The ecology of *Encephalartos lanatus* in Middelburg district, Mpumalanga Province (South Africa)

Nonele Memory Sigasa

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Supervisor: Dr T. N. Suinyuy

Co-supervisor (s): Prof W. Otang-Mbeng

: Dr A. Magadlela

School of Agricultural Science

Faculty of Agriculture and Natural Sciences

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UNIVERSITY OF MPUMALANGA

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Declaration

I, Nonele Memory Sigasa, hereby declare that the dissertation submitted to the University of Mpumalanga is my original work and has not been submitted to other institutions previously. Additionally, all sources I have used have been properly cited and acknowledged through detailed references.

Student: Ms N.M. Sigasa

Signature…… Date…….

23/04/2025

Supervisor: Dr T.N. Suinyuy

Signature…… Date…….

23/04/2025

Co-supervisor(s): Dr A. Magadlela

Signature…… Date…….

23/04/2025

Co-supervisor(s): Prof W. Otang-Mbeng

Signature…… Date…….

Dedication

My late grandmother, Dorothy Ngobe, and my late mother, Angelinah Sigasa, who nurtured and shaped me into the woman I am today, consistently emphasized the importance of prioritizing education above all else. I am indebted to both of you for everything, even though you are not here to witness my achievements. Ngiyabonga kakhulu; sengathi umoya wenu ungaqhubeka uphumule ngokuthula.

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List of Abbreviations

MYA=Million years ago

SA= South Africa

SANBI= South African National Biodiversity Institute

NBSAP= National Biodiversity Strategy and Action Plan

MBSP= Mpumalanga Biodiversity Sector Plan

CITES= Convention on International Trade in Endangered Species of Wild Fauna and Flora

N_2= Dinitrogen

PGPB= Plant Growth Promoting Bacteria

NEMBA- National Environmental Management Biodiversity

NT=Near threatened

VU= Vulnerable

LC= Least Concern

EN= Endangered

CR= Critically Endangered

EX= Extinct in the Wild

IUCN= International union for conservation of nature

BNF= Biological nitrogen fixation

PCR= Polymerase chain reaction

BLAST= Basic local alignment search tool

PCA= Principal component analysis

NH_3= Ammonia

Ca= Calcium

Mg= magnesium

P= Phosphorus

K= Potassium

Cu= Cupper

TCP= Tricalcium phosphate

TAE= Tris-acetate-EDTA

NaOH= Sodium hydroxide

NR= Nitrate reductase

ISR= Induction of systematic resistance

IAA= Indole-3-acetic acid

EPS= Extracellular polymeric substances

T-DNA= Transferred DNA

Publication(s) related to the project research

1. Sigasa, M.N., Yessoufou, K., Magadlela, A., Otang-Mbeng, W. and Suinyuy, T.N. 2023. Population structure of an African cycad: Fire may stimulate the coning phenology of *Encephalartos lanatus* (Zamiaceae) and also predispose its cones to damage. *Diversity*, 15(10): 1075. Doi:10.3390/d15101075
2. Sigasa, M.N., Magadlela, A., Otang-Mbeng, W. and Suinyuy, T.N. The impact of microbes and their associated extracellular enzymes on *Encephalartos lanatus* thriving under harsh environmental conditions in Middelburg, Mpumalanga (Prepared for the Journal: Symbiosis)

General overview of chapters in this dissertation

The dissertation comprises five chapters, organized as follows:

Chapter 1: It provides an overview of the urgent need to conserve cycads, highlighting the crucial ecosystem services they provide. It also emphasizes the importance of the study, outlining the aim, objectives, and research questions of the study.

Chapter 2: This chapter provides a comprehensive literature review on several aspects related to cycads. These include the description/ classification of cycad species; geographical distribution of cycads; importance of cycads regarding ethnobotany, ornamentals, and food; conservation status of cycads; South African biodiversity policy; functional role of cycads in the ecosystem; and soil enzyme activities.

Chapter 3: It focuses on the population structure, effect of fire on life stages and reproductive structure of *Encephalartos lanatus* cones in Botshabelo Nature Reserve.

Chapter 4: It focuses on measuring the average soil nutrients status of the soil, identifying bacteria in the coralloid roots as well as the rhizosphere and non-rhizosphere soils of *Encephalartos lanatus*, examines the enzyme activities linked with these bacteria and further looks into their impact on the elevation of soil nutrients levels in Botshabelo cultural village.

Chapter 5: Outline the highlights and future recommendations of the present study.

Abstract

Cycads are long-lived, slow-growing gymnosperms that were once widely distributed but today are limited to the tropical and subtropical regions of the world. Cycads are of conservation importance because of their unique ecology. First, they are the only gymnosperms that provide an important ecosystem service of nutrient cycling, such as nitrogen fixation, through their association with nitrogen-fixing bacteria present in specialized coralloid roots that are similar to root nodules in legumes. Second, they have unique life histories based on their growth. They are a group of dioecious gymnosperms that occur in various life stages within a population. Third, they are the most threatened plant group on Earth, with over 50 % of the more than 300 species facing a high risk of extinction. The extinction is well noted for the African *Encephalartos* species, which are threatened by habitat loss through transformation, frequent fires, over-exploitation for landscaping and medicinal purposes, reproduction failures and other natural courses like herbivory and poor dispersal, which all affect the population health and structure. There is little knowledge on the ecology of *Encephalartos* cycads, and their extinction will lead to a decline in ecosystem services, and also. information about the diversity of nitrogen-fixing bacteria and other associated microbes in the roots and soils of *Encephalartos* species, and knowledge of their contribution to soil nutrient improvement is limited. This study, therefore, aimed to investigate the effects of disturbances/threats to *E. lanatus* health and population structure. Additionally, the study investigates the associated roots and soil microbes and the effects of *E. lanatus* on soil nutrient status. *Encephalartos lanatus* in a protected area in Botshabelo, Mpumalanga, is prone to fire, herbivory, and habitat conversion, and therefore serves as a model species for the aim of the study. The effects of fire and herbivory on *E. lanatus* health and population structure were assessed by counting and measuring all plants (seedlings, juveniles, adults, coning) in burnt and unburnt plots in Botshabelo. The plants were sexed based on the presence of live cones, remnants of cones from a previous season, or seedlings under the adult plant. Cone damage was assessed by counting and recording cones that were completely or partially damaged by fire and/or herbivores. For soil nutrient status, soil samples were collected from the rhizosphere of a total of 20 adult *E. lanatus* plants (10 each from burnt and unburnt plots), and 20 control samples were collected 5 m away from each target plant, and analyzed for soil nutrients (nitrogen [N], phosphorus [P], potassium [K], calcium [Ca], magnesium [Mg], manganese [Mn], copper [Cu], zinc [Zn], pH, acid saturation, total cation exchange, exchangeable acidity, and organic carbon [C]), enzyme activities using β-(D)-glucosaminidase, β-glucosidase, and phosphatase (alkaline & acid).

Furthermore, the rhizosphere and non-rhizosphere soils, together with coralloid roots, were analyzed for N-fixing, N-cycling, and P-solubilizing bacteria and other associated microbes. The data obtained for the effect of fire and herbivory on population structure was analyzed using analysis of covariance (ANCOVA) and analysis of variance (ANOVA) in RStudio. A two-sample t-test in Statistix 10 software was used to analyse the differences in nutrient concentration and enzyme activities in the rhizosphere and non-rhizosphere soils. The coralloid roots, rhizosphere and non-rhizosphere soils were analysed for their microbial composition. The study revealed that the population follows a "J" structure with a greater ($P < 0.01$) number of adults compared to any other life stage, and *E. lanatus* populations that were affected by fire produced a higher number of cones and were more prone to baboon damage than the cones in the unburnt sections. Rhizosphere soils had a significantly higher concentration ($P < 0.05$) of Mg and Mn than non-rhizosphere soils. In addition, both the rhizosphere and non-rhizosphere soils had significantly similar concentrations ($P > 0.05$) of N, P, K, Ca, Cu, Zn, pH, acid saturation, total cation exchange, exchangeable acidity, and organic C. Specifically, ten bacteria families were identified in the *E. lanatus* rhizosphere, non-rhizosphere soils, and coralloid roots. The Burkholderiaceae and Rhizobiaceae were the most dominant microbial families in both soils. The enzymes β-(D)-glucosaminidase and alkaline phosphatase were higher in the rhizosphere than in the non-rhizosphere soils but not significant ($P > 0.05$). In contrast, acid phosphatase, nitrate reductase and beta-glucosidase were significantly higher ($P < 0.05$) in the rhizosphere than in the non-rhizosphere soils. In conclusion, the study provides evidence that *E. lanatus* face challenges in their natural habitat. However, *E. lanatus* have developed coping mechanisms to withstand the harsh environment and host N-fixing, N-cycling and P-solubilizing microbes that assist the plants to thrive in nutrient-poor conditions. Generally, the current findings show that the identified N-fixing and nutrient-cycling bacteria, and their associated enzymes in *E. lanatus* coralloid roots, rhizosphere, and non-rhizosphere soils account for soil nutrient status improvement in the *E. lanatus* nutrient-poor ecosystem.

Keywords: Coralloid roots; *Encephalartos lanatus*; fire; nitrogen-fixing bacteria; non-rhizosphere soils; rhizosphere; soil nutrient

Chapter 1

General Introduction

1.1 Background

Cycads are the oldest living gymnosperms that have persisted from the mid-Permian (~270 MYA) (Mankga *et al*., 2020), therefore labelled as living fossils (Nagalingum *et al*., 2011). Among the three families, cycads comprise 355 species in 10 genera (Vessey *et al*., 2005; Mankga *et al*., 2020). The genera of cycads include *Zamia, Dioon, Microcycas, Cycas, Ceratozamia, Encephalartos, Stangeria, Bowenia, Lepidozamia* and *Macrozamia* (Calonje *et al*., 2019). Cycads are the most threatened plant group, with over 60% of the plants threatened with extinction (Mankga and Yessoufou, 2017). Among the genera, the *Encephalartos* genus that is indigenous to Africa and consists of more than 60 species (Mankga *et al*., 2020) is the most threatened cycad group (Mankga and Yessoufou, 2017). Most importantly, South Africa is home to the largest number of *Encephalartos* cycad species, making it a regional centre of cycad diversity with more than 30 species (Donaldson, 2003; Yessoufou *et al*., 2017; Mankga *et al*., 2020). *Encephalartos* species in South Africa tend to grow in forest, savanna and grassland habitats (Donaldson, 2008; Suinyuy *et al*., 2013). The forest species such as *E. villosus* and grassland species such as *E. friderici-guilielmi*, *E. ghellinckii* and *E. lanatus* are habitat-restricted, while savanna species such as *E. altensteinii* and *E. natalensis* tend to be more tolerant and can occur in different habitats with forest, savanna and grassland characteristics (Donaldson, 1993, 2008). Grassland *Encephalartos* species are prone to fire (Donaldson, 1995) which has been identified as a threat to cycad survival (Mankga and Yessoufou, 2017) and may therefore impact the grassland cycads negatively. For example, fire has been shown to kill cycad pollinators (Terry *et al*., 2008; Thom *et al*., 2015), leaves, cycad adults and seedlings (Negrón-Ortiz and Gorchov, 2000). This may negatively impact cycad reproduction and disrupt photosynthesis, nutrient uptake, and the population structure. However, like in other plants, fires can also be beneficial to cycads. For example, cycads regenerate by producing new leaf flushes and cones after fire (Donaldson, 1995; Negrón-Ortiz and Gorchov, 2000; Liddle, 2004; Swart *et al*., 2019). Although studies have investigated the effects of fire on cycads (e.g. Donaldson, 1995; Swart *et al*., 2019), knowledge about their impact on population structure is limited and requires investigation.

Cycad habitats are characterized by nutrient-poor soils (Álvarez-Yépiz *et al*., 2011), and they provide the ecosystem service of nutrient cycling to cope in these nutrient-poor habitats. For example, cycads possess three distinct types of roots: taproots, lateral roots and specialized roots known as coralloid roots (Yamada *et al*., 2012; Zheng *et al*., 2018). The primary function of the taproot is to facilitate the assimilation of nutrients. In contrast, the lateral root primarily

serves as a nutrient storage organ, while the coralloid roots house nitrogen (N) fixing bacteria for plant use (Zheng *et al*., 2018). These microbes are capable of breaking down the triple bond of atmospheric dinitrogen (N_2) through a process called biological N_2 fixation (BNF) and converting it into ammonia (NH_3) (Zuberer, 2021), a nutrient that cycads utilize for their survival and growth (Gutiérrez-García *et al*., 2018; Hashidoko *et al*., 2019; Chang *et al*., 2019; Suárez-Moo *et al*., 2019; Ndlovu *et al*., 2023; Motsomane *et al*., 2023). Despite the knowledge of the role of N-fixing bacteria in sustaining cycads, few studies have investigated the diversity of microbes present in the coralloid roots of cycads (Yamada *et al*., 2012; Gutiérrez-García *et al*., 2018; Chang *et al*., 2019; Suárez-Moo *et al*., 2019), and such studies on *Encephalartos* are limited (e.g., Ndlovu *et al*., 2023; Motsomane *et al*., 2023, 2024). Furthermore, soil microorganisms release extracellular enzymes that break down and convert complex molecules into readily available nutrients that may be utilized by plants and microbes (Zungu *et al*., 2020; Ndlovu *et al*., 2023). The enzymes involved in N cycling include urease, acetylglucosaminase and *N*-acetyl-β-D-glucosaminidase (Trotsenko *et al.*, 2001; Lobakova *et al*., 2003; Gehringer *et al.*, 2010). *N*-acetyl-β-D-glucosaminidase (Trotsenko *et al.*, 2001; Lee *et al*., 2020) and β-D-glucosidase cycle carbon (Ndlovu *et al*., 2023). Phosphatases (alkaline and acid) are crucial for the cycling of phosphorus (Turner *et al*., 2002). Studies on cycad-microbe symbiosis have focused mostly on microbes present in the coralloid roots of cycads, with limited efforts on the investigation of extracellular enzymes released by these soil microorganisms.

Over 65 % of the world's cycads are currently facing a high risk of extinction on a global scale (Mankga and Yessoufou, 2017; Yessoufou *et al*., 2017), and this extinction risk is highest in *Encephalartos* cycads, which is an African endemic (Mankga and Yessoufou, 2017). Out of 65 *Encephalartos* species in Africa, South Africa is home to 37 species (> 50 %), of which 73 % are threatened with extinction (Donaldson, 2008) as compared to the global 63 % (IUCN, 2013). Although all *Encephalartos* species are listed in Appendix 1 of the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora (Donaldson, 2008), South Africa is still facing the imminent threat of losing the majority of its cycad species. Regardless of the implementation of numerous conservation efforts and enforcement of restrictive legislation, the population of cycads in their native habitat continues to decline (Martínez-Domínguez *et al*., 2020; Janse van Rensburg *et al*., 2023) leading to the loss of the important ecosystem services they provide in their habitats (Donaldson, 2003; Ndlovu *et al*., 2023).

Mankga and Yessoufou (2017) identified nine factors that are contributing to the high risk of extinction. Seven factors are directly associated with human actions. These factors include habitat loss, fire, grazing, deforestation, overcollection, medicinal usage and alien invasive plants. In addition, one threat relates to the biological aspect of cycads, particularly the lack of reproduction (reproduction failure). Also, climate change manifests as either flood or drought. In addition, cycads are considered to be slow-growing species (Watkinson and Powell, 1997), experience slow reproduction rates, and often reproduce infrequently with unusual recruitment (Raimondo and Donaldson, 2003).

Therefore, conserving cycads is crucial not only for their cultural significance (Mapitsa, 2023) but also for their role in supporting ecosystem function that is threatened by fire. These roles include cycling of nutrients (Ndlovu *et al*., 2023), carbon sequestration (Ma *et al*., 2009), serving as food and shelter for birds and animals (Donaldson, 2008), complex mutually beneficial interactions with insects (Toon *et al*., 2020), and host arbuscular mycorrhizae that influence biogeochemical processes in their micro-habitat (Marler and Calonje, 2020).

1.2 Rationale of the study

Grassland cycads are prone to fire, suggesting that they are fire-adapted or may have evolved with fire. Previous studies showed that fire promotes the growth of leaves in the Australian cycad *Macrozamia riedlei* (Baird, 1977; Dolva and Scott, 1982) as well as affecting the timing of cone production in *Macrozamia communis* (Baird, 1977; Beaton, 1982; Pate, 1993). Fire also plays a crucial role in the process of seedling recruitment within the *Cycas armstrongii* population (Watkinson and Powell, 1997). This suggests that fire can be used as a management tool for fire-adapted cycads. However, it is important to investigate the impact of fire on ecology with an emphasis on the population structure of a cycad species before applying it as a management tool on a cycad such as *E. lanatus* that occurs in grasslands that burn regularly. Previous studies in South Africa have only investigated the impact of fire on coning in *E. cycadifolius* (Donaldson, 1995), leaves and adult *E. transvenosus* (Grobbelaar *et al*., 1989). The *Encephalartos lanatus* population in Middelburg, South Africa, burns regularly and would serve as a model plant to investigate how fire affects its population structure.

Cycads grow in nutrient-deficient soils and have developed special adaptations to survive in harsh environments. Studies show that cycads have established a symbiotic relationship with cyanobionts, known for their ability to convert atmospheric N to plant usable NH_3 (Chang *et*

al., 2019). The diversity of microbes associated with the coralloid roots of *Ceratozamia* (Lobakova *et al*., 2003), *Cycas* (Costa *et al*., 1999; Cuddy *et al*., 2012), *Dioon* (Guitierrez *et al*., 2018; Suárez-Moo *et al*., 2019), *Lepidozamia* (Cuddy *et al*., 2012) and *Macrozamia* (Costa *et al*., 2004; Gehringer *et al*., 2010; Cuddy *et al*., 2012) cycads that grows in the Americas and Australia have been documented and identified. Furthermore, Marler and Calonje (2020) found increased concentrations of N, carbon (C), and phosphorus (P) in the rhizosphere soils of *Cycas micronesica* and *Zamia integrifolia* compared to the surrounding soil. This indicates that cycads play a significant role in soil nutrient contributions. Previous studies in the African cycads found that the coralloid roots of *Encephalartos* species and *Stangeria eriopus* in South Africa can fix N (Grobbelaar *et al*., 1986), which is attributed to the presence of cyanobacteria, predominantly dominated by *Nostoc* species (Grobbelaar *et al*., 1987). These studies solely focused on the association between *Encephalartos* species and N-fixing bacteria without examining their relationship with bacteria involved in N cycling, P solubilizing and C cycling. In addition, the studies did not look at the role of these *Encephalartos* species in enhancing soil nutrient levels and did not explore the role played by the activity of enzymes in the soil. All the plants used in these studies were from botanical garden collections, which do not reflect the natural habitats of the plants, and it was, therefore, difficult to determine if the microbes were from the plants' natural habitat or botanical garden. Botanical garden plants are often irrigated, sprayed with pesticides, and receive nutrient inputs through the application of synthetic fertilizers, which may influence the cyanobionts' composition.

Ndlovu *et al.* (2023) and Motsomane *et al.* (2024) examined N_2 fixation, P solubilizing and N cycling microbial diversity and associated soil enzyme activities in *Encephalartos natalensis* and *E. villosus* growing in their natural habitats. For both *E. natalensis* and *E. villosus,* they identified similar taxa of microbes in the rhizosphere, non-rhizosphere soils, and coralloid roots. These genera include *Variovorax, Paenibacillus, Neobacillus, Bacillus*, *Lysinibacillus, Rhizobium, Pseudomonas, Paraburkholderia* and *Chitinophaga*. Additionally, Ndlovu *et al.* (2023) in their study also found microbial genera such as *Sphingomonas, Phyllobacterium, Olivibacter, Beijerinckia, Methylobacterium, Novosphingobium, Massilia* and *Gottfrieda*. In contrast, a study by Motsomane *et al.* (2024) showed the presence of different microbial taxa, including *Enterobacter, Peribacillus, Cupriavidue, Dyella, Stenotrophomonas, Burkholderia, Caulobacter, Hymenobacter,* and *Bradyrhizobium* in *E. villosus*.

Regarding the activity of enzymes, Ndlovu *et al.* (2023) documented the enzymatic activity in both the rhizosphere and non-rhizosphere soils of *E. natalensis*. The results obtained in the

study showed that the activity of β-D-glucosaminidase and acid phosphatase enzymes was comparable in both the rhizosphere and non-rhizosphere soils. The authors also reported a higher nitrate reductase enzyme activity in the rhizosphere compared to the non-rhizosphere soils. In addition, they found a higher alkaline phosphatase activity in the non-rhizosphere compared to the rhizosphere soils (Ndlovu *et al*., 2023). A different study carried out on *E. villosus* found that the β-D-glucosaminidase, nitrate reductase and phosphatases (acid and alkaline) enzyme activity in the rhizosphere and non-rhizosphere soils of Rhebu and Oceanview were not statistically significant (Motsomane *et al.*, 2024).

Both *E. natalensis* and *E. villosus* occur in disturbed savanna and scarp forest, respectively, and the diversity of microbes associated with cycads in the different habitats vary. This suggests that specific microbes may be associated with specific cycad species and habitat types. The target species in this study, *E. lanatus*, occurs in nutrient-poor grassland habitats that experience regular fires and grazing and may also have different microbial diversity. Therefore, knowledge of the diversity of nutrient-cycling microbes associated with the *E. lanatus* ecosystem and its contribution to soil nutrient improvement in nutrient-deficient grassland habitats is limited and requires investigation.

Among the 10 genera, *Encephalartos* is the only cycad with the highest risk of extinction (Mankga and Yessoufou, 2017). Successful cycad conservation efforts should include the processes and systems that sustain the plants *in situ* and the functioning of their ecosystem. The gap in knowledge on cyanobionts that function in nutrient cycling is, therefore, a barrier to the development of an effective conservation and management plan for *E. lanatus*. In addition, understanding *E. lanatus* cyanobionts-symbiotic interactions aligns with the mission statement of the Mpumalanga Biodiversity Sector Plan (MBSP) of 2015, which focuses on having an adequate representation of biodiversity in Mpumalanga being adaptively conserved, sustainably managed, and restored wherever applicable, while safeguarding all species and ecosystems (Lötter, 2015). Moreover, the information obtained from this study will enhance the knowledge of biodiversity and the enhancement of ecosystem services, which aligns with South Africa's National Biodiversity Strategy and Action Plan (NBSAP) 2015–2025.

1.3 Aim and Objectives

The study aims to investigate the ecology of the African cycad *E. lanatus* in Botshabelo Nature Reserve, Middelburg, in Mpumalanga Province.

The objectives of the study include the following:

a) To investigate the effects of fire on the population structure of the African cycad *E. lanatus* Stapf & Burtt Davy (1926).
b) To assess the rhizosphere and non-rhizosphere soil characteristics (nutrient concentration, total exchange acidity, total cations, and pH) of *E. lanatus*.
c) To identify the N-fixing, P-solubilizing, and N-cycling bacteria present in the *E. lanatus* coralloid roots, rhizosphere, and non-rhizosphere, and assay their extracellular enzyme activities.

1.4 Research question(s)

a) What is the abundance/population status of *E. lanatus* in the Botshabelo Cultural Village?
b) How does fire affect the reproductive structures and life stages of *E. lanatus*?
c) What is the contribution of *E. lanatus* to the enhancement of soil fertility?
d) Which microbial species inhabit the coralloid roots, rhizosphere, and non-rhizospheric zone of the *E. lanatus* plant?

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Chapter 2

Literature Review

2.1 Description/ classification of cycad species

Cycads' origin dates back to around 300 million years ago (MYA) (Hendricks, 1987; Mankga *et al*., 2020a). They reached their highest level of diversity during the Jurassic period and maintained a constant level of diversity throughout the Cretaceous period (Condamine *et al*., 2015). The existing cycads are classified into three families, namely Zamiaceae, Cycadaceae and Stangeriaceae (Osborne *et al.*, 2012), with a total of ten genera and more than 300 species (Mankga *et al*., 2020a). The family Cycadaceae consists exclusively of the genus *Cycas*, while Stangeriaceae consist of two genera, namely *Stangeria* and *Bowenia* (Donaldson, 2003). The Zamiaceae family is exceptionally diversified and has a wide distribution, consisting of seven genera (*Dioon, Encephalartos, Lepidozamia, Ceratozamia, Macrozamia, Zamia,* and *Microcycas*) (Donaldson, 2003).

Cycads are dioecious, generally exhibiting distinct variations in the size and shape of the cones between male and female individuals of the same species (Grobbelaar, 2004). Cycads have the ability to naturally reproduce through seeds and suckers (Demiray *et al*., 2017). For example, the germination of *Cycas revoluta* seeds can take anywhere from 3 to 9 months to begin, and once initiated, the germination process can continue for a year or longer (Benjelloun *et al*., 2020). Under optimal conditions, which include warm, moist conditions, well-drained soil, sufficient sunlight, water and nutrients, the cycad plant requires a minimum of 10-12 years to reach full maturity (Kaviani *et al*., 2014). In addition, cycads have underground (subterranean) or aboveground (Arborescent) stems. Some species, such as *Lepidozamia hopei* and *Encephalartos transvenous,* can grow to a height of 13-18 m and have a tree-like structure (Arborescent). Others, like *Zamia pumila*, are smaller shrubs with underground (subterranean) stems (Webb and Osborne, 1989). Arborescent plants often experience full development of their leaves and megasporophylls within around eight weeks (Marler *et al*., 2020). When it comes to male plants, a higher cone production rate is exhibited per plant per season (Grobbelaar *et al.,* 1989). Cycads require 3-10 years to reach sexual maturity (Demiray *et al*., 2017). However, the majority of the fast-growing *Encephalartos* species found in South Africa, such as *Encephalartos altensteinii, Encephalartos ferox, Encephalartos transvenous* and *Encephalartos natalensis,* require approximately 10-15 years to reach the size at which they produce cones when cultivated from seeds in a garden setting (Grobbelaar, 2004). Cycad roots are typically shallow and fleshy, lacking the tendency to penetrate deep into the soil (Jones, 1993). Furthermore, cycads have a notable characteristic of having extremely slow growth rates

(Raimondo and Donaldson, 2003), typically generating a single pair of new leaves yearly (Whitelock, 2002).

2.2 Geographical distribution of cycads

Cycads were previously abundant throughout a wide range of areas, but the remaining cycads are now limited to tropical and subtropical regions (Calonje *et al*., 2019). America, Africa, Australia and southern Asia are the four regions that constitute the epicentre of cycad diversity (Donaldson, 2003). The *Cycas* genus had the most rapid diversification and has a wide distribution (Yessoufou *et al*., 2017; Mankga *et al*., 2020b). According to fossil data, Asia is identified as the original location of the genus (Mankga *et al*., 2020b). The genus *Cycas* showed a southern distribution, extending from Asia to Australia, eastern Africa and the Pacific Islands (Hill, 2004). *Encephalartos* is a member of the Zamiaceae family, which consists of 65 species and is found exclusively in Africa (Whitelock, 2002; Donalson, 2003; Yessoufou *et al.,* 2014; Condamine *et al.,* 2015). Within South Africa, the genus is spread in a linear pattern along the coast of South Africa, stretching from the Eastern Cape to KwaZulu-Natal and then extending inland into the Mpumalanga and Limpopo Provinces (Donaldson, 2008). The *Dioon* genus consists of fourteen species, with thirteen species found in Mexico (Vovides, 1990; Prado *et al*., 2016) and one species found in Honduras (Prado *et al*., 2016). *Bowenia* is native to Australia and consists of two species, namely *Bowenia spectabilis* and *B. serrulata* (Whittaker, 2004; Hill *et al*., 2019). *Stangeria* is a monospecific genus that is indigenous to Africa (Whitelock, 2002). The genera *Zamia, Ceratozamia,* and *Microcycas* are found in America, while *Lepidozamia* and *Macrozamia* occur in Australia (Jones, 1993).

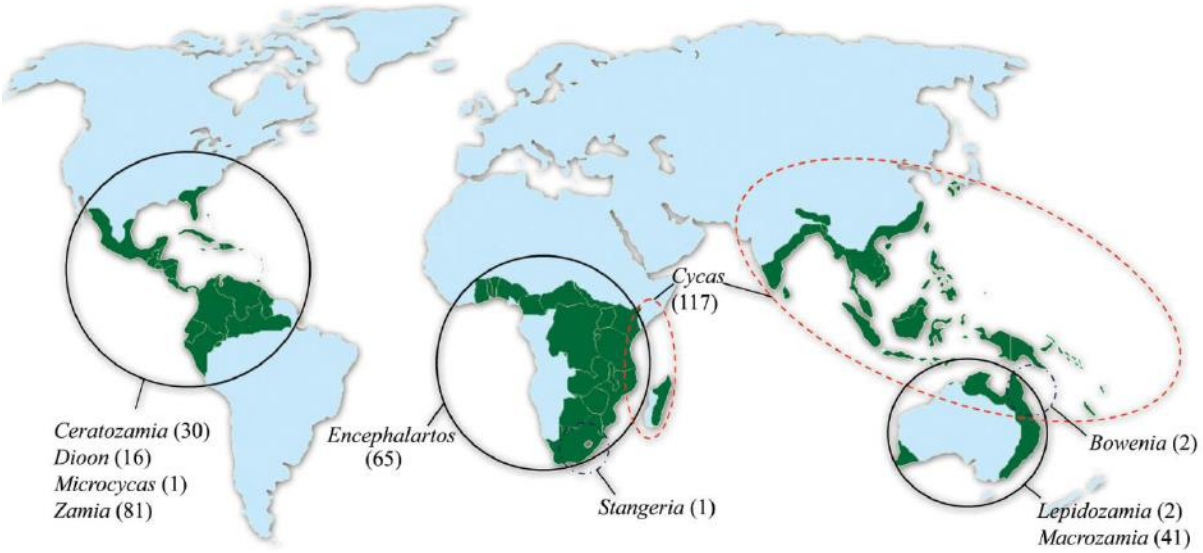

Figure 1: Picture depicting the distribution of cycads (Calonje *et al.*, 2019).

2.3 Importance of cycads regarding ethnobotany, ornamentals, and food

Cycads have been used for ages by various cultural groups in tropical and subtropical regions as a food source, particularly during periods of famine (Donaldson, 2003; Bonta *et al*., 2019). Although these plants are toxic, they are nonetheless important due to the high starch content in their stems and seeds, which makes them a great source of nutrients (Radha and Singh, 2008). The utilization of *Dioon* species in Mexico contributed to the survival of numerous individuals during the prolonged drought (Martínez *et al*., 2020). Cycads are also used as ornamentals for special occasions, especially the leaves (Vovides *et al*., 2010; Bonta *et al*., 2019). *Encephalartos transvenous* leaves and cones are utilized as ornamental elements on tables during special events, while the stems are cultivated close to residences for decorative reasons (Ravele and Makhado, 2009). The utilization of plant-based materials for medicinal purposes is an old practice that was used by our forefathers and has gained popularity in recent years. A few cycad plant species are facing an extinction dilemma as a consequence of the widespread harvesting encouraged by this practice (Williams *et al*., 2013). It is believed that approximately 60% of the South African human population seeks the services of traditional healers, who predominantly utilize plant-based materials as medicinal remedies to treat a wide range of illnesses in their patients (van Wyk *et al*., 2009). Cycads are used as medicine to treat stomach problems, stroke, heart attacks (Ravele and Makhado, 2009), hypertension (Ndawonde *et al*., 2007) as well and breast cancer (Bamigboye *et al*., 2017). Roots, leaves, and

bark are the most commonly utilized materials for medicinal reasons (Ndawonde *et al*., 2007). The upper portion of the *Encephalartos transvenous* bark is collected based on the traditional belief that it possesses magical properties and has the ability to repel evil spirits (Bamigboye *et al*., 2017).

2.4 Conservation status of cycads

Cycads worldwide are currently facing a conservation crisis, making them the most endangered plant group (Hoffman *et al.,* 2010). They have been recognized as a key species for conservation efforts (Marler and Calonje, 2020). To effectively conserve threatened species, it is essential to have a thorough understanding of the environmental conditions and population dynamics that impact species survival and influence their susceptibility to extinction (Álvarez-Yepíz *et al*., 2019). The decline in cycad populations can be attributed to several factors, including habitat loss, overcollection, fire, reproduction failure, deforestation, grazing, medicinal usage, alien invasive plants and flood and drought occurrences. According to Raimondo *et al.* (2009), all the *Encephalartos* species are included in CITES Appendix I, and 78% of the South African species are categorized as threatened. Out of all the cycad species that are native to South Africa, three are categorized as Extinct in the wild (EX), twelve species are Critically Endangered (CR), four species are Endangered (EN), nine species are Vulnerable (VU), seven species that are Near Threatened (NT), and three species are Least Concern (LC) (Bland *et al.,* 2017). The species investigated in this study (*Encephalartos lanatus*) is classified as NT based on the International Union for Conservation of Nature (IUCN) Red List (Donaldson, 2003).

2.5 South African Biodiversity Policy

Cycads are safeguarded by international, national, and provincial laws. The national legislations that govern biodiversity and offers safeguarding measures for diverse species encompassing cycads are [1] National Environmental Management: Biodiversity Act of 2004 (NEMBA). This Act is responsible for the regulation of the cycad trade, the implementation of Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora, the establishment of a Scientific Authority of South African National Biodiversity Institute (SANBI), the administration of the authority to publish a list of endangered cycads, and the establishment of penalties for violation of the Act (DEA, 2013); [2] Threatened or Protected Species (TOPS) Regulations, 2007 administers the authorization system in accordance with the NEMBA legislation (Donaldson, 2008); [3] Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Regulations 2020- when actions involving importing

or exporting listed species happen, this law applies since every cycad species is classified under Appendix I, which bans any trade involving plants that have been collected from the wild, and Appendix II, which permits trade only with a license (Osborne, 1990; Stiles, 1997; Donaldson, 2003); [4] National Environmental Management: Protected Areas Act 57 of 2003 (NEMPAA) - the purpose of this law is to establish specifically designated region for the conservation of cycads species, with the aim of safeguarding their protection and ensuring their long term existence (DEA, 2013); [5] National Environmental Management Act 107 of 1998 (NEMA) establishes the guidelines for the environmental rights of all South African people and outline the principles for safeguarding cycads in South Africa (DEA, 2013). The aforementioned rules and regulations are implemented by Provincial legislation that presently regulates the conservation of cycads. All the provincial regulations are enforced to regulate the trade and transportation of cycads among provinces. The authorities grant permits to individuals who wish to export cycads inside the province, as well as to researchers who intend to perform studies on cycad plants. These legislations incorporate [1] The Natal Conservation Ordinance No. 15 of 1974 (Osborne, 1990); [2] The Limpopo Environmental Management Act No 7 of 2003; [3] The Mpumalanga Nature Conservation Act No 10 of 1998; [4] Mpumalanga Tourism and Parks Agency; [5] The Ciskei Nature Conservation Act No 10 of 1987 and [6] Transkei Decree No 9 of 1992.

2.6 Functional role of cycads in the ecosystem

2.6.1 Food and shelter

Cycads are keystone species that play a key role in maintaining the ecosystem. Cycads provide food and shelter for birds and wild animals (Donaldson, 2008). They are also known for being poisonous, nonetheless, wild animals and insects have evolved a mechanism to neutralize the toxins (Schneider *et al*., 2002). Therefore, the primary consumers of cycad leaves are predominantly Lepidopteran larvae, encompassing both butterflies and moths (Whitaker and Salzman, 2020). These larvae possess a natural resistance to the poisons found in cycad leaves and can store and utilize them as part of their survival mechanisms (Bayliss *et al*., 2009). Staude (2001) reported sixteen species of moths belonging to six distinct genera that were observed feeding on the leaves of African cycads, and the most abundant genus among them was *Callioratis*. The female moth deposits her eggs on delicate, recently emerged cycad foliage, and the larvae then feed on all the tender leaves after hatching (Grobbelaar, 2004). Monkeys have developed specialized eating mechanisms that allow them to get the recently sprouted tender leaves, which have lower poisonous hydrogen cyanide compared to older leaves (Nowak

and Lee, 2011). Zanzibar red colobus monkeys consume the foliage of the arborescent cycads, displaying a preference for fresh, soft leaves rather than mature ones (Nowak and Lee, 2011). Porcupines are known to use *Encephalartos* stems as a source of nourishment and hydration (Goode, 1989).

2.6.2 Cycad's role in nitrogen (N_2) fixation

Due to a lack of nutrition in ancient soils 300 million years ago, cycads evolved a mechanism to host cyanobionts to survive in nutrient-poor environments (Chang *et al*., 2019). Given that cycads and cyanobacteria have existed since prehistoric times, it is possible that their symbiotic association and coevolution began millions of years ago (Chang *et al*., 2019). They have developed a specific root structure known as coralloid roots, which house symbiotic cyanobacteria capable of N_2 fixation for plants (Grobbelaar *et al.*, 1987). The formation of coralloid roots occurred in cycads before being colonized by cyanobionts (Lindblad and Bergman, 1990; Costa *et al*., 1999). Pre-coralloid roots develop and generate papillose tissue (Ahern and Staff, 1994), which during maturation, undergoes degeneration of the papillose tissue sheath and is replaced by a thin outer layer that forms dispersed lenticels (Lindblad, 2009; Chang *et al*., 2019). The N-fixing microorganisms in the soil enter the coralloid roots using dermal breaks in the outer layer (Costa *et al*., 1999). Soon after the N-fixing microorganisms move to the inner layer's roots, the coralloid roots develop a swollen and branched structure with two equal divisions (dichotomously branched) (Chang *et al*., 2019). The infected roots exhibit an increased diameter, and the cyanobacterial zone is located at the junction between the apparent outer and inner cortex (Chang *et al*., 1988). The mutualistic relationship is controlled by regulatory genes (Ahern and Staff, 1994). The cycad secretes hormogonium-inducing substances, which stimulate the N-fixing microorganisms to transform into mobile filaments known as hormogonia (Lindblad, 2009; Chang *et al*., 2019). Hormogonia infiltrate the coralloid roots of cycads using chemotaxis, and the coralloid root of cycad generates hormogonium-repressing factors to facilitate the development of heterocyst in the microbes, which is necessary for N_2 fixation (Chang *et al*., 2019). Under conditions of insufficient soil N supply, such as dunes and rocky outcrops with a lot of salt content (Grove *et al*., 1980), cycads establish a symbiotic relationship with the N-fixing bacteria by producing a signal that triggers the formation of hormogonia, as mentioned earlier (Hashidoko *et al*., 2019). In the cycad species *Cycas revoluta*, this signal has been identified as 1-palmitoyl-2-linoleoyl-sn-glycerol (Hashidoko *et al*., 2019).

According to Yamada *et al*. (2012), *Nostoc* is the predominant type of bacteria strain found in Cycadaceae, while *Anabaena* and *Calonthrix* species establish symbiotic relationships less commonly. A research study on coralloid roots that were sampled from cycads grown in a botanical garden among the genera *Cycas, Zamia,* and *Encephalartos* found that coralloid roots associated with one strain of *Nostoc* (Costa *et al*., 1999). Another study carried out in the natural habitat of cycads confirmed the presence of *Nostoc* strain in coralloid roots (Gehringer *et al*., 2010). However, a recent study identified bacterial species from the genera *Nostoc, Burkholderia, Mesorhizobium, Bradyrhizobium,* and *Caulobacter* present in coralloid roots of wild *Dioon* cycad populations (Gutiérrez-García *et al*., 2018). The following microbe genera were identified in *E. natalensis* coralloid roots: *Lysinibacillus, Bacillus, Paenibacillus* and *Beijerinckia* (Ndlovu *et al.,* 2023). Motsomane *et al.* (2024) reported the presence of *Lysinibacillus, Bacillus, Paenibacillus* and *Enterobacter* in *E. villosus* coralloid roots growing in Oceanview, Eastern Cape, South Africa.

2.7 Soil enzyme activities

Soil microbes secrete extracellular enzymes that are crucial for breaking down organic matter and facilitating the global cycles of carbon (C), phosphate (P), and nitrogen (N). These enzymes also serve as indicators of soil health and fertility (Daunoras *et al*., 2024). Soil extracellular enzymes serve as catalysts and mediators in a variety of biochemical processes occurring in soil, including nutrient mineralization, cycling, decomposition, and the formation of soil organic matter (Sudhakaran and Ravanachandar, 2020). Glycosidase, specifically α-galactosidase, is an enzyme that catalyzes the hydrolysis of disaccharides, α-D-galatopyranosides in soil (Zhang *et al*., 2020). *N*-acetyl-β-D-glucosaminidase and asparaginase accelerate the conversion of asparagine into aspartic acid and NH_3 and break down chito-oligosaccharides by hydrolysis (Hill *et al*., 1967; Mega *et al*., 1972; Parham and Deng, 2000). This has a comprehensive impact on the process of N mineralization and leads to an enhanced uptake of N by plants (Turner *et al.,* 2002). β-glucosidase is an enzyme that plays a vital part in the carbon cycle by breaking down small carbohydrates, which serve as the main energy sources for soil microbes (Nannipieri *et al*., 2011). Nitrate reductases (NR) play an essential role in the nitrogen cycle by converting nitrate into nitrite (Sibevieh *et al*., 2021). Phosphomonoesterase plays a vital part in the biogeochemical cycling of P by acting as a catalyst for the hydrolysis of phosphate monoesters, leading to the formation of free phosphate, which is necessary for biological absorption (Caldwell, 2005). Phosphatases solubilize

insoluble cation-bound P complexes that are not soluble, making them accessible for plant absorption (Yokoyama *et al.*, 2018; Turner and Wright, 2014).

Ndlovu *et al.* (2023) studied the enzyme activity in both the rhizosphere and non-rhizosphere soils of *E. natalensis*. The findings indicated that the activities of β-D-glucosaminidase and acid phosphatase enzymes were similar in both the rhizosphere and non-rhizosphere soils. The author also found elevated nitrate reductase enzyme activity in the rhizosphere relative to the non-rhizosphere. Furthermore, they observed elevated alkaline phosphatase activity in the non-rhizosphere relative to the rhizosphere. The enzyme activity of β-D-glucosaminidase, nitrate reductase and phosphatases (acid and alkaline) in the rhizosphere and non-rhizosphere soils of Rhebu and Oceanview were not statistically significant, according to the findings of a different study that was conducted on *E. villosus* (Motsomane *et al.*, 2024).

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Chapter 3

EFFECTS OF FIRE ON THE POPULATION STRUCTURE OF THE AFRICAN CYCAD *ENCEPHALARTOS LANATUS* STAPF & BURTT DAVY (1926)

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Population Structure of an African Cycad: Fire May Stimulate the Coning Phenology of *Encephalartos lanatus* (Zamiaceae) and Also Predispose Its Cones to Damage

Memory N. Sigasa [1], Kowiyou Yessoufou [2,*], Anathi Magadlela [3], Wilfred Otang-Mbeng [4] and Terence N. Suinyuy [4,*]

[1] School of Agricultural Sciences, Faculty of Agriculture and Natural Sciences, University of Mpumalanga, Mbombela 1200, South Africa; 201716267@ump.ac.za

[2] Department of Geography, Environmental Management and Energy Studies, University of Johannesburg, Johannesburg 2006, South Africa

[3] School of Life Sciences, College of Agriculture, Engineering and Science, University of Kwazulu-Natal (Westville Campus), Durban 4000, South Africa; anathimagadlela@icloud.com or magadlelaA@ukzn.ac.za

[4] School of Biology and Environmental Sciences, Faculty of Agriculture and Natural Sciences, University of Mpumalanga, Mbombela 1200, Wilfred.mbeng@ump.ac.za

*Correspondence: kowiyouy@uj.ac.za (K.Y.); tsuinyuy@gmail.com or Terence.Suinyuy@ump.ac.za (T.N.S.)

Abstract

Cycads are the most threatened group in the plant kingdom. Fire is identified as one of the major factors heightening cycad extinction risk. However, compared to South American cycads, we know little about how fire negatively affects the demography of African cycads. Here, we collected a snapshot of demographic data on the largest known population of South Africa's cycad species, *Encephalartos lanatus,* in unburnt and regularly burnt habitats. We fitted several statistical models to investigate the effects of fire on the population structure of *E. lanatus*. First, we found that the population follows a 'J' structure, with more adults than any other life stage. Contrary to popular belief, this 'J' structure may not necessarily imply that the future of the population is at risk, given that *E. lanatus* is a long-lived species. Second, we found that the abundance of adults explains 25% of the abundance of seedlings but does not predict the abundance of suckers, perhaps suggesting the adults ensure preferential seedlings rather than clonal recruitment. Third, irrespective of life stages, the subpopulation in fire-prone habitats is, in terms of size, proportionately lower than the subpopulation in unburnt areas, suggesting that fire may negatively affect the dynamic of the population. However, fire is not linked to differences in sex ratio across the population; not only do fire-prone subpopulations have more cones, but they also tend to have more damaged cones than unburnt populations. In general, although we raised some limitations of the present study, we also inferred that fire may shape the observed 'J' structure of the population of *E. lanatus*, but, contrary to traditional belief, the 'J' structure is not enough to raise concern about the future of the population. A population dynamics study is required to determine if the future of the population is at risk.

Keywords: African cycads; fire; population structure

3.1 Introduction

In the plant kingdom, cycads, with their unique evolutionary history (Nagalingum *et al*., 2011; Yessoufou *et al*., 2014; Condamine *et al*., 2015) and unique shared morphological features between ferns and angiosperms (Norstog and Nicholls, 1997; Brenner *et al*., 2003), are the most threatened taxonomic group. Once widely distributed, particularly in the Mesozoic era (Hermsen *et al*., 2009), cycads now exhibit a patchy distribution in tropical and subtropical regions (Nagalingum *et al*., 2011). This restricted geography predisposes them to a high risk of extinction, whereby 70% of all cycad species are threatened with high extinction risk (Mankga and Yessoufou, 2017; Yessoufou *et al*., 2017). It is well-established that extinction risk is linked to ecological and biological factors (Sodhi *et al*., 2008; Yessoufou *et al*., 2012) as well as evolutionary history (Davies *et al*., 2011; Daru *et al*., 2013). Specifically, for cycads, nine threats were recently identified, including, in order of importance, habitat loss, overcollection, fire, reproduction failure, deforestation, medicinal usage, grazing, flood/drought, and alien invasive species (Mankga and Yessoufou *et al*., 2017). The question then arises: How does each of these nine factors heighten the extinction risk of cycads? Fire is the third-largest reported threat to cycads (Mankga and Yessoufou *et al*., 2017). Fire, through various aspects, e.g., frequency, intensity, duration, and timing, shapes the population structure of several long-lived species by influencing several demographic processes, e.g., survival, fecundity, and growth (Ahlgren and Kozlowsila, 1974; Bazzaz, 1984; Whelan, 1995). To survive the effects of fire, these species resprout (Pausas *et al*., 2018). However, since surface buds can easily be killed by the heat produced by fire, plants have to keep their buds away from fire heat if they are to survive (Pausas *et al*., 2018). Therefore, the ability of plants to resprout depends on the locations of their buds, e.g., above-ground (thick insulating barks; (Pausas, 2017)) or below-ground (roots, root crown, rhizomes, etc.; (Pausas *et al*., 2016)), using soil as a heat insulator (Auld and Bradstock, 1996). In summary, fire plays positive roles by stimulating reproduction, seed release and germination, and/or vegetative growth of several species (Gill, 1975, 1981; Hartnett and Richardson, 1989; Brewer and Platt, 1994; Spier and Snyder, 1998). Fire is required for the germination of the seeds of some savanna species (Christensen, 1977) and seedling recruitment (Menges, 1995).

However, existing studies of the effects of fire on cycads are old, and most of these studies focused on New World cycads. These studies reveal that fire stimulates leaf formations in the Australian cycad *Macrozamia riedlei* (Baird, 1977; Dolva and Scott, 1982), in *Cycas media*

(Ornduff, 1991), as well as coning phenology in *Macrozamia communis* (Baird, 1977; Beaton, 1982; Pate, 1993). Coning phenology and leaf production were also reported to be stimulated in the African cycad *Encephalartos transvenous* in South Africa (Grobbelaar *et al*., 1989). Fire is also linked to seedling recruitment in the population of *Cycas armstrongii* (Watkinson and Powell, 1997). Furthermore, fire stimulates the fixation of specific nutrients by cycads. For example, Grove *et al*. (1980) reported that in the leaves of the cycad *Macrozamia riedlei,* there were significantly higher concentrations of nitrogen and phosphorus after fire. The same study reported significantly higher concentrations of phosphorus, potassium, and zinc in coralloid roots of the same cycad species after fire (Grove *et al*., 1980). In total, 35 kg of nitrogen per hectare was fixed by *Macrozamia riedlei* in 5-7 years of prescribed fires (Grove *et al*., 1980). In the absence of fire, higher concentrations of calcium, sodium, and chlorine in the leaves of *Macrozamia riedlei* were reported (Grove *et al*., 1980). Cycads, in particular, are adapted to fire-prone ecosystems (Tang, 1990; Griffiths *et al*., 2005; Preece *et al*., 2007; Clarke *et al*., 2013). How, then, could fire be listed among the forces that threaten the survival of cycads?

We set two hypotheses to explain how fire may predispose cycads to extinction. Our first hypothesis is that some important life stages for the population growth of a given species may be more vulnerable to fire effects, thus stressing out the population dynamics of the species. For example, 33-63% of seedling deaths in South Africa's population of *Encephalartos latifrons* were related to fire, while other life stages of the same species showed stronger resilience to fire (Swart *et al*., 2019). An early study reported fire-driven mortality of up to 50% of adults of Australia's *Cycas armstrongii* (Liddle, 2004). Our second hypothesis is that fire may mediate damage to cones (the cycad reproductive structures), thus affecting important demographic processes, e.g., fertility, which may eventually lead to population decline that heightens the extinction risk of cycads. Evidence supporting this hypothesis was provided for South America's cycads. For example, Fawcett and Norstog (1993) linked fire to damaged cones of the genus *Zamia*, leading to low fecundity (Beaton, 1982), which may eventually cause the collapse of its population (Griffiths *et al*., 2005). Furthermore, fire seems to mediate the herbivory of cycad seeds, leading to the mortality of some adult individuals of *Zamia pumila* (Negrón-Ortiz and Gorchov, 2000). For example, herbivory activities of the larvae of *Seirarctia echo* are boosted following fire, resulting in several plants of *Z. pumila* being defoliated (Negrón-Ortiz and Gorchov, 2000). This defoliation led to the deaths of some adults while promoting leaf production in other life stages (Negrón-Ortiz and Gorchov, 2000). Also, the boosted activities of the herbivore *Seirarctia echo* led to more damaged cones and the death

of some seeds of *Z. pumila*, thus resulting in indirect negative effects of fire on the reproduction of this cycad species (Negrón-Ortiz and Gorchov, 2000). However, in comparison with the South American cycads, we do not know much about how Africa's populations of cycads interact with fire. The cycad genus *Encephalartos*, with its 65 species, is endemic to Africa (Yessoufou *et al*., 2014; Yessoufou *et al*., 2017), with South Africa being its centre of diversity (Yessoufou *et al*., 2017).

The aim of the present study is to investigate fire in relation to the demography of an African cycad population, *Encephalartos lanatus* Stapf & Burtt Davy (1926), in South Africa. We tested two hypotheses: (i) some important life stages for population growth of this species may be more vulnerable to fire; (ii) fire mediates damage to cones, thus affecting important demographic processes.

3.2 Materials and Methods

3.2.1 Locality and Study Species

The present study on *Encephalartos lanatus* was conducted from May 2021 to September 2022 in the Botshabelo Nature Reserve, Middelburg, where the largest stands of the natural population of *E. lanatus* occur. The reserve falls within the mesic Highveld grassland bioregion and is home to several small herbivores. The landscape is a fire-prone habitat that experiences annual fires, aiming to promote grass growth for the herbivores. Because of this regular burning, *E. lanatus* is exposed to frequent annual winter fires, which may affect the population structure and the fecundity of the plants.

Encephalartos lanatus is an endemic cycad to South Africa and occurs in the Highveld grasslands and sandstone outcrops around the catchment area of the Olifants River in Middelburg, the Witbank and Bronkhorstspruit districts of Mpumalanga, and the Gauteng provinces of South Africa (Giddy, 1984; Goode, 1989). In its native geography, fire occurs in winter, which is dry with temperatures ranging between -6 and 22°C, and summer temperatures vary from 9 to 32°C. The area is characterized by thunderstorms with summer rainfall of between 500 mm and 625 mm (Giddy, 1984).

Encephalartos lanatus is a medium- to fairly large-sized cycad that grows individually and in clumps with an erect or reclining stem of 1-3 m tall and 25 cm in diameter (Giddy, 1984; Goode, 1989). *E. lanatus* is a frost-hardy, fire-adapted, and drought-resistant plant. Its population size is estimated to be >10,000 mature individuals, and the species is classified as "near threatened" according to IUCN criteria (Osborne, 1995; Donaldson, 2003). The

immature leaves of *E. lanatus* are hairy (wool-like), greyish, and have a curled tip, while the grown leaves are greyish-green in colour and measure 60 to 80 cm long (Osborne *et al*., 2012). The plants' cones in the winter and immature male and female cones are dense and woolly and turn yellow as they mature (Giddy, 1984).

3.2.2 Data Collection

During the years 2021 and 2022, we measured the demographic data of *E. lanatus* in the largest known natural population of the species. First, transects were defined following different directions: north-> south-> north. Second, along each transect, plots were set up such that two consecutive plots were separated by at least 7 m. Twenty plots were established in total. Next, plots close to roads or the edge of the reserve were not considered to avoid edge effects (green plots in Figure 1). Plots that fall in a position where there is no *E. lanatus* (red plots in Figure 1) were also avoided. Finally, only plots that were away from the roads or the reserve's edges and which fell at positions where *E. lanatus* is found were surveyed (black plots in Figure 1). Each of those plots was 30 m × 20 m in the accessible yearly burnt and unburnt sections of the Botshabelo Nature Reserve. Individuals of *E. lanatus* were categorized as burnt based on signs of recent fires, which include scorched leaves, a black trunk/stem, and debris and soot lying around the plant. The unburnt individuals did not have any of the characteristics recorded in the burnt section, and there was no burn debris from the surrounding vegetation. In total, 20 plots (10 in the burnt and 10 in the unburnt sections) were set, which covered the entire accessible population.

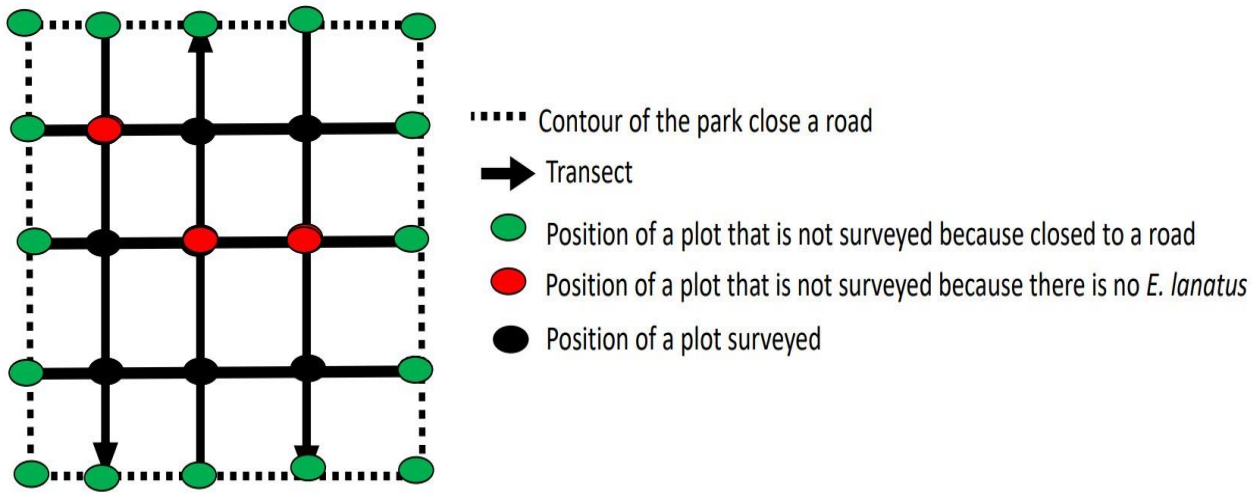

Figure 1. Graphical representation of the design of data collection. The minimum distance between two consecutive plots is 7 m.

In each plot, we recorded the number of *E. lanatus* and the number of stems per individual plant. The plants were categorized into adults, juveniles, suckers (sprouts), and seedlings. Following Hall and Walter (2013), plants on which the longest leaf measured from petiole base to farthest leaflet tip was ≤0.5 m were classified as seedlings, while those with the longest leaf measuring >0.5 m and ≤1 m were grouped as juveniles, and plants with a leaf length of ≥1 m were grouped as adults. Adult plants were sexed whenever possible based on the type of cones they bore and on other indicators like old cone scales and stalks. Those adults for which the determination of sex was not possible were classified as "undetermined". Plants with the status of 'Undetermined sex' were not included in the analysis of sex ratio in this study. Adult plants, sprouts, and juveniles had their stem height measured with a measuring tape.

All cones on plants in the 20 plots were counted, sexed, and assessed for damage. Male cones were considered damaged if parts of the cones were removed or completely broken off from the plant, and also if they were partially burnt by fire or some sporophylls were removed and the remaining cones were on the plant. Female cones were considered to be damaged when immature ovules/seeds were completely or partially removed from the cones and if an immature cone was burnt or completely or partially broken off from the plant. All the collected data are presented in Table S1.

3.2.3 Data Analysis

All the analyses were conducted in R (see R scripts used as Supplemental Information).

Population structure (Tables S1 and S2).

To assess the distribution of life stages across the population, we fitted a simple ANOVA, using the proportion of individuals in each stage (log-response) as the response variable and life stages as the predictor (data in Table S1). To determine the structure of the population, we plotted a box plot with a trend line. We further analyzed the population structure by exploring whether the abundance of one life stage predicts that of another stage (data in Table S2). To this end, we fitted a negative binomial Generalized Linear Model since the response variable is count data (abundance); a negative binomial was preferred to account for overdispersion.

Effects of fire on population structure.

We investigated the effects of fire in four ways. First, we tested whether the proportion of each life stage varied between burnt and unburnt plots in the habitat (data in Table S2). This was performed by fitting an ANCOVA, using proportion as the response variable and life stages

and fire occurrence as the co-variates. Second, we tested the effects of fire on cone production (data in Table S2) by fitting a simple ANOVA, using the number of cones produced as the response variable and the fire occurrence (burnt and unburnt plots) as the predictor. Third, we tested whether fire predicts cone sex ratio distribution (data in Table S2) by fitting a simple ANOVA with the cone sex ratio as the response variable and fire occurrence as the predictor. Finally, we tested whether fire predicts the number of damaged cones (cone herbivory by baboons; data in Table S3). This was conducted by fitting a negative binomial Generalized Linear Model using the number of cones as the response variable and fire and states of the cones (damaged and undamaged) as predictors.

3.3 Results

3.3.1 Population Structure

First, we found that the population of *E. lanatus* follows a 'J' structure (Figure 2) with a significantly higher proportion of adults than any other life stage (ANOVA, DF = 3, F = 42.82, $p < 0.001$; Figure 2). In this structure, juveniles are the least represented in proportion. We further found no link between the abundance of seedlings and suckers, juveniles and suckers, adults and suckers, or between adults and juveniles ($p > 0.05$). However, we found that juveniles and adults explain 48% ($\beta = 0.09 \pm 0.025$, $p < 0.001$) and 25% ($\beta = 0.12 \pm 0.05$, $p = 0.01$) of the changes in the abundance of seedlings, respectively.

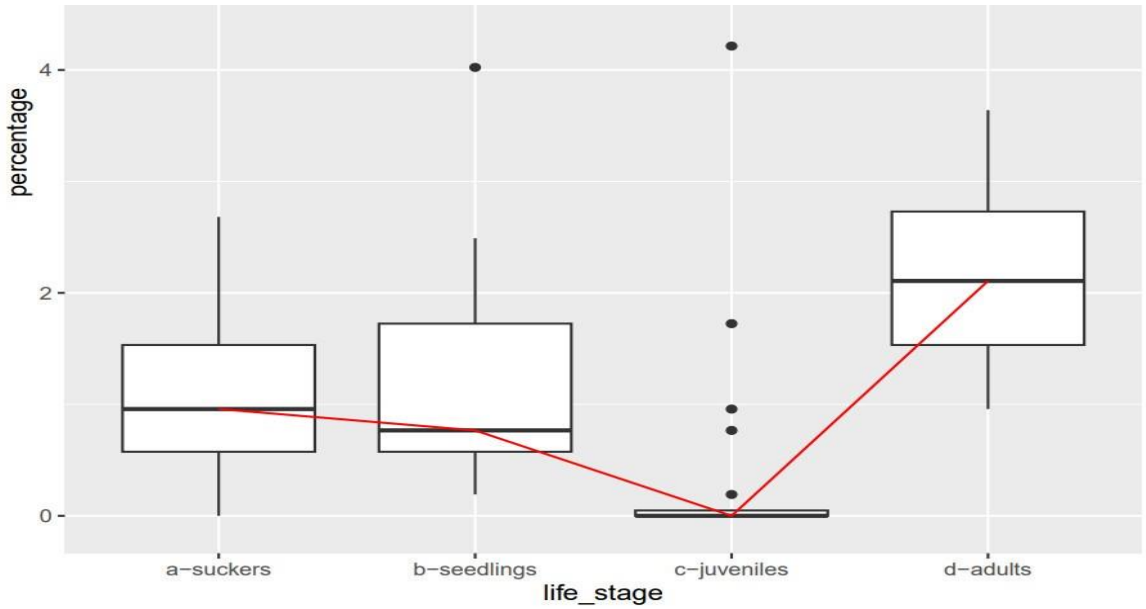

Figure 2. Population structure of *Encephalartos lanatus*

3.3.2 Effects of Fire on Population Structure

We found that irrespective of the life stages, the population in fire-prone habitats is proportionately lower than the population in unburnt areas (ANCOVA, DF = 1, F value = 3.603, p = 0.06; Figure 3), although this difference is only marginal, suggesting that fire may negatively affect the dynamic of the population.

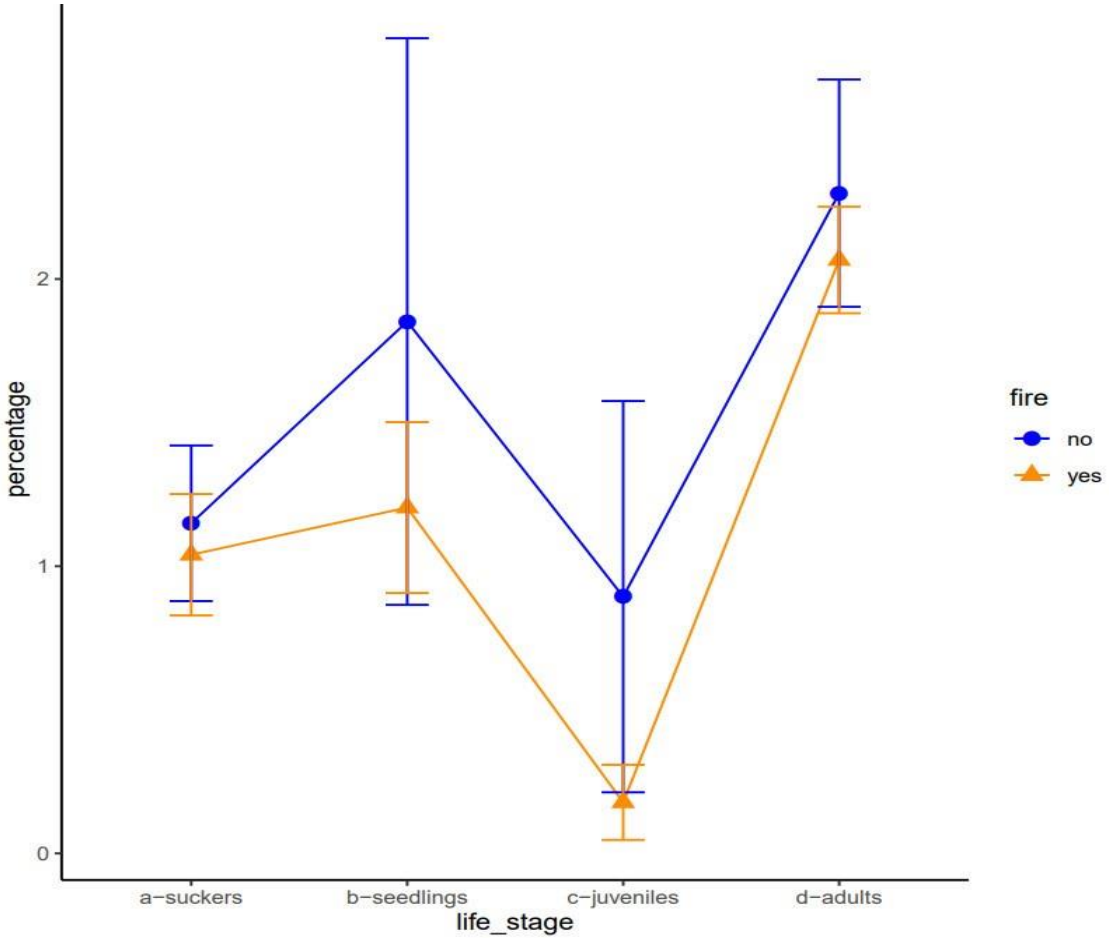

Figure 3. Distribution of life stages in relation to fire occurrence.

To understand the role of fire, we investigated whether fire affects the reproductive organs, i.e., the cones of the species. We found that fire is not linked to differences in sex ratio across the entire population (ANOVA, DF = 1, F = 0.013, p = 0.912). However, we found that fire-prone populations not only possess more cones (ANOVA, DF = 1, F = 13.38, p = 0.0018; Figure 4a), but they also tend to have more damaged cones than unburnt populations (negative GLM; β = 1.77 ± 0.61, p = 0.0035; Figure 4b).

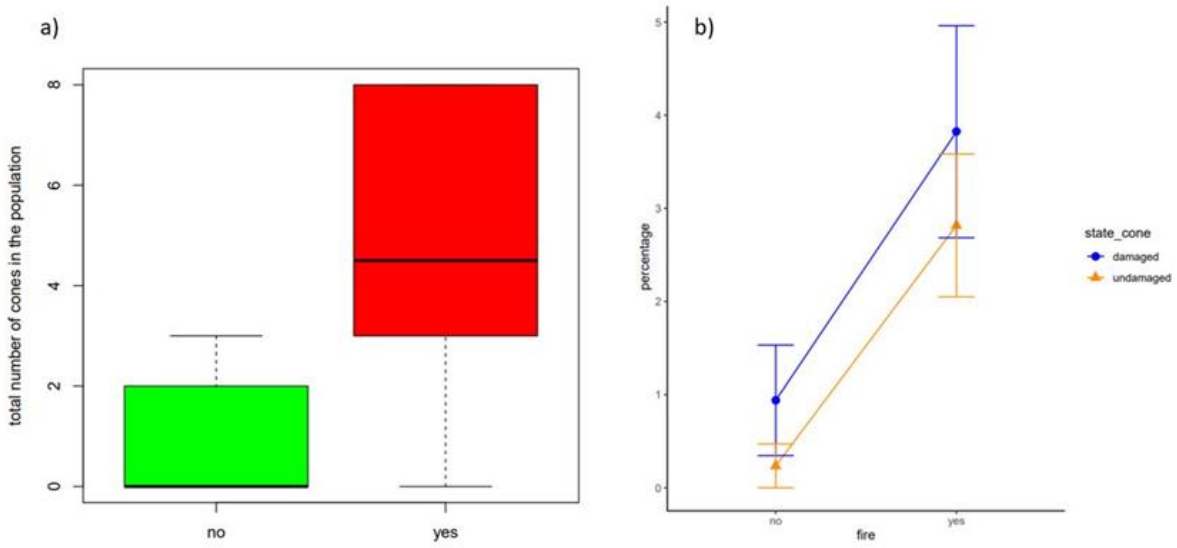

Figure 4. Effects of fire on the cone phenology of *Encephalartos lanatus*. (**a**) Comparison of the total cones between fire-prone areas and unburnt areas; (**b**) comparison of the state of cones (damaged versus undamaged) between fire-prone and unburnt areas.

3.4 Discussion

In the face of the ongoing biodiversity crisis (Ceballos *et al*., 2015), especially the cycad diversity crisis (Daru *et al*., 2013; Yessoufou *et al*., 2017), it is critically important to understand the dynamics of cycad populations. In the present study, we found that the population of *E. lanatus* follows a 'J' structure, suggesting that we have only a few seedlings and juveniles and a larger proportion of adults. This structure, a priori, is a concern for the future of the population of *E. lanatus*, which is the largest known population of this species in Africa. It is, a priori, a concern because this structure departs from the reverse J-shaped structure (see (Vovides, 1990) for cycad genus *Dioon*), i.e., the negative exponential growth model traditionally believed to characterize old-growth forests in an equilibrium state (Hough, 1932; Meyer and Stevenson, 1943; Meyer, 1952; Lorimer, 1980; Kimmins, 1987; Leak, 1996; Veríssimo *et al*., 1992; Dauber *et al*., 2005; Cancino and Gadow, 2002; Zimmerman and Kormos, 2012). From an ecological perspective, the reverse J-growth model implies that strong seedling recruitment is a prerequisite to ensuring positive population growth (FAO, 1994; Ashton and Macintosh, 2002; Bosire *et al*., 2008). Also, ecologically, it implies equal mortality rates across all life stages in a population. The 'J' structure that we found in our study site, therefore, implies a disproportionately higher mortality rate for early life stages (e.g., seedlings and juveniles).

However, the J-shaped trajectory that we found for *E. lanatus* may not necessarily predict negative growth for its population. The reason is that the traditional belief in the reverse J-growth trajectory as indicative of positive growth does not take into consideration the life history of the species at hand. Such consideration is critical since long- and short-lived species respond differently to perturbations ((Silvertown *et al*., 1993); see also Gaoue and Yessoufou (2019)). This is because long-lived species, unlike short-lived ones, invest heavily in long-term survival strategies (Morris *et al*., 2008). According to life history theory, the survival of reproductive adults drives long-term population growth for long-lived species (Silverton *et al*., 1993; Schmidt *et al*., 2011; Adler *et al*., 2014; Gaoue and Yessoufou, 2019). As such, low proportions of early life stages, such as those we found in the present study for a long-lived species like *E. lanatus*, would have limited impacts on the long-term population dynamics of this cycad species (see (Gaoue and Yessoufou, 2019)).

In contrast, low seedling survival rates may be critical in determining the short-term growth trajectory (see (Gaoue, 2016), suggesting that, in the short term, the 'J' structure that we found in this study may be a red flag, which can be quickly dismissed because long-term growth matters most. Long-term growth helps to contribute to the total biodiversity of an ecosystem when it comes to supporting a broad variety of different organisms. Considering these alternative scenarios (the 'J' structure may or may not be a concern), there is a need for a population dynamics study to clarify whether the 'J' structure we found here should be considered a red flag for the largest known population of *E. lanatus*. Such a study should seek to understand which life stages, when lost or heavily perturbed, would severely impact the population dynamics of the long-lived species *E. lanatus*. See Gaoue and Yessoufou (2019) for comprehensive population dynamics.

Pending such research, we employed a basic approach for a preliminary stand on the question. We found that juveniles and adults explain 48% and 25% of the seedling subpopulations, respectively. This implies, perhaps, that the adult subpopulations are ensuring the persistence of a quarter (25%) of seedlings in the population, which is consistent with the life-history theory predictions that the survival of adults is critical for the positive growth of long-lived species. Surprisingly, adults did not predict suckers. This is surprising because adults generate suckers through vegetative or asexual reproduction. Our finding that adults predict seedlings but do not predict suckers may indicate that *E. lanatus* prefers sexual reproduction over vegetative reproduction strategies. However, a recent study demonstrated that when a sexual reproduction strategy is compromised, e.g., through intense fruit harvesting, tropical trees shift

from a sexual to a clonal reproduction strategy (Gaoue *et al*., 2018). Our claim that sexual reproduction strategies may still be predominant in our population of *E. lanatus* over clonal or asexual reproduction would, therefore, imply that seedling recruitment in our population of *E. lanatus* may not yet be at risk.

We found that irrespective of the life stages, the fire-prone subpopulation is proportionately lower than the subpopulation in unburnt areas, suggesting that fire may negatively affect the dynamic of the population. This negative effect may be linked to perturbation of reproduction strategies (Mankga and Yessoufou, 2017) through, for example, less cone production in fire-prone habitats, bias in sex ratio patterns, or substantial cone damage. For example, on bias in sex ratio patterns, Negrón-Ortiz and Gorchov (2000) reported an increase in the production of more female cones by *Zamia pumila* in fire-prone habitats. However, we found that fire-prone populations have more cones than not, suggesting that fire may stimulate cone phenology. Also, we found no correlation between fire and patterns of cone sex ratio across the population, suggesting that fire may not alter sex ratio patterns in a way that negatively affects the population of *E. lanatus*. Nevertheless, we found that fire-prone populations suffer a heavier burden of damaged cones than unburnt populations, meaning that the lower number of seedlings found in the population may be the result of more damaged cones. Negrón-Otiz and Gorchov (2000) reported that fire reduced the survival of seeds of *Zamia pumila* in America but did not kill seedlings. Similar evidence of the negative effects of fire was reported for other cycad genera. Swart *et al*. (2019) reported 33-63% of fire-driven seedling deaths in South Africa's population of *Encephalartos latifrons*, while Liddle (2004) reported a death rate of 50% for adults of *Cycas armstrongii* in Australia due to fire (Liddle, 2004), which at the same time stimulates seedling recruitment of the species (Watkinson and Powell, 1997).

The answer to our initial question of how fire could be listed among threatening forces to cycad survival is that fire mediates damage to cycad cones. However, based on field observations, cone damage in the population of *E. lanatus* that we study is caused by baboons and not directly by fire. Specifically, baboons prefer to attack cones in burnt habitats because it is easier for baboons to escape human attacks or other dangers in burnt habitats where visibility from a distance is higher than in unburnt habitats full of grasses, behind which humans can hide to surprise baboons (see (Eby *et al*., 2014)). As a result of this baboon behaviour, there are more damaged cones in burnt plots than in unburnt ones.

However, there are some limitations to our investigations, which can be used to inform future studies. First, we did not explore what the net outcome of the fire effect was, that is, whether there was a net loss or gain of cones in the burnt plots when damaged by baboons. Also, we have taken a very simplistic approach to fire impacts, focusing on the dichotomy between burnt and unburnt populations. Within the life history of cycads in general, including *E. lanatus*, it is much more realistic to explore cycad population structures in terms of fire regimes rather than simply burnt and unburnt populations. As such, it is important to interrogate what fire regimes would likely promote population persistence, seed production, and seedling emergence, and what regimes might be detrimental in the long term in terms of population dynamics. Finally, one can interrogate the importance of the 'J' structure for the dynamics of a species, which can resprout after fire even if all above-ground biomass is removed in very high-intensity fires. As such, the interpretation of the structure as just one snapshot in time could be misleading. However, a very recent interesting study just demonstrated that, in heavily harvested ecosystems in an Amazon rainforest, a liana species, *Banisteropsis caapi*, boosts clonal growth to persist and ensure its survival in response to intense anthropogenic pressure, but this increased clonal growth did not prevent the population from declining (Coe and Gaoue, 2023). In the context of the present study, even though *E. lanatus* resprouts after fire, this is no guarantee of a positive growth of its population. In conclusion, fire has mixed effects on the population of *E. lanatus*: it stimulates cone production and, at the same time, creates a safe haven for baboons' attacks on the cones of *E. lanatus.* Therefore, a priori, keeping baboons away from the near-threatened species of *E. lanatus* (Donaldson, 2020) would be advisable to reduce the proportion of damaged cones. An important recommendation from this observation on the baboons is that the population, or part of the population, be fenced to exclude baboons so that one can test for the effect of baboons vs. fire on cone production. This could be a way of managing this threatened population by removing a key threat. Ecologically, baboons may help preserve *E. lanatus* populations by damaging their cones. Baboons may be controlling the population to ensure sustainable growth, similar to prey-predator interactions. To clarify whether the future of this population is not at risk despite the fire, a population dynamics study similar to that of Gaoue and Yessoufou (2019) is needed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/artiicle/103390/d15101075/s1, Table S1: Number of individuals per life stage (called 'data2' in the R script); Table S2: All demographic data collected in this study (called 'data' in the R script); Table S3: Number of individuals with cone state (damaged/undamaged) in burnt versus unburnt habitats (called 'data3' in R scripts); R scripts used for data analyses are included as supplemental information.

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Chapter 4

DIVERSITY OF MICROBES IN THE CORALLOID ROOTS, THEIR ACTIVITIES AND THE CONTRIBUTION OF *ENCEPHALARTOS LANATUS* TO SOIL NUTRIENT STATUS IN BOTSHABELO, MPUMALANGA, SOUTH AFRICA

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The impact of microbes and their associated extracellular enzymes on *Encephalartos lanatus* thriving under harsh environmental conditions in Middelburg, Mpumalanga

Memory Sigasa[1], Anathi Magadlela[2,4], Wilfred Otang-Mbeng[3] and Terence N. Suinyuy[2,3]*

[1]School of Agricultural Sciences, Faculty of Agriculture and Natural Sciences, University of Mpumalanga, Mbombela 1200, South Africa

[2]School of Life Sciences, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

[3]School of Biology and Environmental Sciences, Faculty of Agriculture and Natural Sciences, University of Mpumalanga, Mbombela 1200, South Africa

[4]School of Biological and Agricultural Sciences, Faculty of Natural and Applied Sciences, Sol Plaatje University, Kimberly 8301, South Africa

***Corresponding author: Terence.Suinyuy@ump.ac.za**

Abstract

Plant development is usually dependent on the nutrition provided by minerals in the soil and the presence of microorganisms in the rhizosphere or root zone. Cycads developed coralloid roots that house N-fixing bacteria that interact with plants and play a crucial role in enhancing plant productivity and health. However, the diversity of microbes associated with cycads and knowledge of how they affect soil fertility, and the functioning of the ecosystem exist only for a few cycads. The study investigates the nutrient status of the soil and assays the soil enzyme activities of the *Encephalartos lanatus* rhizosphere and non-rhizosphere soils. The study further identified the nutrient-cycling microbes in the coralloid roots of *E. lanatus*, rhizosphere and non-rhizosphere soils. Rhizosphere soils had a significantly higher concentration of Mg and Mn than non-rhizosphere soils. In addition, both the rhizosphere and non-rhizosphere soils had significantly similar concentrations of N, P, K, Ca, Cu, Zn, pH, Acid saturation, total cation exchange, Exchangeable acidity and organic C. The *N*-acetyl-β-D-glucosaminidase and alkaline phosphatase were higher in the rhizosphere than in the non-rhizosphere soils, but not significantly. In contrast, acid phosphatase and nitrate reductase were significantly higher in the rhizosphere than in the non-rhizosphere soils. Specifically, ten bacteria families were identified in the *E. lanatus* rhizosphere, non-rhizosphere soils, and coralloid roots. However, Burkholderiaceae and Rhizobiaceae were the most dominant microbial families in both soils. The research addressed how cycads preserve the ecosystem by facilitating the cycling of P, N and C nutrients. Hence, their conservation must be given exceptional attention, owing to their crucial role in providing ecosystem services in habitats with insufficient nutrients to maintain plant growth.

Keywords: Coralloid roots; cycads; *Encephalartos lanatus*; enzymes; microbes; soil nutrients

4.1 Introduction

Cycads are gymnosperms of Permian origin that were dominant during the Mesozoic (Norstog and Nicholls, 1997). Extant cycads are today restricted to the tropics and subtropics. *Encephalartos* is one of the slow-growing cycads native to Africa and comprises 65 species (Griffiths *et al.,* 2005; Yessoufou *et al*., 2014). According to Condamine *et al*. (2015), 37 *Encephalartos* species are endemic to South Africa. Consequently, South Africa is a significant hotspot for cycads. Cycads are unique plants because of their conservation status. They have a restricted distribution and are the most threatened plant group on Earth with over 70% of the over 300 extant cycad species threatened with extinction (Mankga and Yessoufou, 2017), mostly due to habitat loss, overcollection or poaching (for horticulture and medicinal purposes), fire and reproduction failure (Mankga and Yessoufou, 2017; Swart *et al*., 2019; Ndlovu *et al*., 2023). They have a unique evolutionary history (Nagalingum *et al*., 2011; Yessoufou *et al*., 2017) and life strategies (e.g. dioecious nature of plants, with male and female thermogenic cones that emit volatile odour and are insect-pollinated) (Terry *et al*., 2008). Finally, they are the only members of the gymnosperm family that produce coralloid roots, which host endophytic bacteria that fix nitrogen (N) that play a role in soil nutrient improvement (Ndlovu *et al*., 2023) and possibly help the plant to survive. Therefore, the conservation of cycads is of paramount significance because losing them would result in losing the vital evolutionary history and ecosystem services they offer, such as the cycling of nutrients (Ndlovu *et al*., 2023) and carbon (C) sequestration (Ma *et al.,* 2009).

Cycads grow in nutrient-deficient environments (Gutiérrez-García *et al*., 2018; Ndlovu *et al*., 2023) and the lack of essential nutrients in the soil, particularly N and phosphorus (P), prevents plants from growing (Ågren *et al*., 2012; Yoneyama *et al*., 2012; Wang *et al*., 2012; Bano and Iqbal, 2016; Matiwane *et al*., 2019; Tariq *et al*., 2022). For instance, P is present in the soil but not in a soluble form. As a result, the majority of the plants are not able to exploit it (Wang *et al*., 2012; Magadlela *et al*., 2014), because soils that are acidic and rich in cations bind it in a form that cannot be assimilated by plants (Wang *et al*., 2012; Magadlela *et al*., 2020; Zungu *et al*., 2020).

Coralloid roots of cycads are host to N-fixing microorganisms like *Nostoc* (Lindblad, 2009; Gehringer *et al*., 2010; Gutiérrez-García *et al*., 2018; Chang *et al*., 2019; Pecundo *et al*., 2021). The N-fixing microbes have the ability to break down the atmospheric triple bonds utilizing the nitrogenase enzyme to convert atmospheric N into usable forms such as NH_3 and nitrate, which will promote plant development (Chang *et al*., 2019).

Additionally, cycads benefit from the favourable impacts that bacteria have on them to survive and grow in adverse conditions (Pecundo *et al*., 2021). Moreover, coralloid roots and their associated bacteria might have been a crucial primitive characteristic that allowed cycads to thrive and adjust to the changing environments over millions of years (Gehringer *et al*., 2010; Gutiérrez-García *et al*., 2018; Zheng and Gong, 2019; Motsomane *et al*., 2023a; Ndlovu *et al*., 2023). According to Ndlovu *et al*. (2023), these microorganisms can cycle N, fix N, and solubilize P. In addition, soil microorganisms secrete extracellular enzymes that hydrolyze and transform polymeric compounds into easily accessible nutrients for use by flora and microbes (Zungu *et al*., 2020; Ndlovu *et al*., 2023). These extracellular enzymes also control how nutrients like N, C and phosphate are mineralized and cycled on land (Zungu *et al*., 2020; Khalid *et al*., 2021; Ndlovu *et al*., 2023).

Several studies have evaluated microbes linked with cycads' coralloid roots, rhizosphere, and non-rhizosphere soils. Gutiérrez-García *et al*. (2018) and Suárez-Moo *et al*. (2019) reported similar microbes in the coralloid roots such as *Nostoc*, *Rhizobium*, *Burkholderia* and *Bradyrhizobium* for the *Dioon* genus. An earlier study revealed that coralloid roots of 33 *Encephalartos* spp and *Stangeria eriopus* in South Africa could fix N (Grobbelaar *et al*., 1986), which was due to the presence of the cyanobacteria dominated by *Nostoc* species (Grobbelaar *et al*., 1987). Costa *et al*. (1999) further showed that coralloid roots of *E. natalensis* and *E. villosus* host similar cyanobionts, but each species had a diversity of cyanobionts, suggesting that the cyanobionts in the same cycads may have different functions.

Nonetheless, all these studies are confined to the correlation between *Encephalartos* species and nitrogen-fixing bacteria, without investigating their relationship with N-cycling, P-cycling and N-fixing bacteria. Furthermore, the studies did not investigate the role of these *Encephalartos* species in the enhancement of soil nutrient levels, nor did they evaluate the enzyme activities in their rhizosphere and non-rhizosphere soils. The plants that were utilized in the research projects were obtained from botanical garden collections, which do not properly represent the plants' natural habitats; consequently, it was challenging to ascertain whether the microorganisms were from the plants' natural environment or botanical gardens. The plants that are grown in botanical gardens are usually supplied with water, sprayed for pests and diseases, and given nutritional inputs in the form of fertilizers, all of which have the potential to influence the composition of the microorganisms that are present in the soils.

The most recent studies in the South African cycads *Encephalartos* species by Motsomane *et al*. (2023a, 2023b, 2024) and Ndlovu *et al.* (2023) investigated microbes associated with coralloid roots, rhizosphere and non-rhizosphere soil, and assayed soil enzyme activities of *E. natalensis* and *E. villosus* rhizosphere and non-rhizosphere soils in their natural habitats. Ndlovu *et al*. (2023) found microbes in the coralloid roots of the following genera: *Beijerinckia*, *Bacillus*, *Lysinibacillus* and *Paenibacillus* thus identified as microorganisms capable of both solubilizing P and fixing N. Ndlovu *et al*. (2023) also identified several species from the *Bacillus*, *Paraburkholderia* and *Caballeronia* genera as involved in cycling N. Additionally, the *Caballeronia* genus was found to be capable of P solubilization in the rhizosphere. In non-rhizosphere soils, *Phyllobacterium*, *Olivibacter*, *Sphingomonas* and *Bacillus* were identified as N cycling, while *Methylobacterium* and *Paraburkholderia* were identified as being involved in both P solubilization and N_2 fixation.

Motsomane *et al*. (2023a) also reported microbes in the coralloid roots, rhizosphere, and non-rhizosphere soils of *E. villosus* plants. According to Motsomane *et al*. (2023b) findings, *Lysinibacillus* and *Peribacillus* were identified as bacteria capable of solubilizing P. *Rhizobium*, *Paenibacillus* and *Enterobacter*, on the other hand were found to be N fixing microbes, and *Stenotrophomonas* is involved in N cycling in the coralloid roots (Motsomane *et al*., 2023a). *Pseudomonas*, *paraburkholderia*, *Caballeronia* and *Burkholderia* have been identified as P-solubilizing bacteria in the rhizosphere. *Variovorax* and *Caulobacter* are involved in N-cycling (Motsomane *et al*., 2023b). *Cupriavidus* was identified as an N-fixing bacteria. *Paraburkholderia* and *Burkholderia* were identified by the authors as N-fixing bacteria in the non-rhizosphere. In addition, *Dyella* was reported as a N-cycling bacteria. Moreover, *Variovorax* and *Paraburkholderia* were identified as microorganisms that have the ability to solubilize P and fix N. This is evidence that cycads house comparable microbial species.

Both *E. natalensis* and *E. villosus* are disturbed savanna and scarp forest cycads, respectively, but other South African cycads, such as *E. lanatus*, occur in grassland habitats that are deficient in nutrients. Similar to the disturbed savanna and scarp forest cycads, the grassland cycad *E. lanatus* may flourish in nutrient-poor soils because of their ability to develop symbiotic associations with N-fixing and other nutrient-cycling bacteria that may occur in the grassland. However, knowledge of the diversity of nutrient-cycling microbes associated with the *E.*

lanatus ecosystem and its contribution to soil nutrient improvement in nutrient-deficient grassland habitats is limited and requires investigation.

The grassland cycad in question, *E. lanatus* is indigenous to South Africa and found exclusively within the provinces of Gauteng and Mpumalanga (Sigasa *et al*., 2023). According to Bösenberg (2022), *E. lanatus* has been reclassified as "vulnerable" based on the criteria B1ab(v)+ 2ab(v) of the International Union for Conservation of Nature (IUCN) Red List. This requires that adequate conservation measures are developed for *E. lanatus* populations in the wild to prevent them from being endangered or threatened with extinction, which may lead to a decline in important ecosystem services. On the other hand, the lack of information regarding cyanobionts that play a role in the cycling of nutrients is a significant challenge in the way of the establishment of efficient conservation and management strategies for *E. lanatus* plants.

Additionally, understanding how *E. lanatus* and cyanobionts interact through symbiosis coordinates with Mpumalanga's goal statement. The objective of the Mpumalanga Biodiversity Sector Plan (MBSP) of 2015 is to provide for the protection of all the species and ecosystems that are found within the province. This is accomplished through the implementation of strategies such as adaptive conservation, sustainable management, and restoration when they are required (Lötter, 2015). More importantly, the research data that is gathered from this study will contribute to the enhancement of knowledge regarding biodiversity and the improvement of ecosystem services, which is in alignment with South Africa's National Biodiversity Strategy and Action Plan (NBSAP) 2015–2025. An investigation of the role of microorganisms on *E. lanatus* will provide insights into the impact of the mutually beneficial relationship between microbes and *E. lanatus* on soil nutrient enrichment. The purpose of this study was to identify the microbes inhabiting the rhizosphere, non-rhizosphere soils and the coralloid roots of *E. lanatus*. Thereafter, the nutritional status of the soil was correlated with the enzyme activity of *E. lanatus* from both the rhizosphere and non-rhizosphere soils. Our main hypothesis is that the rhizosphere, non-rhizosphere soils and coralloid roots contain a diversity of microbes besides N-fixing bacteria.

4.2 Materials and Methods

4.2.1 Sites for collecting soil samples

The study was conducted in the Botshabelo Nature Reserve located in the Botshabelo Cultural Village, Middelburg District in Mpumalanga Province (coordinates are omitted due to cycads conservation concerns). The reserve lies within the mesic highveld grassland bioregion, which

experiences rainfall in summer. In Botshabelo, which is in the northern region of Middelburg, the annual rainfall ranges from 700 mm to 725 mm. The underlying geology in the area is rocky, and the dominant vegetation is *Eragrostis lehmanniana, Eragrostis bergiana, Enneapogon brachystachyus, Chloris virgate* and *Cynodon inompletus* grass species. The area experiences an average annual winter temperature that is approximately 15.5°C, while the average summer temperature is around 27.2°C. The area is disrupted by the presence of baboons that feed on *Encephalarto*s *lanatus* plants and leave droppings and urine in the soil.

4.2.2 Soil sampling

Sampling was done as in previous studies by Marler and Krishnapillai (2018) and Marler and Calonje (2020) on *Cycas* in Guam. Sampling areas were selected randomly, and soil samples were collected on two sides of the *Encephalartos lanatus* plants. The targeted soil samples were labelled as rhizosphere soils and collected from under the canopy of *Encephalartos lanatus*, between the stem and drip line of the leaves, whereas the control soil samples were collected 5 meters away from the canopy of *Encephalartos lanatus* plants and labelled as non-rhizosphere soils. Soil from the rhizosphere and non-rhizosphere of *Encephalartos lanatus* was collected in four main compass directions: North, South, East and West. In the rhizosphere, the soil was collected around the plants at depths of 0-10 cm and 10-20 cm. To prevent harm to the subterranean stem, soil from the rhizosphere was collected at a distance of 50 cm from the plant and at the point where the leaves' canopy drip. Both the rhizosphere and the non-rhizosphere soils had 10 replicates. Before sampling, various measurements were taken, including the number of leaves, crown diameter, leaf length, stem diameter, plant height, juvenile, and seedlings. These measurements were recorded using a tape measure. The soil was sampled using a 15 cm wide hand auger and transferred into clear self-sealing plastic bags. It was then loosened and left to air dry in two of the containers that are kept at the University of Mpumalanga's farm (Mbombela Campus), South Africa. Once dried, the soil was taken to the University of Mpumalanga's laboratory and passed through a 2mm sieve to remove any plant debris.

4.2.3 Analysis of Soil Properties

Twenty subsamples of soils from both rhizosphere and non-rhizosphere were sent to the Department of Agriculture and Rural Development Analytical Services (Cedara) for the analysis of N, P, potassium (K), zinc (Zn), magnesium (Mg), manganese (Mn), calcium (Ca), copper (Cu), pH, acid saturation, total cation exchange, exchange acidity, and organic C. The

total N concentration in the soil was measured by an Automated Dumas dry combustion technique utilizing a LECO TruSpec CN (LECO Corporation, Michigan, USA; Matejovic, 1996). To quantify P, K, Zn, Mn, and Cu, an atomic absorption technique was utilized. This entailed using the ambic-2 extraction solution comprising 0.25 M NH_4CO_3, 0.01 M Na_2EDTA, 0.01 M NH_4F, and 0.05g L^{-1} Superfloc (N100), with the pH adjusted to 8 using concentrated NH_3 solution. Subsequently, 25 ml of the solution was mixed with 2.5 ml of soil, and the mixture was agitated utilizing a multiple stirrer set at 400 rpm for ten minutes. The extracts were then filtered with the Whatman no. 1 paper. To measure the concentration of Ca and Mg in the soil, a similar procedure was implemented, but 25 ml of 1 M KCl solution was used as a substitute for the ambic-2 solution. In addition, 20 ml of 0.0365 M $SrCl_2$ was mixed with 5 ml of the filtrate. Next, determine the extractable acidity by titrating 10 ml of the filtrate with 0.005 M NaOH after diluting 10 ml of deionized water with two to four drops of phenolphthalein. The organic C was quantified using mid-infrared spectroscopy. The pH of the soil was measured through the utilization of 10 millilitres of soil solution with 25 millilitres of 1 M KCl solution, then used a multiple stirrer to mix at 400 rpm for five minutes. A gel-filled combination was used to determine the suspension of the pH while agitating. For a comprehensive explanation of the techniques, refer to Manson *et al*. (2020).

4.2.4 Collection and sterilization of coralloid roots

Coralloid roots were randomly collected from *E. lanatus* mature plants in Botshabelo Cultural Village in Middelburg, Mpumalanga Province. The coralloid roots were rinsed with distilled water, then disinfected with 70% (v/v) ethanol for 30 seconds, and soaked in 3.5% (v/v) sodium hypochlorite solution for three minutes. Afterwards, the roots were washed with distilled water ten times and stored inside 100 ml centrifuge tubes filled with 15% glycerol and preserved at 4°C before sequencing and extraction of bacteria.

4.2.5 Extraction, sequencing, and amplification of bacteria from E. lanatus rhizosphere, non-rhizosphere soils and coralloid roots

Soil serial dilutions were conducted to extract bacteria from soil subsamples (rhizosphere and non-rhizosphere soils), and coralloid roots were submerged in 15% glycerol and squashed with sterile tips to extract the bacteria. A hundred microliters of dilution and 10 µl of coralloid root suspension were inoculated in clean Petri dishes that contained nutrient agar. There were three types of nutrient agar (media). The first one is tricalcium phosphate (TCP), which was used to inoculate phosphate-solubilizing bacteria. The second one was Simmons citrate agar, which was used to inoculate N-cycling bacteria. The last nutrient agar is called Jensen's, which was

used to inoculate nitrogen-fixing bacteria. Each selective medium dish was replicated three times and incubated for 3-7 days at 30°C. Pure colonies of bacteria were obtained through subculturing.

After bacterial extraction, a Polymerase chain reaction (PCR) was used to amplify a small segment of pure bacterial colonies utilizing the 16S rRNA gene primers 63F (5' CAGGCCTAACACATGCAAGTC 3') and 1387R (5'-GGGCGGTGTGTACAAGGC 3') following the procedure outlined by Magadlela *et al.* (2017). The PCR amplification was carried out utilizing an EmeraldAmpGT Master Mix under the following conditions: Initial denaturation at 94°C for five minutes, followed by thirty cycles of denaturation at 94°C for 30 seconds, 30 seconds of annealing at 55°C, 2 minutes of extension at 72°C and the final elongation was performed at 72°C for 10 minutes. The outcomes were seen in 1% (w/v) agarose gel electrophoresis conducted at 100 V for 60 minutes utilizing a Tris-acetate-EDTA (TAE) buffer under ultraviolet light. The amplified products were then sent to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria) in South Africa for sequencing. The obtained DNA sequences from Inqaba were subjected to Nucleotide BLAST (NCBI) to find related bacteria in the GenBank database. https//:www.ncbi.nih.gov.

4.2.6 Enzyme activities

The enzyme activities of alkaline phosphatase, acid phosphatase, beta-glucosidase and N-β-D-acetyl-glucosaminidase were measured utilizing the fluorescence-based technique shown by Motsomane *et al*. (2023a) and Ndlovu *et al*. (2023) and the results were expressed as nmol $h^{-1}g^{-1}$. Ten grams of soil and hundred millilitres of autoclaved water were placed inside jars wrapped with foil and homogenized in a shaker at a constant speed for 2 hours and kept overnight at 4°C. Before putting in the substrates, the supernatants were put onto black 96-well microplates. The sample run comprised 200 µl soil aliquot plus 50 µl substrate, which were close to reference standards (200 µl soil aliquot + 50 µl standard), quench standard (200 µl soil aliquot + 50 µl standard). Sample control (200 µl soil aliquot + 50 µl Buffer), negative controls (200 µl buffer 50 µl) and blanks (250 µl buffer). After two hours of incubation at room temperature, 0.5M NaOH was used to stop the reaction. The fluorescent absorbance was then quantified at 450 nm on a Glomax Multi Plus microplate reader. Before measuring acid phosphatase enzyme activity, the buffer and standard were adjusted to a pH of 5.

The nitrate reductase activity was determined using a modified approach developed by Bruckner *et al.* (1995) and expressed as µmol $h^{-1}g^{-1}$. An Erlenmeyer flask coated with foil was

filled with 1 ml of 25 mM KNO_3, 4 ml of 0.9 mM 2,4-dinitrophenol, and 5 ml of autoclaved water. Afterwards, five grams of soil were added to the solution, and the flask was swirled and placed in an incubator for 24 hours at 30 °C. After incubation, 10 ml of 4 M KCl was added to the mixture and swirled thoroughly, then filtered with Whatman number 1 filter paper into a new wrapped flask. To initiate the enzymatic reaction, 2 ml of the infiltrate was added to NH_4Cl buffer that had a pH of 8.5 and 0.8 ml of colour reagent containing 1% of Sulfanilamide, 1 N HCl and 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD). The solution was then incubated for half an hour at 30 °C. To measure the absorbance at 520 nm, an Agilent Cary 60 UV-Vis spectrophotometer was used.

4.2.7 Statistical analysis

The data on soil properties and enzyme activities in the rhizosphere and non-rhizosphere were subjected to a two-sample t-test to test for significant differences through Statistix 10 software, and the Shapiro-Wilk test was used to determine the normality of the residual distribution. The data which were not normally distributed ($p \leq 0.05$) were transformed with $Log10(X + 1)$ and arcsine $\sqrt{(X \div 100)}$ for % variables. The homogeneity of variance assumption was tested using the F-test. Principal component analysis (PCA) was used to look into the relationship between soil nutrients and the corresponding enzyme activities in both the rhizosphere and non-rhizosphere soils of *E. lanatus*. The PCA was carried out utilizing RStudio software (version 3.6.0) with the assistance of the gg plot statistics package and the pr comp function.

4.3 Results

4.3.1 Soil properties

Certain measured variables, as determined by the Shapiro-Wilk normality test, did not follow a normal distribution and were transformed accordingly. In general, the rhizosphere had a higher concentration of macronutrients than the non-rhizosphere soils. The differences were, however, not statistically significant. Mg was the only element to exhibit a statistically significant difference (Table 1). The concentration of micronutrients, such as Mn, was significantly higher in the rhizosphere compared to the non-rhizosphere soils. Rhizosphere soils had a higher concentration of Cu than non-rhizosphere soils, but the difference was not statistically significant. Furthermore, the concentration of Zn was higher in the non-rhizosphere soils, while there was no significant difference when compared to the rhizosphere soils. The level of total cation exchange, exchangeable acidity and acid saturation were higher in the rhizosphere, while the pH level was higher in the non-rhizosphere soils. Nevertheless, the differences were not significant (Table 1). Conversely, the organic C content was higher in the

rhizosphere soils compared to the non-rhizosphere soils, but again, the differences were not significant (Table 1).

Table 1: Soil properties from *E. lanatus* under rhizosphere and non-rhizosphere soils. The outcomes are shown as mean ± SE, n=10

Soil properties		Rhizosphere	Non-rhizosphere
Macro-nutrients ($mg.kg^{-1}$)	Primary nutrients		
	Nitrogen	156.68±27.80[a]	124.88±20.58 [a]
	Phosphorus	13.81±2.94 [a]	8.92±2.29 [a]
	Potassium	1.13±0.01 [a]	1.12± 0.01[a]
	Secondary nutrients		
	Calcium	0.66±0.14 [a]	0.51±0.11 [a]
	Magnesium	1.29±0.12[a]	1.08±0.02[b]
Micro-nutrients ($mg.kg^{-1}$)	Manganese	16.96±5.19[a]	10.58±1.94[b]
	Copper	1.15±0.23 [a]	0.89±0.19 [a]
	Zinc	0.86±0.13 [a]	0.89±0.18 [a]
Soil relative acidity	pH (KCL)	3.84±0.03 [a]	3.92±0.05 [a]
	Acid saturation (%)	65.10±4.68 [a]	65.00±6.86 [a]
	Total cation exchange (cmol/kg)	3.09±0.31 [a]	2.42±0.22 [a]
	Exchangeable acidity (cmol/kg)	1.98±0.24 [a]	1.62±0.27 [a]
Other properties	Organic Carbon (%)	3.52±0.33 [a]	3.21±0.28 [a]

Column means with different letters indicate significant differences in soil chemical properties (two-sample t-test, $P \leq 0.05$)

4.3.2 Identification of E. lanatus microbes from coralloid roots, rhizosphere and non-rhizosphere soils

4.3.2.2 Molecular identification of the isolated bacteria in coralloid roots

Five microbial strains were extracted from the coralloid roots of *E. lanatus* (Table 2). The bacteria responsible for solubilizing P were *Actinoallomurus sp.* and *Pseudomonas putida*. The microbes responsible for N cycling were identified as *Paraburkholderia sp.* and *Burkholderia sp*, whereas one strain of *Bacillus licheniformis* was identified as an N-fixing bacteria. Regarding the microbial activities, it was observed that 20% of microbes were engaged in N_2 fixation, 40% were involved in P solubilization, and the other 40% were associated with N cycling.

Table 2: Bacteria extracted from *E. lanatus* coralloid roots

Family	Scientific name	Accession number	Similarity (%)	Function(s)	Reference(s)
Pseudomonadaceae	*Pseudomonas putida*	OK037572.1	99.21	P-solubilizing	Costa-Gutierrez *et al.* 2022
Thermomonosporaceae	*Actinoallomurus sp.*	KX533973.1	99.15		
Burkholderiaceae	*Burkholderia sp.*	MK559018.1	99.82	N-fixing	Ndlovu *et al.* 2013
	Paraburkholderia sp.	MG182888.1	99.40	P-solubilizing	Wilhelm *et al.* 2020
Bacillaceae	*Bacillus licheniformis*	MK418575.1	84.08	P-solubilizing	Won *et al.* 2019; Rojas *et al.* 2001
				N-fixing	Won *et al.* 2019

4.3.2.3 Molecular identification of the isolated bacteria in rhizosphere soils

A total of sixteen microbial strains were extracted from the rhizosphere soils of *E. lanatus*, as indicated by Table 3. The bacteria responsible for solubilizing P were *Enterobacter cloacae, Burkholderia cenocepacia, Massilia phosphatilytica, Paraburkholderia sp., Burkholderia cepacian, Massilia sp., Paraburkholderia sabiae* and *Paraburkholderia fungorum*. The bacteria involved in N cycling were identified as *Caballeronia concitans* and *Pantoea agglomerans*. The microorganisms responsible for N_2 fixation were identified as *Mucilaginibacter gossypiicola, Rhizobium tropici, Rhizobium miluonense, Agrobacterium rhizogenes* and *Paraburkholderia lacunae*. Within the rhizosphere, a significant portion of 37.5% of the microbial community showed a role in N_2 fixation, whereas 50% of the microbes were engaged in P solubilization. Additionally, a smaller portion of 12.5% of the bacterial population was shown to be associated with N cycle processes.

Table 3: Bacteria extracted from *E. lanatus* rhizosphere soils

Family	Scientific name	Accession number	Similarity (%)	Function(s)	Reference(s)
Rhizobiaceae	*Rhizobium miluonense*	MH236279.1	99.90	N-fixing	Rouhrazi *et al.* 2016
	Rhizobium tropici	MT539147.1	99.12	N-fixing	Rouhrazi *et al.* 2016
	Rhizobium miluonense	MT409530.1	97.43	N-fixing	Rouhrazi *et al.* 2016
	Agrobacterium rhizogenes	MN712240.1	96.28	Induce hairy root system	Sawada *et al*., 1993
Sphingobacteriaceae	*Mucilaginibacter gossypiicola*	NR_116406.1	94.48	Growth Promoter	Vasconcelos *et al.* 2022
Oxalobacteriaceae	*Massilia phosphatilytica*	MK519185.1	98.61	P-solubilizing	Li *et al.* 2019
	Massilia sp.	MH031738.1	96.46	P-solubilizing	Wan *et al.* 2020

Enterobacteriaceae	*Pantoea agglomerans*	MN758864.1	96.79	P-solubilizing	Lorenzi *et al.* 2022; Saadouli *et al.* 2021
				N-fixing	Lorenzi *et al.* 2022
	Enterobacter cloacae	KY660471.1	94.88	N-fixing	Ji *et al.* 2020; Zhang *et al.* 2022
				P-solubilizing	Ji *et al*. 2020
Burkholderiaceae	*Burkholderia cenocepacia*	OR098458.1	97.80	P-solubilizing	You *et al.* 2020
	Caballeronia concitans	NR_145603.1	97.51	N-fixing	Maquia *et al.* 2020
	Paraburkholderia sp.	MZ031499.1	95.25	N-fixing P-solubilizing	Wilhelm *et al.* 2020

Burkholderia cepacia	MN691351.1	97.92	N-fixing	Menard *et al.* 2007
			P-solubilizing	Wan *et al.* 2020
Paraburkholderia sabiae	MK139731.1	98.12	N-fixing	Hug *et al.* 2023; Ndlovu *et al.* 2023
Paraburkholderia fungorum	CP099647.1	99.34	N-fixing P-solubilizing	Raihan *et al.* 2022
Paraburkholderia lacunae	NR_165707.1	99.40	N-fixing	Beukes *et al.* 2021

4.3.2.4 Molecular identification of the bacteria isolated in non-rhizosphere soils

A total of fourteen microbial strains were extracted from the non-rhizosphere soils of *E. lanatus,* as shown in Table 4. The bacteria responsible for solubilizing P were identified as *Rhizobium tropici, Bacillus licheniformis, Trinickia* sp., *Paraburkholderia* sp. and *Bacillus velezensis.* The bacteria involved in N cycling were identified as *Paraburkholderia sp., Paraburkholderia bengalensis, Burkholderia* sp. and *Paraburkholderia atlantica*. The microorganisms responsible for N_2 fixation were identified as *Paenibacillus sp., Paraburkholderia caledonica, Paraburkholderia sp., Rhizobium sp., Rhizobium tropici, Rhizobium nepotum* and *Terriglobus roseus* (Table 4). The proportion of P-solubilizing bacteria in the non-rhizosphere soils was found to be 42.9%. The overall number of N-fixing bacteria accounted for 42.9%, while those involved in N cycling accounted for 28.6%. Based on the Simpson's Diversity Index, it was observed that rhizosphere soils displayed greater (D=0.98) species diversity in comparison to non-rhizosphere soils (D=0.37).

Table 4: Bacteria extracted from *E. lanatus* non-rhizosphere soils

Family	Scientific name	Accession number	Similarity (%)	Function(s)	Reference(s)
Rhizobiaceae	*Rhizobium tropici*	MT539147.1	99.65	N-fixing	Rouhrazi *et al.* 2016
	Rhizobium nepotum	MT533807.1	99.14	N-fixing	Ghorpade and Gupta 2018
	Rhizobium sp.	MT269297.1	98.95	N-fixing	Ndlovu *et al.* 2013
Acidobacteriaceae	*Terriglobus sp.*	KX555420.1	85.61		
Bacillaceae	*Bacillus licheniformis*	ON954648.1	99.67	P-solubilizing	Won *et al.* 2019; Rojas *et al.* 2001
				N-fixing	Won *et al.* 2019
	Bacillus velezensis	MH040972.1	98.15	P-solubilizing	Shi *et al.* 2022
				N-fixing	Shi *et al.* 2022
Paenibacillaceae	*Paenibacillus sp.*	KY229692.1	94.09	P-solubilizing	Khan *et al.* 2020
				N-fixing	
Pseudomonadaceae	*Pseudomonas koreensis*	MT409540.1	96.81	P-solubilizing	Gu *et al.* 2020

Burkholderiaceae	*Paraburkholderia sp.*	MT269305.1	99.66	P-solubilizing	Wilhelm *et al.* 2020
	Paraburkholderia sp.	OM144561.1	97.67	P-solubilizing	Wilhelm *et al.* 2020
	Paraburkholderia bengalensis	MT912698.2	97.51	N-fixing	Nag *et al.* 2022
	Trinickia sp.	LC661717.1	99.83	P-solubilizing	Damo *et al.* 2022
	Paraburkholderia atlantica	MK690530.1	96.31	N-fixing	Paulitsch *et al.* 2020
	Burkholderia sp	MK704288.1	97.39	N-fixing	Mattos *et al.* 2005

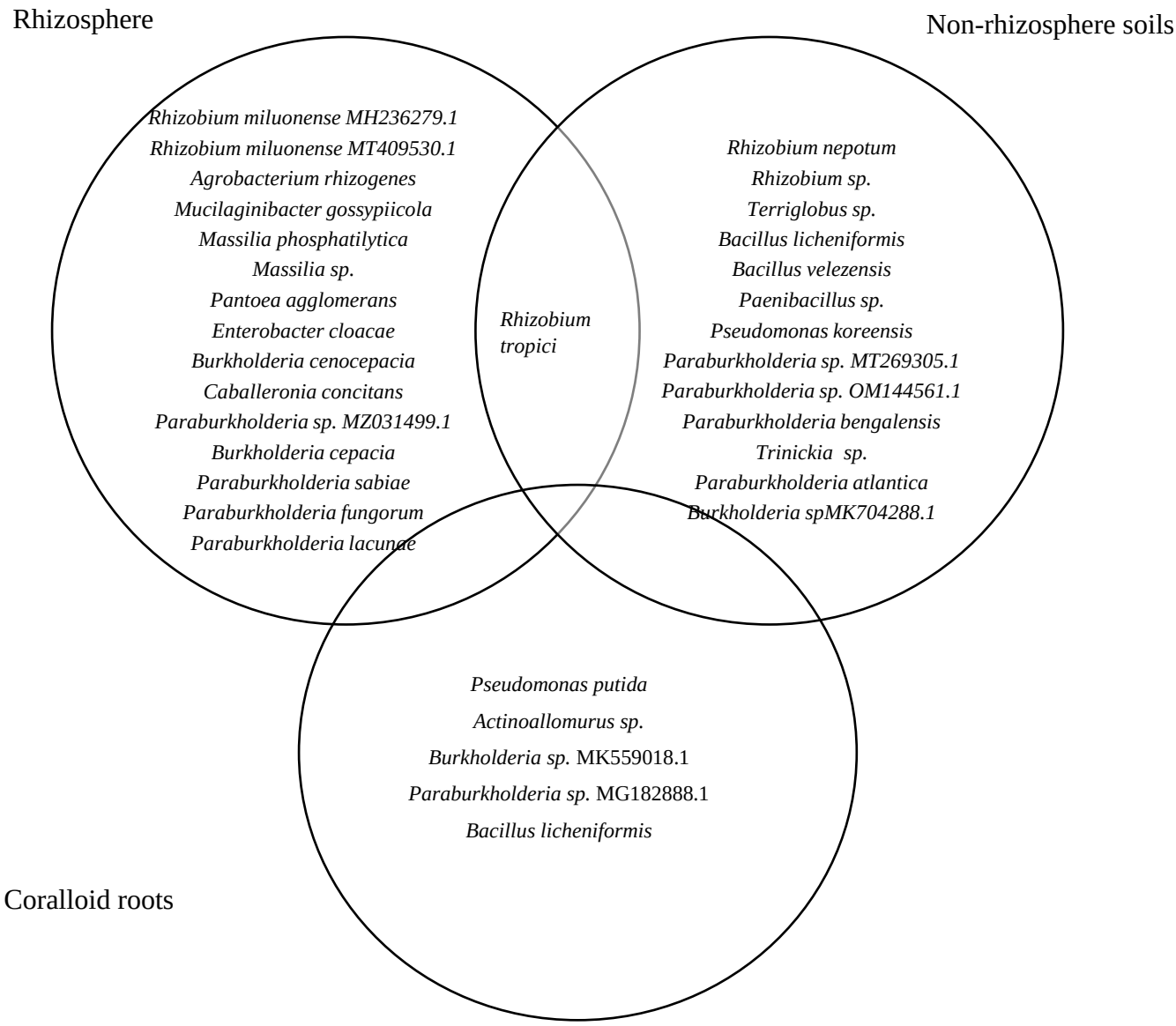

Figure 1: Venn diagram depicting the similar and different bacteria found in the coralloid roots, rhizosphere and non-rhizosphere soils of *Encephalartos lanatus.*

4.3.3 Soil extracellular enzyme activities

The enzyme activities of acid phosphatase, alkaline phosphatase, nitrate reductase and β-(D)-glucosaminidase were found to be the highest in the rhizosphere soils, whereas the non-rhizosphere soils exhibited the lowest enzyme activities (Table 5). In contrast, the activity of β-glucosidase was the highest in non-rhizosphere soils, as shown in Table 5. The activities of acid phosphatase, β-glucosidase and nitrate reductase enzymes exhibited significant differences in both the rhizosphere and non-rhizosphere soils. However, there was no

significant difference was observed in alkaline phosphatase and β-(D)-glucosaminidase activities (Table 5) in both soil samples.

Table 5: Soil enzyme activities of *E. lanatus* in the rhizosphere and non-rhizosphere soils. The outcomes are shown as mean ± SE, n=10

Enzyme Activity	Rhizosphere	Non-rhizosphere
Acid Phosphatase ($nmolh^{-1}g^{-1}$)	0.49±0.03[a]	0.40±0.04 [b]
Alkaline phosphatase ($nmolh^{-1}g^{-1}$)	0.52±0.03 [a]	0.46±0.24 [a]
Nitrate reductase ($\mu molh^{-1}g^{-1}$)	3.61±0.08 [a]	3.32±0.20 [b]
N-acetyl-β-D-glucosaminidase ($nmolh^{-1}g^{-1}$)	0.44±0.03 [a]	0.43±0.03 [a]
β-glucosidase ($nmolh^{-1}g^{-1}$)	0.49±0.03 [a]	0.51±0.03 [b]

Column means with different letters indicate significant differences (two-sample t-test, $P \leq 0.05$)

4.3.4 Relationships between soil characteristics of E. lanatus in both the rhizosphere and non-rhizosphere soils

The two principal component analyses (PCA) accounted for 62.8% of the variation in soil characteristics and their corresponding enzymes. PC1 accounted for 45.2% of the overall variation, while PC2 accounted for 17.6% of the total variation. The clusters revealed that the soil characteristics and enzymes were comparable in both the rhizosphere and the non-rhizosphere soils. There was a negative correlation observed between the concentration of P in the soil and the activity of both acid and alkaline phosphatases. The enzymes nitrate reductase and *N*-acetyl-β-D-glucosaminidase also revealed a negative relationship with N concentration. The nitrate reductase exhibited a positive correlation with soil pH. Acid phosphatase was positively correlated with β-glucosidase. The levels of N and P were positively correlated to organic C and total cation exchange.

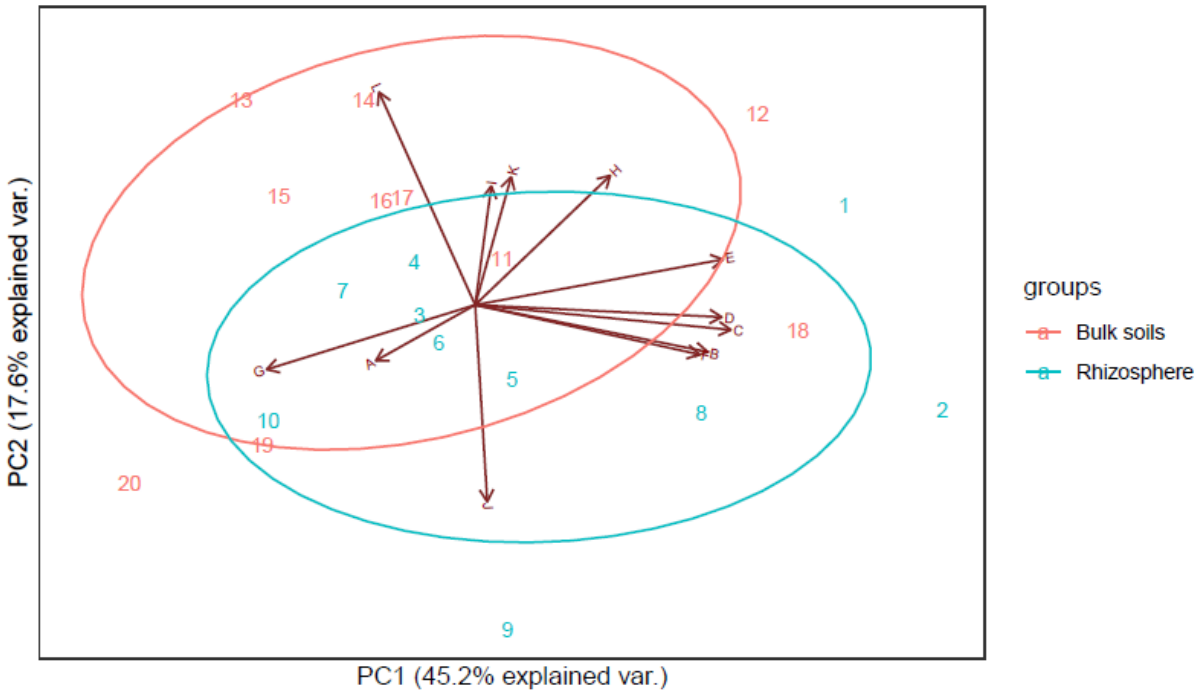

Figure 2: Relationship between the soil traits and extracellular enzyme activities of *E. lanatus* rhizosphere and non-rhizosphere soils. The following is a representation of soil attributes: A = nitrate reductase, B = nitrogen concentration, C = phosphorus concentration, D = organic carbon, E = exchangeable acidity, F = total cation exchange, G = pH, H = acid saturation, I = acid phosphatase, J = alkaline phosphatase, K = β-glucosidase, L = N-acetyl-β-D-glucosaminidase. Principal component analysis (PCA).

4.4 Discussion

The presence of nutrients in the soil can be found in varying amounts and has a crucial role in the development of plants. However, several nutrient elements have relatively low solubility, making them inaccessible to plants (Rafique *et al*., 2022). Hence, it is important to investigate the biological, physical and chemical properties of the soil to understand if soil can preserve and restore ecological processes (Mir *et al*., 2023).

The present study focused on determining the characteristics of the soil samples, as well as isolating bacteria from coralloid roots and soils and subsequently evaluating their enzyme activities. The results showed that the rhizosphere and the non-rhizosphere had significant similarities in terms of the concentration of N, P, K, Ca, Cu, Zn, pH, acid saturation, total cation exchange, exchangeable acidity, and organic C. These results are in contradiction to previous studies in *E. natalensis* (Ndlovu *et al.,* 2023) and *C. micronesica* (Marler and Krishnapillai, 2018), where the concentrations of macro and micronutrients were greater in the rhizosphere

than in the surrounding soils. Therefore, it is reasonable to propose that this phenomenon could be associated with the disruption caused by fire, given the historical susceptibility of Botshabelo Cultural Village to fire incidents. Botshabelo is a natural reserve that is subject to fires that burn regularly, and these fires are utilized as a management tool to conserve the ecosystem. (Bayne *et al*., 2019). The soils and coralloid roots for this study were collected immediately following the fire in the area, as the grass remains were still visible on the ground and the *E. lanatus* trunks were scorched.

Other variables, like pH, can also account for the observed differences. The pH observed in the soils from the current study is extremely acidic, while the soils studied by Ndlovu *et al*. (2023) were slightly acidic, which may have created an environment that was better suited for *E. natalensis* plant growth. In the research work by Ndlovu *et al*. (2023), the soil pH was 5.75 in the rhizosphere and 5.14 in the non-rhizosphere soil. In the current study, the soil pH was 3.84 and 3.92 in the rhizosphere and non-rhizosphere soils.

This study also presents results indicating a higher concentration of Mn in the rhizosphere soils of *E. lanatus* compared to the studies conducted by Maler and Calonje (2020) on *C. micronesica* and Ndlovu *et al*. (2023) on *E. natalensis*. Huang *et al*. (2016) conducted a study on sugarcane plantlets, testing the effect of Mn on strongly acidic soils. Their results provided evidence supporting the assertion that the accumulation of Mn in the soil is attributed to the presence of acidic soils with a pH of 4.48 (Huang *et al*., 2016). Based on the findings of this research, it is plausible to suggest that the enhanced presence of Mn in the rhizosphere may have been influenced by fire events, leading to an excessive buildup of this element due to the combined effects of temperature and soil acidity. Moreover, this result provides evidence supporting the notion that pH and Mn have a substantial impact on hindering the uptake of other nutrients (P and Ca) in the soil by plants (Alejandro *et al*., 2020).

The concentration of Mg was discovered to be significantly higher in the rhizosphere as compared to the non-rhizosphere soils. One possible explanation for the sustained elevation in Mg levels is the lack of impact from the blaze. According to Agbeshie *et al*. (2022), previous studies have reported that certain elements are prone to wildfire, resulting in a reduction in Mg and P levels due to the processes of volatilization and oxidation. The study by Agbeshie *et al*. (2022) reported that fire either enhances or has no effect on the concentration of P, K, Ca and Mg, while typically decreasing the concentration of S and N. However, the study yielded distinct results. The study revealed a high concentration of N in the rhizosphere compared to

the non-rhizosphere soils, which lacks statistical significance. In a similar vein, Chungu *et al*. (2019) also reported a high concentration of total N in burned sites, which accelerated the growth of *Eucalyptus grandis* plants in comparison to unburned sites.

Nevertheless, the bioavailability of nutrients in the soil can be enhanced by the presence of microbes. Microbes play a crucial role in preserving soil health and facilitating the cycling of nutrients (Khoshru *et al*., 2023). The capacity of soil-dwelling microbes and their associated extracellular enzyme activities to transform minerals from an insoluble state to a soluble form, facilitating plant uptake, has been well-documented (Rafique *et al*., 2022). Several recent studies have documented the prevalence of *Acidobacteria* and *Alphaproteobacteria* microbes in acidic soils (Msimbira and Smith, 2020). These microbes have been found to play a significant role in aiding plants to withstand various environmental challenges, notably drought and inadequate nutrient levels (Msimbira and Smith, 2020).

However, in this study, a total of ten microbial families, consisting of fifteen genera, were successfully isolated from the coralloid roots, rhizosphere and non-rhizosphere soils. The genera referred to in this context are *Bacillus*, *Mucilaginibacter, Rhizobium, Agrobacterium, Paraburkholderia, Paenibacillus, Terriglobus, Actinoallomurus, Pseudomonas, Enterobacter, Burkholderia, Massilia, Trinickia, Caballeronia* and *Pantoea*. All the different microorganisms present in the soil samples and coralloid roots of *E. lanatus* are significant in terms of safeguarding and promoting its growth and development. The Burkholderiaceae and Rhizobiaceae bacteria families were found to be the ones with the greatest abundance in both soil samples.

Ichikawa *et al*. (2023) and Madhaiyan *et al*. (2021) have demonstrated that *Paraburkholderia* exhibits a significant influence on enhancing plant growth by engaging in N_2 fixation and the solubilization of insoluble P. The genera *Paenibacillus, Rhizobium, Enterobacter,* and *Pseudomonas* are well known for their ability to produce Indole-3-acetic acid and siderophore, not to mention their capacity for P solubilization and N_2 fixation (Motsomane *et al*., 2023b). Previous studies have established a correlation between these genera and *Dioon* cycad (Gutiérrez-García *et al*., 2018). *Massilia* shares certain characteristics with *Paenibacillus, Rhizobium, Enterobacter* and *Pseudomonas,* with the exception of its inability to fix N (Ofek *et al*., 2012). Additionally, it has been observed that the stimulation of plant development can be attributed to the formation of auxins and gibberellins, as noted by Ran *et al*. (2023). According to Damo *et al*. (2022), it has been confirmed that the bacteria belonging to the genera

Trinickia, Burkholderia, Paraburkholderia, Pseudomonas, Enterobacter and *Pantoea* possess the ability to solubilize P and render it accessible for absorption by plants.

Grady *et al*. (2016) reported that *Paenibacillus* exhibits the ability to endure fluctuations in soil pH while also regulating phytopathogens through the induction of systematic resistance (ISR). *Bacillus* and *Agrobacterium* have been identified as significant contributors to the process of P solubilization, as noted by Li *et al*. (2021). Shah *et al*. (2021) identified *Bacillus, Enterobacter, Burkholderia, Agrobacterium, Paenibacillus, Rhizobium* and *Pseudomonas* as microorganisms that possess the ability to solubilize P. The solubilization process transforms insoluble P into a soluble form within the soil, making it accessible for plant uptake and utilization. As stated by Shah *et al*. (2021), *Pseudomonas, Bacillus, Enterobacter, Burkholderia* and *Rhizobium* have been identified as producers of indole-3-acetic acid (IAA), while *Pseudomonas* and *Burkholderia* generate cytokinins. Additionally, Mumtaz *et al*. (2017) found that these bacteria are capable of solubilizing Zn. *Bacillus* is a ubiquitous bacteria that can thrive in a variety of environments (Kashyap *et al*., 2019). Noteworthy attributes of *Bacillus* include fixing atmospheric N and solubilising various minerals such as K, P and Zn (Ahmad *et al*., 2017). Additionally, it is involved in the generation of phytohormones and siderophores (Ahmad *et al*., 2017; Mumtaz *et al*., 2017; Saxena *et al*., 2020).

According to Vasconcelos *et al*. (2022), *Mucilaginibacter* bacteria produce extracellular polymeric substances (EPS), which play a vital role in the precipitation of metals and the enhancement of plant growth under polluted and unfavourable environmental conditions. *Actinoallomurus* is known to generate isoflavonoids, Type I polyketide synthase and non-ribosomal peptide synthetase genes (Pozzi *et al*., 2011). Isoflavonoids are derived from flavonoids, which are secondary metabolites and have been found to possess diverse biological activities and play significant roles in plant physiology. For instance, it regulates cellular proliferation, offers defence against both biotic and abiotic stressors also attracts insects for plant pollination (Dias *et al*., 2021). *Agrobacterium* is one of the soil-detrimental bacteria that induce gall development by entering plant tissues and attaching itself to the cell wall, thus producing excessive amounts of auxin and cytokinin due to the transferred DNA (T-DNA) (Finer *et al*., 2016). On the other hand, *Agrobacterium rhizogenes* is known to induce hairy roots by passing on a portion of their plasmid to a plant host, which able the plasmid to alter the plant's genome expressing genes that help the plant survive in adverse conditions (Sawada *et al*., 1993; Satuti *et al*., 2000). *Terriglobus* are chemo-organoheterotrophic (Pascual *et al*., 2015).

As noted by Ekenler and Tabatabai (2004), soil microbes are responsible for the secretion of extracellular enzymes. These enzymes have been recognised as significant mediators and facilitators in numerous biochemical processes (Błońska *et al*., 2017; Ndabankulu *et al*., 2022; Santos *et al*., 2022). The significance of these processes lies in their role in preserving soil quality and facilitating the effective operation of ecosystems, as evidenced by the findings of Błońska *et al*. (2017), Ndabankulu *et al*. (2022) and Santos *et al*. (2022). The processes encompass the cycling of vital elements, including P, N, and C, as outlined by Ndlovu *et al*. (2023). Also, the process of nutrient mineralization is linked to the decomposition and renewal of soil organic matter, alongside the breakdown of xenobiotics (Błońska *et al*., 2017; Sudhakaran and Ravanachandar, 2020). The enzymes acid and alkaline phosphatase play a crucial role in the process of P mineralization and cycling within soil ecosystems (Ndlovu *et al*., 2023). In addition, the production of P is enhanced by the enzymatic action of phosphatase, which catalyses the hydrolysis of phosphoric acid monoester, resulting in the formation of phosphate ions (Ndlovu *et al*., 2023).

Acid and alkaline phosphatase are essential for the process of P cycling (Ndlovu *et al*., 2023). The study revealed that there is a significantly higher concentration of acid phosphatase in the rhizosphere compared to the surrounding non-rhizosphere soils. Furthermore, the alkaline phosphatase levels were higher in the rhizosphere in comparison to the non-rhizosphere soils. Nevertheless, the results were not significantly different.

The enzyme *N*-acetyl-β-D-glucosaminidase is known to have a significant impact on both the N cycle (Acosta-Martínez *et al*., 2019; Ullah *et al*., 2019) and the C cycle (Acosta-Martínez *et al*., 2019; Ndlovu *et al*., 2023). Furthermore, it has been noted that *N*-acetyl-β-D-glucosaminidase is responsible for facilitating the transformation of asparagine into aspartic acid and NH_3 alongside its role in the hydrolysis of chito-oligosaccharides (Hill *et al*., 1967; Mega *et al*., 1972; Parham and Deng, 2000) along with the hydrolysis of chitin, resulting in the release of amino sugars. According to Acosta-Martínez *et al*. (2019), amino sugars serve as significant contributors to the mineralization of N in the soil. The level of *N*-acetyl-β-D-glucosaminidase enzyme was higher in the rhizosphere than in the non-rhizosphere soils, but the difference was not statistically significant.

β-glucosidases are a group of enzymes that play an essential role in C cycling by facilitating the breakdown of low-molecular-weight carbohydrates (Nannipieri *et al*., 2011; Ullah *et al*., 2019). The enzymatic function of glucosidase provides an important understanding of the

mechanism involved in the breakdown of cellulose, which is commonly acknowledged as the primary polysaccharide found in natural environments (Acosta-Martínez *et al*., 2019). β-glucosidase does this by liberating saccharides glycosides and catalysing the breakdown of cellulose, cellotetraose into cellobiose, which can further undergo further conversion into glucose (Zungu *et al*., 2020). The concentration of β-glucosidase was higher in the non-rhizosphere soils compared to the rhizosphere soils. Nitrate reductase is an enzyme essential for the fixation of nitrate (Krywult and Bielec, 2013). The results showed that the activity of nitrate reductase was significantly higher in the rhizosphere as compared to the non-rhizosphere, and Ndlovu *et al.* (2023) reported similar results.

The PCA analysis showed a negative association between enzymes and their corresponding nutrients. For example, the acid and alkaline phosphatase showed a negative correlation with the concentration of P. *N*-acetyl-β-D-glucosaminidase and nitrate reductase had a negative correlation with N. Nevertheless, the elevated levels of acid phosphate, nitrate reductase and β-glucosidase enzymes indicate that the cycling of P, N and C was occurring in the Botshabelo Nature Reserve, which has nutrient-deficient soils, facilitating the absorption of nutrients by the *E. lanatus* and neighbouring plants, hence promoting their growth. The negative association could be attributed to the acidic pH levels in the soils. According to Wang *et al*. (2012), Magadlela *et al*. (2020), Zungu *et al*. (2020), Ndabankulu *et al*. (2022) and Berza *et al*. (2022) acidic soils contain a high level of cations such as iron and aluminium establishing a bind with P, rendering it inaccessible for plant absorption. This elucidates the relationship between soil P and total cations in the principal component analysis (PCA). The findings of the present study, conducted in extremely acidic soils, indicate that the activity of acid phosphatase was the highest in the rhizosphere.

In addition, the levels of alkaline phosphatase were high in the rhizosphere compared to the non-rhizosphere soils but were non-significant. Thus, the study of Turner (2010) provides evidence for this conclusion that acid phosphatase enzyme is prevalent in soil with a pH below 4 since they reported a high level of acid phosphatase in acidic soils. The acidity of the soil has an important impact on the amount of organic C it contains. This is because soil acidity controls the availability of nutrients, the breakdown of organic matter and other soil processes (Wibowo and Kasno, 2021). Nevertheless, organic C, N and P were revealed to be negatively correlated with pH. This indicates that a lower soil pH promotes the buildup of organic matter and the encouragement of microbial activity in decomposing the organic materials (Wibowo and Kasno, 2021).

4.5 Conclusions

Botshabelo, situated in the Mpumalanga Province (Middelburg), is a cultural site that is susceptible to both positive and negative impacts on the soil's properties caused by fire. The fire has modified the natural dynamics of cycad-microorganism interactions, where the rhizosphere soils are richer in microorganisms, enzyme activities and accessibility of nutrients to plants. However, cycads and associated microbes preserve the ecosystem by facilitating the cycling of nutrients. Therefore, their protection requires exceptional consideration due to their significant contribution to ecosystem services in environments with inadequate nutrient levels for sustaining plant development.

Author contributions

M.S. and T.N.S collected samples from the field. M.S. carried out all the research experiments, analyzed the results and wrote the paper. T.N.S and A.M. funded the study. W. M, T.N.S. and A.M. proofread the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable

Data Availability Statement

Data and materials are accessible upon request.

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Conflict of Interest

The authors declare that they have no competing interests.

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Chapter 5

General Conclusions and Recommendations

5.1 General conclusions

Cycads represent a lineage of gymnosperm plants that have existed for an extensive period. Given their evolutionary history, they have evolved to be able to survive and even benefit from fire, which has both positive and negative effects on their population and reproductive structure. Cycads play an essential role in ecosystem services (Ndlovu *et al*., 2023; Motsomane *et al*., 2024) and are recognized as one of the most endangered groups of plants that have managed to survive extinction (Donaldson, 2011; Álvarez-Yepíz *et al*., 2019). They are found in environments such as dunes, rocky outcrops, and areas prone to recurrent fires, which are not conducive to plant growth (Álvarez-Yepíz *et al.*, 2014). Their ability to thrive in these ecosystems is thought to be significantly associated with their ability to form symbiotic relationships with BNF cyanobacteria (Zheng *et al.*, 2018; Zheng and Gong, 2019).

The results of the study showed that several factors may hinder the development or growth of *E. lanatus* plants. These factors include damage of plant reproductive and vegetative structures by baboon, irregular fires that burn the plants and the and nutrient-deficient soils. Nevertheless, *E. lanatus* has evolved mechanisms that allow the plant to persist in challenging conditions by harbouring microbes for survival. The presence of these microbes, together with their extracellular enzymes, renders insoluble nutrients accessible for *E. lanatus* utilization. Conversely, fire facilitates the regeneration and cone production of *E. lanatus*.

5.2 Recommendations for future work

Having a greater understanding of cycad reproduction is of paramount importance for conservation groups (Calonje *et al*., 2011). This knowledge may aid in developing strategies to enhance the reproductive success of cycads by providing them with the necessary resources and optimal conditions. In the future, it will be important to carry out a study on pollination syndrome and the impact of fire on pollinators in order to gain significant knowledge regarding the reproduction of cycads and population dynamics. Additionally, it will also be advisable to assess the optimal nutrient levels for cycad growth. This investigation may reveal that what we currently perceive as low or high nutrient concentrations may be suitable for cycad growth. Consequently, the plants may not rely on the microbes present in the soil or coralloid roots but rather utilize the nutrients already available in the soil to enhance their growth.

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