMW36770	*E. tetragona*	South Africa	JF947187

[a]Isolates collected and sequenced in this study.

[b]Ex-type strains

[c]CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), South Africa; CBS = the Centraalbureau voor Schimmelcultures, The Netherlands; CCF = Culture Collection of Fungi, Czech Republic.

TABLE II: Fixed base pair differences, and their positions, between *G. serotectus* and *G. ubusi* isolates.

Identity	Culture Number	ITS																												
		52	**53**	**55**	**56**	**57**	**64**	**65**	**83**	**92**	**98**	**133**	**158**	**172**	**176**	**188**	**189**	**190**	**192**	**219**	**228**	**229**	**424**	**427**	**458**	**462**	**466**	**468**	**484**
Gondwanamyces serotectus	CMW36767	-	C	G	A	T	G	A	T	-	T	-	C	T	A	A	C	T	G	A	-	-	C	T	T	G	T	C	C
G. serotectus	CMW34100	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G. serotectus	CMW36768	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G. serotectus	CMW34101	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gondwanamyces ubusi	CMW36769	C	G	A	T	A	A	G	C	T	C	A	T	A	G	C	T	A	A	C	A	A	T	A	G	T	C	G	A
G. ubusi	CMW36700	C	G	A	T	A	A	G	C	T	C	A	T	A	G	C	T	A	A	C	A	A	T	A	G	T	C	G	A

TABLE III: Number of fixed base pair differences between species of *Gondwanamyces*.

	G. serotectus	*G. ubusi*	*G. capensis*	*G. proteae*	*G. scolytodis*	*G. cecropiae*(CCF3568)	*G. cecropiae*(CCF3565)
G. serotectus	0	19	53	56	85	92	78
G. ubusi	19	0	54	53	70	77	72
G. capensis	53	54	0	9	72	74	69
G. proteae	56	53	9	0	67	70	65
G. scolytodis	85	70	72	67	0	38	35
G. cecropiae(CCF3568)	96	77	74	70	38	0	2
G. cecropiae(CCF3565)	94	72	69	65	35	2	0

TABLE IV: Morphological differences between *G. serotectus*, *G. ubusi* and their closest neighbours *G. capensis* and *G. proteae*.

	Conidiophore	Conidiogenous cells	No. of Conidiogenous cells	Conidia	Rhizoids	Reference
G. serotectus	141.0 × 7.8 µm, l/w 18.0	8.9 × 3.5 µm, l/w 2.6	4–10	8.4 × 4.2 µm, l/w 2.0	17.4 × 6.6 µm, l/w 2.6	This study
G. ubusi	108.0 × 6.8 µm, l/w 15.9	11.3 × 3.9 µm, l/w 2.9	3–7	10.0 × 3.5 µm, l/w 2.9	20.9 × 6.7 µm, l/w 3.1	This study
G. capensis	65.6 × 3.9 µm, l/w 16.8	9.5 × 5.3 µm, l/w 1.8	5–12	5.0 × 3.8 µm, l/w 1.3	no information available	Wingfield & Van Wyk 1993
G. proteae	132.0 × 8.0 µm, l/w 16.5	9.0 × 5.0 µm, l/w 1.8	7–12	5.0 × 3.0 µm, l/w 1.7	13.0 × 5.0 µm, l/w 2.6	Wingfield *et al.* 1988

FIG. 1. Symptoms of disease on *E. ingens* in the field and after inoculation with *Gondwanamyces serotectus*. (A) Diseased *E. ingens* tree at the Capricorn Toll Plaza region in the Limpopo Province showing greying and collapse of succulent branches (B) & (D) rotting and discoloration of the succulent branches on the exterior and internal core (C) insect tunnels in a succulent branch of *E. ingens*. (E) Internal rot of succulent branch six wk after inoculation with *Gondwanamyces serotectus*. (F) Control inoculation showing no internal rot.

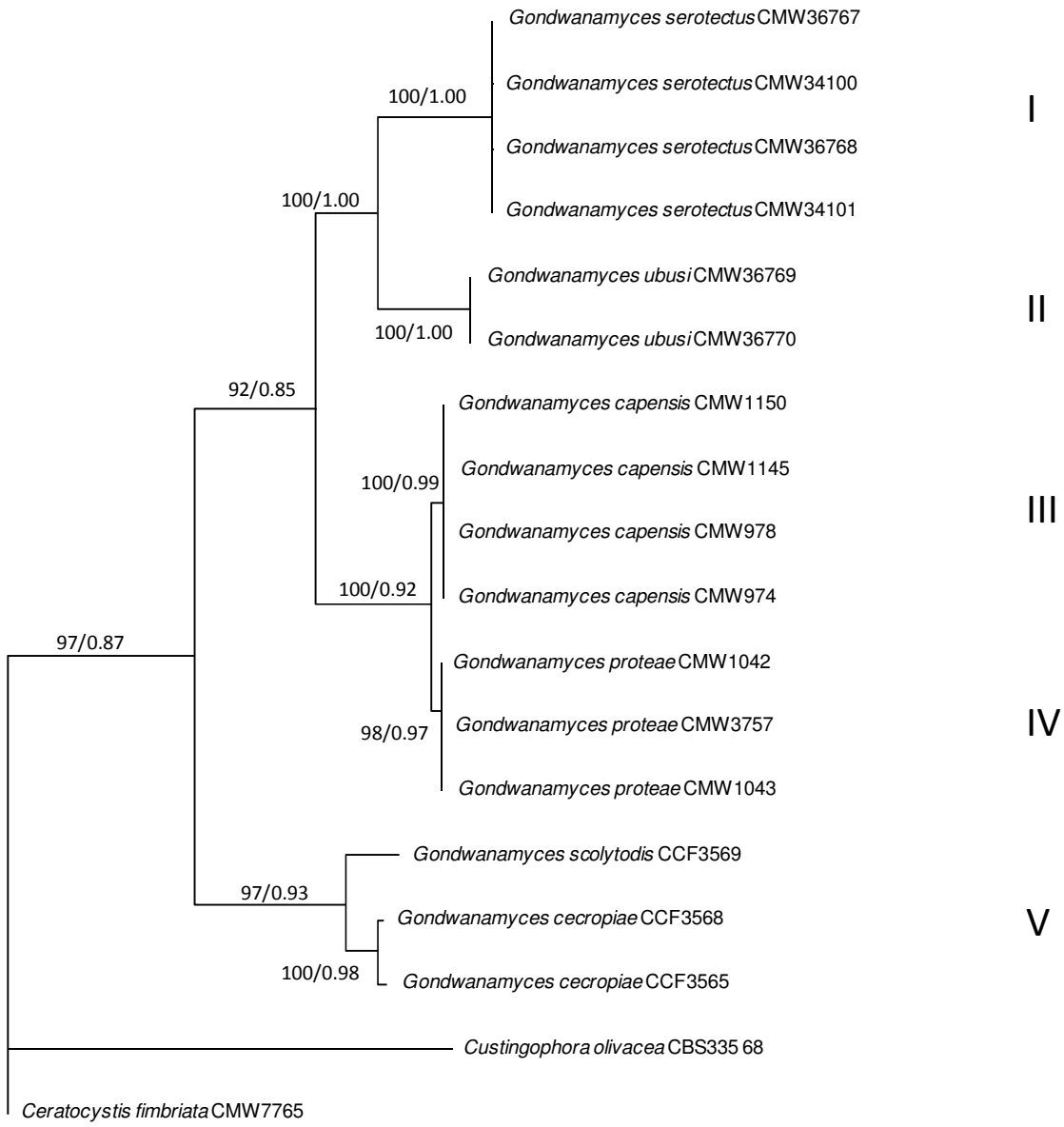

FIG. 2. The most parsimonious tree obtained from Maximum Parsimony analyses of ITS sequence data of the representative taxa of the genus *Gondwanamyces*. Numbers at the nodes indicate maximum likelihood bootstrap and Bayesian MCMC posterior probabilities.

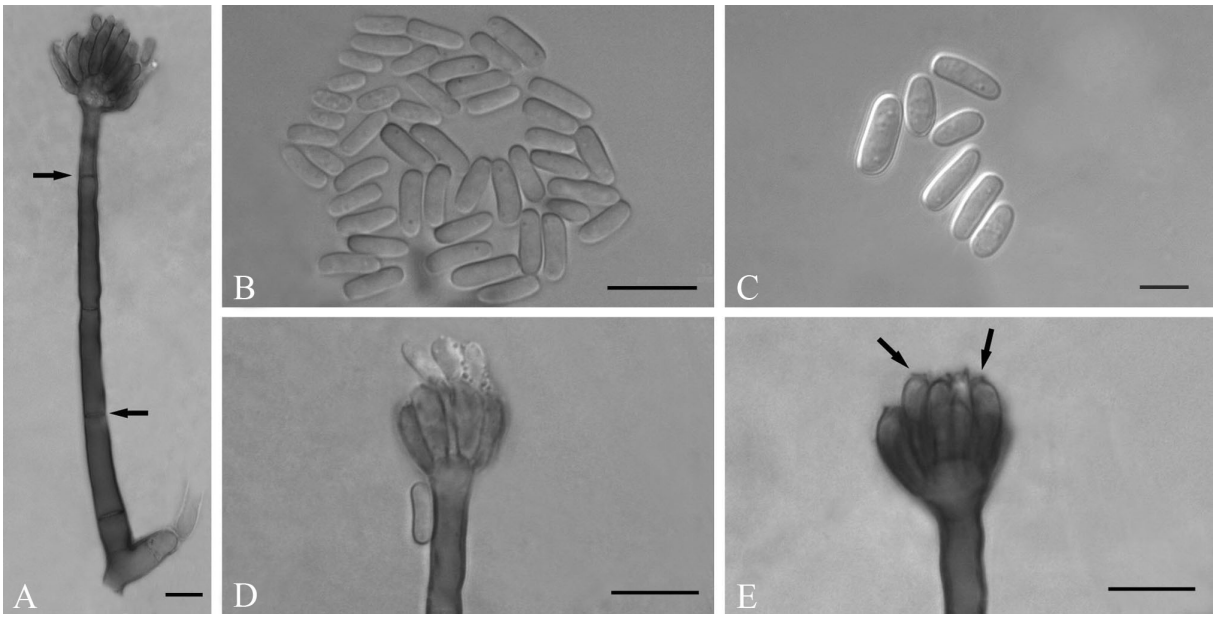

FIG. 3. *Gondwanamyces serotectus* (A) Conidiophore showing foot cell and sinuate stipe. (B) Obovate conidia. (C) Conidia of variable size. (D) Conidiophore with phialidic conidiogenous cells and newly produced conidia. (E) Conidiophore with conidiogenous cells showing phialides with distinct collarettes. Bars: A, B, D, E = 10 µm; C = 5 µm.

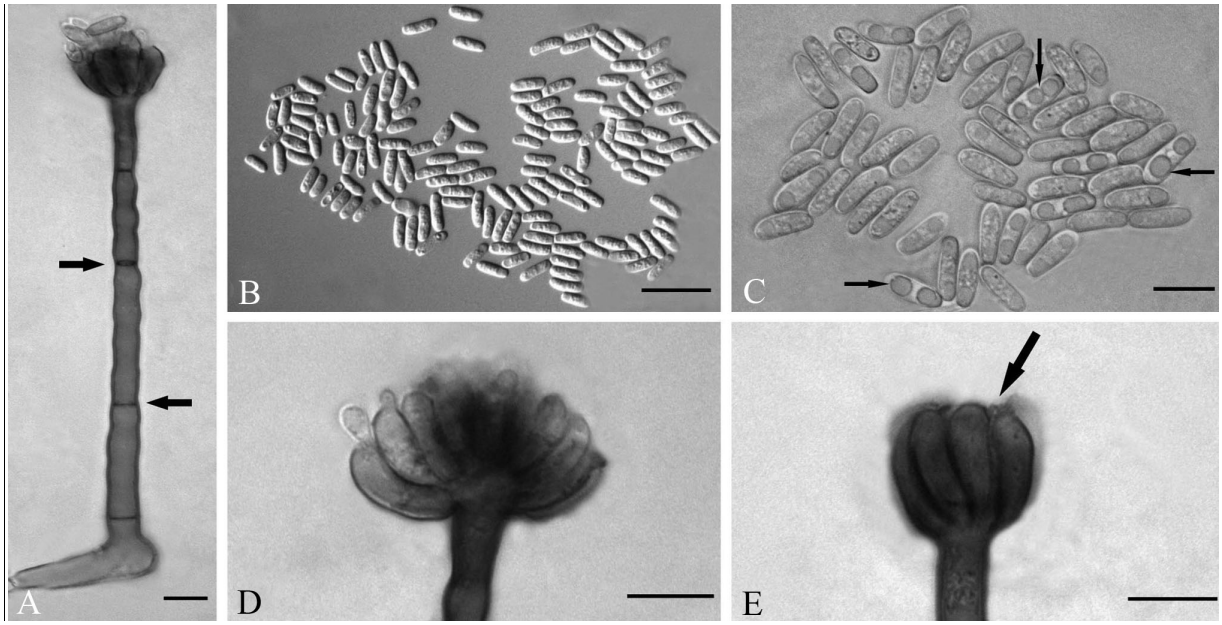

FIG. 4. *Gondwanamyces ubusi* (A) Conidiophore showing foot cell and sinuate stipe. (B) Obovate conidia. (C) Conidia with distinct guttules. (D) Conidiophore with conidiogenous cells and newly produced conidia (E) Conidiophore with phialidic conidiogenous cells. Bars: A, C, D, E = 10 µm; B = 20 µm.

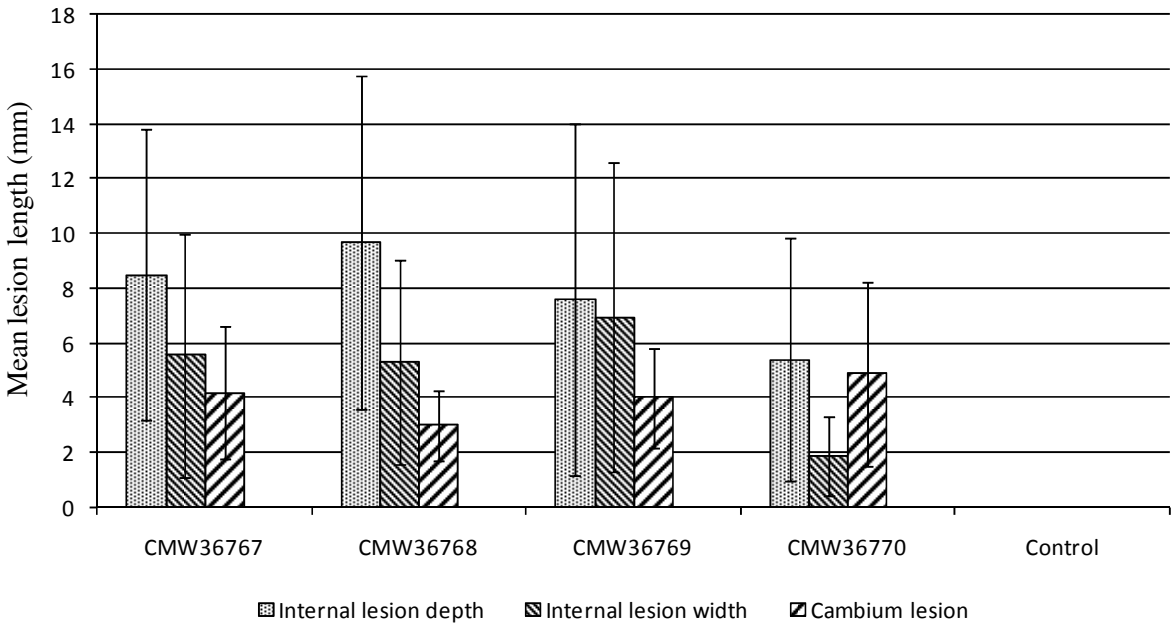

FIG. 5. Histogram of mean lesion lengths (mm) resulting from inoculations with two isolates of *G. serotectus* (CMW36767, CMW36768) and *G. ubusi* (CMW36769, CMW36770) used in the *E. ingens* pathogenicity trails. Bars indicate 95 % confidence limits for each isolate.

Chapter 3

Lasiodiplodia species associated with dying *Euphorbia ingens* in South Africa

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ABSTRACT

Various species of *Euphorbia* occur in South Africa, including herbaceous, succulent and woody types. The largest of the succulent *Euphorbia* spp. in South Africa is *Euphorbia ingens*. These trees have been dying at an alarming rate in the Limpopo Province during the course of the last 15 years. Investigations into the possible causes of the death have included the possible role of fungal pathogens. Amongst the fungi isolated from diseased trees were species in the Botryosphaeriaceae. The aim of this study was to identify these fungi using morphology and DNA sequence data of two gene regions (TEF-1α & ITS). Results showed that *Lasiodiplodia theobromae* and *Lasiodiplodia mahajangana* were present, representing the first report of *Lasiodiplodia* species on a succulent *Euphorbia* species. Pathogenicity studies showed that these *Lasiodiplodia* species can cause infections on healthy *E. ingens* trees, implicating them as contributors to the decline of *E. ingens.*

Keywords: Botryosphaeriaceae, candelabra trees, tree diseases, insect infestations, climate change

1. INTRODUCTION

The genus *Euphorbia* includes more than 2100 species worldwide. *Euphorbia* species are known to vary dramatically in morphology and range from large woody trees to shrub like herbaceous plants and succulent cactus like plants (Palgrave *et al.* 2002, PBI Euphorbia project, www.euphorbiaceae.org). There is a high diversity of woody to succulent euphorbias in Southern Africa, with the largest of these species being *Euphorbia ingens* E. Meyer: Boissier (Palgrave *et al.* 2002, Gildenhuys 2006). *E. ingens* and similar species are characterised by woody main stems and fleshy succulent branches, giving the trees a candelabrum shape (Van Wyk and Van Wyk 1997, Palgrave *et al.* 2002, Gildenhuys 2006). *E. ingens* is known only to occur in Africa with high densities in Southern Africa (Palgrave *et al.* 2002, Gildenhuys 2006).

In the last 15 years, there have been alarming reports of large-scale decline and death of *E. ingens* trees in South Africa. Mortality of these trees has been particularly severe in the Limpopo Province. Symptoms associated with the death of trees include greying and spots on the succulent branches, infestation by branch and stem boring insects and brown to blue discolouration of the internal tissues of the branches and woody main stems (Roux *et al.* 2008, 2009). Preliminary investigations into the cause of this disease have yielded various fungi including species of Botryosphaeriaceae (Roux *et al.* 2008, 2009).

The Botryosphaeriaceae are known as opportunistic pathogens that cause cankers and death of numerous tree species, especially after periods of drought, frost, hail damage and other environmental conditions leading to stress (Punithalingam 1980, Slippers and Wingfield 2007). They are also known to be endophytes, infecting healthy trees and only causing disease after the onset of stress (Smith *et al*. 1996). In South Africa, fungi in the Botryosphaeriaceae are common, and often cause disease of especially commercially grown plantation trees

(Laughton 1937, Swart *et al.* 1985, Smith *et al.* 1996, Roux and Wingfield 1997), native tree species such as *Pterocarpus angolensis* DC. (Mehl *et al*. 2011) and *Syzygium* species (Pavlic *et al*. 2007).Various species are also known to occur on, and cause disease of fruit trees in the genera *Malus*, *Pyrus*, *Prunus*, *Populus*, *Syzygium* and *Vitis* (van Niekerk *et al.* 2004, Damm *et al*. 2007, Pavlic *et al.* 2007, Slippers *et al.* 2007). There are, however, no reports of Botryosphaeriaceae from succulent *Euphorbia* species.

The aim of this study was to identify species of Botryosphaeriaceae collected during studies of dying *E. ingens* trees in the Limpopo Province of South Africa. We also tested the pathogenicity of the isolates on healthy *E. ingens* trees to consider their possible involvement in tree death.

2. MATERIALS AND METHODS

2.1. Collection of samples and isolations

Isolates were collected from diseased *E. ingens* (FIG. 1A) at four sites in the Limpopo Province during 2009. Isolations were made from blue-black discoloured wood (FIG. 1B) in the main woody stems of the trees, as well as from necrotic tissue and insect tunnels in the succulent branches. Isolations were also made from insects collected from rotting succulent branches and the woody main stems. Direct isolations were made from the plant material taken from the leading edges of lesions using a sterile scalpel. These tissue samples were plated on 2 % Malt Extract Agar (MEA) (15 g agar and 20 g malt extract 1 L^{-1} distilled water; Biolab, Merck, Midrand, South Africa) with streptomycin (0.4 g L^{-1}; Sigma-Aldrich, St. Louis, USA). Isolations were made from insects by crushing them onto water agar (15 g L^{-1} distilled water; Biolab) and incubating the plates for six weeks at 20 °C. Cultures from insects were purified by transferring mycelium to fresh 2 % MEA. A second set of isolates was obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology

Institute (FABI), University of Pretoria which were collected previously, from similar symptoms (Roux *et al.* 2008, 2009), from diseased *E. ingens* trees in the Limpopo Province. Purified single-spore isolates from plant tissue and insects were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.2. Culture and Morphological Characteristics

Isolates were plated onto 1.5 % Water Agar (WA; 15 g L^{-1} distilled water; Biolab) containing sterilized pine needles to induce the formation of fruiting bodies. Cultures were incubated at 20 °C under near-ultra violet (UV) light. Characteristic fungal structures (conidia, conidiogenous cells, paraphyses, conidiomata) were viewed using a Zeiss light microscope fitted with an Axiocam digital camera with Axiovision 3.1 software (Carl Zeiss Ltd., Germany). The fungal structures were placed on glass microscope slides and mounted in 75 % lactic acid. Colors of resultant cultures were determined using the color notations of Rayner (1970).

2.3. DNA extraction and Polymerase Chain Reaction (PCR) amplification

Isolates, representing different collection sites and culture morphology, were grown for six days on 2 % MEA, prior to DNA extraction. DNA extraction followed the protocol of Möller *et al.* (1992), after mycelium was scraped from the surfaces of the cultures and freeze dried for 24 hours in 2 ml Eppendorf tubes. DNA concentration was determined using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

Polymerase chain reaction was used to amplify the internal transcribed spacer regions and the 5.8S gene using the primers ITS 1 and ITS 4 (White *et al*. 1990) and the Translation Elongation Factor 1-α (TEF 1-α) gene region using the primers EF1-F and EF1-R (Jacobs *et*

al. 2004). PCRs were done using an Applied Biosystems Veriti thermocycler (Applied Biosystems, Foster City, USA) following the protocols described by Mohali *et al.* (2007).

PCR products were viewed using an agarose gel (2 %; Whitehead Scientific, Cape Town, South Africa), loaded with GelRed (Anatech, USA), and visualised under UV illumination. The size of the PCR products was estimated using a 100 bp DNA molecular marker (O'RangeRuler™ 100bp DNA ladder, Fermentas Life Sciences, Vilnius, Lithuania). Sephadex G-50 columns (1 g in 15 ml distilled water; SIGMA, Steinheim, Germany) were used to purify the amplified products in preparation for sequencing.

2.4. DNA sequencing

Confirmed PCR products were sequenced with an ABI3700 DNA analyzer (Applied Biosystems) using a Big Dye Cycle Sequencing kit Version 1.1 (Applied Biosystems). Sequences were edited based on forward and reverse sequences using Mega Version 4.0 (Tamura *et al*. 2007). To confirm gene identity and obtain related sequences, the correctly edited sequences were placed in the nucleotide database *blastn* (National Centre for Biotechnology Information, www.ncbi.nih.nlm.gov). Mafft version 5.851 (Katoh *et al.* 2002) was used to align the sequences from this study and those of closely related species obtained from the blast results. Phylogenetic analysis of each data set was done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2002) and phylogenetic trees were constructed using random stepwise addition and tree bisection and reconstruction as branch swapping algorithms, based on heuristic searches. Bootstrap and maximum parsimony analyses were run using a 1000 replicates (Felsenstein 1985). A partition homogeneity test was used to determine whether the ITS and TEF 1-α sequence data sets could be combined (Farris *et al*. 1995, Huelsenbeck *et al*. 1996). Two separate phylogenetic analyses, one including all the recently described species of *Lasiodiplodia* and another

considering only the clades including the isolates from this study were conducted. Prior to the partition homogeneity test, data sets of individual gene regions were analysed separately. The data sets were rooted with the GenBank sequences of *Botryosphaeria sarmentorum* A.J.L. Phillips, Alves & Luque (CBS 12041) and *Lasiodiplodia gonubiensis* Pavlic, Slippers & M.J. Wingf. (CMW 14078) (TABLE I).

Bayesian analysis was used to determine the posterior probabilities of each dataset (ITS, TEF 1-α) based on the Monte Carlo Markov Chain (MCMC) method. A jModelTest 0.1.1 (Posada 2008) was used to determine the most appropriate nucleotide substitution model. The best-fitting models for the ITS and TEF 1-α datasets, based on Akaike Information Criterion (AICc), were determined for the complete analysis (TPM1: ITS, K80+G: TEF 1-α) and the specific analysis (TIM2+G: ITS, K80+G: TEF 1-α). The Bayesian analysis was run on MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) and trees were recorded every 100 generations based on four chains producing 5 000 000 generations. The likelihood data were used in graphical analysis to estimate the burn-in values for each dataset. Mega Version 4.0 (Tamura *et al.* 2007) was used to produce consensus trees from the two analysed datasets from which the posterior probabilities were determined.

2.5. Pathogenicity trials

Two isolates (CMW36766, CMW36765) obtained in this study were used to inoculate healthy *E. ingens* trees in the North West Province. Cultures were first grown on 2 % MEA for five days and then used to inoculate wooden toothpicks first soaked in Malt Extract (20 g malt extract L^{-1} distilled water) and then placed on the surface of MEA in Petri dishes. Mycelium-colonised toothpicks, and sterile toothpicks for the controls, were inserted into the succulent branches (five branches for each isolate) to a depth of 3 mm. After six weeks the results were determined by measuring the surface lesions, cambium lesions and internal lesions after

cutting branches in half at the point of inoculation. Isolations were made on MEA from inoculated tissue to comply with Koch's postulates. To determine significance between means, a student's t test was done with $P < 0.05$ as being significant. Since there was no variance in the controls, the data for each isolate were Bonferroni-corrected for multiple comparisons ($\alpha = 0.05$). All tests were conducted using JMP version 9.0.2 (SAS institute 2011).

3. RESULTS

3.1. Collection of samples and isolations

Fungal isolates resembling the Botryosphaeriaceae were obtained from three of the 23 trees sampled in 2009. Five isolates were obtained from the CMW culture collection (collected 2007/8) and originated from diseased trees at the National Game Breeding Centre at Mokopane. Of the eight isolates resembling Botryosphaeriaceae, six isolates originated from Mokopane, one from Capricorn and one from the Louis Trichardt area. Six of these were from diseased plant material and the remaining two were isolated from insects infesting diseased tissue.

3.2. Culture and morphological characteristics

Fungal structures showed typical features of *Lasiodiplodia* species, with aseptate, hyaline conidia, becoming dark brown and septate with striations as they matured. Cultures were white with abundant, fluffy aerial mycelium which became an olivaceous grey (23''''b) with time (10 days). Pycnidia were produced after five days on the WA with sterilized pine needles and were black in colour, unilocular, solitary, immersed in the media and were formed on the top surfaces of the pine needles (FIG. 2).

3.3. DNA sequence analyses

The ITS and TEF 1-α datasets were combined based on a value of P = 0.350 (complete *Lasiodiplodia* species group, 43 taxa) and P = 0.140 (specific *Lasiodiplodia* species clade, 29 taxa) obtained from the partition homogeneity test done in PAUP (FIGS 3, 4). MP analyses of the individual gene region data sets did not give a good resolution in terms of species identity and with P > 0.05, trees were combined for this study. The MP analysis for the combined datasets for the complete *Lasiodiplodia* group (characters = 655, 9 % of characters parsimony informative) and the specific *Lasiodiplodia* species clade (characters = 649, 2 % of characters parsimony informative) generated 7 (TL = 182, CI = 0.780, RI = 0.832, RC = 0.649) and 100 trees respectively (TL = 53, CI = 0.849, RI = 0.855, RC = 0.726), with similar topology for both groups. Both of the combined datasets had strong Bayesian support with statistically significant values. However bootstrap analysis produced trees with limited resolution, possibly due to similarity in the DNA sequences investigated, not resolving the final identity of the species. Bayesian analysis produced trees with high resolution and was used as the final model to identify the species (Douady *et al.* 2003). Burn-in values were obtained for all analyses (burn-in values: complete analysis; 52 and specific analysis; 122).

In the final phylogenetic analyses, the data set including all described *Lasiodiplodia* spp. gave rise to seven clades while three clades emerged for the data set containing only selected *Lasiodiplodia* spp. The complete *Lasiodiplodia* data set did not show a good resolution but indicated that the isolates from this study resided in clade one. Analyses of the reduced *Lasiodiplodia* data set showed that isolates represented *Lasiodiplodia mahajangana* Begoude, Jol. Roux, Slippers (CMW36765) and *Lasiodiplodia theobromae* (Patouillard) Griffon & Maubl (CMW26225, CMW26592, CMW26593, CMW26594, CMW26595, CMW36766, CMW37026) with strong Bayesian support (FIGS 3, 4). *L. mahajangana* was isolated from blue stain in the wood from one tree (FIG. 1B) near the Capricorn Toll Plaza (S23 21.910 E29

44.621), while *L. theobromae* was isolated from diseased plant material (CMW26225, CMW26592, CMW26593, CMW26594, CMW26595) form Mokopane (S24 10.291 E29 01.131) as well as the insects *Cyrtogenius africus* Wood (CMW36766) and *Cossonus* Claireville (CMW37026) from Mokopane and Last Post Private Nature Reserve (S23 17.738 E29 55.467) (Louis Trichardt site), respectively.

3.4. Pathogenicity trials

L. mahajangana (CMW36765) and *L. theobromae* (CMW36766) produced lesions on the exterior, cambium and internal core of healthy *E. ingens* branches (FIG. 1C). The most severe damage caused by the fungi was in the internal core of the succulent branches which had rotten. Lesions on the exterior were conspicuous at the point of inoculation with necrotic tissue and a black discharge. *L. mahajangana* and *L. theobromae* lesions were brown and circular at the points of inoculation with necrotic tissue in the internal core. The control also had small brown circular lesions at the point of inoculation but had no signs of discoloration in the cambium or internal core (FIG. 1D) of the succulent branches. Statistical analysis did not show significant differences in pathogenicity between species except for the cambium lesion length with P values of 0.025 (DF = 12.51), 0.1303 (DF = 18.00) and 0.4261 (DF = 14.06) for the cambium lesion, internal lesion depth and internal lesion width data, respectively (FIG. 5). Isolations from the sites of inoculation yielded *L. theobromae* and *L. mahajangana* based on characteristic morphological features.

4. DISCUSSION

Results of this study showed that two species of *Lasiodiplodia*, *L. theobromae* and *L. mahajangana* are associated with die-back symptoms on *E. ingens*. These fungi were identified based on morphological characteristics and DNA sequence comparisons. They are

both well-known from trees in Southern Africa (Crous *et al.* 2000, Burgess *et al.* 2003, Pavlic *et al.* 2007, Begoude *et al.* 2010), but have not previously been reported from *E. ingens*.

L. mahajangana is a recently described species from healthy branches of *Terminalia catappa* L. in Madagascar (Begoude *et al.* 2010). The current study represents only the second report of this fungus and very limited information is, therefore, available regarding its possible origin or importance. In this study *L. mahajangana* was isolated from blue stain in the wood of the main stem of an *E. ingens* tree. Blue stain is a common symptom of wood infected by species in the Botryosphaeriaceae, resulting from the dark colour of the mycelium of these fungi (Slippers and Wingfield 2007). This study thus suggests that *L. mahajangana* has a potentially wide distribution in Africa, including a diverse host range. Its impact and importance will, however, only become known with further surveys and more in depth studies.

L. theobromae was obtained from diseased plant material and insects collected from the internal parts of dying *E. ingens* trees. These insects, *Cyrtogenius africus* (Curculionidae: Scolytinae), and a *Cossonus* sp. (Curculionidae: Cossoninae) were not surface disinfected and inoculum of *L. theobromae* could have been on their surfaces or related to tissue that they had consumed and so occurring in their guts. The Botryosphaeriaceae typically disperse via rain splash and are not adapted to insect dispersal. But species such as *L. theobromae* have previously been isolated from insects such as *Hypocryphalus mangiferae* Stebbing (Scolytinae) after surface sterilization, implying that it might be carried in the gut or mycangia (Masood *et al.* 2010). Both insect families have previously been associated with succulent *Euphorbia* species (Wollaston) (Jordal 2006, 2009).

Species delineation within the genus *Lasiodiplodia*, and particularly within what is known as *L. theobromae*, is recognised to be problematic and in need of detailed re-evaluation. In the

past three years, eight cryptic species have been described within the genus *Lasiodiplodia*, including several within what was previously known as *L. theobromae* (Alves *et al.* 2008, Pavlic *et al.* 2008, Abdollahzadeh *et al.* 2010, Begoude *et al.* 2010). Where five years ago only five species of *Lasiodiplodia* was known, this number has now reached 14. Phylogenetic analyses, based on ITS and TEF 1-α gene sequences, in the current study, showed poor resolution in the *L. theobromae* clade (FIGS 3, 4) with limited differences between isolates of *L. theobromae* and *L. hormozganensis* Abdollahzadeh, Zare & A.J.L. Phillips. Similarly, conidial sizes for isolates obtained from *E. ingens*, varied from those of previous descriptions of *L. theobromae* (TABLE II). Additional analyses will be needed to resolve this group more clearly, either by including other gene regions or using specific microsatellite markers to consider the problem at a population level.

Inoculation studies, using *L. theobromae* and *L. mahajangana*, showed that both these fungi have the potential to cause disease on *E. ingens*. Both produced extensive internal rot of the succulent branches of these trees, within six weeks. It was not surprising to find that *L. theobromae* and *L. mahajangana* were able to cause disease symptoms on *E. ingens*. Previously, *L. theobromae* was shown to be pathogenic to *Eucalyptus* clones (GC540) (Pavlic *et al.* 2007), grapevines (Úrbez-Torres *et al.* 2008, Úrbez-Torres and Gubler 2009) and *T. catappa* (Begoude *et al.* 2010). Similar to results in the study by Begoude *et al.* (2010), in our study, *L. mahajangana* produced smaller lesions in artificial inoculation studies than *L. theobromae*.

Since environmental and other stress factors play an important role in the epidemiology of diseases caused by fungi in the Botryosphaeriaceae, the symptoms observed on dying *E. ingens* trees in South Africa could, at least in part, be attributed to *L. theobromae* and *L. mahajangana*. A study by Van der Linde (2011) indicated that increased temperature and decreased rain in the study areas in the Limpopo Province over the last 40 years may be a

possible stress factor for *E. ingens*. It does appear that a link to an environmental and/or an anthropogenic trigger initiated the sudden and severe decline of these trees, in combination with various pathogens and insects. This study presents the first report of *Lasiodiplodia* on a succulent *Euphorbia* species. Further and more extensive surveys will be required to fully understand the diversity and distribution of Botryosphaeriaceae on native *Euphorbia* trees and to establish the possible triggers enabling these fungi to attack and thrive on these trees.

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TABLE I: Isolates of Botryosphaeriaceae used in this study and obtained from *Euphorbia ingens* and GenBank

Species	Culture Number	Host	Origin	GenBank Accession Number	
				ITS	TEF-1α
Botryosphaeria sarmentorum	CBS120.41	*Pyrus communis*	Norway	AY573207	AY573224
Lasiodiplodia citricola	IRAN1521C	*Citrus* sp.	Iran	GU945353	GU945339
L. citricola	IRAN1522C	*Citrus* sp.	Iran	GU945354	GU945340
L. crassispora	CMW13488	*Eucalyptus urophylla*	Venezuela	DQ103552	DQ103559
L. crassispora	WAC12533	*Santalum album*	Australia	DQ103550	DQ103557
L. crassispora	UCD27Co	Grapevines	USA	GU799457	GU799488
L. gilanensis	IRAN1501C	Unknown	Iran	GU945352	GU945341
L. gilanensis	IRAN1523C	Unknown	Iran	GU945351	GU945342
L. gonubiensis	CBS115812	*Syzygium cordatum*	South Africa	DQ458892	DQ458877
L. gonubiensis	CMW14078	*S. cordatum*	South Africa	AY639594	DQ103567
L. hormozganensis	IRAN1498C	*Mangifera indica*	Iran	GU945356	GU945344
L. hormozganensis	IRAN1500C	*Oleo* sp.	Iran	GU945355	GU945343
L. iraniensis	IRAN921C	*Mangifera indica*	Iran	GU945346	GU945334

L. iraniensis	IRAN1502C	*Juglans* sp.	Iran	GU945347	GU945335
L. mahajangana	CMW36765	*Euphorbia ingens*	South Africa	JN098457	JN098464
L. mahajangana	CMW27801	*Terminalia catappa*	Madagascar	FJ900595	FJ900641
L. mahajangana	CMW27818	*T. catappa*	Madagascar	FJ900596	FJ900642
L. mahajangana	CMW27820	*T. catappa*	Madagascar	FJ900597	FJ900643
L. margaritacea	CBS122519	*Adansonia gibbosa*	Australia	EU144050	EU144065
L. margaritacea	CBS122065	*Adansonia gibbosa*	Australia	EU144051	EU144066
L. parva	CBS494.78	Cassava-field soil	Colombia	EF622084	EF622064
L. parva	CBS456.78	Cassava-field soil	Colombia	EF622083	EF622063
L. parva	CBS356.59	*Theobroma cacao*	Sri Lanka	EF622082	EF622062
L. plurivora	STEU-5803	*Prunuss alicina*	South Africa	EF445362	EF445395
L. plurivora	STEU-4583	*Vitis vinifera*	South Africa	AY343482	EF445395
L. pseudotheobromae	CBS116459	*Gmelinea arborea*	Costa Rica	EF622077	EF622057
L. pseudotheobromae	CBS374.54	*Coffea* sp.	Zaire	EF622080	EF622059
L. pseudotheobromae	CBS447.62	*Citrus aurantium*	Suriname	EF622081	EF622060
L. rubropurpurea	CBS118740	*Eucalyptus grandis*	Australia	DQ103553	DQ103571

L. rubropurpurea	CMW15207	*E. grandis*	Australia	DQ103554	DQ103572
L. theobromae	CMW36766	*Euphorbia ingens*	South Africa	JN098457	JN098465
L. theobromae	CMW37026	*E. ingens*	South Africa	JN098458	JN098466
L. theobromae	CMW26225	*E. ingens*	South Africa	JN098459	JN098467
L. theobromae	CMW26592	*E. ingens*	South Africa	JN098460	JN098468
L. theobromae	CMW26593	*E. ingens*	South Africa	JN098461	JN098469
L. theobromae	CMW26594	*E. ingens*	South Africa	JN098462	JN098470
L. theobromae	CMW26595	*E. ingens*	South Africa	JN098463	JN098471
L. theobromae	CBS111530	Unknown	Unknown	EF622074	EF622054
L. theobromae	CMW30105	*Syzygium cordatum*	Zambia	FJ747642	FJ871116
L. theobromae	CMW30104	*S. cordatum*	Zambia	FJ747641	FJ871115
L. theobromae	CMW28317	*Terminalia catappa*	Cameroon	FJ900602	FJ900648
L. theobromae	CMW28319	*T. catappa*	Cameroon	FJ900603	FJ900649
L. theobromae	IRAN1233C	*Mangifera indica*	Iran	GU973868	GU973860
L. theobromae	IRAN1496C	*M. indica*	Iran	GU973869	GU973861
L. venezuelensis	WAC12539	*Acacia mangium*	Venezuela	DQ103547	DQ103568

L. venezuelensis	WAC12540	*A. mangium*	Venezuela	DQ103548	DQ103569

TABLE II: Conidial measurements comparing *Lasiodiplodia theobromae* isolates from *Euphorbia ingens* and previous studies.

Conidial size (µm)	Host	Reference
*20–30 × 10–15	Unknown	Punithalingam 1976
26.2–28.8 × 14–14.4	Unknown	Alves *et al*. 2008
22.5–26 × 12.5–15	*Terminalia catappa*	Begoude *et al.* 2010
22.4–24.2 × 12.9–14.3	*Mangifera indica*	Abdollahzadeh *et al.* 2010
18.1–21.3 × 11.6–13.3	*Euphorbia ingens*	This study

* Indicates the first description of the anamorph state of *Lasiodiplodia theobromae*.

FIG. 1. Disease symptoms on *Euphorbia ingens* trees. (a) Dying *Euphorbia ingens* trees near Mokopane in the Limpopo Province. (b) Blue stain in wood from which *Lasiodiplodia mahajangana* was isolated. (c) Internal lesion produced by *Lasiodiplodia theobromae* (CMW36766) on the succulent branches of *Euphorbia ingens* during the pathogenicity trial. (d) Healthy control inoculation showing no disease development.

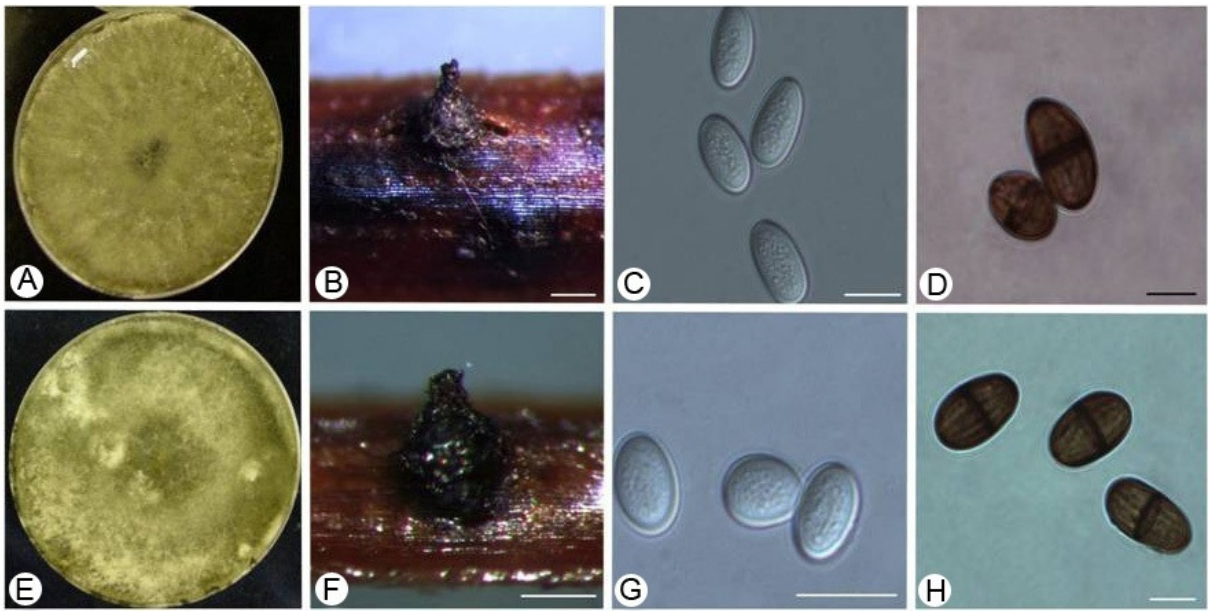


FIG. 2. *Lasiodiplodia mahajangana* and *Lasiodiplodia theobromae* culture and conidial morphology. (a) Culture morphology of *L. mahajangana*. (b) Pycnidium of *L. mahajangana*, with short neck, on sterile pine needle. (c) Immature conidia of *L. mahajangana* with typical ellipsoid to ovoid shape. (d) Mature conidia of *L. mahajangana* being one septate with characteristic striations. (e) Culture morphology of *L. theobromae*. (f) Pycnidium of *L. theobromae*. (g) Immature conidia of *L. theobromae*. (h) Mature conidia of *L. theobromae*. Bars: b, f = 200 µm; c, g = 20 µm; d, h = 10 µm.

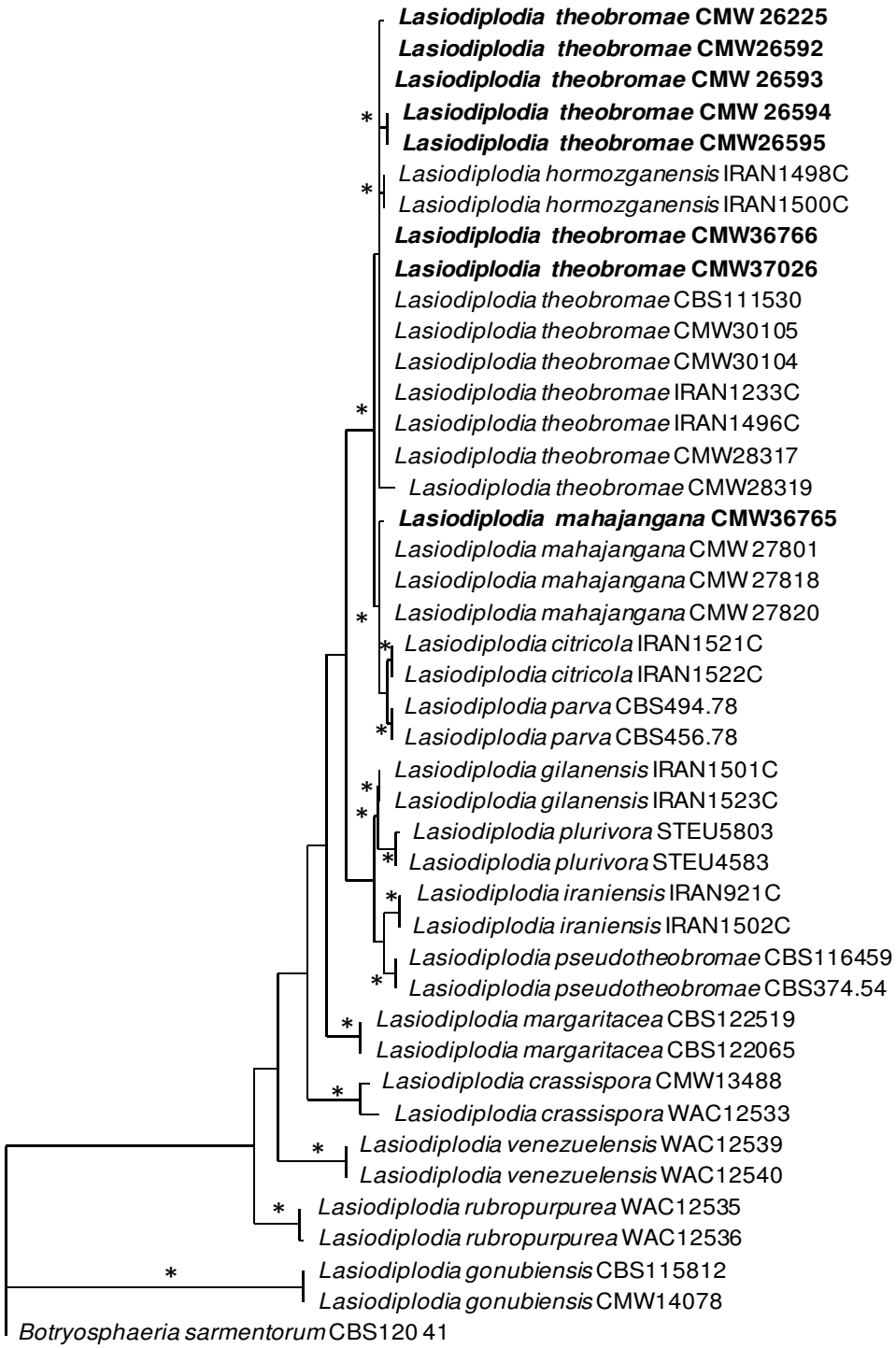


FIG. 3. One of the most parsimonious trees obtained from maximum parsimony analyses of the combined sequences of ITS and TEF 1-α (complete) of representative taxa of *Lasiodiplodia*. Isolates in bold were collected in this study and stars at the nodes indicate posterior probabilities higher than 0.90.

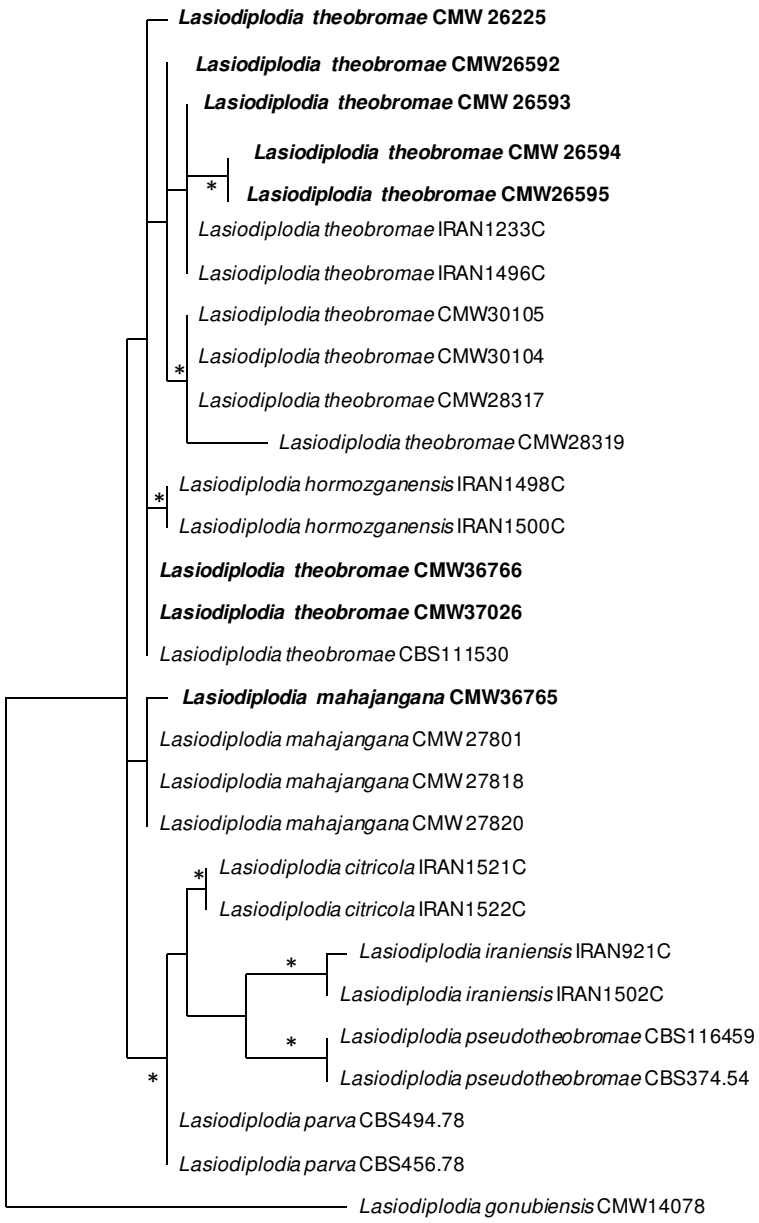
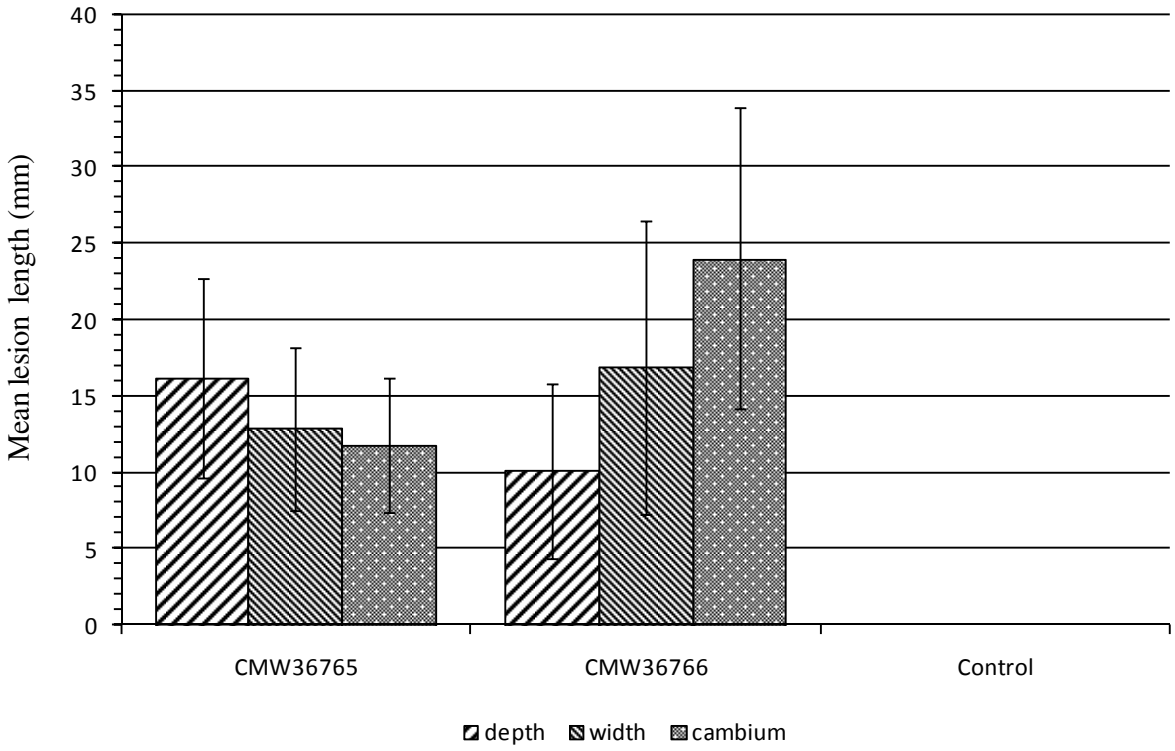


FIG. 4. One of the most parsimonious trees obtained from maximum parsimony analyses of the combined sequences of ITS and TEF 1-α (clade specific) of the representative taxa of *Lasiodiplodia*. Isolates in bold were collected in this study and stars at the nodes indicate posterior probabilities higher than 0.90.



Lasiodiplodia isolates used in pathogenicity trials

FIG. 5. Histogram of mean lesion lengths (mm) resulting from inoculations with isolates of *Lasiodiplodia mahajangana* (CMW36765) and *Lasiodiplodia theobromae* (CMW367660) used in the *Euphorbia ingens* pathogenicity trails. Bars indicate 95 % confidence limits for each isolate.

Chapter 4

Die-off of giant tree Euphorbias in South Africa: Symptoms and relationships to climate

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This chapter has been submitted for publication as: Van der Linde JA, Roux J, Wingfield MJ, Six DL. Die-off of giant *Euphorbia* trees in South Africa: Symptoms and relationships to climate. (Submitted to *Diversity and Distributions*, 19 August 2011)

ABSTRACT

Euphorbia ingens is the largest of the succulent tree euphorbias in Southern Africa. In South Africa, it is most abundant in the north, especially in the Limpopo and North West Provinces. In the mid-nineties, *E. ingens* trees were noted to show symptoms of disease, and by 2000 severe mortality had begun to occur in the Limpopo Province. Pilot studies were unable to detect the involvement of abiotic factors in the death of these trees. Additional studies in 2007 and 2008 documented a high diversity of fungi and insects on dying *E. ingens* but no clear relationship between these biotic factors and the death was found. This led us to investigate the possible involvement of changes in climate as a trigger leading to the death of *E. ingens*. Four sites within the severely affected Limpopo Province were included and two sites in the North West Province where *E. ingens* was less affected. Nine linear transects were established in each site. Each tree within a transect was scored as alive or dead and as mature or juvenile. Beetle and moth damage, animal damage, fire damage as well as spots or lesions on the succulent branches were scored as present or absent. Fruit quantity and gray discoloration of branches were ranked by quantity and severity. Weather data from 1969 – 2008 were obtained for each site. Trees in the Limpopo Province were more severely affected by disease and insects and exhibited higher levels of mortality compared to trees in the North West Province. Temperature and precipitation over the 40 year period analyzed, showed a greater upward trends in temperature and downward trends in precipitation in the Limpopo Province compared with the North West Province. Estimates of potential evapo-transpiration and water balance indicated an increasing water demand while precipitation has remained the

same or is decreasing. The dramatic death of *E. ingens* appears to be linked to increasing moisture deficits resulting in tree stress, which in turn allows opportunistic pathogens and insects to increase in severity, ultimately contributing to tree mortality.

Keywords: *E. ingens*, mass mortality, Limpopo Province, climate change, evapo-transpiration

1. INTRODUCTION

Euphorbia ingens is a large woody succulent tree known only from Africa where it has a wide distribution across Botswana, Lesotho, Mozambique, South Africa, Swaziland and Zimbabwe. This species occurs in high densities in localized sites in the northern provinces of South Africa (Gauteng, KwaZulu-Natal, North West, Limpopo) with especially high abundances in the Limpopo Province (Gildenhuys 2006, Palgrave 2002, Van Wyk & Van Wyk 1997). In the last 10 to 15 years, high levels of mortality of *E. ingens* have been observed in the Limpopo Province (Malan 2006, Roux *et al.* 2008, 2009). No previous declines or die-offs of *E. ingens* are known to have occurred. Therefore, this striking and rapid mortality is of great concern. While the cause of this mortality is not known, insects, pathogens and climate change have all been speculated to play a role (Malan 2006, Roux *et al.* 2008, 2009).

Tree die-offs have been increasingly reported since the 1970's (Raffa *et al.* 2008, Allen *et al.* 2010). Various tree species around the globe have been affected (Fisher 1997, Fensham & Holman 1999, Suarez *et al.* 2004, Tsopelas *et al.* 2004, Foden *et al.* 2007, Hogg *et al.* 2008). Die-offs have occurred in a variety of ecosystems indicating that this phenomenon is not limited to one type of environment (Allen *et al.* 2010). Tree mortality is increasingly being linked to the occurrence of drier conditions or more variable rainfall patterns linked to climate change (Wardle & Allen 1983, Dezzeo *et al.* 1997, Liang *et al.* 2003, Lwanga 2003, Clark 2004, Jurskis 2005, Bigler *et al.* 2006, Allen 2009). Drier conditions can lead directly to mortality through effects on tree physiological functions, or indirectly through effects on tree vigor and defences, which favour attacks by insects and pathogens, leading to rapid tree deaths and even development of epidemics (Konkin & Hopkins 2009, Allen *et al.* 2010).

Plant physiological functions can also be affected by fluctuations in weather. For example, high levels of rainfall followed by warm dry periods can indirectly lead to stress and/or mortality. Microorganisms and plant roots rapidly deplete the gaseous oxygen present in waterlogged soils creating an anoxic environment retarding energy utilization and leading to reduced mineral and water uptake and the death of fine roots, resulting in severe stress to plants and sometimes death (Sinclair & Lyon 2005). During this period plants also become increasingly susceptible to pathogens (Sinclair & Lyon 2005) such as *Phytophthora* root rot (Frankel 2007, 2008). When such abnormally wet periods are followed by dry periods, trees struggle to meet their water needs due to the death of roots caused by oxygen deprivation or disease, resulting in high levels of mortality.

Shifting precipitation patterns are accompanying global warming in some areas (Peters 1990, King & Harangozo 1998, Bergstrom & Chown 1999, Pounds *et al.* 1999). Such shifts can result in precipitation occurring at times other than when trees most need it, creating physiological stress, and lowered resistance to insects and disease, which may ultimately lead to increased levels of tree mortality. However, in areas experiencing no change in amount or timing of precipitation, warming may still result in stress, and ultimately mortality, due to longer growing seasons and higher demand for water with no concurrent increase in moisture (Dale *et al.* 2001, Sinclair & Lyon 2005, Stone *et al.* 2007, Sturrock *et al.* 2011).

Initial studies on the die-off of *E. ingens* were conducted at the Biodiversity Conservation Centre of the National Zoological Gardens (NZG), Mokopane, Limpopo Province, in which the main disease symptoms in affected trees was found to be gray discoloration of normally green succulent branches (Malan 2006). In this study, Malan (2006) was unable to link symptom development and eventual tree death, precipitation (due to limited data), fire, or soil erosion. The possibility that insects or pathogens were responsible for the die-off led to a more detailed study at the same site (Roux *et al.* 2008, 2009). Various disease symptoms were

found on trees at the site including blue stain of the woody stems of diseased trees, as well as spots and subsequent rotting of the succulent branches. Insect infestations were also found in both the woody and succulent parts of the plants (Roux *et al.* 2008, 2009). From affected portions of trees, fungi belonging to the Botryosphaeriaceae, Microascales, Ophiostomataceae and the Teratosphaeriaceae were isolated and insects in the Pyralidae and Curculionidae were collected (Roux *et al.* 2008, 2009, Van der Linde *et al.* 2011a, Van der Linde *et al.* 2011b). However, while a number of fungi and insects were found infesting affected trees, none appeared to be the primary cause of mortality.

Because no single insect or pathogen stood out as being a major driver in *E. ingens* mortality, we hypothesized that climate change (changes in temperature and/or precipitation regimes) may be the primary cause of decline and mortality of *E. ingens* in Southern Africa. Anthropogenic warming is already occurring in Africa where temperatures are predicted to increase by three to seven degrees Celsius by the year 2100. Concurrently, rainfall is expected to either increase or decrease, depending on region, by approximately 20% during the 21st century (Boko *et al.* 2007, Houniet *et al.* 2009). Changes in environmental conditions such as changing temperatures and rainfall amounts or timing may stress *E. ingens* trees, particularly those occurring at their ecological margins, leading directly to mortality through physiological stress or indirectly through responses by secondary insects and pathogens.

The objectives of this study were to determine whether temperature or precipitation patterns are related to insect activity, symptoms of disease, and degree of mortality of *E. ingens* in South Africa. Our study was an attempt to determine the main factors underlying the decline of *E. ingens* and perhaps identify an environmental trigger that has led to die-offs of *E. ingens* in South Africa.

2. MATERIALS AND METHODS

2.1. Study sites

The study was conducted in South Africa from August 2009 to April 2010 at sites in the Limpopo Province (Euphorbia drive, Homestead, Last Post, Keith Johnson), where the die-off is very severe, and the North West Province (Enzelsberg, Wolfaan), where the die-off of *E. ingens* trees is less apparent (FIG. 1 and TABLE I). Sites were chosen to represent a range of tree mortality, from very high to low.

2.2. Assessments of symptoms and mortality

At each site, nine linear belt transects (50m x 100m) were established. Locations of transects were recorded using a Global Positioning System (GPS) (Garmin 60 series, South Africa). Due to the variable distribution in density of *E. ingens* trees, transects were placed in areas where a reasonably high number of trees were present.

Within each transect *E. ingens* trees were tagged using the GPS. The diameters of the tagged trees were measured at breast height (approximately 1.4 m; DBH). In each transect, numbers of juvenile (no fruit production) and adult trees (fruit production), and dead and live trees were recorded. Damage by beetles, moths and animals were scored separately as binary variables (present/absent). The percentage of fruit carried by the trees was scored based on a ranking system of one to three: (1) ≤ 25 – 50%, (2) 50 – 75% and (3) 75 – 100% of branches with fruit. Gray discoloration was also scored based on a ranking system: (1) primary tier braches discoloured, (2) primary and secondary tier branches discoloured, (3) primary, secondary, and tertiary tier branches discoloured, with primary branches representing the lowest, oldest branches.

2.3. Weather data

Monthly precipitation and temperature (minimum and maximum) data were obtained from 1969-2008 (Enzelsberg, Wolfaan, Keith Johnson, Last Post) and 1996-2008 (Euphorbia drive, Homestead) from the South African Weather Service (www.weathersa.co.za) (TABLE II).

2.4. Data Analysis

Differences in mortality among sites and the main symptoms associated with *E. ingens* decline (graying) were analyzed using Kruskal-Wallis one-way ANOVA on ranks using medians for independent variables (moth damage, bird damage, insect damage, basal damage, grey discoloration, spots, mature and juvenile trees, fruit quantity) averaged by transect (n=9 per site). Principal components analysis (PCA) was used to visually detect potential relationships among the three symptoms observed to be most commonly associated with decline (graying, spots, and moth damage) and weather variables (monthly minimum and maximum temperatures, and monthly precipitation). Weather data for PCA was partitioned by season for the four decades preceding the establishment of transects (Keith Johnson, Last Post, Enzelsberg, Wolfaan) or the last decade preceding establishment (Euphorbia Drive/Homestead for which earlier temperature data were not available).

To determine whether temperature and/or precipitation varied by decade (Keith Johnson, Last Post, Enzelsberg, Wolfaan), we used two-way ANOVAs with site, decade and month as potential sources of variation and minimum or maximum monthly temperatures or monthly precipitation as predictor variables. For Keith Johnson and Last Post, maximum and minimum temperature and precipitation data was normal and possessed equal variances and ANOVAs were conducted on raw data. For Enzelsberg and Wolfaan, maximum and minimum, or minimum temperature data, respectively, failed to meet assumptions of normality.

Transforming with (ln +1) (to account for zeros in the data) failed to normalize these data. Likewise, for all four sites, precipitation data were not normal nor did they exhibit equal variances. When transformed using (ln+1), data for Keith Johnson passed the test for equal variances but not normality. The same transformation for data for Enzelsberg and Wolfaan failed to normalize data or equalize variances. While data in these cases violated some assumptions of ANOVA, the large sample size allowed the use of this more robust test. Therefore, two-way ANOVAs were conducted on transformed or raw data depending on their distributions. Mean separations for one-way ANOVAs were conducted using the Tukey Test. Mean separations for two-way ANOVAs were conducted using the Holm-Sidak method. All analyses, except PCA, were conducted using SigmaStat within SigmaPlot 11.2 (Systat Software, Inc., San Jose, CA). PCAs were conducted using Community Analysis Package (CAP) (Pisces conservation Ltd., Lymington, UK).

To investigate whether temperature or precipitation have changed over time at the study sites in ways that may have influenced the decline of *E. ingens*, 40-year means for maximum and minimum temperature and precipitation were calculated for the four sites for which long-term weather data were available (Keith Johnson, Last Post, Enzelsberg, Wolfaan) or the last decade for Euphorbia Drive/Homestead. Annual deviations from these means were then calculated and plotted over time. An index of water balance for each of the sites was also calculated. This index is based on the difference between precipitation and potential evapo-transpiration (PET) and was included to provide information on how changes in water balance in these systems may relate to the die-off. The index is calculated using monthly means of daily temperature, total monthly precipitation and day length adjusted for latitude. Its derivation is fully described in Thornthwaite (1948). Deviations from the mean water balance index over time were calculated and plotted to determine whether water balances at the sites

were becoming more negative and whether there is a threshold water deficit that may help predict the onset of mortality at a site.

3. RESULTS

3.1. Variables associated with *E. ingens* die-off

For several of the variables initially considered, e.g. fire history of the sites and farm management type, there was no apparent relationship with disease and die-off. However, greying, spots, and moth damage appeared to be associated with die-off and these were included in further analyses (TABLE III).

Mortality of *E. ingens* varied significantly among sites ($H = 21.56$, $df = 5$, $P<0.001$). The mean proportion of dead trees was highest at the adjacent Homestead and Euphorbia Drive sites ($\bar{x} = 0.307$, $SE = 0.120$ and $\bar{x} = 0.235$ $SE = 0.112$, respectively) followed by Enzelsberg ($\bar{x} = 0.137$, $SE = 0.049$). The mean proportion of dead trees was low at Last Post and Wolfaan ($\bar{x} = 0.026$, $SE = 0.015$ and $\bar{x} = 0.050$, $SE = 0.028$, respectively) and almost non-existent at Keith Johnson ($\bar{x} = 0.008$, $SE = 0.008$).

The degree of greying present on trees was significantly different among sites ($H = 43.67$, $df = 5$, $P<0.001$). The mean ranking of greying (measured on a scale from 0-3) was highest at Euphorbia Drive ($\bar{x} = 1.89$, $SE = 0.058$) followed by Last Post ($\bar{x} = 1.33$, $SE = 0.84$) and Homestead ($\bar{x} = 1.24$, $SE = 0.11$). Greying was intermediate at Keith Johnson ($\bar{x} = 0.549$, $SE = 0.152$) and least apparent on trees at Wolfaan ($\bar{x} = 0.22$, $SE = 0.09$) and Enzelsberg ($\bar{x} = 0.03$, $SE = 0.01$).

Moth damage was found to be significantly different between sites ($H = 29.21$, $df = 5$, $P<0.001$). Trees with the highest proportion of moth damage were found at the two sites in Mokopane, Euphorbia drive ($\bar{x} = 0.889$, $SE = 0.111$) and Homestead ($\bar{x} = 0.733$, $SE = 0.073$).

Moth damage was also relatively high at Enzelsberg ($\bar{x}$ = 0.652, SE = 0.076) and Last Post ($\bar{x}$ = 0.616, SE = 0.033). Low levels of moth damage were found to occur at Keith Johnson ($\bar{x}$ = 0.431, SE = 0.063) and Wolfaan ($\bar{x}$ = 0.122, SE = 0.060).

Spots on the green tissue were found to be present at high levels at all of the sites (H = 23.50, df = 5, P = 0.003) with the highest occurrences being at Euphorbia drive ($\bar{x}$ = 1.000, SE = 0.000) and Last Post ($\bar{x}$ = 0.981, SE = 0.009). High levels of spots were also found at Homestead ($\bar{x}$ = 0.940, SE = 0.026) followed by Enzelsberg ($\bar{x}$ = 0.782, SE = 0.048) and Wolfaan ($\bar{x}$ = 0.761, SE = 0.082).

3.2. Relationship between climate and *E. ingens* die-off

Results of ANOVAs comparing monthly means for maximum and minimum temperature and precipitation among decades (decade 1 = 1969-1978, decade 2 = 1979-1988, decade 3 = 1989-1998, decade 4 = 1999-2008) for each site (except Euphorbia Drive/Homestead for which long-term data were missing for temperature) indicated significant differences in environmental conditions between the sites for these periods (TABLE IV). For both the Last Post and Keith Johnson sites, mean maximum temperatures during the second, third and fourth decades were significantly warmer than those occurring in the first decade. At these sites, the fourth decade was significantly warmer than the first and second decades, and the third was warmer than the first decade. There was no significant difference in precipitation among decades. At Enzelsberg, temperature was significantly different between decades. However, at this site, maximum temperatures were warmest in decades two and three, and minimum temperatures showed no change by decade. Precipitation was significantly lower in the fourth decade than in the first. There was no interaction between decade and month for Last Post, Keith Johnson, or Enzelsberg. At Wolfaan, decade four was warmer than decades one, two or three, and there was a significant difference among decades and a significant

interaction between decade and month for minimum temperatures. Decade four exhibited significant warming from decades one, two and three and these differences were due to increased minimum temperatures in May through August.

For the Euphorbia Drive/Homestead sites, deviations from the ten year mean showed a clear trend in warming for maximum temperatures with the first three years below the mean, the fourth year at the mean and the final six years well above the mean (FIG. 2). Deviations from the ten-year mean for minimum temperatures were much more variable and showed no clear trend. For the Keith Johnson and Last Post sites (FIGS. 3, 4), there were clear trends of increasing maximum and minimum temperatures over the forty year period. In both cases, the period from 1969 through 1981 was relatively cool, while the period from 2002 through 2008 was relatively warm, and the period in between was highly variable. For Keith Johnson, 15 of the first 20 years fell below the forty year average temperature, while in the latter twenty years, 14 years (maximum temperature) or 12 years (minimum temperature), were higher than the40 year average. At Last Post, trends in temperature closely mirrored those at Keith Johnson. For Wolfaan (FIG. 5), 12 years in the first twenty year period were below the 40 year average for maximum temperature, while the trend reversed in the later twenty years, where 11 years out of twenty were above average. For minimum temperatures, 19 of the first 20 years were below the 40 year average, while 10 were below this average for the latter twenty year period and the last four years showed a striking increase. Enzelsberg (FIG. 6) differed from all other sites in that there was no consistent increase in maximum or minimum temperatures over the 40 year period.

Precipitation showed an overall decline or increased inter-annual variability at each site over the 40 year time period that was analyzed. At Homestead/Euphorbia Drive, equal numbers of years were above or below the 40 year mean in the first 20 years but 12 of 8 fell below the 40 year mean in the latter 20 year period (FIG. 2). For Keith Johnson, 13 of the first 20 years were

above the 40 year mean, while 13 of the latter 20 years were below this mean (FIG. 3). Similarly, at Last Post, 11 of the first 20 years were above the 40 year mean and 12 of the latter 20 years were below the mean (FIG. 4). At Wolfaan, precipitation did not change appreciably (FIG. 5). At Enzelsberg, precipitation decreased slightly with equal years above and below the 40 year mean in the first 20 year period and 12 below for the second 20 year period (FIG. 6). The last 7 years at this site all fell below the 40 year mean.

Because temperature and precipitation interact to determine the actual amount of water available to plants for growth and maintenance, PET was calculated for each year for each of the study sites and for which both temperature and precipitation data were available. Total annual precipitation was then subtracted from the PET calculations to produce an index of water balance (WB). This index is an estimate based on the input of water to the system minus that which would be lost if the area was uniformly covered with vegetation given particular monthly mean temperatures adjusted for day length for a given latitude. Euphorbia Drive/Homestead, by far, exhibited the greatest (most negative value) mean PET followed by Enzelsberg, Last Post, Keith Johnson, and Wolfaan (TABLE V). Estimates of WB indicated a greater moisture deficit (more negative values) at sites with high levels of symptoms and mortality (Euphorbia Drive/Homestead, Keith Johnson, Last Post) than at the two relatively healthy sites (Wolfaan, Enzelsberg) (TABLE V). For the Euphorbia Drive/Homestead site (FIG. 7), only ten years of temperature data were available, greatly restricting our ability to detect trends over time. However, relative to other sites, PET was highest at this site indicating a very high demand for moisture by vegetation. At Keith Johnson (FIG. 8), PET increased in the last decade. While water balance (deficit) on average did not increase at this site, it became highly erratic in the last decade. At Last Post (FIG. 9), PET remained relatively constant over the 40 year period, although the WB deficit increased over the last decade. At Wolfaan (FIG. 10), both WB and PET increased greatly in the last decade relative to previous decades. PET

did not increase noticeably at Enzelsberg (FIG. 11). In the mid-80s and from 2002-2008, this site exhibited higher WB deficits than during other periods. Both of these periods coincided with low precipitation. However, WB deficits remained relatively low over all years. Only Enzelsberg (in 1976 and 2001) and Wolfaan (in 1997 and 2000) showed years where water balance was positive (FIGS. 10, 11)

There was no strong upward or downward trend in WB for Euphorbia Drive/Homestead (FIG. 12). Keith Johnson had overall lower WB deficits for years at the beginning of the 40 year period analyzed with 8 of the first 10 years above the mean (FIG. 13). The last ten years were highly variable with considerable swings above and below the mean. At Last Post, the first ten years were mainly above the mean and the last ten years mainly below the mean with the period between highly variable (FIG. 14). Enzelsberg exhibited the greatest variability in WB over time, although, like Last Post, the last 7 years were all below the mean (FIG. 15). At Wolfaan, the first ten years were mainly above the mean and the last eleven years all below the mean, with the period between highly variable (FIG. 16).

3.3. Principal Components Analyses

PCAs were run using data for branch greying, spots on the green tissue, moth damage and weather data for each decade for Last Post, Keith Johnson, Enzelsberg and Wolfaan, and for the last decade for Euphorbia Drive/Homestead. PCA plots were done for all sites except Euphorbia Drive/Homestead for decades three (1989-1998) and four (1999-2008). These two decades were chosen because climatic influences on the decline may have begun in the decade(s) immediately preceding the development of obvious symptoms and widespread mortality. In the ordination plot for the fourth decade (including all sites), sites with the highest tree die-off cluster with spots, moth damage, greying, higher maximum temperatures and lower precipitation, particularly in the dry winter and fall periods while the healthier sites

cluster with higher precipitation and lower temperatures. The ordinations for the third and fourth decades that do not include the Euphorbia Drive/Homestead site (FIGS. 17A, B) mostly agree with one another and the PCA conducted for all sites for decade four (FIG. 17C). For decade three, the sites exhibiting the greatest degree of decline clustered with higher maximum and minimum temperatures, lower precipitation, greying, spots and moth damage while the relatively healthy sites clustered with lower temperatures and greater precipitation. The first two axes accounted for 84% of the variance; adding the third axis brought this estimate to 94%. For decade four, the sites exhibiting the greatest degree of die-off clustered with higher maximum temperatures, lower precipitation, greying and spots while the relatively healthier sites clustered with lower temperatures and greater precipitation. In the ordination for decade four (FIG. 17 D), the first two axes explain 88% of the variance. When the third axis was included, it was possible to account for 96% of the variance. Ordinations run for decades one and two (data not shown) agreed very closely with the ordination for decade three.

4. DISCUSSION

The three main symptoms associated with the die-off of *E. ingens* were graying of the branches, spots on the green tissue and moth damage. These symptoms varied in severity among sites, but were overall much less severe at sites in the North West Province (Wolfaan, Enzelsberg) than in the Limpopo Province. These sites also had higher levels of recruitment (more juveniles) than sites in the Limpopo Province. The main symptom of disease, graying of the branches, was most severe in the Limpopo Province, particularly at the Euphorbia drive/Homestead site which also exhibited the highest level of mortality.

The Limpopo Province sites are approximately 2˚ further north, and experience higher temperatures and lower rainfall, that those in the North West Province. Therefore, these sites

may be marginal for *E. ingens.* If this is true, extended hot dry periods could have pushed the trees past a threshold where they are no longer able to survive. Our analyses of weather patterns over the last 40 years indicated that maximum and minimum temperatures have increased in the Limpopo Province and may be driving the die-offs. The greatest change in maximum temperatures occurred at Euphorbia drive/Homestead (with a 1.97˚C increase), the site with the most obvious symptoms and most severe mortality. Maximum temperatures in the North West Province did not increase greatly, although Wolfaan exhibited a rise of about 0.63˚C. However, minimum temperatures at Wolfaan increased dramatically in the last decade (about 2.66˚C). Even though temperatures increased in the North West Province over the 40 year period, the mean annual maximum and minimum temperatures were still lower overall compared to the Limpopo Province. As temperatures increase, so does evapo-transpiration. If precipitation does not increase to meet this increase in demand for moisture by plants then stress may occur resulting directly in mortality if stress becomes severe or indirectly due to increased susceptibility to insects and pathogens (Stone *et al.* 2007, Dukes *et al*. 2009, Tubby *et al*. 2010, Sturrock *et al.* 2011). In cases where precipitation declines at the same time temperature increases, this effect may be even more pronounced. In this study, sites with higher temperatures, higher PET and higher WB deficits exhibited the greatest degree of symptoms and mortality. While the data are not conclusive that greater water stress is the proximate cause of the die-off, they indicate that climate is playing a substantial role.

Principle component analysis indicated that the main symptoms associated with die-off (greying of stems, moth damage and spots on green tissue) were most closely associated with sites experiencing the hot and dry autumn and winter periods (Fig. 17). Autumn and winter are the dry seasons for this portion of South Africa. Increases in temperature and decreases in precipitation during this period may increase stress on trees leading to greater susceptibility to insects and disease, and ultimately, mortality. Unfortunately, temperature data for Euphorbia

Drive/Homestead, the site most severely impacted by the die-off, were available only from 1996-2009. Die-offs were first noticed in the mid-to-late 90s at this site, suggesting that an environmental trigger for the die-off likely occurred in the previous decade (1989-1998). This trigger may have been related to increased temperatures (and concurrent increased demand for water) as increases in temperature were observed at the other sites in the Limpopo Province during that period.

We were not able to definitively determine the actual cause or trigger of the *E. ingens* die-offs being observed in South Africa. However, the die-offs appear to be the result of the interaction of many factors including temperature, precipitation and various biotic components (particularly, insects and fungi). Additional work that includes a greater number of sites spanning a range of symptoms, mortality, and weather conditions will contribute to a better understanding of the links between weather conditions and climate change and die-offs of *E. ingens*.

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TABLE I: Characteristics and locations of study sites in the Limpopo and North West Provinces.

Site Number	Site Name	GPS coordinates	Elevation (m)	Locality/Province	Land Usage	Vegetation type/density	Soil	Fire History
1	Enzelsberg	S25 22.817 E26 15.975	1100-1200	Enzelsberg/North West	Private Game Farm	Savanna/Very Dense	Rocky/Clay	No recent history
2	Wolfaan	S25 46.411 E27 36.404	1200-1300	Rustenburg/North West	Old Overgrown Farm/Between Citrus Orchids	Savanna/Dense	Sandy	Last fire: 2009
3	Euphorbia Drive	S24 09.923 E29 02.275	1200-1300	Mokopane/Limpopo	Game Breeding Farm	Savanna/Sparse	Clay	No recent history
4	Homestead	S24 10.437 E29 03.266	1200-1300	Mokopane/Limpopo	Old Overgrown Farm	Savanna/Dense	Clay	Last fire: 2001
5	Keith Johnson	S23 21.910 E29 44.621	1000-1100	Polokwane/Limpopo	Domestic Cattle Farm	Savanna/Sparse	Sandy	No recent history
6	Last Post	S23 17.738 E29 55.467	900-1000	Louis Trichardt/Limpopo	Private Game Farm	Savanna/Sparse to Dense	Rocky/Clay	No recent history

TABLE II: Weather stations used for each site in the Limpopo and North West Provinces.

Province	Site	Rainfall station	Temperature station	*Distance from site (km)
Limpopo	Homestead	Palmer Estate	Mokopane	17.5/6
Limpopo	Euphorbia drive	Palmer Estate	Mokopane	17.5/6
Limpopo	Keith Johnson	Mara-Pol	Mara	49/29.5
Limpopo	Last Post	Mara	Mara	39.5/39.5
North West	Wolfaan	Brits/Hartbeespoortdam	Buffelspoort II AGR	18/19
North West	Enzelsberg	Tuscany	Marico	17/16

* Rainfall/Temperature station distance from site

TABLE III: Mortality, % mature vs juvenile trees, and scores for symptoms on live trees in each transect at each study site.

Site	Transect	Moth Damage (%)	Grey (mean rank)	Spots (%)	Mature (%)	Mortality (n in each transect)
Euphorbia drive (n = 189)	1	100	2.00	100	100	5
	2	100	1.78	100	100	5
	3	100	1.50	100	100	4
	4	100	1.78	100	100	9
	5	100	2.00	100	100	5
	6	100	2.00	100	100	2
	7	100	2.00	100	100	6
	8	100	1.96	100	100	5
	9	0	2.00	100	90	1
Total Mortality						42 (22%)
Homestead (n = 277)	1	41	1.02	80	76	1
	2	70	1.00	90	95	1
	3	67	0.93	0	93	0
	4	58	1.38	100	100	1
	5	62	1.05	100	86	3
	6	100	1.25	88	94	15
	7	94	1.94	94	100	39
	8	*	*	*	*	24
	9	94	1.56	100	100	15
Total Mortality						99 (36%)
Last Post (n = 172)	1	54	1.35	100	100	0
	2	57	1.71	93	86	0
	3	68	1.16	94	97	1
	4	63	0.88	96	79	0
	5	54	1.23	100	100	2
	6	54	1.15	100	100	0
	7	62	1.46	100	92	0
	8	85	1.55	100	100	0
	9	57	1.5	100	93	1
Total Mortality						4 (2%)
Keith Johnson (n = 182)	1	36	0.48	72	60	0
	2	23	0.33	81	30	0
	3	33	0.33	100	67	0
	4	72	0.33	89	78	2
	5	35	0.30	56	43	0
	6	42	0.33	58	50	0
	7	19	0.19	47	38	0
	8	60	1.13	93	87	0
	9	68	1.52	100	94	0
Total Mortality						2 (1%)

Enzelsberg	1	45	0.03	74	58	0
(n = 224)	2	55	0.03	69	52	1
	3	73	0.05	68	45	4
	4	55	0.07	59	59	3
	5	64	0.00	64	45	2
	6	93	0.00	100	73	10
	7	27	0.00	87	60	2
	8	75	0.11	96	79	6
	9	100	0.00	87	53	1
Total Mortality						29 (13%)
Wolfaan						
(n = 627)	1	28	0.03	90	79	14
	2	33	0.00	100	100	3
	3	46	0.72	85	87	0
	4	1	0.19	89	33	8
	5	0	0.02	41	22	0
	6	0	0.13	49	8	2
	7	2	0.02	42	5	1
	8	0	0.53	89	33	1
	9	0	0.35	100	55	0
Total Mortality						29 (5%)

***No scores were obtained for transect eight, since all the trees in this site were dead.**

n = total number of trees in transect.

Variables: Damage = moth damage, internal feeding on succulent branches (rated as present/absent), Grey: grey discoloration occurring on succulent branches (rated from 0-3), Spots: various types of yellow and white spotting occurring on the succulent branches (rated as present/absent, Mature: refers to proportion of adults within each transect.).

TABLE IV: ANOVA tables for comparisons of monthly mean maximum, minimum temperatures (degrees ˚C) and precipitation (mm) for four decades (1969-1978, 1979-1988, 1989-1998, 1999-2008) at each of five sites in South Africa used in this study. For least square means for a particular ANOVA, values followed by a different letter are significantly different at P< 0.05.

Source of variation	DF	F	P	Least square means (SE) by decade	
Last Post					
Maximum temperature					
Decade	3	11.84	<0.001	69-78	26.72 (0.14)a
Decade x month	33	1.84	0.70	79-88	27.39 (0.14)b
Total	431			89-98	27.55 (0.15)bc
				99-08	27.85 (0.13)bd
Minimum temperature					
Decade	3	7.41	<0.001	69-78	12.07 (0.10)a
Decade x month	33	0.985	0.50	79-88	12.18 (0.10)b
Total	455			89-98	12.45 (0.10)c
				99-08	12.67 (0.10)cb
Precipitation					
Decade	3	2.02	0.11	69-78	46.38 (3.67)a
Decade x month	33	0.81	0.77	79-88	36.42 (3.67)a
Total	478			89-98	37.43 (3.69)a
				99-08	37.50 (3.67)a
Keith Johnson					
Maximum temperature					
Decade	3	11.75	<0.001	69-78	26.74 (0.13)a
Decade x month	33	0.82	0.76	79-88	27.36 (0.13)b
Total	479			89-98	27.45 (0.13)bc
				99-08	27.85 (0.13)c
Minimum temperature					
Decade	3	8.40	<0.001	69-78	12.08 (0.10)a
Decade x month	33	0.92	0.597	79-88	12.16 (0.10)a
Total	478			89-98	12.56 (0.10)b
				99-08	12.66 (0.10)b
Precipitation					
Decade	3	1.04	0.37	69-78	38.96 (3.93)a
Decade x month	33	0.55	0.98	79-88	38.15 (3.93)a
Total	475			89-98	31.06 (4.00)a
				99-08	40.00 (3.93)a
Euphorbia Dr./Homestead					
Precipitation					
Decade	3	0.54	0.65	69-78	48.27 (3.36)a
Decade x month	33	1.54	0.03	79-88	46.32 (3.35)a
Total	476			89-98	42.33 (3.37)a
				99-08	46.06 (3.35)a
Signif. decade x month interactions (p<0.05):		Jan 69-78, 89-98 wetter than 99-08 Mar 69-78 wetter than 99-08 Nov 69-78, 79-88, 89-99 drier than 99-08			

Enzelsberg					
Maximum temperature					
Decade	3	8.29	<0.001	69-78	26.89 (0.18)a
Decade x month	33	0.85	0.71	79-88	27.93 (0.17)b
Total	359			89-98	27.52 (0.17)b
				98-08	26.86 (0.18)a
Minimum temperature					
Decade	3	3.59	0.01	69-78	11.72 (0.13)a
Decade x month	33	0.47	0.99	79-88	12.29 (0.14)b
Total	347			89-98	11.77 (0.12)a
				99-08	11.81 (0.12)a
Precipitation					
Decade	3	3.14	0.03	69-78	52.80 (4.12)a
Decade x month	33	1.33	0.11	79-88	42.97 (4.12)a
Total	479			89-98	55.26 (4.12)a
				99-08	46.58 (4.12)a
Wolfaan					
Maximum temperature					
Decade	3	5.07	0.002	69-78	26.09 (0.14)a
Decade x month	33	1.02	0.44	79-88	26.10 (0.14)a
Total	479			89-98	26.14 (0.14)a
				99-08	26.75 (0.14)b
Minimum temperature					
Decade	3	60.72	<0.001	69-78	11.08 (0.16)a
Decade x month	33	0.88	0.67	79-88	11.20 (0.16)a
Total	478			89-98	11.50 (0.16)a
				99-08	13.68 (0.17)b
Precipitation					
Decade	3	0.51	0.68	69-78	54.87 (4.15)a
Decade x month	33	0.89	0.65	79-88	50.93 (4.15)a
Total	477			89-98	57.18 (4.17)a
				99-08	57.28 (4.17)a

TABLE V: Mean (SE) maximum and minimum temperature (˚C), precipitation (cm), potential evapo-transpiration (PET) and water balance (WB) (PET minus precipitation) at six study sites exhibiting various levels of die-off of *Euphorbia ingens* in South Africa

Site	Decade	Max. Temperature	Min. Temperature	Precipitation	PET	WB
Euphorbia Dr	1969-1978	-	-	56.58 (2.46)	-	-
Homestead	1979-1988	-	-	55.59 (3.96)		
	1989-1998	-	-	47.42 (2.02)	-	-
	1999-2008	27.81 (0.34)	13.61 (0.19)	55.27 (4.70	-102.60 (1.54)	-47.33 (5.45)
	Total period	-	-	54.02 (1.78)	-	-
Keith Johnson	1969-1978	26.72 (0.16)	12.07 (0.15)	47.02 (4.88)	-92.24 (1.79)	-45.22 (4.94)
	1979-1988	27.39 (0.20)	12.18 (0.24)	44.71 (4.89)	-95.87 (1.46)	-51.16 (5.57)
	1989-1998	27.57 (0.23)	12.45 (0.18)	40.14 (3.23)	-96.42 (1.78)	-56.28 (3.75)
	1999-2008	27.86 (0.25)	12.66 (0.17)	45.18 (7.16)	-97.25 (1.49)	-52.07 (7.80)
	Total period	27.38 (0.12)	12.34 (0.10)	44.26 (2.55)	-95.44 (0.85)	-51.18 (2.81)
Last Post	1969-1978	26.72 (0.16)	12.07 (0.15)	55.66 (4.12)	-94.66 (1.97)	-38.97 (4.05)
	1979-1988	27.39 (0.20)	12.18 (0.24)	43.70 (4.94)	-98.20 (1.65)	-54.49 (5.93)
	1989-1998	27.55 (0.26)	12.45 (0.18)	44.89 (4.67)	-96.09 (1.07)	-51.78 (5.11)
	1999-2008	27.85 (0.22)	12.66 (0.17)	45.00 (5.35)	-95.95 (1.20)	-51.16 (5.46)
	Total period	27.38 (0.12)	12.34 (0.10)	47.31 (2.44)	-96.22 (0.76)	-48.96 (2.66)
Wolfaan	1969-1978	26.09 (0.24)	11.08 (0.08)	54.88 (3.21)	-90.75 (0.84)	-35.88 (3.45)
	1979-1988	26.16 (0.18)	11.21 (0.11)	51.73 (2.69)	-90.35 (0.78)	-38.62 (2.62)
	1989-1998	26.20 (0.33)	11.50 (0.13)	57.10 (2.61)	-89.20 (1.65)	-32.10 (3.42)
	1999-2008	26.72 (0.24)	13.65 (0.80)	56.69 (6.93)	-99.43 (3.36)	-48.24 (4.13)
	Total period	26.29 (0.13)	11.86 (0.26)	55.10 (2.07)	-92.43 (1.15)	-38.71 (1.91)
Enzelsberg	1969-1978	26.97 (0.28)	11.67 (0.10)	63.36 (5.12)	-94.66 (1.95)	-30.75 (6.48)

1979-1988	27.98 (0.25)	12.10 (0.30)	51.57 (4.83)	-98.19 (1.64)	-46.33 (6.37)
1989-1998	27.32 (0.25)	11.87 (0.16)	66.33 (6.04)	-96.69 (0.86)	-29.77 (6.48)
1999-2008	27.27 (0.30)	11.66 (0.17)	55.90 (5.60)	-95.45 (1.26)	-38.18 (6.63)
Total period	27.39 (0.14)	11.83 (0.10)	54.29 (2.76)	-96.24 (0.75)	-36. 26 (3.30)

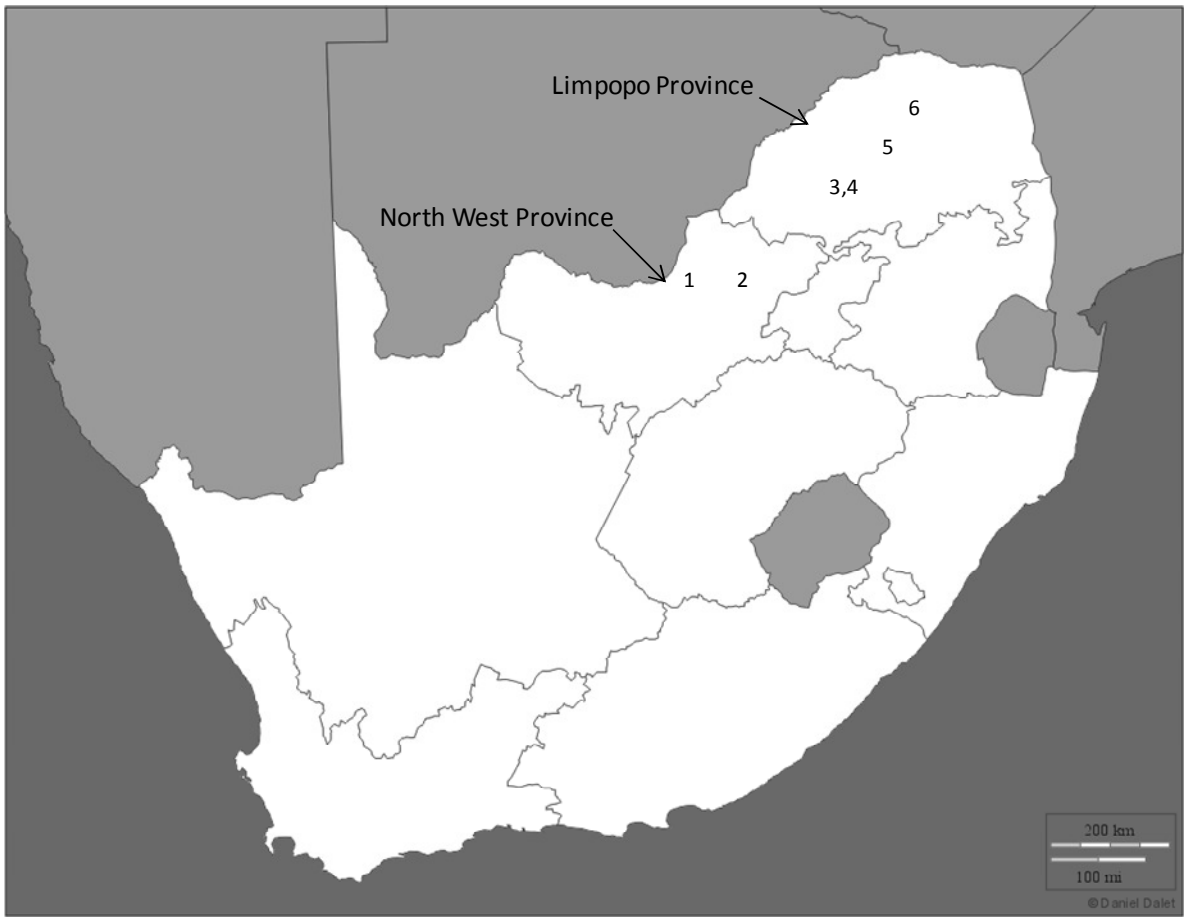
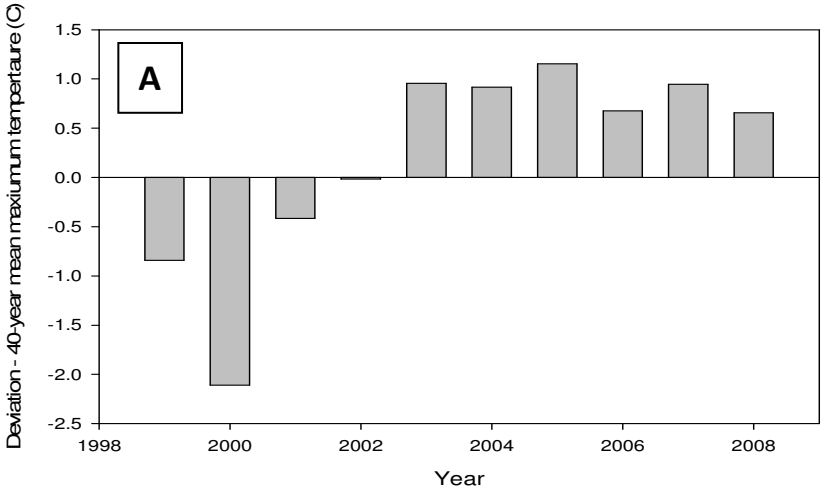
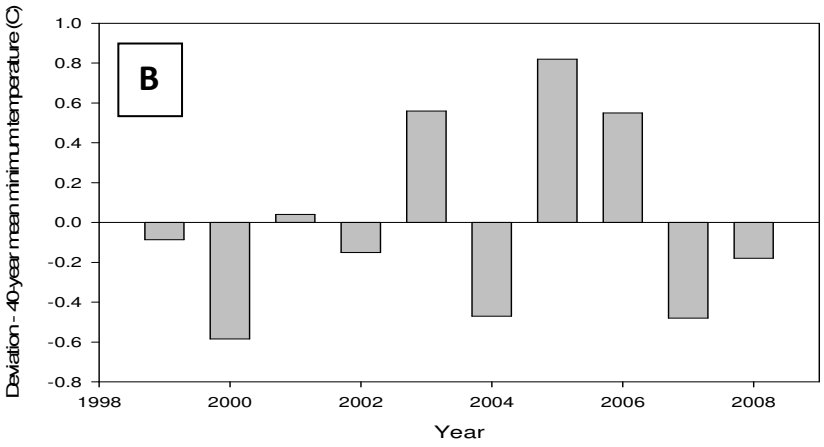
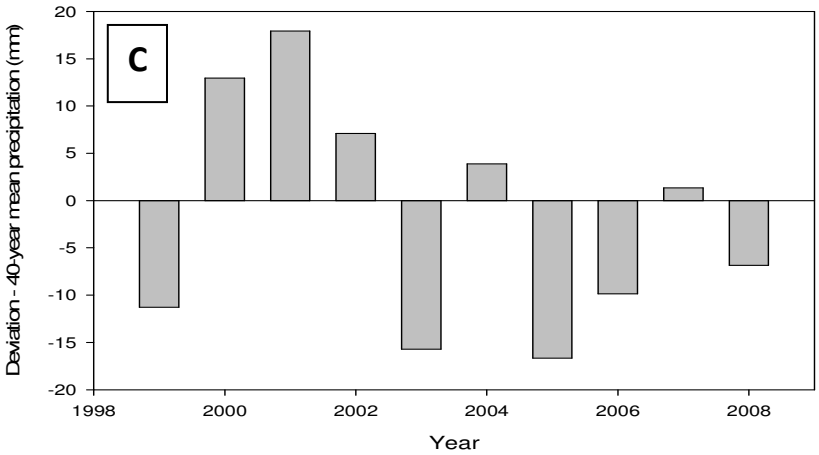


FIG. 1. Map of South Africa showing locations of study sites used investigating die-off of *Euphorbia ingens*. 1 = Enzelsberg, 2 = Wolfaan, 3 = *Euphorbia* drive, 4 = Homestead, 5 = Keith Johnson and 6 = Last Post.







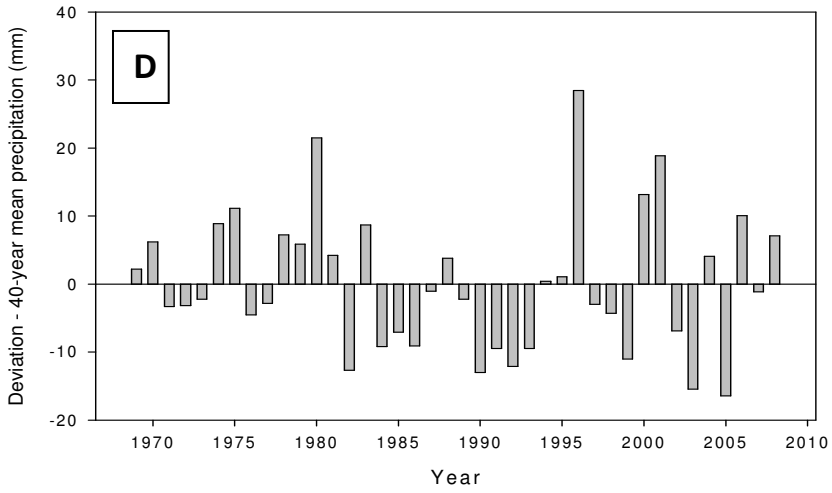
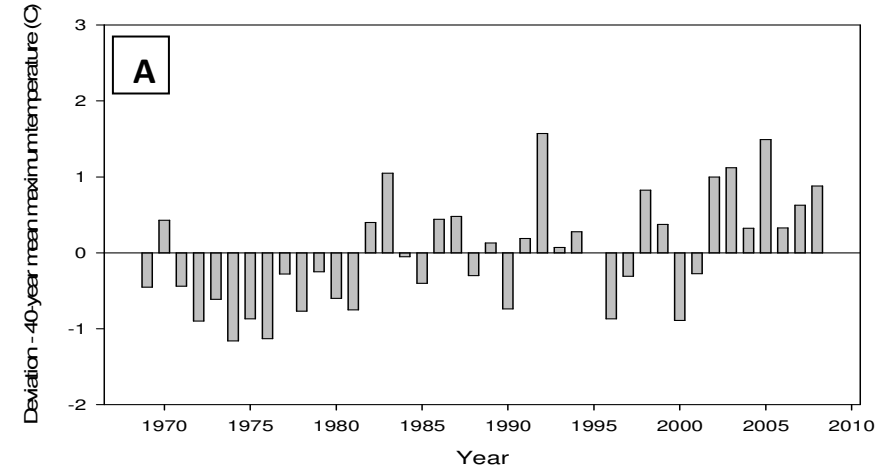
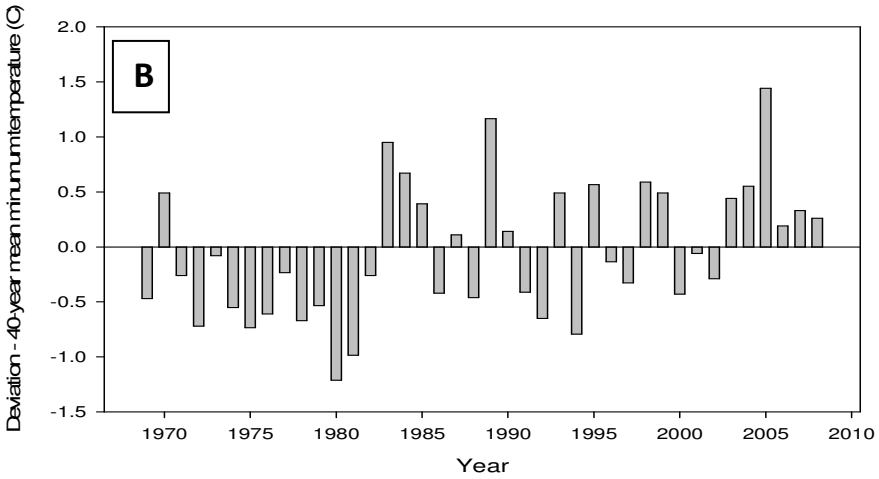


FIG. 2. Deviations from the 10 year mean for A) mean annual maximum temperature, B) mean annual minimum temperature, and C) mean annual precipitation for the period from 1999-2008, and for D) from the forty year mean for mean annual precipitation for the period from 1969-2008 at the Euphorbia Drive/Homestead study sites, South Africa.





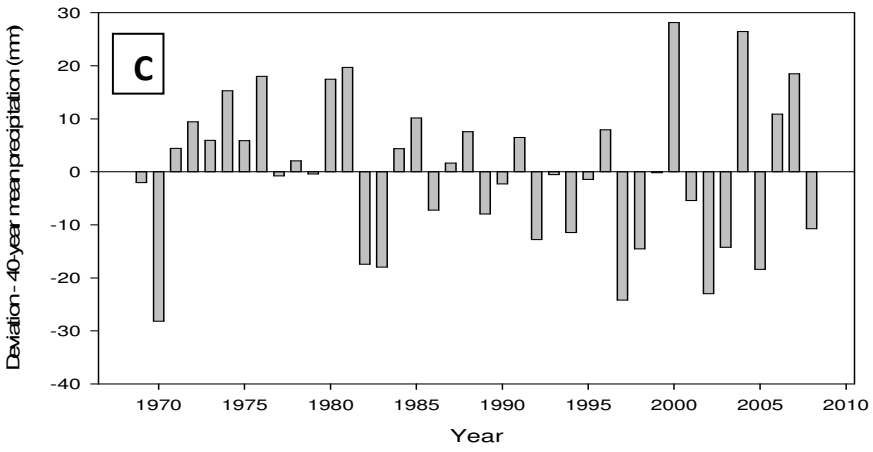
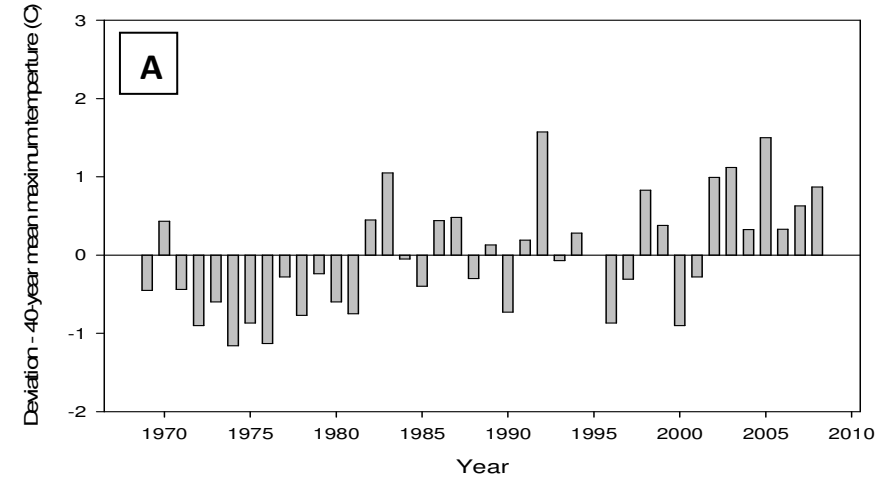
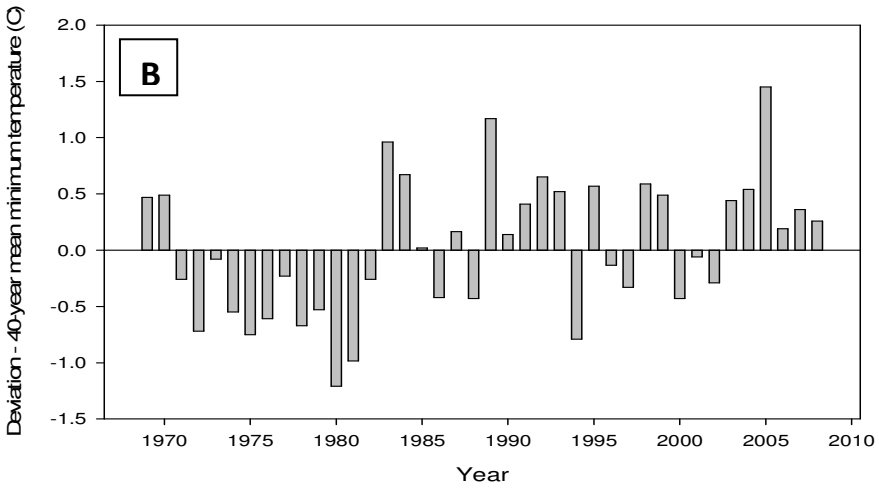


FIG. 3. Deviations from the 40 year mean for A) mean annual maximum temperature, B) mean annual minimum temperature, and C) mean annual precipitation for the period from 1969-2008 at the at the Keith Johnson study site, South Africa.





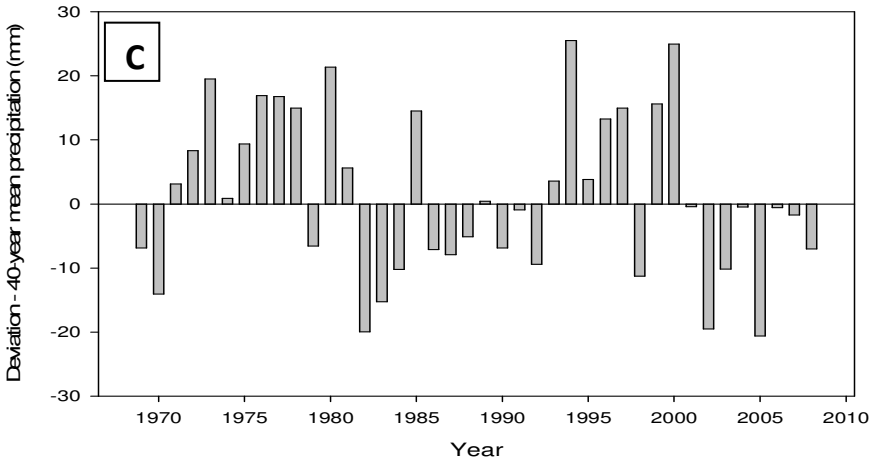


FIG. 4. Deviations from the 40 year mean for A) mean annual maximum temperature, B) mean annual minimum temperature, and C) mean annual precipitation for the period from 1969-2008 at the at the Last Post study site, South Africa.

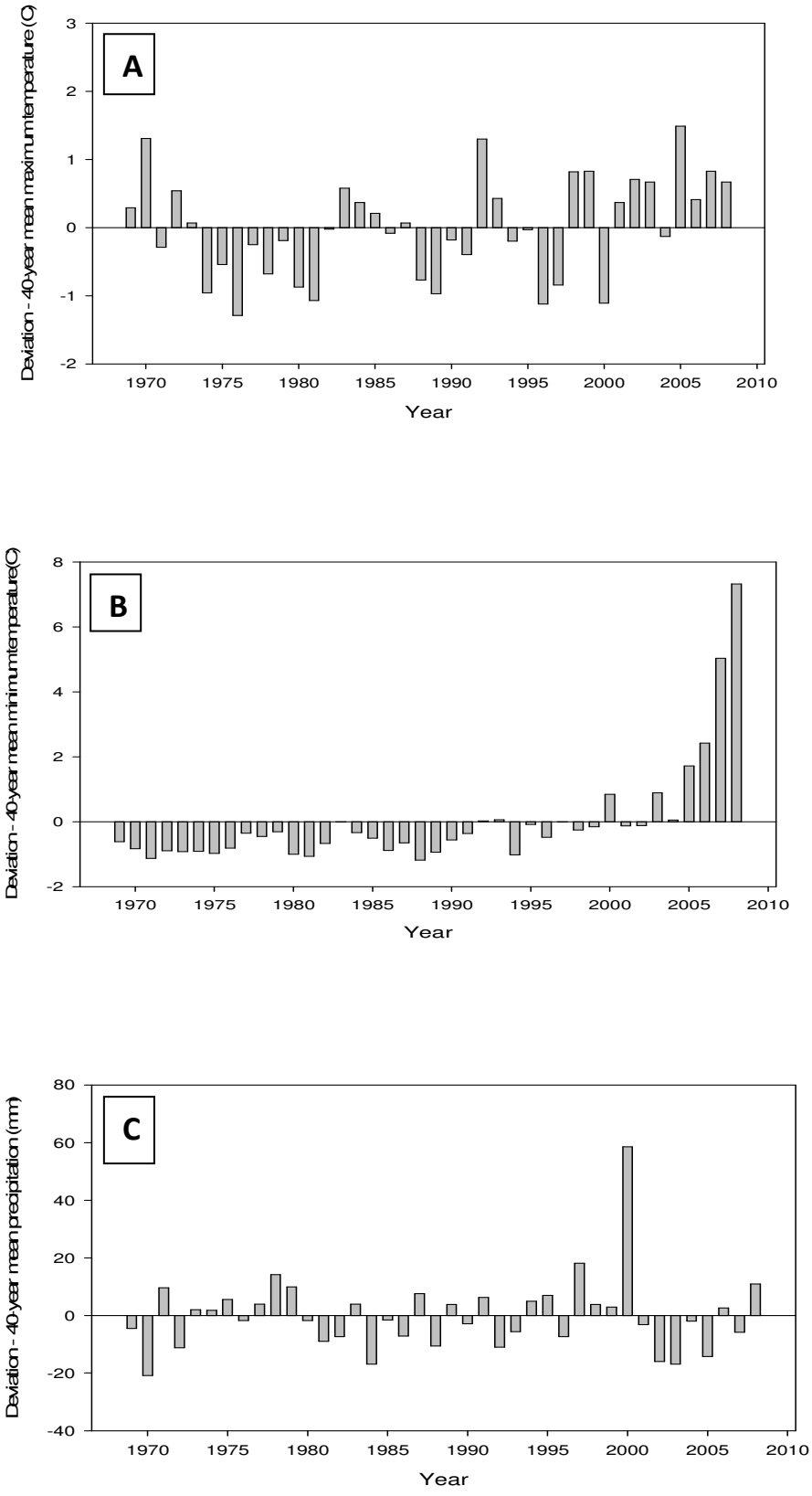


FIG. 5. Deviations from the 40 year mean for A) mean annual maximum temperature, B) mean annual minimum temperature, and C) mean annual precipitation for the period from 1969-2008 at the at the Wolfaan study site, South Africa.

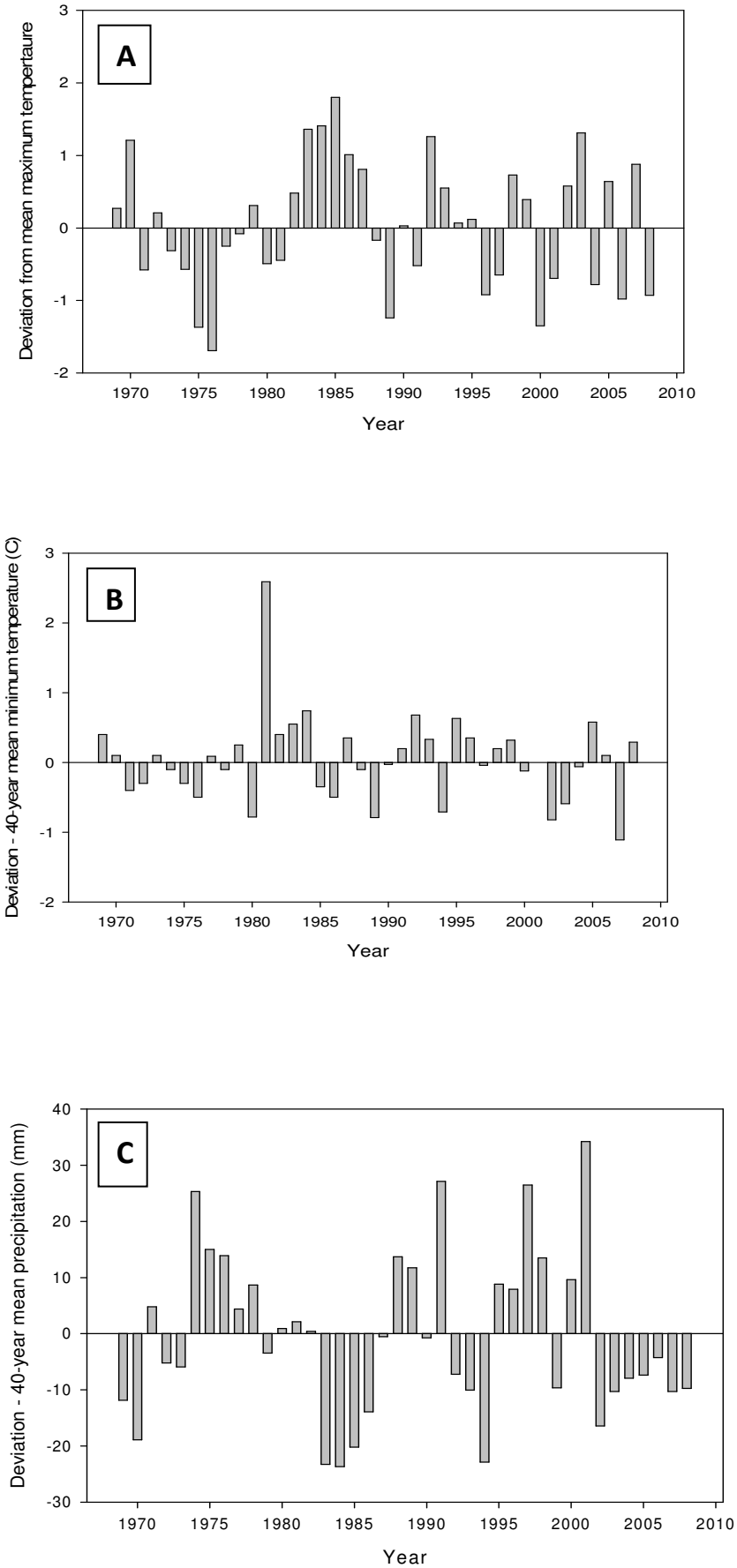


FIG .6. Deviations from the 40 year mean for A) mean annual maximum temperature, B) mean annual minimum temperature and C) mean annual precipitation for the period from 1969-2008 at the at the Enzelsberg study site, South Africa.

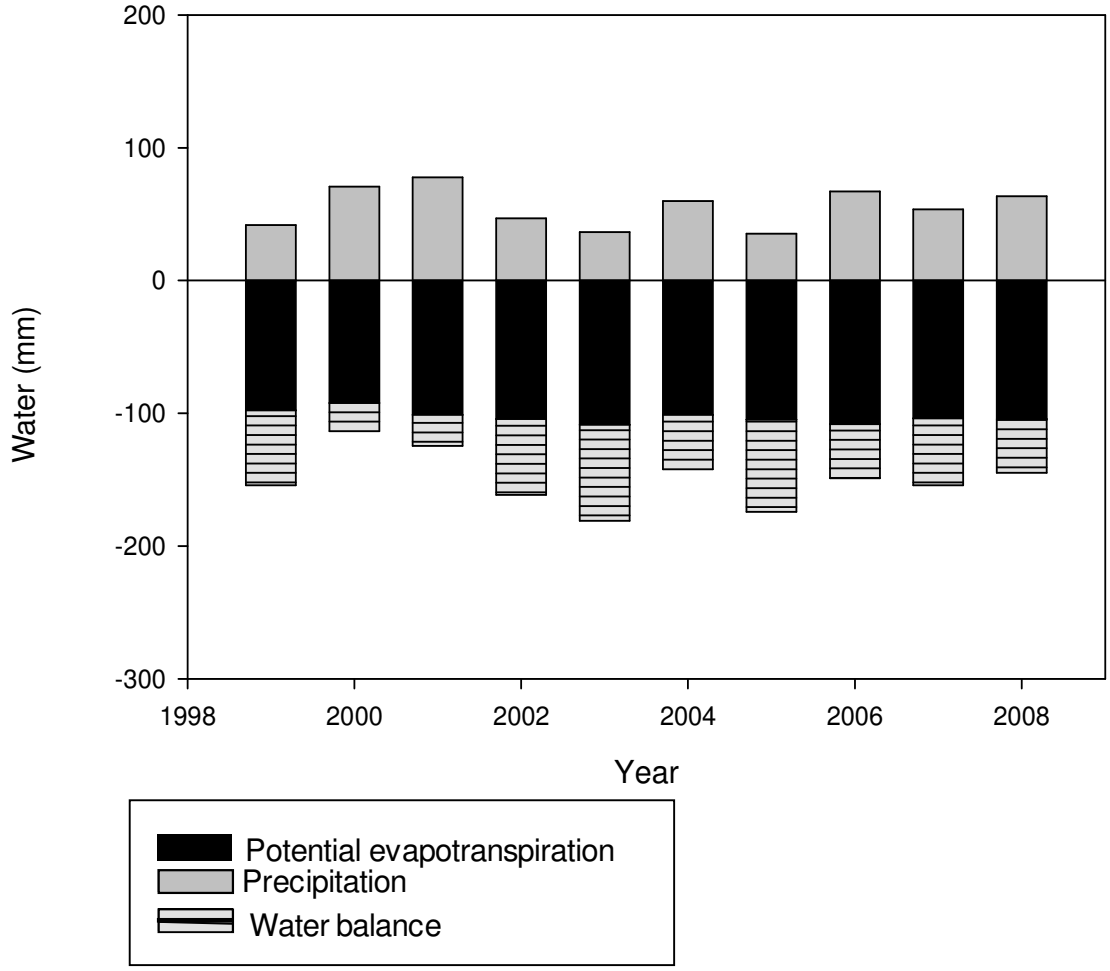


FIG. 7. Annual precipitation and estimates of potential evapo-transpiration (PET) and water balance [precipitation (water input) – PET (water demand/output)] (mm) for the period from 1999-2008 for the Euphorbia Drive/Homestead study sites.

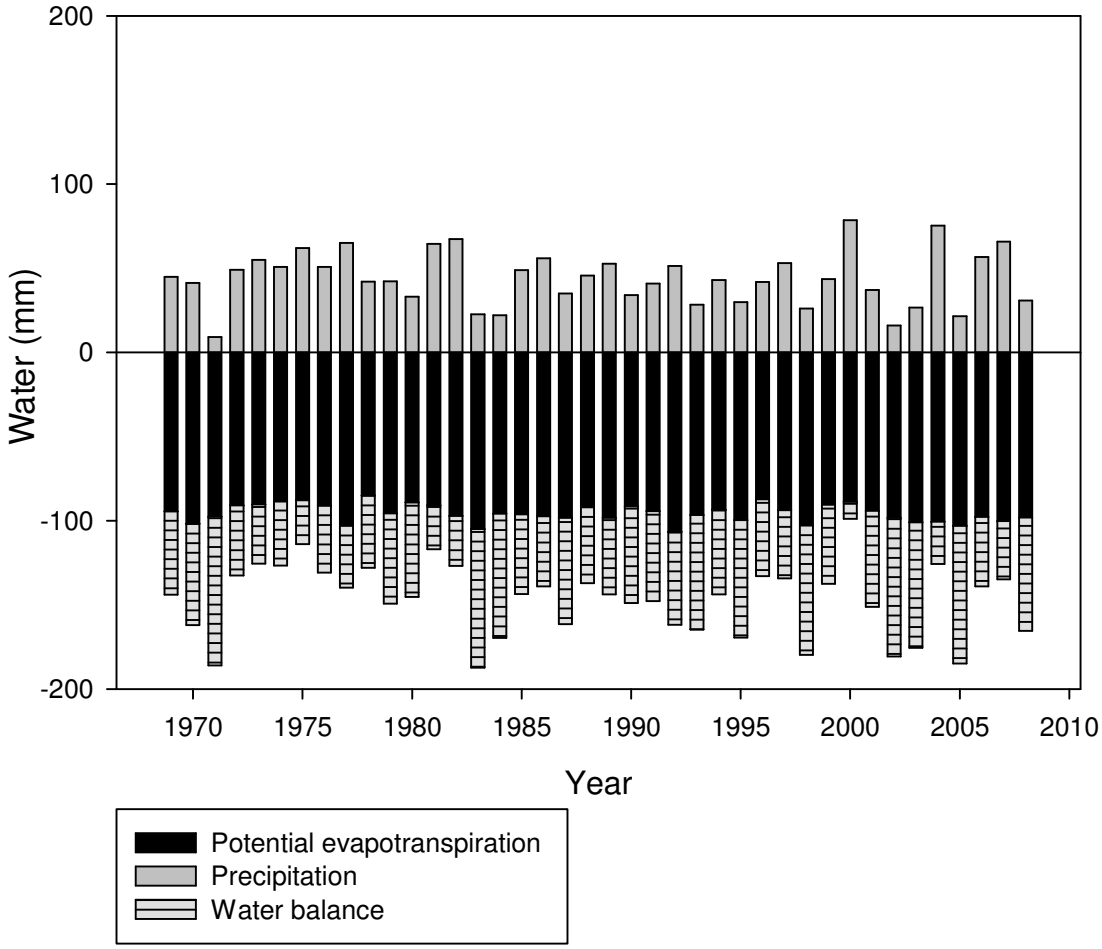


FIG. 8. Annual precipitation and estimates of potential evapo-transpiration (PET) and water balance [precipitation (water input) – PET (water demand/output)] (mm) for the period from 1969-2008 for the Keith Johnson study site.

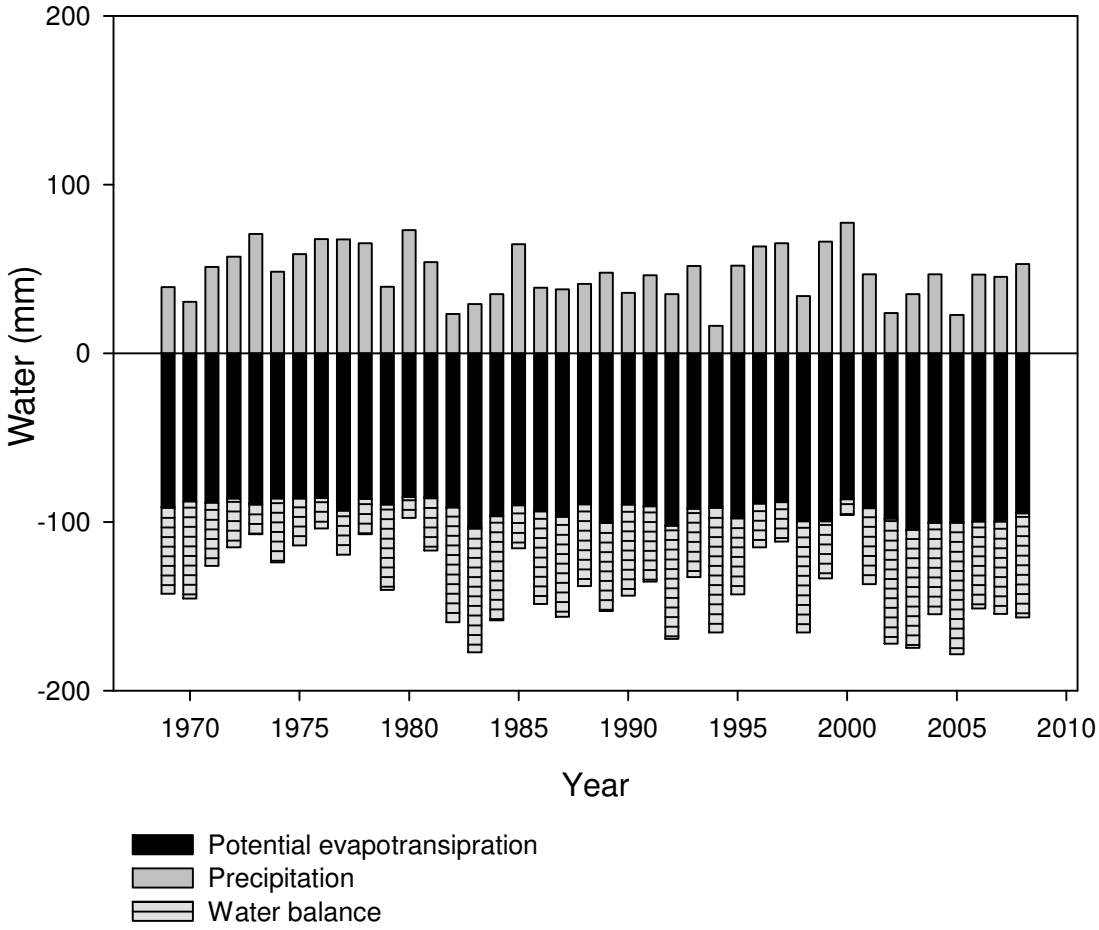


FIG. 9. Annual precipitation and estimates of potential evapo-transpiration (PET) and water balance [precipitation (water input) – PET (water demand/output)] (mm) for the period from 1969-2008 for the Last Post study site.

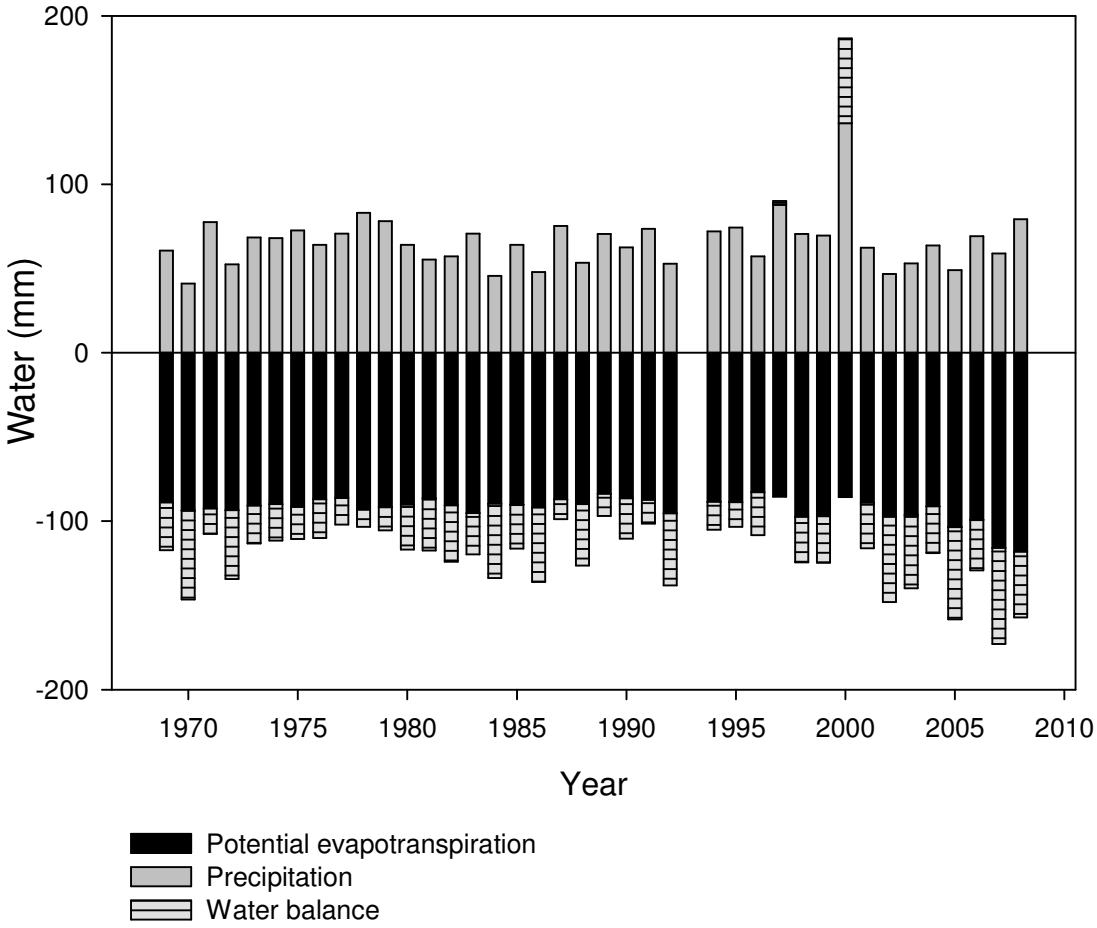


FIG. 10. Annual precipitation and estimates of potential evapo-transpiration (PET) and water balance [precipitation (water input) – PET (water demand/output)] (mm) for the period from 1969-2008 for the Wolfaan study site. 1993 weather data was only partial so was not used.

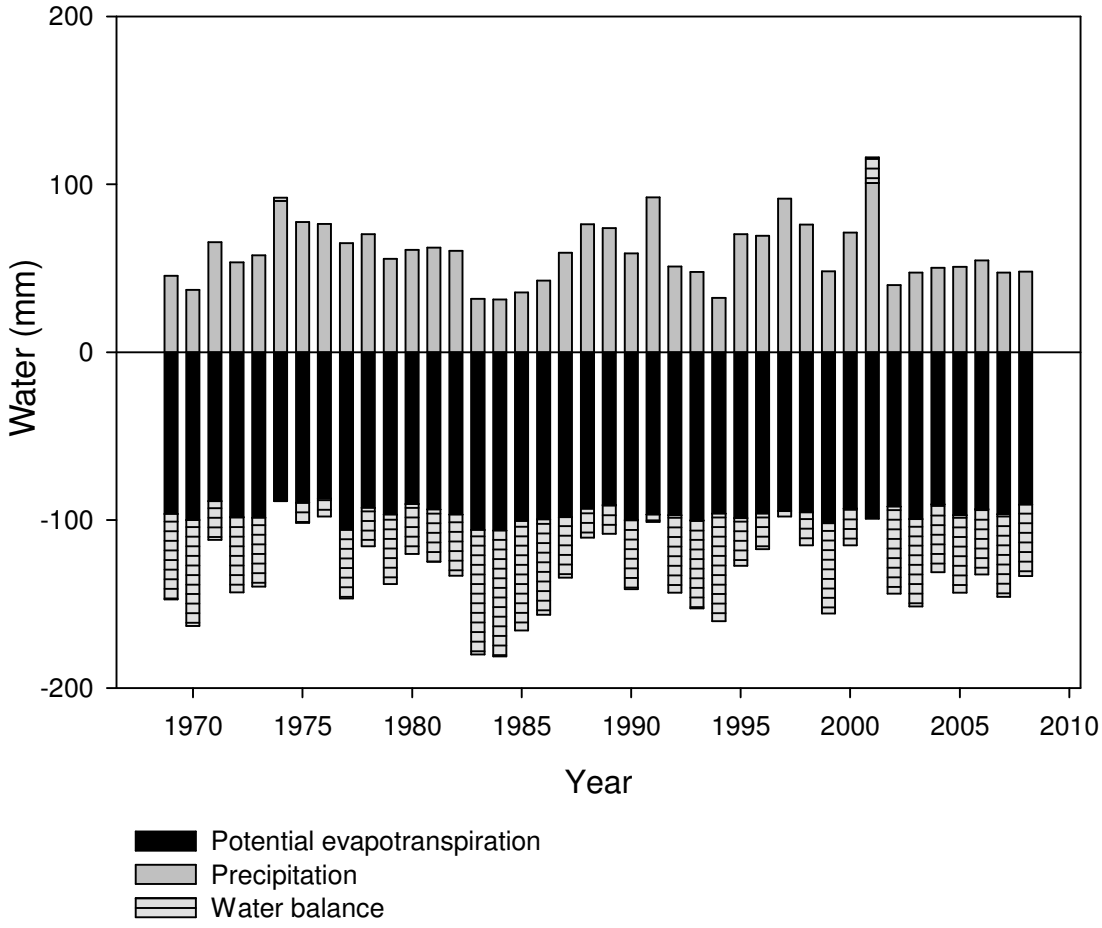


FIG. 11. Annual precipitation and estimates of potential evapo-transpiration (PET) and water balance [precipitation (water input) – PET (water demand/output)] (mm) for the period from 1969-2008 for the Enzelsberg study site.

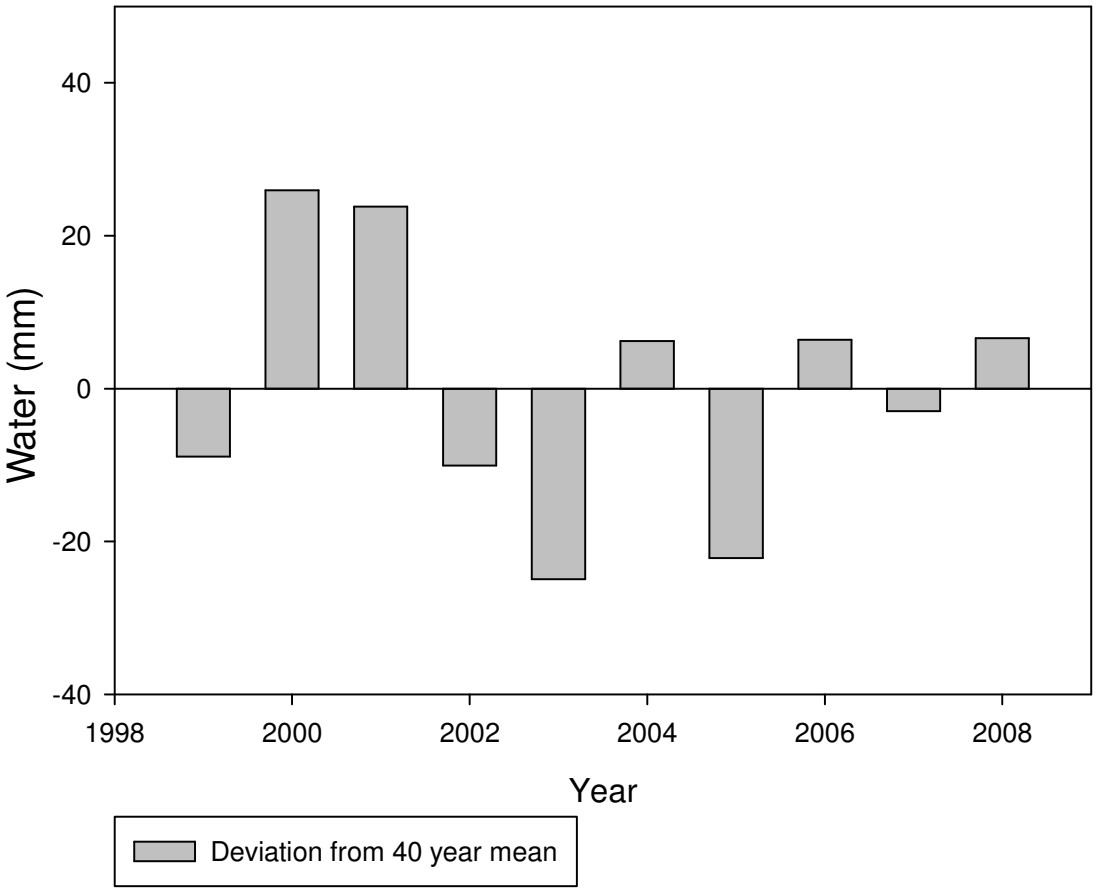


FIG. 12. Annual deviations from the ten-year mean (1999-2008) of water balance (precipitation - potential evapo-transpiration, mm) estimates for the Euphorbia Drive/Homestead study sites.

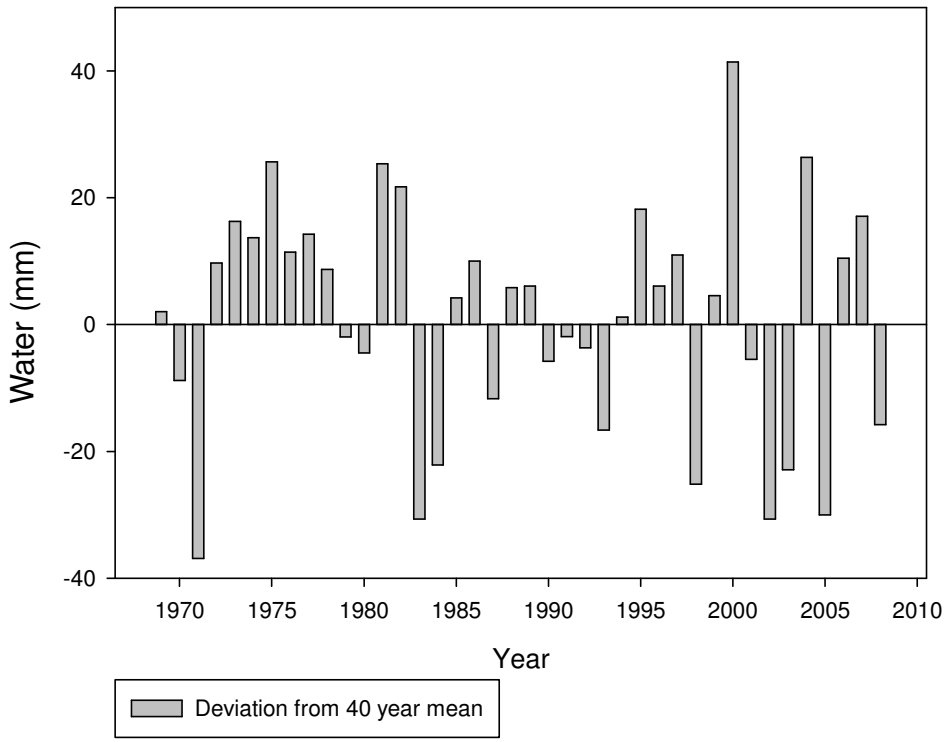


FIG. 13. Annual deviations from the forty-year (1969-2008) mean of water balance (precipitation - potential evapo-transpiration, mm) estimates for the Keith Johnson study site.

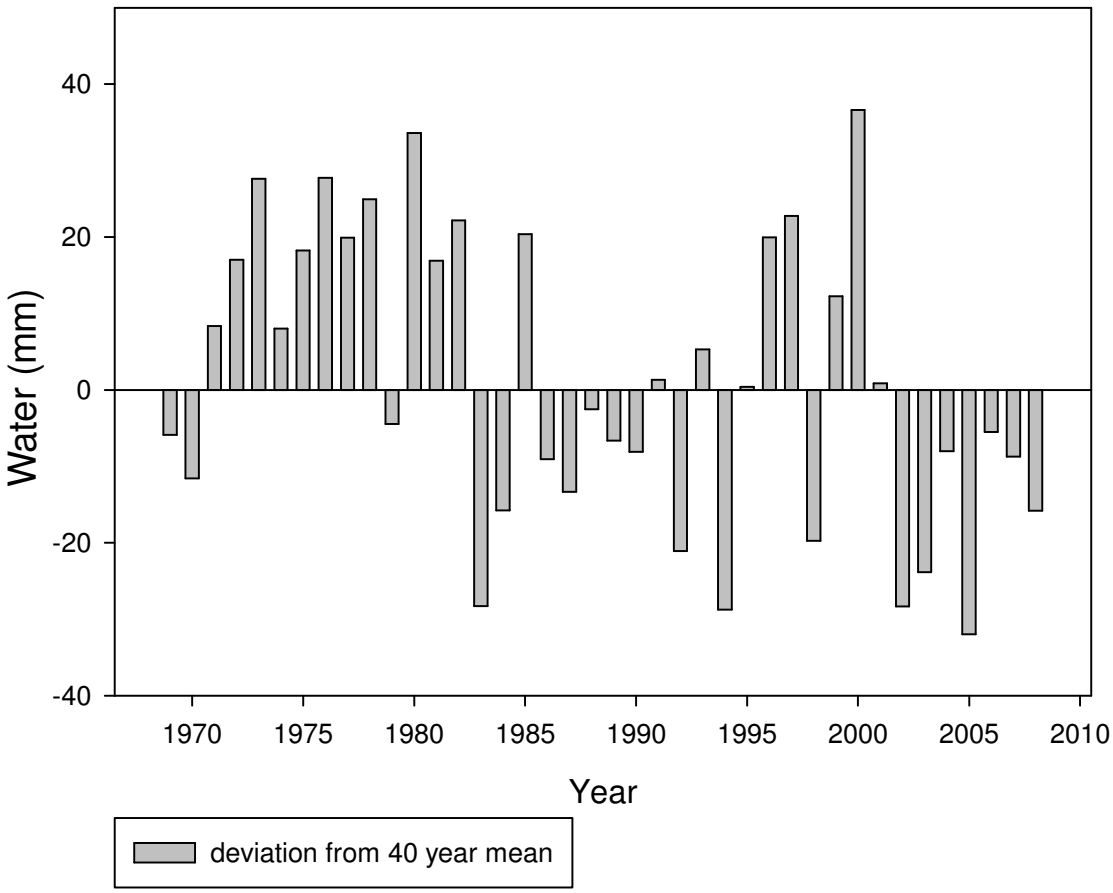


FIG. 14. Annual deviations from the forty-year mean (1969-2008) of water balance (precipitation - potential evapo-transpiration, mm) estimates for the Last Post study site.

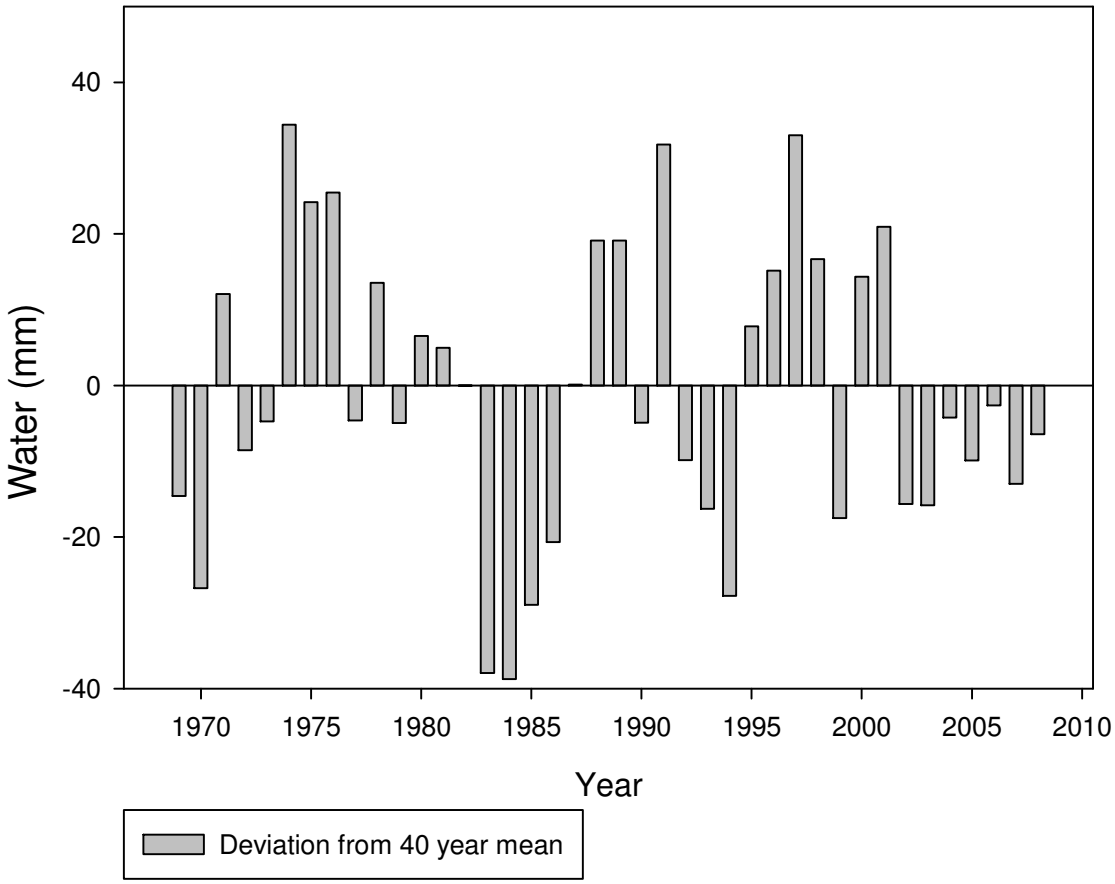


FIG. 15. Annual deviations from the forty-year mean (1969-2008) of water balance (precipitation - potential evapo-transpiration, mm) estimates for the Enzelsberg study site.

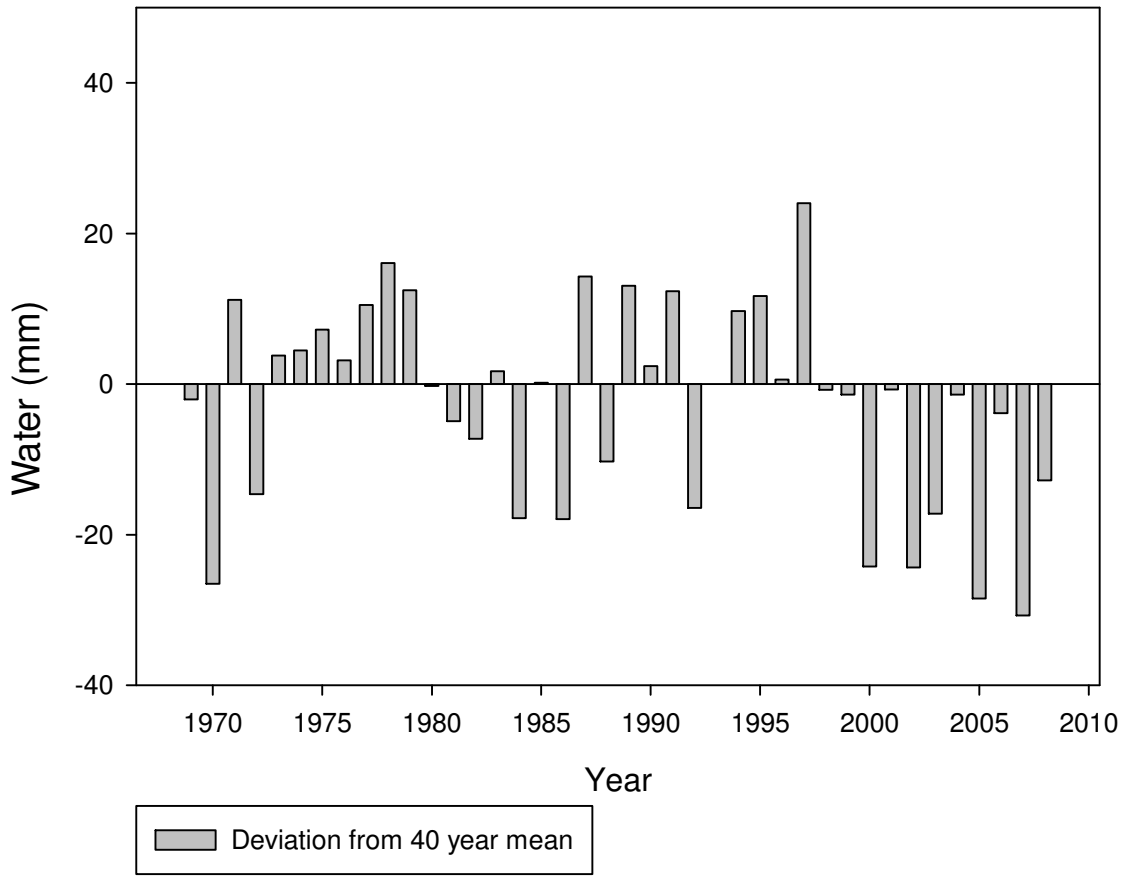
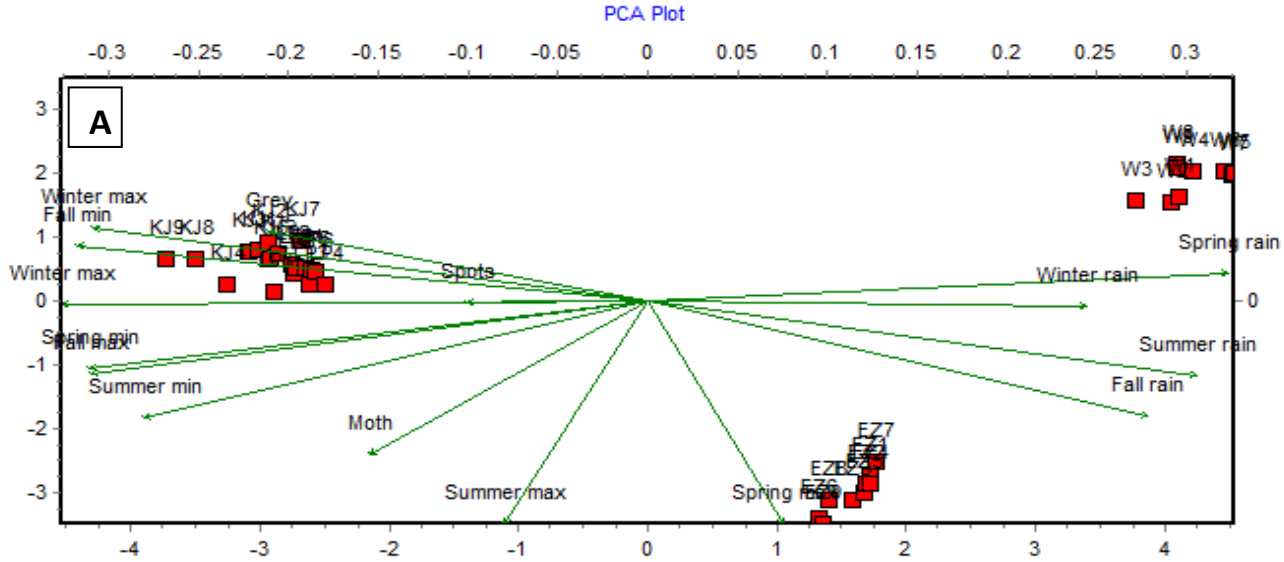
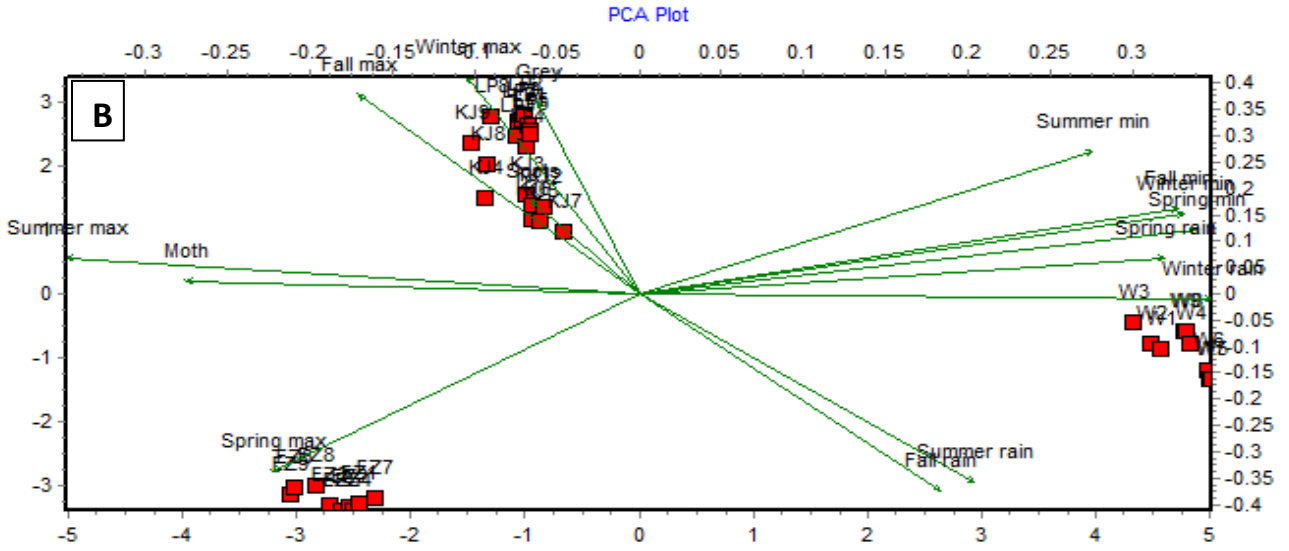


FIG. 16. Annual deviations from the forty-year mean (1969-2008) of water balance (precipitation - potential evapo-transpiration, mm) estimates for the Wolfaan study site.





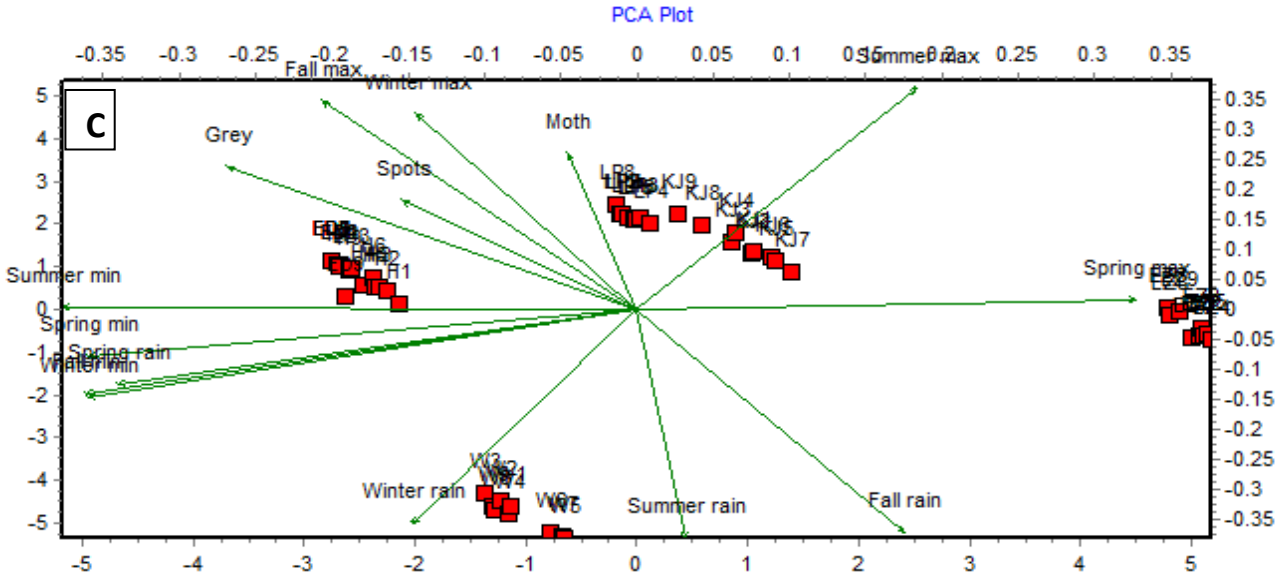


FIG. 17. PCA plots for A) for the decade from 1969-1978, and B) and the decade from 1979-1988 for the Last Post, Keith Johnson, Enzelsberg, and Wolfaan sites, and C) the decade from 1999-2008 for all sites (including Euphorbia Drive and Homestead). Variables included were the degree of spotting and graying of *Euphorbia* trees, the presence of moth damage, mean annual maximum and minimum temperatures, and mean annual precipitation. Spotting, graying and moth damage were averaged by transect (N=9 per site).

Chapter 5

Possible factors leading to the large – scale mortality of *Euphorbia ingens* in the Limpopo Province, South Africa

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ABSTRACT

The plant genus *Euphorbia* is one of the most diverse in the world with over 2100 species, including both succulent and woody plants. In South Africa, the largest of the succulent tree-like euphorbias is *Euphorbia ingens*. In the last 10 to 15 years, high levels of mortality of these trees have been observed in the Limpopo Province of South Africa. The main symptoms include rotting and browning of the succulent branches, grey discoloration, various spots and lesions on succulent branches, blue stain of the main woody stems as well as insect damage. Affected trees generally die within a few years. In the past five years a number of studies have been undertaken to investigate the possible causes of this disease, looking at both biotic (fungal and insect) and abiotic factors. This review aims to summarize all available information regarding the death of *E. ingens* trees in the Limpopo Province, including the history of the disease and results from field observations on disease development and possible causal agents over the past two years.

Keywords: Abiotic, climate change, pathogens, die-offs, Limpopo Province

1. INTRODUCTION

The genus *Euphorbia* (Euphorbiaceae) is very diverse, comprising more than 2100 species that occur in various regions of the world and grow in a wide variety of soil types and landscapes (Van Wyk & Van Wyk 1997, Palgrave 2002, PBI Euphorbia project, www.euphorbiaceae.org). The genus includes herbaceous, woody and succulent species, ranging in size from small herbs (e.g. *Euphorbia fusiformis* Buch.-Ham. ex. D. Don) and shrubs (e.g. *Euphorbia tirucalli* L.) to large trees (e.g. *Euphorbia cooperi* N.E. Brown: Berger var. *cooperi*) (Van Wyk & Van Wyk 1997, Palgrave 2002). Internationally, the best known species in the genus is probably *Euphorbia pulcherrima* Willd.: Klotzsch, a well-known ornamental plant used during Christmas season in the United States, Europe and other countries (PBI Euphorbia project, www.euphorbiaceae.org). The most characteristic properties of *Euphorbia* species are the release of a milky latex when damaged, as well as flowers being reduced into distinct bundles of inflorescences known as cyathia (Palgrave 2002, PBI Euphorbia project, www.euphorbiaceae.org).

A high diversity of *Euphorbia* species are found in South Africa, including representatives of all growth forms, from herbaceous to woody (Palgrave 2002, Van Wyk & Van Wyk 1997). Of these, the tree-like species are found in five of the provinces, with *E. cooperi*, *E. ingens* E. Meyer: Boissier, *E. tetragona* Haw and *E. tirucalli* being the most prominent (Palgrave 2002, Gildenhuys 2006). These species have a main woody stem that support succulent cactus like branches (Palgrave 2002, Van & Van Wyk 1997). *E. ingens*, also known as the tree *Euphorbia* or naboom (mokgoto; Northern Sotho), is the largest of these trees, reaching up to 10m in height (Gildenhuys 2006).

E. ingens occurs in different habitats (bushveld, savanna and tropical areas) with differing topographies across Botswana, Mozambique, South Africa, Swaziland and Zimbabwe

(Gildenhuys 2006, Palgrave 2002, Van Wyk & Van Wyk 1997). The largest populations of *E. ingens* in South Africa are found in the Limpopo and North West Provinces with smaller numbers found in KwaZulu-Natal and the Gauteng Province (FIG. 1) (Gildenhuys 2006). The trees are of significant importance ecologically and provide multiple goods and services such as provision of moisture and nutrients to animals during periods of drought (Brown *et al*. 2003, Dudley 1997, Heilmann *et al*. 2006, PBI Euphorbia project, www.euphorbiaceae.org). *E. ingens*, and other succulent, tree-like *Euphorbia* species, are also important to humans who use the latex for fishing and medicines (Hall & Whitehead 1927, Getahun 1976, Johnson *et al*. 1999, Natarajan *et al*. 2005).

Over the past ten to 15 years, *E. ingens* trees have been dying in large numbers in certain parts of, especially the Limpopo Province, South Africa. Symptoms of disease were first observed on *E. ingens* in the Limpopo Province in the mid to late nineties (Mark Howitt pers. comm., National Zoological Gardens, National Game Breeding Centre, Mokopane), however, deaths have also been observed in the Mpumalanga Province and it is feared that the survival of species is threatened.

In this paper, we summarize information pertaining to the deaths of *E. ingens* trees in the Limpopo Province. Furthermore, the possible reasons for the tree deaths, as suggested by numerous scientists and interested parties, including ecological conditions that might predispose trees to damage by fungi and insects, are considered. We also include observations and data collected during a two-year study of *E. ingens* trees in the Limpopo and North West Provinces to support some of our conclusions.

2. HISTORY OF TREE DEATH IN THE LIMPOPO PROVINCE

2.1. Insects and Pathogens on *E. ingens*

In 2006, a survey of *E. ingens* was conducted at the Biodiversity Conservation Centre (NZG) of the National Zoological Gardens in Mokopane (Potgietersrus), to consider the possible involvement of abiotic factors in the sudden decline of these trees (Malan 2006). These included soil type, soil erosion, fire damage, temperature, and rainfall. The study also provided an evaluation of overall tree condition and recruitment at the site. A transect, 900 m by 50 m, including a total of 225 *E. ingens* trees, was evaluated. Trees in the early stages of decline exhibited a general greying of the succulent branches while trees in the most advanced stage of decline had died and collapsed completely. All trees in the population were found to be in some stage of decline, with 25% already dead at the time of the evaluation (Malan 2006). Additionally, Malan (2006) found that all but one of the trees in the transect were adults.

Malan (2006), suggested that the decline of *E. ingens* trees was due to several possible factors. These included damage due to baboons (*Papio ursinus* Kerr) and vervet monkeys (*Cercopithecus aethiops* L.) that eat the branch terminals and fruit of these trees, preventing the development and release of seed into the environment (Malan 2006). The author further suggested that an insect or pathogen could be causing the death of the trees. This was considered likely since fire and drought did not appear to be the causal factors.

Roux *et al.* (2008, 2009), characterized symptoms of disease on *E. ingens* at the Game Breeding Centre in Mokopane and attempted to identify fungi and insects possibly involved in the death of *E. ingens*. Reported symptoms included grey discoloration of the succulent branches, spotting and lesions on the external areas of the succulent branches, blue stain in the main woody stems and rotting of internal cores of the succulent branches (FIG. 2). From these

symptoms various fungi were isolated including *Cibiessii* spp., *Fusarium* spp., *Graphium* sp., *Gondwanamyces* sp., *Lasiodiplodia* spp., *Phoma* sp. and a *Podospora* sp. Insect infestations were also found in the woody stems (Curculionidae) and succulent branches (Pyralidae) of dying trees (Roux *et al.* 2008, 2009).

Studies during 2009 and 2010 at multiple sites in the Limpopo Province to further characterise disease symptoms and the factors associated with the death of *E. ingens* considered both abiotic and biotic factors. Three main areas were studied, namely the National Game Breeding Centre in Mokopane (Euphorbia drive and Homestead) (S24 10.291 E29 01.131), a domestic farm in the vicinity of the Capricorn area (Keith Johnson site, S23 21.910 E29 44.621) and a private game farm close to Louis Trichardt (Last Post, S23 17.738 E29 55.467) (FIG. 3). At all four sites, symptoms of disease similar to those reported by Roux *et al.* (2008, 2009) were found. These included various spots and scars on the external areas of the succulent branches, internal rotting of the succulent branches and gray discoloration of the healthy succulent branches (FIG. 2).

A high diversity of fungi were isolated from diseased areas on *E. ingens* in the Limpopo Province and a number of insects belonging to different genera were collected. The most commonly isolated fungi resided in the Botryosphaeriaceae, Cordycipitaceae, Microascales, Nectriaceae, Ophiostomataceae and the Teratosphaeriaceae (TABLE I). In the Botryosphaeriaceae, *Lasiodiplodia mahajangana* Begoude, Jol. Roux, Slippers and *Lasiodiplodia theobromae* (Patouillard) Griffon & Maubl were identified (Van der Linde *et al.* 2011a). The Microascales included a previously unknown species described as *Gondwanamyces serotectus* van der Linde, Jol. Roux. (Van der Linde *et al.* 2011b). Insects included *Cyrtogenius africus* Wood (Coleoptera: Curculionidae, Scolytinae), *Cossonus* sp. Claireville, *Stenoscelis* sp. Wollaston (Coleoptera: Curculionidae, Cossoninae) and *Megasis* sp. Guenée (Lepidoptera: Pyralidae) (FIG. 4A).

2.2. Disease development over time 2009 – 2010

To obtain a longer term view of *E. ingens* mortality in the Limpopo Province and a more defined understanding of symptom development over time, we monitored the progression of disease symptoms on 48 specifically tagged *E. ingens* trees. The trees selected for observation, at the sites previously studied in the Limpopo Province and listed above, were of different sizes, age and levels of symptom development. Trees were monitored every three months over a two-year period. At the time of each visit, symptoms of disease were scored and photos taken from a set point to monitor the progress of disease symptoms. Over the two-year period there were no recoveries or deaths from the disease. Disease symptoms, however, became severe, especially the grey discoloration of the succulent branches (FIG. 4B). Remaining green healthy regions of the succulent branches became paler over the two years of monitoring, with one of the trees having collapsed branches (FIGS. 4C, D) due to extensive rotting of the internal core.

3. ALTERNATIVE THEORIES REGARDING *E. INGENS* DEATHS

During the two years of observation of dying *E. ingens* trees in the Limpopo Province, discussions with farmers, botanists and ecologists, resulted in a number of theories regarding the death of these trees. These included suggestions of climate change-driven mortality, population range changes through source and sink type changes, changing fire regimes and other anthropogenic impacts. In the following sections, the likelihood of some of these causal factors being involved are discussed in greater detail.

3.1. Land management & fire

Land management, including grazing and fires, has been proposed as a possible factor linked to the decline of *E. ingens* populations. Factors associated with land management have a direct influence on the vegetation type present within a specific area. It has been hypothesized that *E. ingens* might prefer areas of high density, especially in their juvenile phase, and survive better in densely vegetated areas underneath canopies of larger trees (Mark Howitt & Rentia Malan pers. comm., Elsa van Wyk, Plant Herbarium, Plant Sciences, University of Pretoria pers. comm.). It is thought that underneath the canopies of co-occurring trees, a micro-climate exists that might favour juvenile *E. ingens* trees (Thrash 1998). In this case, nutrients may be at higher concentrations and soil moisture may be retained due to the shading (Turner *et al.* 1966, Vandermeer 1980). The "surrogate" plants may also protect young trees from animal herbivory and being trampled on (Thrash 1998).

The presence, or alternately the absence, of fire have both been hypothesized to be directly linked to the decline of *E. ingens* (Alf Sephton & Coert Geldenhuys pers comm.). It is widely accepted that most ecosystems require some level of fire damage to ensure the productivity and health of plants in the system (Hardison 1976, Katan 2000). Where land management has resulted in changes in the frequency and intensity of fires, it has had detrimental effects on the affected ecosystems, either directly through damage to plants, or indirectly through impacting on nutrient availability and changing the micro-climate of areas (Certini 2005, Parker *et al.* 2006). Fire also plays an important role in the control of tree diseases through reducing pest levels (Parmeter & Uhrenholdt 1975, Hardison 1976, McCullough *et al.* 1998, Parker *et al.* 2006).

A study by Van der Linde *et al.* (2011c), found no direct link was foung between the absence/presence of fire and *E. ingens* die-offs. Two of the sites they studied had been

exposed to fire; Homestead in the Limpopo Province and Wolfaan in the North West Province. Homestead was exposed to a fire that lasted a few days in 2001 (Mark Howitt pers. com). Trees at this site are dying at an alarming rate, compared to trees at Wolfaan, which are more healthy and have a higher recruitment rate. Trees at Wolfaan have previously been exposed to two fires (Alf Sephton pers. com), burning for one day, between 2007 and 2009. The other sites (Van der Linde *et al.* 2011c) which experienced no fire has high levels of *E. ingens* die-offs (Last Post, Euphorbia drive and Keith Johnson – Limpopo Province) and low levels of *E. ingens* die-offs (Enzelsberg – North West Province).

During our two year study of *E. ingens* in the Limpopo and North West Province we included the same sites as those studied by Van der Linde (2011c). We could, however, not find supporting evidence for above mentioned hypothesis. Die-offs of *E. ingens* were observed in both densely vegetated sites and those with sparse vegetation. The vegetation composition at all sites were also similar, being dominated by *Ehretia regida* (Thunb.) Druce, *Grewia flavescence* Juss and *Dichrostachys cinerea* Wight et Arn.

3.2. Climate change and tree death

Apart from changes in fire and land use patterns, or pathogens and insects (Malan 2006, Roux *et al.* 2008; 2009, Van der Linde *et al.* 2011a; b) playing a primary role in the death of *E. ingens*, climate change, or at least short-term climate fluctuations, remain a strong possibility driving the die-off of *E. ingens*. It is predicted that there will be an increase in winter and summer temperatures by three to seven degrees Celsius by the year 2100 in Southern Africa (Boko *et al.* 2007, Houniet *et al*. 2009). Minimum temperatures will more likely display a higher level of increase than maximum temperatures and this will likely lead to more significant changes in temperature during winter than summer (Du Plessis *et al.* 2003). It is also predicted that rainfall will increase or decrease, depending on area, by 20% during the

21st century (Boko *et al.* 2007, Houniet *et al.* 2009). With climate change leading to higher temperatures and more unpredictability in rainfall, the range of some pathogens will increase in South Africa to areas where trees are susceptible to a particular pathogen and lead to severe losses of a particular plant host (van Staden *et al.* 2004).

Forest ecosystems are influenced by disturbances that play an integral role in their structure and function. Natural disturbances maintain the structure and function of forests over time (Dale *et al*. 2001, Logan 2003). However, when disturbances are unnatural or when natural disturbances are altered in their severity or occurrence, they may act to disrupt and alter communities and ecological processes and services, resulting in negative cascading effects that ultimately may result in permanent alterations (Bergeron & Leduc 1998, Dale *et al.* 2001, Logan 2003). Climate change can alter disturbance regimes in such a way that they occur outside of their natural range of variation. These disturbances include fire, insect and pathogen outbreaks (Dale *et al.* 2000, Dale *et al.* 2001, Logan 2003, Williamson *et al*. 2009, Sturrock *et al.* 2011).

Studies by Van der Linde *et al.* (2011c), found an increase in temperature with reduced rainfall in areas of severe *E. ingens* die-offs. Temperature and precipitation data was analysed over a 40 year period at four sites in the Limpopo Province and two sites (areas of less severe die-offs) in the North West Province (TABLE II). Compared to the Limpopo Province the sites in the North West province were cooler with higher rainfall levels over the 40 year period (Van der Linde *et al.* 2011c) (TABLE III). Furthermore, evapo-transpiration and water balance levels were investigated and results showed that trees were under more stress in the Limpopo Province (Van der Linde *et al.* 2011c). This finding might explain the more severe die-offs in the Limpopo Province since the trees will be more vulnerable to pathogen and insect attack due to their state of increased stress.

3.3. Source and Sink population changes

A possible explanation for the die-off of *E. ingens* trees in certain regions of the Limpopo Province which has not been investigated at all relates to the natural population dynamics, such as source-sink, metapopulation and remnant population dynamics, of these trees (Prof. Kevin Balkwill, APES, Wits, pers comm). It has, for example, been proposed that areas where *E. ingens* trees are dying are actually areas which are not optimal for their occurrence, but where they had been able to establish because of temporary favourable conditions. The identification of source-sink, metapopulation and remnant populations in plants are, however, difficult, especially for remnant populations which occur over extended periods of time (Eriksson 1996). Similarly, evidence for source-sink populations in plants are minimal and relating to annuals, while most data for metapopulations relate to biannuals (Eriksson 1996).

4. CONCLUSIONS

E. ingens trees in the Limpopo Province are dying at an alarming rate. Prior investigations have found a high diversity of fungi and insects on the diseased trees (Roux *et al.* 2008; 2009, Van der Linde *et al.* 2011a; b). The roles of these fungi and insects remain uncertain, but it seems that an abiotic factor is involved, stressing the trees and making them more susceptible to these fungi and insects. Analysis of climatic data revealed climate changes, beyond the tree's threshold, in the Limpopo Province, which could have acted as a catalyst to the sudden dying of trees (Van der Linde *et al.* 2011c). Comparisons with trees in healthier sites (North West Province) revealed less severe changes in climate and overall climatic conditions more suited for the trees revealed by evapo-transpiration and water balance levels (Van der Linde et al. 2011c).

The death of *E. ingens* appears to be caused by a combination of biotic and abiotic factors acting in concert. Although Van der Linde *et al.* (2011c) provided the most detailed study into

the die-off of these trees, 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-offs of different species of *Euphorbia* in different provinces. These studies must also include more in depth studies of land management and fire regimes as well as climate data for each province in order to better understand the possible involvement of fungi, insects and the various abiotic factors in the decline of native trees in South Africa.

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TABLE I: Fungi and insects reported from *E. ingens* in South Africa

Fungi isolated	Insect/Symptom	Site
Lasiodiplodia theobromae	Cossoninae and Scolytinae (Curculionidae)	Last Post and Euphorbia drive
L. mahajangana	Blue stain in main woody stem	Keith Johnson
Gondwanamyces serotectus	Cossoninae (Curculionidae)	Last Post and Keith Johnson
Fusarium oxysporum	Plant roots	Last Post
Beauveria sp.	Exterior spotting and lesions	Last Post
Readeriella sp.	Exterior spotting and lesions	Last Post
Aureobasidium sp.	Exterior spotting and lesions	Last Post
Sporothrix sp.	Cossoninae and Scolytinae (Curculionidae)	Last Post and Euphorbia drive
Podospora sp.	Exterior spotting and lesions	Euphorbia drive
Phoma sp.	Exterior spotting and lesions	Euphorbia drive
Graphium sp.	Exterior spotting and lesions	Euphorbia drive

TABLE II: Main symptoms of disease and population structure at six sites in the Limpopo and North West Provinces.

Province	Site	Symptoms and Juvenile tree levels (% total trees studied at each site)			
		Gray discoloration	Moth	Spots and lesions	Juveniles
Limpopo	Euphorbia drive	100	99	100	1
	Homestead	99	68	94	9
	Keith Johnson	39	42	76	42
	Last Post	93	64	93	8
North West	Enzelsberg	4	64	78	41
	Wolfaan	17	7	67	63

Adapted from Van der Linde *et al.* 2011

TABLE III: General summary of temperature and rainfall of the sites investigated in this study.

Province	Site	Mean Annual Maximum Temperature (1969 - 2008) ˚C	Mean Annual Minimum Temperature (1969 - 2008) ˚C	Mean Annual Precipitation (1969 - 2008) mm	Maximum Temperature change ˚C	Minimum Temperature change ˚C
Limpopo	*Euphorbia drive/Homestead	27.8	13.6	545	↑ 1.97	↑ 0.60
	Keith Johnson	27.4	12.3	442	↑ 1.13	↑ 0.60
	Last post	27.4	12.3	473	↑ 1.13	↑ 0.60
North West	Enzelsberg	27.7	11.9	593	↓ 0.01	↑ 0.09
	Wolfaan	26.3	11.9	664	↑ 0.63	↑ 2.66

*Mokopane temperature data is from 1999 – 2008

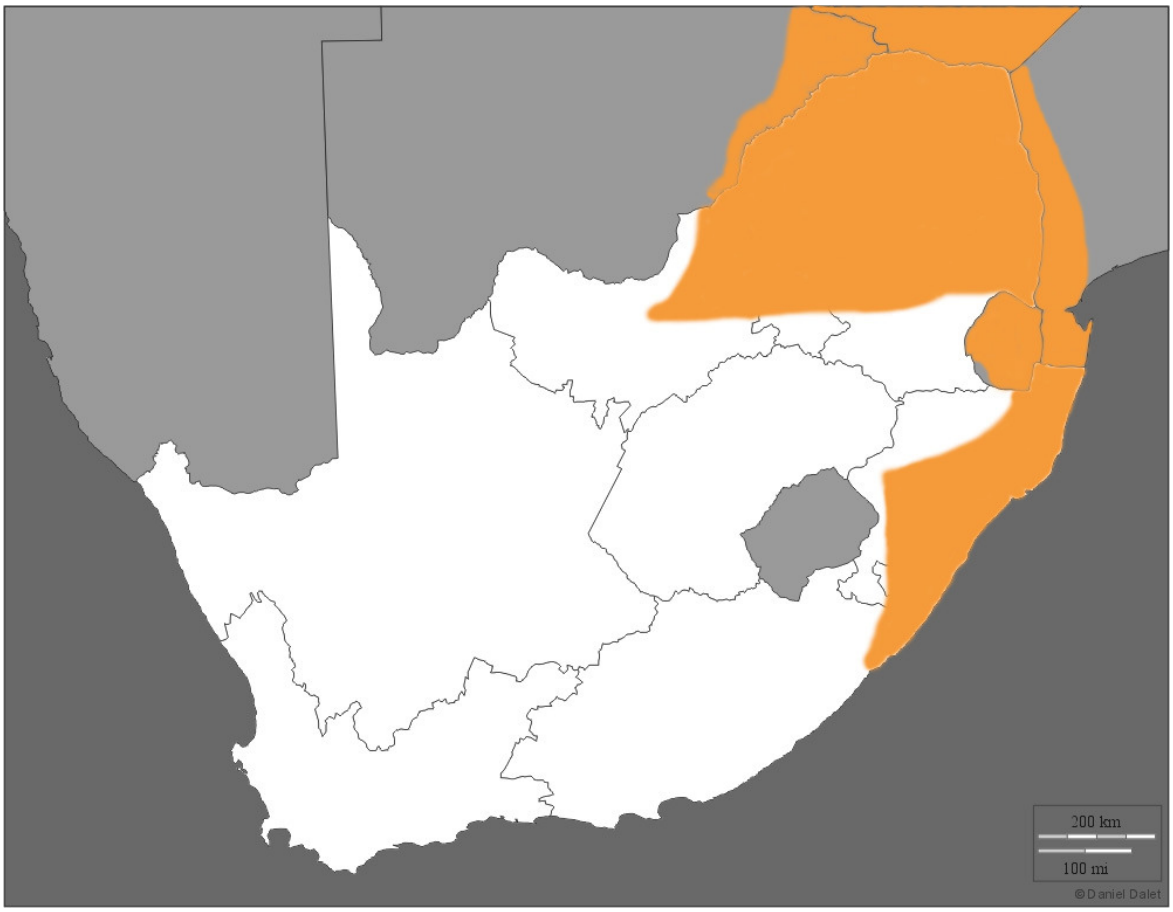

FIG. 1. Map of Southern Africa indicating the areas where *Euphorbia ingens* occur, illustrated by orange shading. (Palgrave 2002, Van Wyk & Wyk 1997).

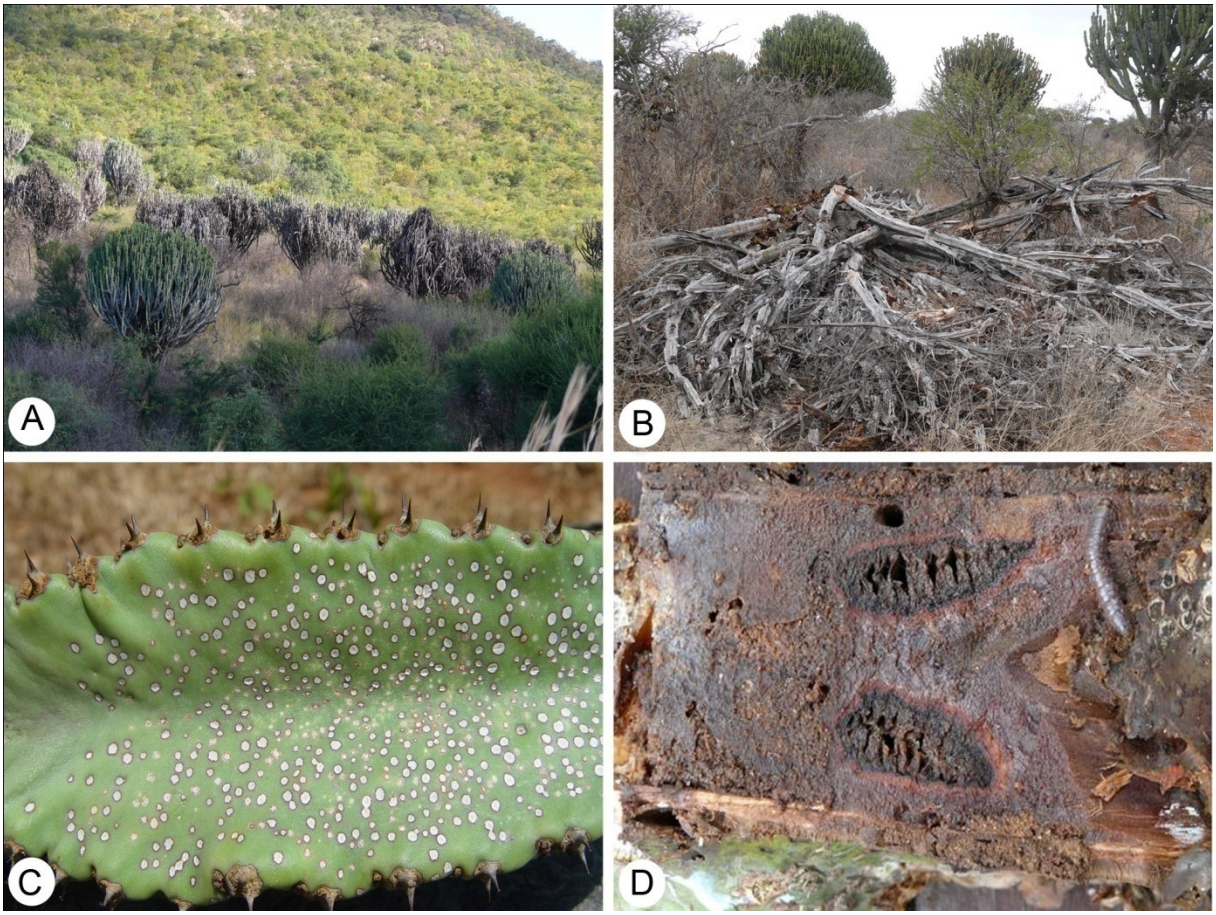

FIG. 2. Mortality of *E. ingens* in the Limpopo Province and symptoms found on diseased and dying *E. ingens* trees. (a) Large-scale mortality of *E. ingens* trees at Mokopane. (b) Dead *E. ingens* trees at Last Post. (c) Typical spotting and lesions on the external area of the succulent branches. (d) Internal rotting and feeding damage by *Megasis* sp.

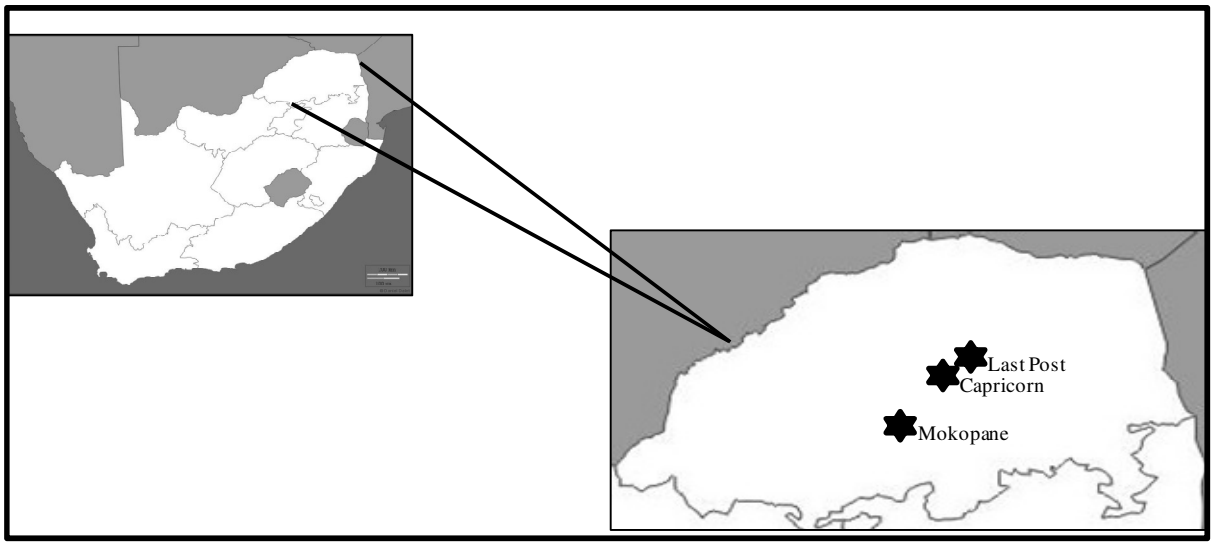

FIG. 3. Map indicating the location of the three sites investigated in the Limpopo Province (Van der Linde *et al.* 2011c).

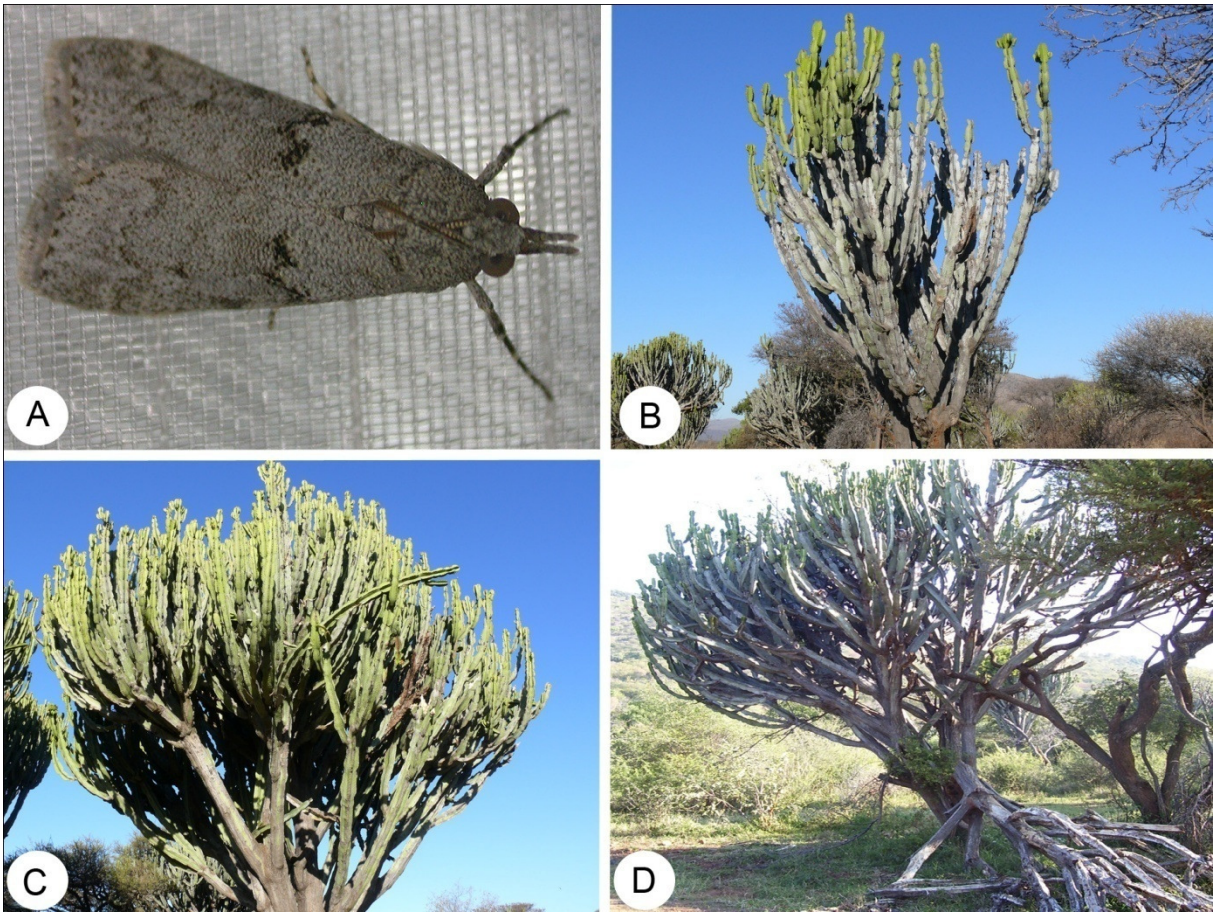

FIG. 4. Adult moth of the feeding larvae and progress of disease symptoms on *E. ingens* from 2009 – 2010. (a) Adult *Megasis* moth. (b) Grey discoloration of the succulent branches. (c) *E. ingens* tree (2009) used in the progress study. (d) Same *E. ingens* tree (2010) with collapsed branch from internal rotting.

SUMMARY

Euphorbia ingens trees are dying and are becoming diseased at an alarming rate in the Limpopo province of South Africa. This study was the first to investigate the sudden death of *E. ingens* in South Africa collectively from both an abiotic and biotic perspective. A high diversity of fungi were isolated and insects collected from dying *E. ingens* trees, including previously unknown species of *Gondwanamyces,* described as *G. serotectus* and *G. ubusi*, *Lasiodiplodia theobromae* and *L. mahajangana*. A moth, *Megasis* sp., was commonly associated with dying trees and could play a role in the death of trees. This is the first study to conduct pathogenicity trials with *Gondwanamyces* spp. on any plant host. Both *Gondwanamyces* spp. and *Lasiodiplodia* spp. used in the pathogenicity studies produced lesions in the succulent branches of *E. ingens* trees, suggesting that both groups of fungi could play a role in the death of trees. Analyses of temperature and precipitation data, over the last 40 years, indicated an average increase of 2 °C in temperature at the sites of severe *E. ingens* decline, with cooler and more stable temperatures in the North West Province. Water balance and evapo-transpiration studies also indicated that the trees are under more stress in the Limpopo Province compared to the North West Province. Dying trees are using more water for cellular functions than they obtain, eventually leading to a negative water balance during the periods of increased temperature. Together with the changing climate, fungi and insects may play an important role in the development of disease. This study provides a solid foundation for further studies regarding native tree decline and the factors that should be considered. To truly understand the role of fungi and insects in the decline of *E. ingens,* further studies should be conducted in different areas of the country investigating other dying *Euphorbia* spp., and studying the relationship between the fungi and insects collected in more detail