Encephalartos ghellinckii -microbe symbiosis, soil nutrient inputs and enzyme activities in KwaZulu-Natal and Eastern Cape grasslands

Mongwadi Tshwane
201826143
orcid.org/ 0000-00-6622-097602

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Supervisor: Dr. T.N Suinyuy

Co-supervisor: Prof A. Magadlela (Sol Plaatje University)

School of Biology and Environmental Sciences

Faculty of Agriculture and Natural Sciences

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UNIVERSITY OF MPUMALANGA

DECLARATION

I, Mongwadi Tshwane, hereby declare that this dissertation is my original work and has not been submitted to any other institution for a degree or any other qualification. All sources of information used in this research have been duly acknowledged.

Student: Mongwadi Tshwane (201826143)

Signature: ……. Date: 19/04/2026

Main Supervisor: Dr. T.N. Suinyuy

Signature: …… ……..... Date: 21/04/2026

Co-Supervisor: Prof. A. Magadlela

Signature: …… Date: 22/04/2025

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LIST OF ABBREVIATIONS

AGPs	- Arabinogalactan-proteins
Al	- Aluminum
AMF	- Arbuscular mycorrhizal fungi
ATP	- Adenosine triphosphate
ASL	-Above sea level
BLAST	- Basic Local Alignment Search Tool
BNF	- Biological nitrogen fixation
C	- Carbon
Ca	- Calcium
CITES	-Convention on International Trade in Endangered Species of Wild Fauna and Flora
ES	-Ecosystem services
Fe	-Iron
IAA	-Indole-3-acetic acid
IUCN	-The International Union for Conservation of Nature
K	-Potassium
KZN	- KwaZulu-Natal
MYA	- Million years ago
N	-Nitrogen
NBSAP	- National Biodiversity Strategy and Action Plan
NCBI	-National Center for Biotechnology Information
NDFA	-Nitrogen derived from atmosphere
NDFS	-Nitrogen derived from soil
NFB	-Nitrogen-fixing bacteria
NH_3	-Ammonia
P	-Phosphorus
PCR	-Polymerase chain reaction
TCP	-Tricalcium phosphate

ABSTRACT

Cycads are an ancient group of gymnosperms, with over 300 species classified into three families and 10 genera. Originating around 300 million years ago, they are now primarily found in tropical and subtropical regions, where they play crucial ecological roles in carbon sequestration, nutrient cycling, and soil stabilization. A key adaptation of cycads is their symbiotic relationship with nitrogen (N)-fixing bacteria in their coralloid roots, which convert atmospheric N into forms such as ammonia that the plants can assimilate for growth. This trait, which predates its occurrence in legumes and enables cycads to thrive in nutrient-deficient soils, such as those in grassland ecosystems. However, most studies on the role of plant symbiotic relationship with N-fixing bacteria have focused on angiosperms, with little knowledge on their relationship with cycads. Also, over 70% of the cycad species are threatened with extinction, and their loss would have significant ecological consequences due to their vital ecosystem services. This study aims to investigate the symbiotic interactions between *Encephalartos ghellinckii* and N-fixing bacteria, the role of soil microbial communities and enzyme activities, in regulating soil nutrient availability and influencing the plant's N source reliance. By examining these factors, this research seeks to understand the contribution of the microbes to the survival of *E. ghellinckii* in nutrient-deficient, acidic grasslands. To achieve this, microbial diversity in the coralloid roots, rhizosphere, and non-rhizosphere soils of *E. ghellinckii* was assessed across four sites (Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano). The study also examined extracellular enzyme activities associated with these microbes and their contribution to soil nutrient availability. Additionally, soil nutritional status, including pH, nutrient concentrations, total cations, and exchange acidity, was analyzed to determine its influence on cycad-microbe symbiosis. The N source preference of *E. ghellinckii* was calculated from atmospheric derived N and plant N concentration. Bacterial strains identified in *E. ghellinckii* coralloid roots, rhizosphere, and non-rhizosphere soils included *Bacillus, Rhizobium*, *Paraburkholderia*, *Enterobacter*, *Caballeronia*, *Klebsiella*, *Pseudomonas*, *Inquilinus*, and *Arthrobacter*. These microbes contributed to N fixation, N cycling, and phosphate solubilization. No significant differences were observed in phosphorus-cycling (acid and alkaline phosphatase), nitrogen-cycling (nitrate reductase), and carbon-cycling (β-glucosidase) enzyme activities between rhizosphere and non-rhizosphere soils. Increased enzyme activity was associated with increased nutrient availability and responses to nutrient limitations, highlighting the complex interactions governing soil nutrient dynamics. Isotope

δ^{15}N analysis revealed that *E. ghellinckii* derived more than 80% of its N from atmospheric fixation, while less than 20% was soil-derived. A positive correlation was observed between soil phosphorus (P) concentrations and the proportion of N fixed from the atmosphere across all sites. These findings highlight the role of microbial symbiosis and associated enzyme activities in enhancing nutrient availability in acidic, nutrient-poor grassland soils. Furthermore, this association facilitates *E. ghellinckii*'s ability to access and utilize atmospheric N, supporting its persistence in nutrient deficient ecosystems.

Keywords: *Encephalartos ghellinckii*, cycads, coralloid roots, nitrogen fixation, soil enzymes, grassland ecosystems.

GENERAL OVERVIEW OF THESIS CHAPTERS

This dissertation consists of five chapters arranged as follows:

Chapter 1 introduces the conservation status of cycads, the ecological and evolutionary significance of *Encephalartos ghellinckii*, and the ecosystem services rendered by cycads. It also presents the problem statement, research questions, aims, and objectives of the study, as well as the justification and relevance of investigating microbial associations and nutrient dynamics in cycad coralloid roots and surrounding soils.

Chapter 2 provides a literature review covering the evolutionary history of cycads, cycad-microbe symbiosis, contrasts with legume-microbe symbiosis, cycad distribution and uses, and the contributions of soil microbes and extracellular enzymes to nutrient availability in grassland ecosystems.

Chapter 3 focuses on the microbial composition of *E. ghellinckii* coralloid roots, rhizosphere, and non-rhizosphere soils across four study sites. This chapter also examines soil nutrient status and extracellular enzyme activities, highlighting the relationships between microbial diversity, soil nutrients, and plant survival in nutrient-depleted soils.

Chapter 4 investigates the physiological adaptations of *E. ghellinckii* to nutrient-poor soils, with a focus on nitrogen acquisition. It examines the species' reliance on atmospheric nitrogen through biological nitrogen fixation, correlations between NDFA in plant leaves and soil nutrient concentrations, and the role of plant-microbe interactions in supporting growth and survival.

Chapter 5 presents the general conclusions of the study, synthesizing key findings from the data chapters, and provides recommendations for future research and conservation strategies for *E. ghellinckii* and other cycads in grassland ecosystems.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Cycadales, commonly known as cycads, are a prehistoric lineage of gymnosperms that originated in the Permian era (Mamay, 1969; Norstog and Nicholls, 1997; Whitelock, 2002). They comprise over 300 species across three families and 10 genera (Donaldson *et al*., 2003). They are the oldest extant seed plants, with the earliest cycad fossils dating back to roughly 300 million years ago (MYA) (Mamay, 1969; Zhifeng and Thomas, 1989; Donaldson, 2003). They were found worldwide during the Mesozoic, but the present-day cycads are thought to have originated roughly 12 million years ago are now confined to isolated populations in tropical and subtropical regions (Figure 1.1) (Donaldson, 2003 Nagalingum *et al*., 2011).

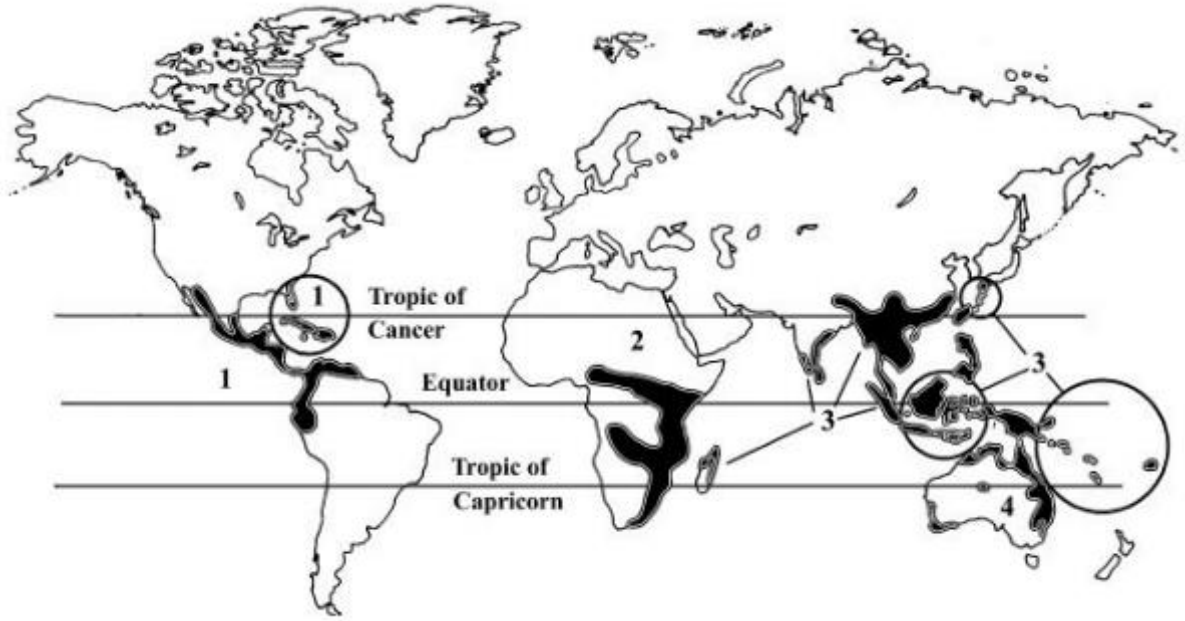

Figure 1.1: Global distribution patterns of cycad genera, grouped as follows: Group 1 (*Ceratozamia, Zamia, Microcycas, Dioon*), Group 2 (*Encephalartos, Stangeria*), Group 3 (*Cycas*), and Group 4 (*Macrozamia, Lepidozamia, Bowenia*) (Hill *et al*., 2003).

This plant group is very important in their ecosystems as they offer essential ecosystem services (ES) such as carbon (C) sequestration, nutrient cycling, and fixed atmospheric nitrogen (N) (Donaldson, 2003, 2008; Ma *et al*., 2009). They are the only gymnosperm known to have a symbiotic association with N-fixing bacteria, hosted in their specialized apogeotropic roots called coralloid roots (Ma *et al*., 2009; Gutiérrez-García *et al*., 2019).

The ability of cycads to fix N through the N-fixing bacteria hosted by the coralloid roots is similar to that of legumes through the rhizobia hosted by nodules (Lindström and Mousavi, 2010; Zheng *et al*., 2018). The *Rhizobium*–legume interaction is the most well-known plant-bacteria symbiotic relationship (Lindström and Mousavi, 2010; Chang *et al*., 2019), however, little is known about other non-legume plants such as cycads that fix atmospheric N (Zheng *et al*., 2018). Cycads date back approximately 300 million years, which implies that the N-fixing process in cycads may have predated the N-fixation process in legumes which were only discovered 65 million years ago (MYA) (Raven, 2002; Zheng *et al*., 2018). Understanding the N-fixing process in cycads through their symbiotic relationship with the N-fixing bacteria might aid in unpacking the evolutionary history of N-fixation through plant-bacteria interactions.

Cycads are highly threatened with extinction with about 70% of the over 300 species facing high extinction risk worldwide (Mankga and Yessoufou, 2017; Yessoufou *et al*., 2017). If not conserved, the essential services they offer may be lost. Mankga and Yessoufou (2017), reported habitat destruction as the leading cause of cycad extinction, a factor earlier highlighted by Dondaldson (2003) for southern African cycad genus *Encephalartos*. Additionally, habitat destruction may indirectly contribute to the decline in cycad populations by increasing their visibility and accessibility to illegal collectors (Donaldson, 2003). According to literature, habitat destruction further has a negative impact on soil fertility and causes changes in the assemblages of plants (Mayfield and Daily, 2005; Fischer and Lindenmayer, 2007; Ohsowski *et al*., 2012). As a result, cycads in these habitats are likely to grow in soils with diminishing nutrient levels. Today cycads occur and thrive in habitats with low or inaccessible nutrients, such as steep outcrops, sclerophyll forests, sand dunes, grasslands, and savannas (Giddy, 1974; Zheng *et al*., 2018; Gutiérrez-García *et al*., 2019).

All extant cycads form symbiotic associations with cyanobacteria that inhabit their coralloid roots (Adams *et al.*, 2006; Usher *et al.*, 2007; Suárez-Moo *et al.*, 2019). These associations have played a crucial role in cycad survival and adaptation to environmental stresses over millions of years (Chang *et al.*, 2019; Gutiérrez-García *et al.*, 2019). Cyanobacteria not only fix atmospheric N for their hosts (Chang *et al.*, 2019; Suárez-Moo *et al.*, 2019), but also produce arabinogalactan-proteins (AGPs) that promote plant growth and development (Majewska-Sawka and Nothnagel, 2000; Seifert and Roberts, 2007; Jackson *et al.*, 2012; Díez *et al.*, 2014). Through the enzyme nitrogenase, they convert dinitrogen (N_2) into ammonia

(NH_3), a form readily available for plant assimilation (Boyd, 2001; Díez *et al.*, 2014). In return, the cycad supplies fixed carbon in the form of fructose to sustain its symbionts (Black *et al.*, 2002; Ekman *et al.*, 2006; Bano and Iqbal, 2016).

Research on cycad–microbe symbioses dates back to the 19th century (Caiola, 1980), although progress has been relatively slow compared to legume–*Rhizobium* and actinorhizal symbioses (Chang *et al.*, 2019). Cyanobionts in coralloid roots are predominantly from the genus *Nostoc*, with other N-fixing genera such as *Calothrix*, *Richelia*, and *Scytonema* also reported (Costa *et al.*, 2004; Gehringer *et al.*, 2010; Gutiérrez-García *et al.*, 2019; Suárez-Moo *et al.*, 2019). Additional bacterial phyla, including Actinobacteria, Bacteroidetes, Proteobacteria, and Verrucomicrobia, have been documented within coralloid roots, although cyanobacteria remain dominant due to their ability to produce secondary metabolites that enhance colonization success (Chang *et al.*, 2019; Suárez-Moo *et al.*, 2019).

Secondary metabolites facilitate both cooperation and competition among microbes and between hosts and symbionts (Vandenkoornhuyse *et al.*, 2015; Massalha *et al.*, 2017; Scherlach and Hertweck, 2018). In cycads, they facilitate communication between coralloid roots and cyanobacteria, ensuring selective colonization by N-fixing partners while restricting other microbes (Adams *et al.*, 2013; Suárez-Moo *et al.*, 2019). As a result, coralloid roots are primarily colonized by nitrogen-fixing cyanobacteria, reflecting their main function in N acquisition (Halliday and Pate, 1976; Zheng *et al.*, 2018; Suárez-Moo *et al.*, 2019).

Although root endophytes are influenced by the soil microbial pool (Vandenkoornhuyse *et al.*, 2015; Suárez-Moo *et al.*, 2019), soil microbial diversity is itself shaped by environmental factors such as pH, precipitation, temperature, elevation, salinity, and latitude (Fierer and Jackson, 2006; Fierer *et al.*, 2012; Gaiero *et al.*, 2013; Keet *et al.*, 2019). Among these, soil pH is widely recognized as the dominant driver, with extreme values supporting the lowest microbial richness compared to neutral soils (Fierer and Jackson, 2006; Fierer *et al.*, 2012; Keet *et al.*, 2019; Lauber *et al.*, 2009). Soil pH also regulates nutrient availability, as acidic soils often contain high concentrations of aluminium (Al) and iron (Fe), which form insoluble complexes with phosphorus (P), thereby reducing P bioavailability (Ferguson *et al.*, 2013; Penn and Camberato, 2019; Barrow and Hartemink, 2023).

Soil microorganisms are therefore central to nutrient acquisition in nutrient-poor ecosystems such as grasslands. They include arbuscular mycorrhizal fungi (AMF), rhizobia, *Bacillus*, and others, which tolerate soil acidity, salinity, and drought while enhancing nutrient uptake (Dodd, 2000; Fisher and Vovides, 2004; Fisher and Jayachandran, 2008; Martínez-Hidalgo and Hirsch, 2017; Hou *et al.*, 2018). Previous studies on cycad rhizospheres have identified a wide range of microbial taxa, including AMF, *Bacillus*, *Rhizobium*, *Burkholderia*, *Myxobacteria*, *Streptomyces*, *Actinomycetospora*, *Bradyrhizobium*, *Planctomyces*, *Rhodobium*, *Amycolatopsis*, *Nostoc*, *Klebsiella*, and *Tolypothrix* (Lobakova *et al.*, 2003; Cuddy *et al.*, 2012; Suárez-Moo *et al.*, 2019). These microbes support plant growth and ecosystem functioning by fixing N, solubilizing P, cycling nutrients, and producing extracellular enzymes (Wardle *et al.*, 2004; Franche *et al.*, 2009; Ferguson *et al.*, 2013; Maymon *et al.*, 2015; Martínez-Hidalgo and Hirsch, 2017; Suárez-Moo *et al.*, 2019; Zungu *et al.*, 2020). Extracellular enzymes such as phosphatases, β-D-glucosidase, dehydrogenase, and β-glucosaminidase further enhance nutrient availability by mineralizing organic matter and mobilizing otherwise inaccessible nutrients (Parham and Deng, 2000; Turner, 2010; Billah *et al.*, 2019; Zhang *et al.*, 2020; Lacava *et al.*, 2021; Zungu *et al.*, 2020).

Grassland environments in South Africa host several cycad species, including *Encephalartos lanatus*, *E. friderici-guilielmi*, and *E. ghellinckii* (Giddy, 1974; Whitelock, 2002; Suinyuy *et al.*, 2009; Sigasa *et al.*, 2023). These habitats are characterized by nutrient-deficient, acidic soils (Nandwa, 2001) and are subject to regular fires used as a management tool (Giddy, 1974; Whitelock, 2002; Carbutt and Kirkman, 2022; Sigasa *et al.*, 2023). While fire plays an ecological role, repeated burning may accelerate soil erosion through the loss of vegetation cover (Lunt and Morgan, 2002; Carbutt and Kirkman, 2022). In addition, cycads in these habitats endure extreme climatic conditions, including frost and snow in winter and high summer temperatures (Giddy, 1984; Goode, 1989; Whitelock, 2002).

The persistence of these species under such conditions may be linked to symbiotic associations with nitrogen-fixing bacteria in their coralloid roots (Zheng *et al.*, 2018; Chang *et al.*, 2019). Among grassland cycads, this association has been studied in *E. lanatus* (Sigasa, unpublished data), but its nature in other species remains poorly understood. This knowledge gap is particularly important for *E. ghellinckii*, which mainly occurs in non-protected areas, with only limited populations in reserves, and is currently listed as Vulnerable under criterion C1 (IUCN, 2022). At present, little is known about the composition and function of microbial communities

associated with the coralloid roots of *E. ghellinckii*, or their role in supplying nutrients to the plant and enriching surrounding soils. Addressing this gap is crucial for understanding how *E. ghellinckii* persists in nutrient-poor, acidic grasslands and for informing effective conservation strategies.

This study therefore investigates (i) the diversity of endophytic bacteria in coralloid roots, (ii) microbial communities in rhizosphere and non-rhizosphere soils, and (iii) extracellular enzyme activities linked to N, P, and C cycling as indicators of soil health (Zungu *et al.*, 2020). By focusing on these aspects, the research provides insight into the strategies enabling *E. ghellinckii* to survive under harsh conditions and contributes to the broader understanding of nutrient cycling in grassland ecosystems.

1.2 Problem statement

Cycads are considered one of the most endangered plant groups globally, with approximately 71% of the over 300 species at high risk of extinction (IUCN, 2022). Habitat destruction, which degrades soil fertility and disrupts plant communities, is a major factor driving this alarming trend (Mayfield and Daily, 2005; Fischer and Lindenmayer, 2007; Ohsowski *et al.*, 2012; Yessoufou, 2017). As a result, many cycad species, including those of the genus *Encephalartos*, are confined to nutrient-poor soils, which significantly contribute to their vulnerability and extinction risk (Giddy, 1974; Whitelock, 2002).

This extinction risk is particularly evident among African *Encephalartos* species, which are adapted to survive in a variety of harsh environmental conditions across diverse ecosystems, including forests, grasslands, and savannas (Giddy, 1974; Donaldson, 2003, 2008). These habitats often experience extreme climatic and environmental stressors, such as nutrient-deficient soils, soil acidity, fire, and drought (Giddy, 1974; Donaldson, 2003, 2008; Mankga and Yessoufou, 2017). South Africa, a global hotspot for cycad diversity (Donaldson, 2003, 2008), faces a critical conservation challenge, with over 50% of its *Encephalartos* species threatened by extinction, and more than 90% classified as species of conservation concern (Rayner and Pires, 2016).

Encephalartos ghellinckii, although classified as vulnerable (IUCN, 2022), exemplifies a species that endures extreme environmental stressors. It persists in acidic, nutrient-deficient grasslands, where it is regularly exposed to fire, frost, snow, and drought (Giddy, 1974;

Whitelock, 2002). Despite these conditions, the species survives, a resilience thought to be facilitated by its symbiotic relationship with cyanobacteria, a feature common in cycads (Zheng *et al.*, 2018; Chang *et al.*, 2019; Gutiérrez-García *et al.*, 2019). However, the composition of cyanobacteria in its coralloid roots and the functional role of these microbial communities in nutrient enhancement and cycling remain poorly understood.

In addition, the contribution of rhizosphere microbes to cycad survival in nutrient-poor grasslands is underexplored. Soil microbes are essential for nutrient solubilization and improved plant nutrient uptake, particularly in ecosystems where soil fertility is low (Millard and Singh, 2010; Mencel *et al.*, 2022). Investigating the interactions between *E. ghellinckii* and its associated microbial communities — in both coralloid roots and soils — is therefore critical for understanding how this species adapts and persists under such challenging conditions.

This research aligns with South Africa's National Biodiversity Strategy and Action Plan (NBSAP) for 2015–2025, which emphasizes the conservation of threatened plant species and recognizes their role in sustaining ecosystem services (Department of Environmental Affairs, 2015). By addressing current knowledge gaps, this study contributes to the development of informed conservation strategies for *Encephalartos* species and offers insights into the microbial interactions that support their survival in nutrient-deficient environments. Such insights may also prove valuable for the conservation of other endangered cycads within the same lineage.

1.3 Aims and objectives of the study

This study aimed to investigate the symbiotic relationship between *E. ghellinckii* and N-fixing bacteria, as well as the role of soil microbial communities and enzyme activities in regulating nutrient availability. The research explored how these interactions influenced the plant's N source reliance and its survival in nutrient-deficient, acidic grassland ecosystems across KwaZulu-Natal, and the Eastern Cape in South Africa.

The objectives of this study are to:

i. Investigated the symbiotic interactions between *Encephalartos ghellinckii* and bacteria in the coralloid roots, as well as the microbial communities in the rhizosphere and non-rhizosphere soils, and their associated enzyme activities, to understand their role in

enhancing soil nutrient availability and supporting the survival of *E. ghellinckii* in grassland ecosystems.

ii. Examined how low nutrient availability and soil acidity influenced the N source reliance of *E. ghellinckii*, specifically comparing its dependence on atmospheric N versus soil-derived N in grassland habitats.

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CHAPTER 2: LITERATURE REVIEW

2.1 Cycad diversity and distribution

Cycads are among the oldest plant groups, with fossil records dating back 280–325 MYA, and were dominant during the Mesozoic before declining in the Cretaceous (Mamay, 1969; Zhifeng and Thomas, 1989; Norstog and Nicholls, 1997; Donaldson, 2003).They were dominant during the Mesozoic, but the mass extinction experienced in the Cretaceous period significantly decreased their populations and distribution (Mamay, 1969; Norstog and Nicholls, 1997; Whitelock, 2002). Currently, they are limited to tropical and subtropical regions worldwide (Donaldson, 2003; Vovides *et al*., 2003). They can be found in small populations on oceanic islands as well as in the warmer parts of Africa, Asia, Australia, and North and South America (Donaldson, 2003).

Cycads are currently believed to be the closest relative to all other existing seed plants (Nixon *et al*., 1994). Research using both morphological and molecular methods established that cycads are monophyletic, meaning they have a single evolutionary origin (Stevenson, 1990; Chase *et al*., 1993). Calonje *et al*. (2013), categorized living cycads into three families: Cycadaceae, Zamiaceae, and Stangeriaceae, encompassing 11 genera and a total of 359 species. The family Cycadaceae consists of only one genus *Cycas,* the Stangeriaceae family has two genera, *Stangeria* and *Bowenia* and the Zamiaceae family is the most diverse as it consists of eight genera namely *Ceratozamia*, *Chigua*, *Dioon, Encephalartos*, *Lepidozamia*, *Macrozamia*, *Microcycas,* and *Zamia* (Stevenson, 1992; Donaldson, 2003; Calonje *et al*., 2013).

Africa is a crucial center for cycad diversity (Figure 2.1), with 69 species identified across the continent (Donaldson, 2003, 2008). South Africa plays a prominent role in this diversity, hosting around 70% of Africa's cycad species (Hill *et al*., 2003). It is recognized globally for its rich cycad flora, ranking third in the number of cycad taxa (Golding and Hurter, 2003; Hill *et al*., 2003). Of the 66 species in South Africa, 65 belong to the *Encephalartos* genus, and one to the *Stangeria* genus (Donaldson, 2003, 2008). The *Encephalartos* species are primarily distributed along the eastern part of the continent, from South Africa up to Sudan, and extend into central and West Africa (Figure 2.1) (Golding and Hurter, 2003; Donaldson, 2008).

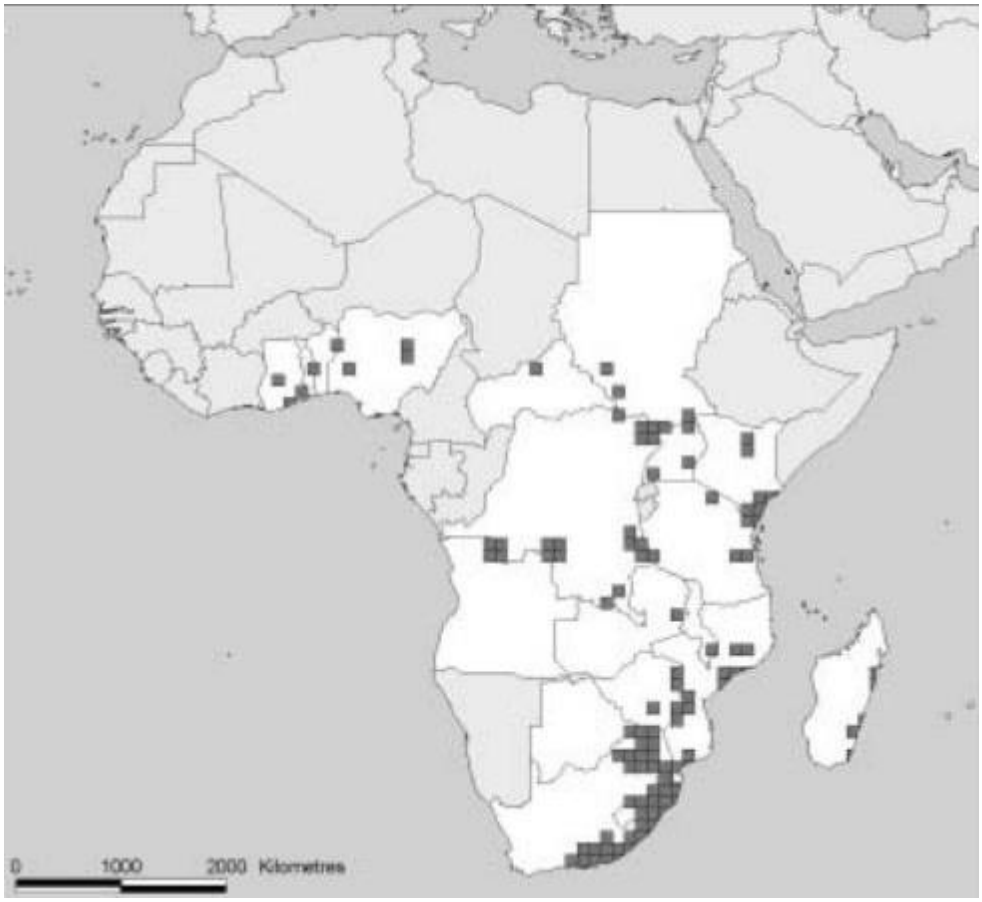

Figure 2. 1: Spatial distribution of cycads in Africa and adjacent Indian Ocean Islands (Donaldson, 2003).

Encephalartos species are mainly found in forests, grasslands, and savannas, and they exhibit adaptations tailored to these specific environments (Donaldson, 2008). The soil cycads generally grow on is acidic and barren (Giddy, 1974; Zheng *et al*., 2018). Similar to other cycad species, their ability to survive in these environmental conditions can be attributed to their symbiotic association with bacteria in the coralloid roots, however, this has not been confirmed for all cycad species.

2.2 *Encepharlatos ghellinckii*: distribution and importance

Encephalartos ghellinckii is a cycad species indigenous to South Africa (Giddy, 1974). It is primarily found in KwaZulu-Natal and the Eastern Cape (Giddy, 1974; Donaldson, 2008; Suinyuy and Johnson, 2021). This cycad species exhibits two distinct morphological forms: the highland or giant form found in the Drakensberg Mountains at elevations of 1500-2700 meters above sea level, and the lowland or dwarf form found at elevations of 500-1400 meters

above sea level, between the Eastern Cape and the Umkomaas River (KZN) (Jones, 1993; Suinyuy and Jonhson, 2021). The highland form can reach heights of three meters with stems 0.5-0.9 meters wide, whereas the lowland form typically grows up to one meter tall with stems ranging from 0.4-0.7 meters wide (Cousins *et al*., 2012; Suinyuy and Johnson, 2021).

The bark and stems of *E. ghellinckii* are traded and utilized for medicinal purposes in South Africa and because of this, there is a high rate of illegal collection of the species from the wild (Cousins *et al*., 2012). *Encephalartos ghellinckii* was recorded as part of the commonly traded species in the two largest traditional markets in South Africa which are found in Johannesburg and Durban (Cousins *et al*., 2012). Additionally, it is utilized and traded for horticultural purposes (Cousins *et al*., 2011). These practices are contributing to a decline in the wild *E. ghellinckii* populations which may lead to extinction in the future (Cousins *et al*., 2011, 2012). Also, in South Africa, it is believed that cycads chase away evil spirits, and if planted at the entrance of a household the spirits will not pass beyond where the cycad tree is planted. This belief is one of the causes of illegal poaching (Cousins *et al*., 2011, 2012).

Encephalartos ghellinckii thrives in grasslands, typically on steep, rocky outcrops (Giddy, 1974; Donaldson, 2008). This demonstrates its adaptation to diverse climatic conditions, including harsh winters with heavy snowfall and frost and mild to hot summers. It has also shown resilience to fire, often thriving in fire-prone habitats (i.e., grasslands), and fire is believed to stimulate cone and leaf development (Giddy, 1974; Goode, 1989; Whitelock, 2002). Frequent fires lead to soil exposure to erosion and leaching, which negatively affect the soil nutrient status. According to Nandwa (2001), South African grasslands are relatively acidic and nutrient-deficient. Thus, the symbiotic association between cycads and cyanobacteria is essential in such habitats as it will aid in nutrient acquisition and further improve soil fertility (Bano and Iqbal, 2016; Chang *et al*., 2019). Additionally, in habits such as grasslands, soil microbes are essential for solubilizing and cycling nutrients making them available for plant assimilation (Zungu *et al*., 2020), which benefits both the *E. ghellinckii* plant and the surrounding vegetation in the grasslands. However, there is no information on microbes hosted by *E. ghellinckii* coralloid roots and the soil microbes that aid their survival in grassland habitats.

2.3 The threatened state of cycads

Cycads are currently among the rarest and most endangered living organisms on Earth (Hoffmann *et al*., 2010) with more than 70% of cycads threatened with extinction (Chaudhry, 2010; Osborne *et al*., 2012; Yessoufou *et al*., 2017). South Africa is regarded as one of the primary regions for cycad diversity, more especially for the *Encephalartos* genus, and 71% of the population is threatened with extinction (Donaldson, 2003, 2008). Currently, most of the cycad genera are listed in CITIES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) in Appendix I and II (Donaldson, 2003, 2008). Emphasizing the need for their conservation. Numerous threats have led to the decline of cycad populations (Donaldson, 2003). While natural mortality plays a role, human activities have been found to significantly accelerate the decline of cycad populations, far outweighing the effects of natural processes (Donaldson, 2003).

Destructive human activities, such as habitat transformation and illegal harvesting, have led to the widespread removal of cycad plants from the wild (Donaldson, 2003). These activities are driven by urban development, afforestation, agricultural use, and excessive cycad collection for medicinal purposes, among other reasons (Donaldson, 2003; Ravele and Makhado, 2010; Mankga and Yessoufou, 2017; Williamson *et al*., 2016). According to Donaldson (2003), habitat destruction further affects cycad populations by making the plants more accessible to illegal harvesters and collectors. Other contributing factors include climate change, invasive species, and natural events such as fires, floods, and droughts (Donaldson, 2003; Mankga and Yessoufou, 2017). The decline in cycad populations is alarming because their extinction would result in the loss of both the plants and their essential ecosystem services they provide. Understanding the factors that influence the extinction risk of cycads is crucial for effective conservation decisions (Mankga and Yessoufou, 2017).

2.4 Cycad symbiotic association with bacteria

2.4.1 Cycad coralloid roots

Coralloid roots are present in all living cycad species and establish a symbiotic relationship with N-fixing bacteria (Grobbelaar *et al*., 1986; Ahern and Staff, 1994; Costa and Lindblad, 2002; Lindblad and Bergman, 2018). The symbiotic association between cycads and bacteria is believed to be ancient and has been crucial for helping cycads adapt to and survive various

environmental conditions over the years (Halliday and Pate, 1976; Usher *et al*., 2007; Chang *et al*., 2019). Coralloid roots develop from the lateral roots and grow into small, coral-like root clusters (Grobbelaar, 1987; Zheng *et al*., 2002; Gutiérrez-García *et al*., 2019). According to Costa and Lindblad (2002), and Lindblad and Bergman (2018), the formation of the coralloid roots is encoded by genes in the cycads. They are apogeotropic roots that grow upward, varying in depth from the soil surface to more than 30 cm beneath the ground (Grobbelaar, 1987; Zheng *et al*., 2002; Chang *et al*., 2019; Gutiérrez-García *et al*., 2019). The fresh weight of the coralloid roots can reach up to 500 g (Ahern and Staff, 1994; Zheng *et al*., 2002).

The developmental stages include root maturation, bacterial colonization, coralloid formation, senescence, and reconstitution (Costa and Lindblad, 2002). The term 'coralloid' is typically used to refer to mature roots that have been invaded, whereas 'precoralloid' denotes uninvaded roots that have the potential to be colonized and transform into coralloid roots (Ahern and Staff, 1994). There are suggestions that coralloid roots may have other functional roles, but N fixation is believed to be their primary function (Grobbelaar, 1987; Zheng *et al*., 2002; Chang *et al*., 2019; Gutiérrez-García *et al*., 2019).

2.4.2 The colonization of bacteria in coralloid roots

There is limited knowledge about the process by which bacteria invade coralloid roots; however, several mechanisms have been proposed (Costa and Lindblad, 2002). Milindasuta (1975) suggests that cyanobacteria from the surrounding soil penetrate the inner layers of coralloid roots through dermal breaks. Nathanielsz and Staff (1975a) propose that invasion may occur through injured root parts, papillose openings in the dermal layer, or lenticels. Another possibility is that the invasion occurs at the base, the growing apex, or the intercalary zone of the coralloid root (Ahern and Staff, 1994).

Once inside the roots, cyanobacteria settle in the algal zone, a specific cortical layer within coralloid roots (Milindasuta, 1975; Costa and Lindblad, 2002; Lindblad and Bergman, 2018). When coralloid roots are cut in cross or longitudinal sections, a distinct dark blue-green band becomes visible (Halliday and Pate, 1976; Milindasuta, 1975; Lindblad and Bergman, 2018). According to Lindblad et al. (1985), this region contains numerous elongated cycad cells linking adjacent cortical layers. These elongated cells are thought to be specialized for metabolite transfer between symbionts (Lindblad et al., 1985).

The presence of cyanobacteria in the root leads to permanent changes in root development and growth (Ahern and Staff, 1994; Costa and Lindblad, 2002). In addition to colonization of the cortical zone by the blue-green algae, another notable effect is reduced elongation and increased radial growth of the roots (Milindasuta, 1975). This pattern is well illustrated in Macrozamia riedlei, where uninvaded roots are longer and thinner compared to invaded roots, which are shorter and thicker (Costa and Lindblad, 2002). Once roots reach this stage, the process becomes permanent, establishing a stable symbiotic association between cycads and cyanobacteria (Ahern and Staff, 1994).

2.4.3 Diversity of cyanobacteria in the coralloid roots

Research on the symbiotic relationship between N-fixing microbes and cycads is not recent, progress has been gradual, and knowledge remains limited for certain cycad species. (Ahern and Staff, 1994; Costa and Lindblad, 2002; Zheng *et al*., 2018; Chang *et al*., 2019). Historically, cyanobacteria isolated from cycad coralloid roots were identified as *Nostoc* or *Anabaena* (Watanabe, 1963; Caiola, 1974, 1980). In recent years, the isolated cyanobacteria predominantly belonged to the *Nostoc* genus, with other studies also identifying the presence of the *Calothrix*, *Richeliea,* and *Scytonema* genera (Grobbelaar *et al*., 1987; Costa and Lindblad, 2002; Gehringer *et al*., 2010; Gutiérrez-García *et al*., 2019). Additionally, *Bacillus* has been found as part of the microbes hosted within the coralloid roots (Gutiérrez-García *et al*., 2019). *Nostoc* has been identified in several cycad species across six genera: *Bowenia, Dioon, Cycas, Macrozamia, Encephalartos,* and *Zamia* (Lindblad *et al*., 1985; Grobbelaar *et al*., 1987; Caiola, 1980). In the Australian cycad *Macrozamia communis*, both *Anabaena* and *Nostoc* were identified (Grobbelaar *et al*., 1987), whereas *Calothrix* was observed in a species of South African *Encephalartos* (Grobbelaar *et al*., 1986; Gehringer *et al*., 2010). However, there are still cycad species in which the microbes within their coralloid roots remain unidentified.

Although cyanobacteria are the dominant coralloid root symbiont, though other endophytes have also been recorded in limited populations (Warshan *et al*. 2018; Zheng *et al*., 2018; Chang *et al*., 2019; Suárez-Moo *et al*., 2019). Suarez-Moo *et al*. (2019) examined bacterial endophytes within the coralloid roots of the genus *Dioon*, as well as in the rhizosphere and surrounding soils. They recorded the presence of cyanobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, and Planctomycetes in the coralloid roots. Similarly, Gutiérrez-García *et al*. (2019), examined the microbial diversity in the coralloid roots of *Dioon* species and identified

multiple bacterial families beyond cyanobacteria, highlighting the presence of additional microbes in these roots.

Numerous soil-dwelling bacteria, including cyanobacteria, exhibit the ability to synthesize specialized metabolites, allowing them to adapt to biotic and abiotic stressors (Warshan *et al*., 2018; Gutiérrez-García *et al*., 2019). These specialized metabolites substantially influence the competitive and collaborative dynamics of microbe-microbe and host-microbe interactions (Massalha *et al*., 2017; Gutiérrez-García *et al*., 2019; Scherlach and Hertweck, 2018).

It is hypothesized that the dominance of cyanobacteria in the coralloid roots may result from specific recognition signals established between cycads and cyanobacteria (Lindblad and Bergman, 2018). According to Zheng *et al*. (2018), there is a transfer of metabolites between the cycad coralloid roots and the cyanobacteria, which is believed to influence the predominance of cyanobacteria in the coralloid roots. The metabolites exuded include cyanobacterial toxins, phenolic compounds, and polysaccharides (Lobakova *et al*., 2004; Gehringer *et al*., 2010). It is suggested that phenolic compounds play a role in the development and functioning of the symbiotic relationship between cyanobacteria and cycads and may partially inhibit the invasion of roots by other endophytic microbes (Lobakova *et al*., 2004; Leghari *et al*., 2016). The involvement of phenolic compounds in the symbiosis of microbes and roots has also been noted in the legume-rhizobia symbiosis (Lobakova *et al*., 2004). Thus, whether released by the host plant or the cyanobacteria, these secondary metabolites appear to facilitate the dominance of cyanobacteria in the coralloid roots (Zheng *et al*., 2018).

2.4.4 The essential role of nitrogen fixation by the bacteria hosted by the coralloid roots

Nitrogen is considered one of the most essential nutrients required for proper plant development and growth, playing a crucial role in the biochemical and physiological functioning of plants (Mahmud *et al*., 2020; Braun *et al*., 2022). Although N is present in the soil, approximately 80% of it occurs in the atmosphere (Mahmud *et al*., 2020; Braun *et al*., 2022). However, plants cannot directly use atmospheric dinitrogen (N_2) because it is chemically inert and does not react with other molecules to form essential compounds (Bano and Iqbal, 2016; Chang *et al*., 2019; Mahmud *et al*., 2020). Atmospheric N_2 can be converted into a form available to plants through biological N fixation (BNF) (Chang *et al*., 2019; Mahmud *et al*., 2020).

Biological N fixation is the conversion of atmospheric N_2 into ammonia through a series of biochemical and microbial processes (Franche *et al*., 2009). Microorganisms, belonging to the *Rhizobium*, *Enterobacter, Bacillus*, *Stenotrophomonas*, and *Peribacillus* genera, play a crucial role in the BNF process (Ahern and Staff, 1994; Costa and Lindblad, 2002; Mahmud *et al*., 2020). These microbes utilize the enzyme nitrogenase to break the strong triple bond between nitrogen atoms in N_2, converting it into ammonia (Bano and Iqbal, 2016; Chang *et al*., 2019). Nitrogenase, a metalloenzyme found in prokaryotes, is essential for catalyzing N fixation (Bano and Iqbal, 2016). The resulting ammonia is a form of N that plants can easily assimilate, promoting plant growth and enhancing soil fertility (Halliday and Pate, 1976; Hoffmann *et al*., 2010).

The BNF process is highly energy-demanding and dynamic (Mahmud et al., 2020). It also requires high levels of phosphorus (P), which is essential for adenosine triphosphate (ATP) production (Míguez-Montero et al., 2020). Although P is abundant in most soils, much of it is unavailable for plant uptake (Vance, 2001). Its unavailability arises from its tendency to form insoluble complexes with cations such as aluminum (Al^{3+}), iron (Fe^{3+}), and calcium (Ca^{2+}) (Vance, 2001; Chen and Liao, 2016), resulting in cation-bound P that plants cannot assimilate (Hinsinger, 2001; Maseko and Dakora, 2013). This phenomenon is particularly common in acidic soils with high cation concentrations (Vance, 2001). Furthermore, soil P availability not only affects the BNF process but also directly influences plant P levels and overall growth (Magadlela et al., 2014, 2016).

The symbiotic relationship between cycads and AMF allows the plants to absorb more nutrients, especially P and water (Fisher and Vovides, 2004). This enhances their access to P in deficient soils, enabling ATP production necessary for the BNF process. AMF absorb soil P through their mycelia, which explore the rhizosphere beyond the nutrient depletion zone of immobile nutrients (Maseko and Dakora, 2013). To further facilitate plant P uptake, AMF release acid and alkaline phosphatases that solubilize bound P in the soil (Asmar et al., 1995; Maseko and Dakora, 2013). The combined association of cycads with N-fixing bacteria and AMF enhances N acquisition, allowing cycads to thrive in arid environments. This symbiosis provides cycads with an advantage over surrounding plants and supports the BNF process, improving both N acquisition and soil nutrient status (Halliday and Pate, 1976; Maseko and Dakora, 2013; Magadlela et al., 2016). However, there is still no information on the diversity of bacteria hosted by *E. ghellinckii* or their role in enhancing nutrient status in grassland ecosystems through atmospheric N_2 fixation.

2.4.5 Soil microbial communities free-living and in association with cycads

South African grasslands are relavitively acidic and nutrient deficient (Nandwa, 2001). Soils with low pH levels typically have low availability of nutrients such as ammonium (NH_4^+), calcium (Ca^{2+}), and potassium (K^+) (Aprile and Lorandi, 2012). However, soil-borne microorganisms play a crucial role in soil amelioration by cycling and solubilizing insoluble soil nutrients, making them accessible for plant uptake (Dodd, 2000). The microbiota prevalent in the soil includes genera such as *Bradyrhizobium, Burkholderia, Paraburkholderia, Pseudomonas, Rhizobium*, *Paenibacillus, and Lysinibacillus* and others such as AMF (Martínez-Hidalgo and Hirsch, 2017; Zungu *et al*., 2020). These microorganisms are known for their plant growth-promoting traits, including N fixation, solubilization of nutrients like P and potassium (K), and phytohormone production (Glick, 2012; Martínez-Hidalgo and Hirsch 2017; Duran-Bedolla *et al*., 2021).

Additionally, these microorganisms secrete extracellular enzymes that serve as catalysts and mediators in biochemical processes such as decomposition, mineralization, nutrient cycling, and the formation of soil organic matter (Lucas *et al*., 2008; Martínez-Hidalgo and Hirsch, 2017; Sudhakaran and Ravanachandar, 2020). These activities further make insoluble terrestrial nutrients available for plant absorbtion (Zungu *et al*., 2020). The extracellular enzyme activities excreted by the soil microbes include urease, phosphatase, β-D-glucosidase, β-D-cellobiohydrolase, dehydrogenase, β-D-glucosaminidase, among others, which aid in these cycling processes (Merino *et al*., 2016; Adetunji *et al*., 2017).

Key extracellular enzymes such as β-D-glucosidase, β-D-cellobiohydrolase, and dehydrogenase mediate carbon cycling by breaking down polysaccharides into glucose, the main microbial energy source (Henriksson et al., 1998; Maymon *et al*., 2015; Zungu et al., 2020). Other enzymes such as β-D-glucosaminidase and asparaginase hydrolyze chito-oligosaccharides and convert asparagine into aspartic acid and ammonia, processes that directly enhance nitrogen mineralization and increase its availability for plant assimilation (Hill et al., 1967; Mega et al., 1972). The activities of acid and alkaline phosphatase enzymes are responsible for the mineralization and cycling of phosphate (Van Aarle and Plassard, 2010). These microbial attributes and their various mechanisms to support plant survival in nutrient-poor environments indicate their role in promoting plant growth (Glick, 2012).

Ndlovu *et al*. (2023), reported that the association of *Encephalartos natalensis* with root and soil microbes enhances soil fertility thus enabling the plant host to survive in nutrient-deprived soils. Also, in the study conducted by Suárez-Moo *et al*. (2019), plant growth-promoting bacteria from the genera *Burkholderia, Rhizobium, Mycobacterium*, and *Rhizobium* were identified in the rhizosphere of cycad species belonging to the genus *Dioon*. This indicates that cycad species interact with plant growth-promoting bacteria both in their roots and the rhizosphere soil to aid in their survival in harsh environmental conditions. However, there is no available information on *E. ghellinckii*, a cycad species that grows and thrives in acidic, nutrient-deprived grasslands. Its survival may be linked to its association with root and soil microorganisms, but this relationship is not yet understood for *E. ghellinckii*.

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CHAPTER 3: ROLE OF CORALLOID ROOT-ASSOCIATED MICROBES, SOIL MICROORGANISMS, AND ENZYME ACTIVITIES IN THE PERSISTENCE OF *ENCEPHALARTOS GHELLINCKII* IN GRASSLAND ECOSYSTEMS

Abstract

Cycads are ancient gymnosperms that provide critical ecosystem services, such as carbon sequestration and nutrient cycling. They are well known for their symbiotic relationships with nitrogen (N)-fixing bacteria, which enhance nutrient availability in nutrient-poor environments. However, there is limited knowledge on the diversity of microbes present in the coralloid roots of cycads, particularly the African cycad *Encephalartos*, and how they contribute to soil nutrient enrichment. *Encephalartos ghellinckii*, a vulnerable species endemic to South Africa, survives in harsh, nutrient-poor grassland soils, potentially relying on microbial associations. Yet, little is known about the microbial communities associated with this species and their role in supporting its survival. This study investigates the symbiotic interactions between *E. ghellinckii* and N-fixing bacteria and the role of soil microbes and enzymes in supporting its survival in grasslands. Coralloid roots, rhizosphere, and non-rhizosphere soil samples of *E. ghellinckii* were collected from Cathedral Peak, Oribi Gorge, Umgano, and Thabankulu. Soil samples were analyzed for bacterial composition, extracellular enzyme activities, and soil properties, such as pH, total cations, nutrient concentrations, and exchangeable acidity. Soils across all sites were acidic and nutrient-poor, with total N showing the highest concentrations, while other essential nutrients such as phosphorus and potassium were relatively low. Bacterial extraction and identification from coralloid roots, rhizosphere, and non-rhizosphere soils revealed diverse microbes from families such as Paenibacillaceae, Enterobacteriaceae, Pseudomonadaceae, Bacillaceae, and Burkholderaceae, exhibiting nutrient-cycling traits such as N fixation, P solubilization, and N cycling. Extracellular enzymes, including β-glucosidase, nitrate reductase, and phosphatases, showed activity across all sites, with nitrate reductase showing the highest activity. These microbial functions support nutrient mineralization and uptake. Overall, the persistence of *E. ghellinckii* in nutrient-deficient, acidic soils maybe facilitated by microbial associations that promote nutrient availability, emphasizing the ecological significance of cycads in maintaining grassland resilience.

Keywords: *Encephalartos ghellinckii*, symbiosis, nitrogen-fixing, extracellular enzyme activities, nutrient cycling, microbial diversity

3.1 Introduction

Cycads are primitive seed plants that have been present for roughly 300 million years (Zhifeng and Thomas, 1989; Whitelock, 2002; Donaldson, 2003). They have undergone minimal evolutionary change, with fossils from millions of years ago closely resembling modern cycads, earning them the title of "living fossils" (Nagalingum *et al*., 2011; Goel and Khuraijam, 2015). This makes them a group of significant evolutionary importance, as they contain valuable insights into plant ecology and evolution (Brenner *et al*., 2003; Ma *et al*., 2009; Goel and Khuraijam, 2015). Cycads play a crucial role in their ecosystems, as they provide essential ecosystem services such as N fixation, carbon (C) sequestration, nutrient cycling, and biodiversity maintenance (Donaldson, 2008; Ma *et al*., 2009; Álvarez-Yépiz *et al*., 2015; Chang *et al*., 2019). However, cycads are currently considered the most endangered plant group in the world, with 71% of the species facing extinction (The International Union for Conservation of Nature (IUCN), 2022). Without conservation efforts, the vital ecosystem services they provide could be lost.

Cycads are the only gymnosperms known to establish a symbiotic relationship with N-fixing bacteria (NFB) residing in their coralloid roots (Zheng *et al*., 2018; Gutiérrez-García *et al*., 2019). All modern cycads possess coralloid roots, suggesting that these structures have existed since the earliest cycad lineages (Raven, 2002). According to Brenner *et al*. (2003), soils from 300 million years ago were significantly low in nutrients. It is believed that cycads survived these harsh conditions over millions of years primarily because of their symbiotic association with N-fixing cyanobacteria (Brenner *et al*., 2003; Gutiérrez-García *et al*., 2019; Zheng *et al*., 2019). Kipp *et al*. (2024), reported that modern cycads are found in habitats with nutrient-deficient soils, and it is believed that their association with NFB enables them to persist and survive in these soil environmental conditions.

One notable species, *Encephalartos ghellinckii*, is native to South Africa and occurs exclusively in grasslands characterized by acidic, nutrient-poor soils, frequent droughts, and harsh climatic conditions (Giddy, 1974; Nandwa, 2001; Donaldson, 2008; Abdel-Hamid et al., 2020). It is found in the Eastern Cape and KwaZulu-Natal provinces, where it exhibits two distinct forms: a highland form that grows at elevations of 1500–2700 m in the Drakensberg Mountains, and a lowland form occurring between 500–900 m above sea level, from the Eastern Cape to the Umkomaas River (Jones, 1993; Suinyuy and Johnson, 2021). Highland individuals can reach heights of up to three meters, while the lowland form is generally shorter, averaging about one meter (Cousins et al., 2012; Suinyuy and Johnson, 2021).

In this study, populations were observed in Cathedral Peak, Oribi Gorge, and Umgano (KwaZulu-Natal), and Thabankulu (Eastern Cape), where some individuals growing on steep cliffside slopes, highlighting the species' remarkable adaptability. These grassland habitats are also susceptible to erosion due to fire-based management practices that remove vegetation cover (Lunt and Morgan, 2002; Carbutt and Kirkman, 2022). The ability of *E. ghellinckii* to persist under such extreme conditions may be linked to its symbiotic association with N-fixing bacteria in its coralloid roots. However, the composition of these microbial communities and their role in supporting the plant's survival remains poorly understood. Given its vulnerable status but continued accessibility in the wild (IUCN, 2022), *E. ghellinckii* presents a valuable opportunity for research and conservation efforts aimed at protecting both the species and the broader ecosystem services cycads provide.

Grobbelaar *et al*. (1986), investigated the N-fixing abilities of 31 South African cycad species and found that *E. ghellinckii*, along with other *Encephalartos* species, is capable of fixing N. Additionally, Grobbelaar *et al*. (1987), identified the bacterial communities in the coralloid roots of 31 *Encephalartos* species. Their findings, consistent with observations in other *Encepharlatos* species from their study, identified a single strain of *Nostoc* in the coralloid roots of *E. ghellinckii*. However, recent studies investigating the diversity of endophytes in the coralloid roots, have identified a more diverse community of microbes belonging to the *Bacillus*, *Burkholderia, Rhizobium, Bradyrhizobium, Mesorhizobium, Amycolotopsis, Devosia, Nostoc* genus (Gutiérrez-García *et al*., 2019; Suárez-Moo *et al*., 2019; Pecundo *et al*., 2021; Motsomane *et al*., 2023; Ndlovu *et al*., 2023; Wang *et al*., 2023). In addition to their ability to fix N, these microbes have been reported to have other plant growth-promoting traits such as phytohormones production, P solubilization, and disease resistance (Liu *et al*., 2023; Ndlovu *et al*., 2023; Zheng and Gong, 2019).

The rhizosphere soils serve as the primary source of the microbes associated with plant roots (Vandenkoornhuyse *et al*., 2015; Suárez-Moo *et al*., 2019). Plant roots release exudates that attract microbes to the rhizosphere and selectively permit the entry of beneficial microbes based on their function (Vandenkoorhuyse *et al*., 2015; Suárez-Moo *et al*., 2019). In natural environments, essential nutrients such as N and P are often bound in organic molecules, making them inaccessible for direct plant uptake (Coyne and Mikkelsen, 2015; Jacoby *et al*., 2017). To access these nutrients, plants depend on the activity of soil microorganisms such as *Rhizobium*, *Bacillus*, and *Klebsiella*, *Actinomycetes, Burkholderia* amongst others, to facilitate the cycling and mineralizing of insoluble nutrients (Coyne and Mikkelsen, 2015; Jacoby *et al*., 2017;

Martínez-Hidalgo and Hirsch, 2017). Furthermore, soil microorganisms secrete extracellular enzymes that degrade polymeric compounds into smaller, soluble molecules, releasing previously inaccessible micro- and macronutrients (Lucas *et al*., 2008; Bell *et al*., 2013; Burns *et al*., 2013; Coyne and Mikkelsen, 2015). This enzymatic activity enhances nutrient availability, allowing plants to absorb these essential nutrients from the soil (Bell *et al*., 2013).

Extracellular enzymes are vital for the mineralization and cycling of essential terrestrial nutrients like N, P, and C (Lucas *et al*., 2008; Bell *et al*., 2013; Burns *et al*., 2013; Coyne and Mikkelsen, 2015). These enzymes can be categorized based on their specific functions, though some can influence multiple nutrient cycles simultaneously (Zungu *et al*., 2020). Soil enzyme activities such as β-D-glucosaminidase, dehydrogenase, asparaginase, β-D-glucosidase, and phosphatase are pivotal for the cycling and mineralization of N, P, and C (Parham and Deng, 2000; Turner, 2010; Billah *et al*., 2019; Zhang *et al*., 2020; Lacava *et al*., 2021), thus making them essential for the overall functioning of plants in natural environments. However, there is no information on the diversity of the soil microorganisms in the rhizosphere soils of *E. ghellinckii* and how they contribute to nutrient-cycling in nutrient-deprived soils.

This study aimed to examine the symbiotic interactions between *E. ghellinckii* and N-fixing bacteria in coralloid roots, alongside soil microorganisms and associated enzyme activities, and their role in enhancing soil nutrition to support *E. ghellinckii* survival in grasslands. This will be achieved by 1) identifying the microbes in the coralloid roots, *E. ghellinckiii* rhizosphere, and non-rhizosphere soils, 2) Assessing the soil nutrition in the rhizosphere and surrounding soil of *E. ghellinckii*, and 3) assaying the extracellular soil enzymes activities responsible for cycling N, P and C in *E. ghellinckii* rhizosphere and non-rhizosphere. This research will also provide valuable insights into the role of plant-microbe interactions in the survival of other cycad species within the same lineage as *E. ghellinckii*, particularly those that are critically endangered and inaccessible to study.

3.2 Material and Methods

3.2.1 Species description and study sites

Soil and root samples of *Encephalartos ghellinckii* were collected from four geographically distinct grassland sites in South Africa: Cathedral Peak, Umgano, and Oribi Gorge in KwaZulu-Natal, and Thabankulu in the Eastern Cape. Cathedral Peak, located in the Drakensberg Mountains at approximately 2085 metres above sea level (asl), is characterised by montane

grassland with cold, dry winters that include frost and occasional snowfall, and a mean annual precipitation (MAP) of about 1400 mm (Hill, 1996; Everson et al., 1998; Morris et al., 2016). Umgano, situated at approximately 1474 metres asl, experiences a temperate climate with an MAP of around 1100 mm (Mucina and Rutherford, 2006; Fisher, 2024), and features rocky grasslands interspersed with scattered shrubs and *Pinus* trees (field observation). Thabankulu, at about 1258 metres asl in the Eastern Cape, is characterised by seasonal temperature fluctuations ranging from 1.9 °C to 31.6 °C, with peak monthly rainfall reaching approximately 153.6 mm (Aveling et al., 2020). The vegetation in this area consists primarily of grassland with scattered shrubs (field observation). Oribi Gorge, the lowest site at approximately 497 metres asl, lies in a subtropical zone with an MAP of about 1176 mm, predominantly during the summer months (Glen, 1996; Bleher et al., 2003). This site is characterised by open grassland vegetation, where *E. ghellinckii* grows in grassy clearings (field observation).

3.2.2 Soil collection and nutrient analysis

The sampled *E. ghellinckii* plants were spaced 20–30 meters apart. Sampling was guided by the cycads collection permits, with seven plants selected from Cathedral Peak, five from Thabankulu, and three each from Oribi Gorge and Umgano. Soil samples were collected from two depths (0–10 cm and 10–20 cm), from rhizosphere and non-rhizosphere soils, making soil type and depth the main factors in this study, with site as another factor. For rhizosphere soils, four subsamples were collected from the four cardinal points around each plant and combined to produce a composite sample. This approach aimed to capture the average soil conditions around each plant. The non-rhizosphere (control) soils were collected five meters away from the target plant, with the direction randomly chosen using the cardinal points (North, South, East, and West). At each control point, four subsamples were taken at the same depths (0–10 cm and 10–20 cm) within one meter of each other and combined to form a composite non-rhizosphere sample.

The soil samples were air-dried and passed through a 2 mm sieve, then sent to the Analytical Services Unit at Cedara College of Agriculture, under the KwaZulu-Natal Department of Agriculture and Rural Development in South Africa, for the analysis of total soil nutrients, cation concentrations, cation exchange capacity, and pH. The characteristic soil analysis was performed following protocols of Manson and Roberts, (2000). The total soil N concentration was measured using the Automated Dumas dry combustion method with a LECO CNS 2000

(Leco Corporation, USA). The atomic absorption method was used to measure soil P and potassium concentrations, where 2.5 ml soil solution was mixed with 25 ml ambic-2 solution at a pH of 8. After that, the mixture was stirred at 400 rpm for 10 minutes using a multiple stirrer and filtered with Whatman no.1 paper. The same methods as above were used to determine the soil calcium and magnesium concentration however, 25 ml 1 M KCl solution was used instead of ambic-2 solution. Soil pH was determined by mixing 10 ml soil solution with 25 ml 1 M KCl solution and stirring at 400 rpm for 5 minutes using a multiple stirrer. The pH of the suspension was measured using a gel-filled combination glass electrode while stirring.

In this study, the design considered two factors: site (Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano) and soil type (rhizosphere versus non-rhizosphere). Although soils were sampled at two depths (0–10 cm and 10–20 cm), these were composited to produce one representative sample per soil type for each plant and therefore depth was not treated as a separate factor in the analysis. This approach ensured consistency in comparing soil nutrients and microbial communities across sites and between rhizosphere and non-rhizosphere soils.

3.2.3 Soil bacterial extraction and identification

Soil samples collected from both the rhizosphere of *E. ghellinckii* and non-rhizosphere areas in Cathedral Peak, Oribi Gorge, Thabankulu, and Umgano were serially diluted, and a spread technique was conducted onto selective media plates. N-fixing bacteria were cultured on Jensen's agar, an N-free medium. Phosphate-solubilizing bacteria were cultured on Pikovskaya's agar media, containing tricalcium phosphate (TCP) as the source of P. Nitrogen-cycling bacteria were cultured on Simmons citrate agar, where citrate was the C source and inorganic ammonium salts provided the sole N source. All selective media plates were replicated three times and incubated for three to five days at 30 °C. Repeated streaking methods were used to obtain pure bacterial colonies.

A portion of the pure bacterial colonies was amplified using polymerase chain reaction (PCR) with 16S ribosomal RNA gene primers: 63F (5'- CAGGCCTAACACATGCAAGTC -3') and 1387R (5'- GGGCGGTGTGTACAA -3'). Polymerase chain reaction (PCR) amplification was carried out with EmeraldAmp GT Master Mix under the following conditions: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 2 minutes, with a final extension step at 72 °C for 10 minutes. The PCR products were separated on a 1 % (w/v) agarose gel via electrophoresis and visualized under UV light to confirm the amplification of

the correct product size. The amplicons were sent to Inqaba Biotechnological Industries (Pty) Ltd, South Africa, for sequencing. The DNA sequences were then edited and compared to known bacterial nucleotide sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov, accessed on 13 August 2024).

3.2.4 Surface sterilization of coralloid roots and extraction of bacterial DNA

Coralloid roots (Figure 3.1), were harvested during soil collection from five *E. ghellickii* plants from each site.

Figure 3. 1: *Encephalartos ghellinckii* plant (A), coralloid roots (B).

After harvesting, the coralloid roots were rinsed with distilled water and stored in ice. At the laboratory, the coralloid roots were surface sterilized with 70 % (v/v) ethanol for 30 seconds and soaked in 3.5% (v/v) sodium hypochlorite solution for 3 minutes. Thereafter, they were rinsed ten times with sterile distilled water and immersed in 100 µl of 15 % glycerol in sterile Eppendorf tubes and stored at 4 °C. To facilitate bacterial extraction, the coralloid roots in 100 µl of 15 % glycerol were crushed with sterile pipette tips. Nitrogen-fixing bacteria were cultured on Jensen's agar, an N-free medium. Phosphate-solubilizing bacteria were cultured on Pikovskaya's agar media, containing tricalcium phosphate (TCP) as the source of P (Figure 3.2). Nitrogen-cycling bacteria were cultured on Simmons citrate agar, where citrate was the C source and inorganic ammonium salts provided the sole N source. All selective media plates were replicated three times (3x) and incubated for three to five days at 30 °C. Repeated streaking methods were used to obtain pure bacterial colonies.

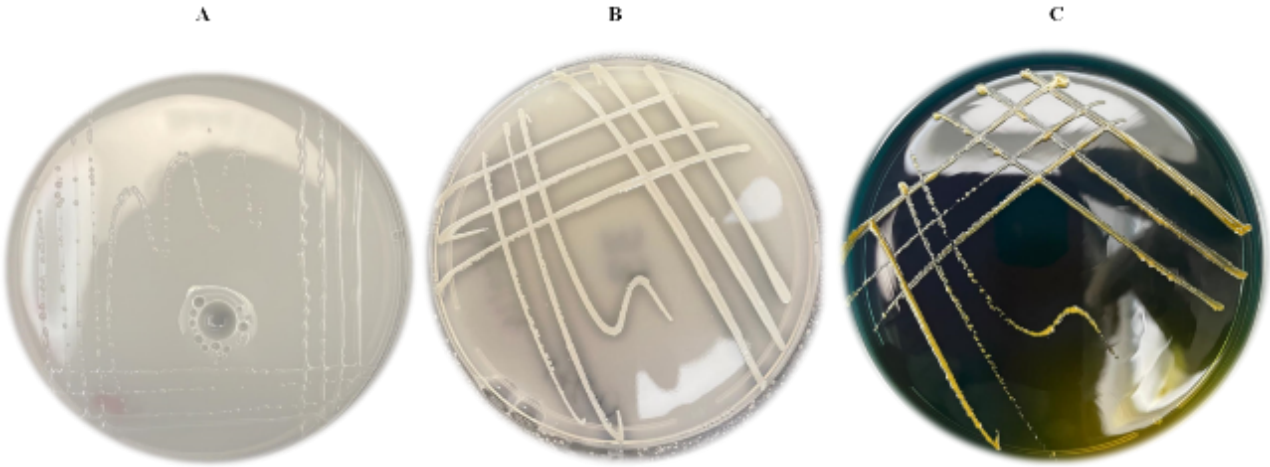

Figure 3. 2: (A) Nitrogen-fixing bacterial colonies cultured on Jensen's medium, (B) N-cycling bacterial colonies on TCP medium, and (C) phosphate-solubilizing bacterial colonies on Simmons citrate medium.

A portion of the pure bacterial colonies was amplified using polymerase chain reaction (PCR) with 16S ribosomal RNA gene primers: 63F (5'- CAGGCCTAACACATGCAAGTC -3') and 1387R (5'- GGGCGGTGTGTACAA -3'). Polymerase chain reaction (PCR) amplification was carried out with EmeraldAmp GT Master Mix under the following conditions: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30

seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 2 minutes, with a final extension step at 72 °C for 10 minutes. The PCR products were separated on a 1% (w/v) agarose gel via electrophoresis and visualized under UV light to confirm the amplification of the correct product size. The amplicons were sent to Inqaba Biotechnical Industries (Pty) Ltd, South Africa, for sequencing. The DNA sequences were then edited and compared to known bacterial nucleotide sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov, accessed on 13 August 2024).

3.2.5 Soil enzyme activities

Carbon cycling (β -glucosidase) and P cycling activities (acid phosphatase, and alkaline phosphatase) were measured using the fluorescence-based method described by Jackson *et al*. (2013) and reported in nmol $h^{-1}g^{-1}$. In this procedure, 10 g of each soil sample was mixed with 100 ml of autoclaved distilled water (dH_2O) and homogenized at medium speed on a shaker for two hours, then stored overnight at 4°C. The supernatants were subsequently transferred to 96-well microplates, for the β-glucosidase, which is the carbon cycling enzyme, 4-MUB-N-acetyl-β-D-glucosidase substrate was added, while 4-MUB-phosphate was used for phosphorus cycling enzymes. The assay included (200µl soil aliquot + 50µl substrate), reference standards (200 µl bicarbonate buffer + 50 µl standard), quench standard (200µl soil aliquot + 50µl standard), sample control (200 µl soil aliquot + 50 µl buffer), negative controls (200 µl buffer + 50 µl substrate), and blanks (250 µl buffer). The 96-well plates were incubated at 25 °C for two hours, after which the reactions were stopped by adding 5 µl of 0.5 M NaOH to each well. Fluorescence was measured at 450 nm with a Glomax Multi Plus microplate reader. For the acid phosphatase activity, the buffer and standard were adjusted to pH 5 before measurement**.** Adjusting the pH of the standard and buffer solutions allows each enzyme's activity to be measured under its optimal conditions, ensuring the most accurate assessment of its efficiency**.**

Nitrate reductase activity (N cycling) was assessed using a modified procedure based on the method developed by Kandeler (1995). Five grams of each soil sample was added in a solution containing 4 mL of 0.9 mM 2, 4-dinitrophenol, 1 mL of 25 mM KNO_3, and 5 mL of autoclaved dH_2O in a 50 mL volumetric flask wrapped in foil. The mixture was thoroughly mixed and incubated at 30 °C for 24 hours in an oven. After incubation, 10 mL of 4 M KCl solution was added to each sample and briefly mixed. The resulting mixture was filtered through (Whatman

no. 1) filter paper. The enzymatic reaction was initiated by combining 2 mL of the filtrate with 1.2 mL of 0.19 M ammonium chloride buffer (pH 8.5) and 800 µL of the colour reagent (1 % sulfanilamide in 1 N HCl and 0.2 % N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD)). Thereafter, the reaction was incubated in an oven at 30 °C for 30 minutes. The absorbance was measured at 520 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA). The nitrite (NO_2) released was quantified as 0.1 µmol h^{-1} g^{-1}.

3.2.6 Statistical analysis

All analyses were conducted using STATISTICA software version 7. One-way analysis of variance (ANOVA) was used to compare the means and assess the significant differences in soil nutrients and enzymatic activity between the rhizosphere and non-rhizosphere soils of *E. ghellinckii* across the four sites: Cathedral Peak, Oribi Gorge, Umgano, and Thabankulu. The normality of the data was evaluated using the Kolmogorov-Smirnov test, and the homogeneity of variances was evaluated using Levene's test. The significant differences of $p \leq 0.05$ between the means were tested by the Tukey HSD tests. A simple linear correlation analysis was performed using the statistical software R to assess the relationship between extracellular soil enzyme activities and soil nutrient levels.

3.3 Results

3.3.1 Soil nutrient concentration and soil relative acidity

Among the primary nutrients, total N exhibited the highest concentration across all study sites (Table 3.1). Within the rhizosphere soils, Umgano had the highest total N concentration, followed by Cathedral Peak, Thabankulu, and Oribi Gorge (Table 3.1). A similar trend was observed in the non-rhizosphere soils, with Umgano again showing the highest total N concentration (Table 3.1). Across all sites, rhizosphere soils generally had higher N concentrations than non-rhizosphere soils, with a significant difference observed only at Cathedral Peak ($p = 0.032$; Table 3.1).

Phosphorus concentrations were relatively low across all study sites (Table 3.1), but rhizosphere soils consistently exhibited higher P concentrations than non-rhizosphere soils. A significant difference was observed at Cathedral Peak ($p = 0.012$; Table 3.1). Potassium (K) had the lowest concentrations among the primary nutrients, with rhizosphere soils showing

higher concentrations than non-rhizosphere soils (Table 3.1). A significant difference in K concentrations between rhizosphere and non-rhizosphere soils was also observed at Cathedral Peak ($p = 0.012$; Table 3.1).

Among the intermediate nutrients, calcium (Ca) concentrations were generally higher in rhizosphere soils compared to non-rhizosphere soils, although these differences were not statistically significant across most sites ($p > 0.05$; Table 3.1). However, a significant difference was observed between Cathedral Peak non-rhizosphere soils and Thabankulu rhizosphere soils ($p = 0.039$; Table 3.1). For magnesium (Mg), Thabankulu soils showed significantly higher concentrations compared to Cathedral Peak ($p = 0.0001$), Oribi Gorge ($p = 0.0020$), and Umgano ($p = 0.0002$) in rhizosphere soils, with similar patterns observed in non-rhizosphere soils ($p < 0.001$; Table 3.1). No significant differences were observed between rhizosphere and non-rhizosphere soils across all sites ($p > 0.05$; Table 3.1).

Among the micronutrients, manganese (Mn) exhibited significantly higher concentrations in Thabankulu compared to Cathedral Peak ($p = 0.0001$), Oribi Gorge ($p = 0.0001$), and Umgano ($p = 0.0001$) in rhizosphere soils (Table 3.1), with a similar pattern observed in non-rhizosphere soils ($p < 0.01$; Table 3.1). Zinc (Zn) and copper (Cu) concentrations showed no significant differences across sites or between rhizosphere and non-rhizosphere soils at any of the study sites (Table 3.1).

Soils across all study sites were acidic, with pH values ranging from 4.09 to 5.61 (Table 3.1). Exchange acidity was significantly higher in Thabankulu rhizosphere soils compared to Umgano ($p = 0.0008$) and Oribi Gorge ($p = 0.0025$), with a similar trend observed in the non-rhizosphere soils ($p = 0.0053$; Table 3.1). No significant differences were observed between Cathedral Peak rhizosphere soils and those from Thabankulu ($p = 0.0543$), Oribi Gorge ($p = 0.7785$), or Umgano ($p = 0.1178$), with a similar trend observed in the non-rhizosphere soils ($p > 0.05$; Table 3.1). The total cation exchange capacity was significantly higher in Thabankulu rhizosphere soils compared to Cathedral Peak ($p = 0.0001$), Oribi Gorge ($p = 0.007$), and Umgano ($p = 0.0007$), with a similar trend observed in the non-rhizosphere soils ($p < 0.01$; Table 1). Organic carbon content was significantly low in Oribi Gorge rhizosphere soils ($p = 0.0001$), compared to the other study sites (Table 3.1), with a similar trend observed in the non-rhizosphere soils ($p = 0.0001$; Table 3.1).

Table 3. 1: Soil nutrient concentrations (mg/kg) and relative acidity (mean ± SE) in the rhizosphere and non-rhizosphere soils of *Encephalartos ghellinckii* from Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano, Eastern Cape and KwaZulu-Natal. Uppercase letters indicate significant differences in nutrient concentrations and relative acidity between sites, while lowercase letters denote variations within sites (One-Way ANOVA, $p \leq 0.05$; CP: n=7; TB: n=5; OR: n=3; UM: n=3). In which, P= Phosphorus, K = Potassium, Total N = Total Nitrogen, Ca = Calcium, Mg = Magnesium, Zn = Zinc, Mn = Manganese, Cu = Copper, and OC = Organic Carbon.

Nutrients	Cathedral Peak		Thabankulu		Oribi Gorge		Umgano	
	Rhizosphere	Non-rhizosphere	Rhizosphere	Non-rhizosphere	Rhizosphere	Non-rhizosphere	Rhizosphere	Non-rhizosphere
Primary nutrients (mg/kg)								
P	8.89 ± 0.76^{Aa}	6.15 ± 0.37^{Bb}	4.81 ± 0.57^{BC}	3.87 ± 0.48^{BC}	3.88 ± 0.60^{BC}	2.50 ± 0.60^{C}	2.83 ± 0.64^{C}	2.42 ± 0.57^{C}
K	0.72 ± 0.08^{Aa}	0.36 ± 0.05^{Bb}	0.23 ± 0.02^{BC}	0.14 ± 0.02^{BC}	0.39 ± 0.11^{BC}	0.20 ± 0.03^{C}	0.50 ± 0.04^{C}	0.33 ± 0.01^{C}
Total N	8973.64 ± 235.98^{Aa}	7215.32 ± 492.82^{Bb}	6800.77 ± 224.26^{B}	6055.69 ± 296.32^{B}	3129.24 ± 57.17^{C}	2635.04 ± 205.72^{C}	9836.07 ± 0.00^{A}	7363.56 ± 1263.50^{AB}
Intermediate nutrients (mg/kg)								
Ca	3.44 ± 1.01^{AB}	2.47 ± 0.64^{B}	5.92 ± 0.53^{A}	5.59 ± 0.667^{AB}	5.97 ± 0.17^{AB}	4.64 ± 0.37^{AB}	4.39 ± 1.06^{AB}	2.38 ± 0.35^{AB}
Mg	1.63 ± 0.25^{B}	1.19 ± 0.15^{B}	15.11 ± 3.53^{A}	17.31 ± 0.93^{A}	5.19 ± 0.38^{B}	3.98 ± 0.50^{B}	2.93 ± 0.59^{B}	1.37 ± 0.22^{B}
Micronutrients (mg/kg)								
Zn	1.09 ± 0.22^{A}	2.19 ± 1.41^{A}	2.74 ± 0.50^{A}	2.06 ± 0.45^{A}	2.56 ± 0.45^{A}	3.70 ± 0.54^{A}	0.40 ± 0.12^{A}	0.04 ± 0.04^{A}
Mn	9.01 ± 0.99^{B}	7.18 ± 1.32^{B}	88.70 ± 6.82^{A}	85.53 ± 15.90^{A}	39.01 ± 3.49^{B}	31.64 ± 2.68^{B}	6.80 ± 1.86^{B}	3.31 ± 0.36^{B}
Cu	3.65 ± 0.18^{A}	4.13 ± 1.24^{A}	1.63 ± 0.21^{A}	1.49 ± 0.34^{A}	2.19 ± 0.31^{A}	3.01 ± 0.57^{A}	0.822 ± 0.14^{A}	1.15 ± 0.12^{A}
Soil relative acidity								
pH	4.09 ± 0.54^{A}	4.11 ± 0.53^{A}	5.51 ± 0.10^{A}	5.61 ± 0.08^{A}	5.06 ± 0.07^{A}	4.83 ± 0.08^{A}	4.50 ± 0.10^{A}	4.43 ± 0.05^{A}
Exchange acidity (cmol/kg)	0.82 ± 0.15^{AB}	0.79 ± 0.23^{ABC}	0.02 ± 0.01^{BC}	0.06 ± 0.01^{C}	0.06 ± 0.01^{BC}	0.07 ± 0.01^{BC}	1.51 ± 0.44^{A}	1.26 ± 0.06^{A}
Total cation exchange (cmol/kg)	5.35 ± 0.42^{B}	4.80 ± 0.64^{B}	21.34 ± 3.65^{A}	23.10 ± 1.17^{A}	11.05 ± 0.54^{B}	9.46 ± 0.58^{B}	8.75 ± 1.15^{B}	5.89 ± 0.84^{B}
Parameter								
OC (%)	6.00 ± 0.00^{A}	6.00 ± 0.00^{A}	6.00 ± 0.00^{A}	6.00 ± 0.00^{A}	3.77 ± 0.9^{B}	3.80 ± 0.50^{B}	6.00 ± 0.00^{A}	6.00 ± 0.00^{A}

3.3.2 Microbes identified from *E. ghellinckii* coralloid roots

Microbes from the coralloid roots of *E. ghellinckii* at Cathedral Peak, Thabankulu, Umgano, and Oribi Gorge were classified into six families: Paenibacillaceae, Enterobacteriaceae, Rhodobacteraceae, Alcaligenaceae, Burkholderaceae, and Moraxellaceae (Figure 3.3). Paenibacillaceae were identified only in Thabankulu, while Rhodobacteraceae and Alcaligenaceae were exclusive to Cathedral Peak (Figure 3.3). Enterobacteriaceae were identified in Thabankulu and Oribi Gorge, with this family accounting for all microbial strains isolated from Oribi Gorge (Figure 3.3)

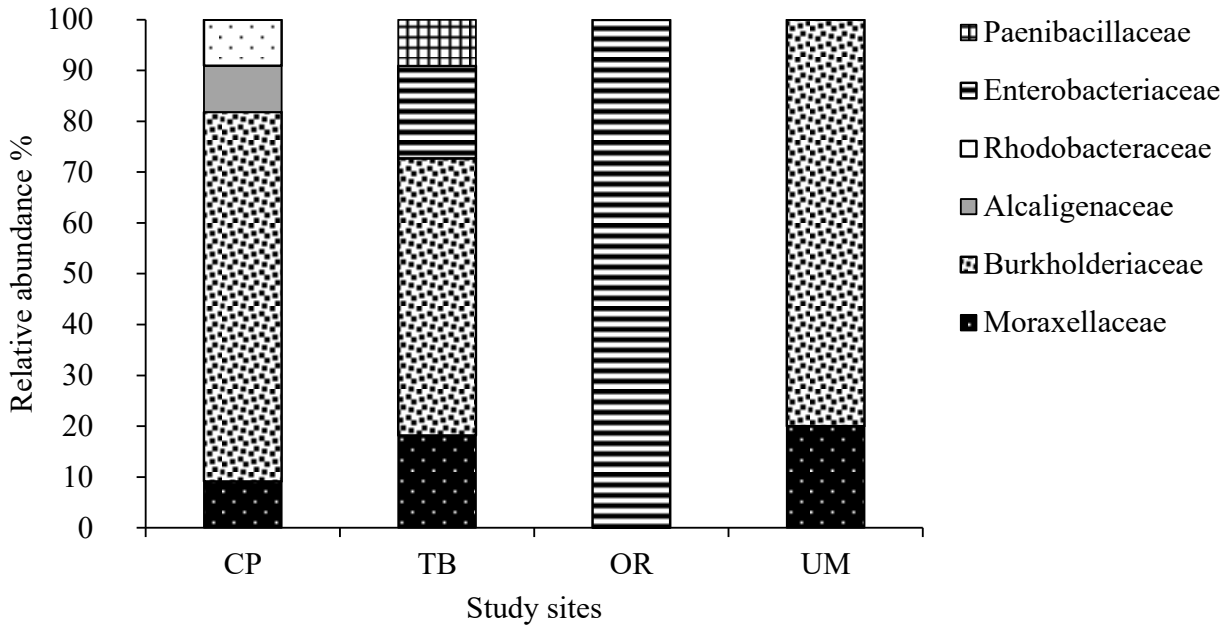

Figure 3. 3 : The species composition of bacteria at the family level from *E. ghellinckii* coralloid roots collected from Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), and Umgano (UM).

Microbes isolated from the coralloid roots of *E. ghellinckii* at Cathedral Peak, Thabankulu, Umgano, and Oribi exhibited the ability to fix N, solubilize P, and cycle N with some strains involved in both N-fixation and P-solubilization (Tables 3.2-3.5).

At Cathedral Peak, 11 microbial strains were isolated, representing five genera: *Acinetobacter*, *Burkholderia*, *Paraburkholderia*, *Achromobacter*, and *Cereibacter* (Table 3.2). The dominant family among these isolates was Burkholderiaceae, which included a majority of the strains (Table 3.2). A similar pattern was observed at Thabankulu, where 11 microbial strains were isolated from six genera: *Acinetobacter*, *Paraburkholderia*, *Caballeronia*, *Trinickia*, *Enterobacter*, and *Brevibacillus* with Burkholderiaceae family being predominant (Table 3.3).

At Umgano, five microbial strains were isolated, belonging to two genera: *Acinetobacter* and *Paraburkholderia* (Table 3.4). The Burkholderiaceae family again dominated these isolates, highlighting its consistent presence across the sites. In contrast, at Oribi Gorge, three microbial strains were isolated, each representing a different genus: *Enterobacter*, *Kosakonia*, and *Klebsiella*, all of which belonged to the Enterobacteriaceae family (Table 3.5). The Burkholderiaceae family was absent from this site (Table 3.5).

Table 3. 2: Microbial diversity in *E. ghellinckii* coralloid roots from Cathedral Peak

Family	Scientific name	Accession number	Similarity %	Functions
Moraxellaceae	*Acinetobacter septicus*	MN725744.1	99.62	P-solubilization (Mujumdar *et al*., 2023)
Burkholderiaceae	*Paraburkholderia fungorum*	MT611735.1	99.61	N-fixing (Raihan *et al*., 2022) P-solubilizing (Napitupulu *et al*., 2023)
	Burkholderia multivorans	OQ926016.1	92.31	P- solubilizing (Li *et al*., 2013; 2018)
	Paraburkholderia graminis	OR831931.1	92.45	N-cycling (Viallard *et al*., 1998)
	Paraburkholderia phytofirmans	OR430493.1	100	N- fixing (Puri *et al*., 2020) P- solubilizing (Aziz *et al*., 2020)
	Paraburkholderia sediminicola	OQ076265.1	100	P- solubilizing (Vanwijnsberghe *et al*., 2021)
	Paraburkholderia nemoris	OR922244.1	00	N- fixing (Vanwijnsberghe *et al*., 2021) P- solubilizing (Vanwijnsberghe *et al*., 2021)
	Paraburkholderia madseniana	OR922229.1	100	P-solubilization (Wilhelm *et al*., 2020)
	Paraburkholderia aromaticivorans	OQ076254.1	96.86	N- fixing (Vio *et al*., 2020)
Alcaligenaceae	*Achromobacter xylosoxidans*	MT040694.1	80.8	N- fixing (Jha and Kumar, 2009) P- solubilizing (Jha and Kumar, 2009)
Rhodobacteraceae	*Cereibacter sphaeroides*	EF633263.1	80.9	N-fixing (Llamas *et al*., 2023) P- solubilizing (Dat *et al*., 2024)

Table 3. 3: Microbial diversity in *E. ghellinckii* coralloid roots from Thabankulu

Family	Scientific name	Accession number	Similarity %	Function
Moraxellaceae	*Acinetobacter septicus*	MN725744.1	99.67	P-solubilizing (Mujumdar *et al*., 2023)
	Acinetobacter guillouiae	OR821296.1	89.1	N-fixing (Kour *et al*., 2023)
Burkholderiaceae	*Paraburkholderia fungorum*	MH972166.1	99.83	N-fixing (Raihan *et al*., 2022) P-solubilizing (Napitupulu *et al*., 2023)
	Paraburkholderia phytofirmans	CP157347.1	100	N- fixing (Puri *et al*., 2020) P- solubilizing (Aziz *et al*., 2020; Puri *et al*., 2020)
	Paraburkholderia sediminicola	PP905144.1	98..08	P- solubilizing (Vanwijnsberghe *et al*., 2021)
	Paraburkholderia graminis	OR831931.1	98.00	N-fixing (Viallard *et al*., 1998)
	Caballeronia mineralivorans	PQ034603.1	98.00	N-fixing (Uroz and Oger, 2017) P-solubilizing (Uroz and Oger, 2017)
	Trinickia caryophylli	CP131478.1	86.9	N-fixing (Estrada-de Los Santos *et al*., 2019)
Enterobacteriaceae	*Enterobacter cloacae*	OR084780.1	100	N-fixing (Ji *et al*. 2020) P-solubilizing (Macedo-Raygoza *et al*., 2019)
	Enterobacter ludwigii	OR079894.1	100	P-solubilizing (Lee *et al*., 2019)
Paenibacillaceae	*Brevibacillus reuszeri*	MT071700.1	100	N-fixing (Çakmakçı, 2019) P-solubilizing (Yildirim *et al*., 2011)

Table 3. 4: Microbial diversity in *E. ghellinckii* coralloid roots from Umgano

Family	Scientific name	Accession number	Similarity %	Function
Moraxellaceae	*Acinetobacter septicus*	MN725744.1	99.81	Solubilize P (Mujumdar *et al*., 2023)
Burkholderiaceae	*Paraburkholderia fungorum*	CP157347.1	100	N-fixing (Raihan *et al*., 2022)
				P- solubilizing (Napitupulu *et al*., 2023)
	Paraburkholderia phytofirmans	OR430493.1	100	N- fixing (Puri *et al*., 2020)
				P- solubilizing (Aziz *et al*., 2020)
	Paraburkholderia aromaticivorans	OQ076254.1	100	N- fixing (Vio *et al*., 2020)
	Paraburkholderia ginsengisoli	CP066075.1	100	N-fixing (Vio *et al*., 2020; Kim *et al*., 2020)
				P- solubilizing (Kim *et al*., 2020)

Table 3. 5: Microbial diversity in *E. ghellinckii* coralloid roots from Oribi Gorge

Family	Scientific name	Accession number	Similarity %	Function
Enterobacteriaceae	*Enterobacter cloacae*	OR084780.1	100	N-fixing (Ji *et al*. 2020)

			P- solubilizing (Macedo-Raygoza *et al*, 2019)
Kosakonia oryzae	OM909321.1	99.35	N-fixing (Peng *et al*., 2009)
Klebsiella vaiicola	MN725749.1	98.61	N-fixing (Li *et al*., 2015)

3.3.3 Soil microorganisms identified from the *E. ghellinckii* rhizosphere and non-rhizosphere

In rhizosphere soils, 11 microbial families were identified, with the highest diversity observed at Umgano (six families), followed by Oribi Gorge (five families), and Cathedral Peak and Thabankulu (four families each) (Figure 3.4A). Non-rhizosphere soils showed lower diversity, containing nine families. Umgano hosted the highest diversity (five families), while Thabankulu had the fewest (three families) (Figure 3.4B).

Across all sites, Pseudomonadaceae was the dominant family in both rhizosphere and non-rhizosphere soils (Figure 3.4A and 3.4B). Burkholderiaceae was present in three of the four sites (Cathedral Peak, Oribi Gorge, and Umgano) in both soil types (Figure 3.4A and 3.4B). Enterobacteriaceae were identified in the rhizosphere soils of all study sites, but in non-rhizosphere soils, they were only found at Cathedral Peak and Umgano (Figure 3.4A and 3.4B).

Several microbial families were observed exclusively at specific sites. Xanthomonadaceae were present in both soil types only at Oribi Gorge (Figure 3.4A and 3.4B). Bacillaceae were exclusive to Thabankulu, occurring in both rhizosphere and non-rhizosphere soils (Figure 3.4A and 3.4B). Similarly, Erythrobacteraceae and Lysobacteraceae were restricted to the rhizosphere soils of Thabankulu, while Rhizobiaceae were confined to the rhizosphere soils at Cathedral Peak (Figure 3.4A).

Unique microbial families were also identified in the non-rhizosphere soils. Pectobacteriaceae was found only at Thabankulu, Flavobacteriaceae at Oribi Gorge, Yersiniaceae at Umgano, and Caulobacteraceae at Cathedral Peak and Thabankulu (Figure 3.4 B).

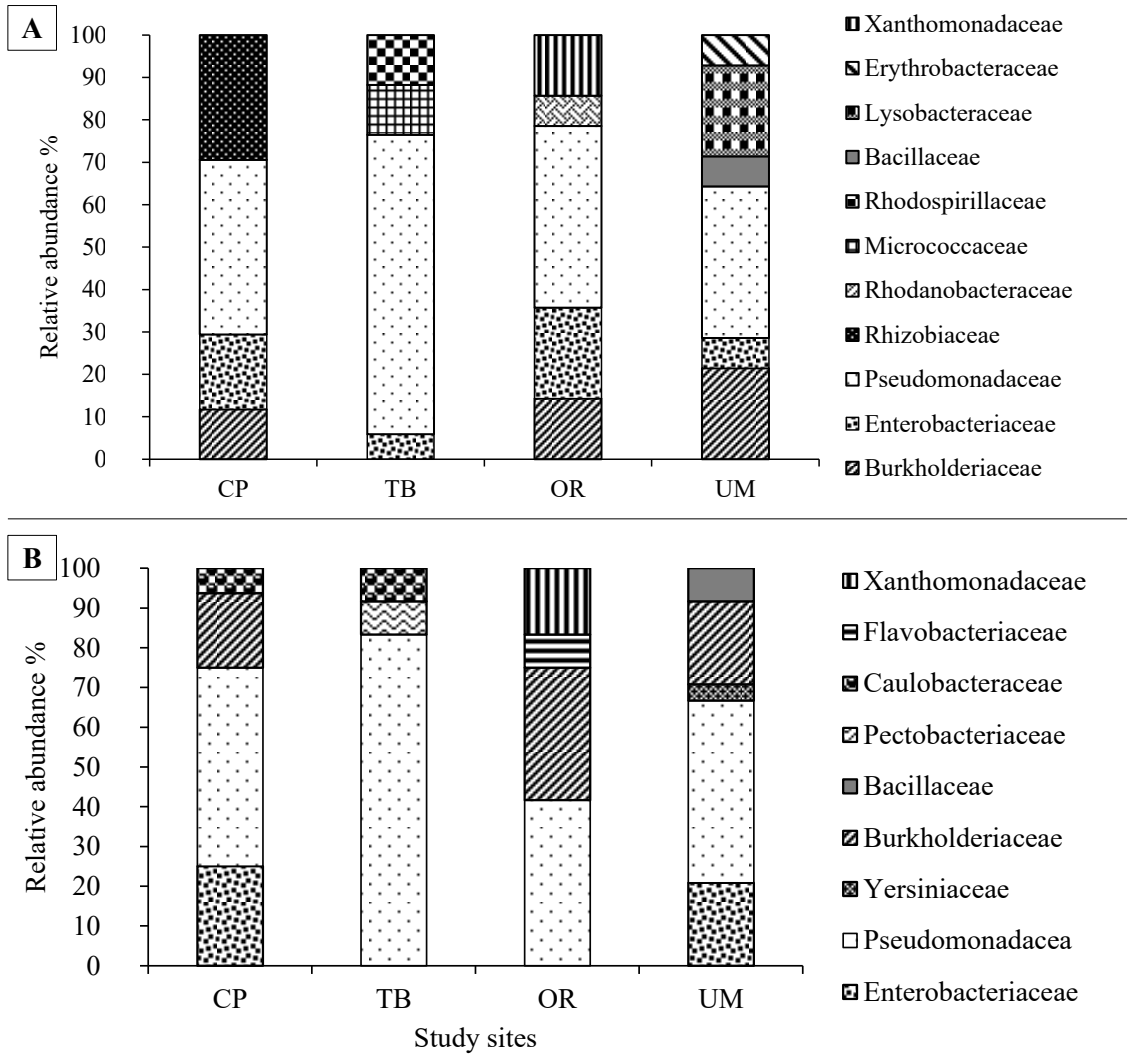

Figure 3. 4: (A) Bacterial species composition at the family level *from E. ghellinckii* rhizosphere soils collected from Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), and Umgano (UM). (B) Bacterial species composition at the family level from non-rhizosphere soils collected from the same locations: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), and Umgano (UM).

Across all study sites, non-rhizosphere soils generally exhibited higher microbial diversity compared to rhizosphere soils. At Cathedral Peak, 17 bacterial strains were isolated from rhizosphere soils, while non-rhizosphere soils consisted of 16 strains (Tables 3.6 and 3.7). Despite the similar strain counts, non-rhizosphere soils exhibited a broader diversity of genera, with seven genera identified compared to five in rhizosphere soils (Tables 3.6 and 3.7). At Thabankulu, the rhizosphere soils contained a higher number of bacterial strains (17) compared to the non-rhizosphere soils (12), but the non-rhizosphere soils had fewer genera, with only 3 identified compared to 5 in the rhizosphere (Table 3.8 and 3.9). In Oribi Gorge and Umgano, a more consistent pattern was observed, with non-rhizosphere soils hosting greater microbial diversity (Table 3.10 to 3.13). At Umgano, non-rhizosphere soils contained 23 strains from 10 genera, while the rhizosphere soils harbored 14 strains from 6 genera (Tables 3.12 and 3.13).

The microbial community composition revealed *Pseudomonas* as the dominant genus across all sites in both rhizosphere and non-rhizosphere soils (Table 3.6 to 3.13). At Cathedral Peak, the rhizosphere soils were primarily dominated by *Pseudomonas*, *Rhizobium*, and *Enterobacter* (Table 3.6 and 3.7). At Thabankulu, *Pseudomonas* was the dominant genus in both the rhizosphere and non-rhizosphere soils (Tables 3.8 and 3.9). At Oribi Gorge, *Pseudomonas* was dominant in the rhizosphere soils, while both *Pseudomonas* and *Burkholderia* dominated the non-rhizosphere soils (Tables 3.10 and 3.11). In Umgano, the rhizosphere soils were mostly dominated by *Burkholderia*, *Stenotrophomonas*, and *Pseudomonas*, while the non-rhizosphere soils were mostly composed of *Paraburkholderia* and *Pseudomonas* (Tables 3.12 and 3.13).

Table 3. 6: Soil microorganisms from *E. ghellinckii* rhizosphere soils in Cathedral Peak

Family	Scientific name	Accession number	Similarity %	Function
Enterobacteriaceae	*Enterobacter mori*	PQ047752.1	99.26	P-solubilizing (Kiprotich *et al*., 2023)
	Enterobacter asburiae	MN691269.1	99.57	N-fixing (Van Chuong *et al*., 2024)
	Enterobacter ludwigii	KM280650.1	99.20	N-fixing (Alkurtany *et al*., 2023) P-solubilizing (Lee *et al*., 2019)
Pseudomonadacea	*Pseudomonas palleroniana*	PQ113825.1	97.20	N-fixing (Suyal *et al*., 2018) P-solubilizing (Adhikari *et al*., 2021)
	Pseudomonas tolaasii	PP911399.1	96.62	N-fixing (Mbonambi *et al*., 2024) P-solubilizing (Mbonambu *et al*., 2024)
	Pseudomonas poae	CP159260.1	100	N-fixing (Jiao *et al*., 2024) P-solubilizing (Jiao *et al*., 2024)
	Pseudomonas monteilii	PQ115154.1	99.72	N-fixing (Li *et al*., 2024) P-solubilizing (Li *et al*., 2024)
	Pseudomonas veronii	PQ097982.1	99.53	N-fixing (Setten *et al*., 2013) P-solubilizing (Haque *et al*., 2020)
	Pseudomonas lactis	PQ108509.1	99.28	N-fixing (Mazari *et al*., 2024) P-solubilizing (Mazari *et al*., 2024)
	Pseudomonas palleroniana	PQ113825.1	99.60	N-fixing (Suyal *et al*., 2018) P-solubilizing (Adhikari *et al*., 2021)

Burkholderiaceae	*Paraburkholderia fungorum*	MT611709.1	100	N-fixing (Raihan *et al*., 2022) P-solubilizing (Napitupulu *et al*., 2023)
	Cupriavidus basilensis	MK934453.1	100	N-fixing (Platero *et al*., 2016)
Rhizobiaceae	*Rhizobium tropici*	MT539147.1	99.22	N-fixing (Gomes *et al*., 2015) P-solubilizing (Kumar and Ram., 2014)
	Rhizobium calliandrae	MH312040.1	97.98	N-fixing (Rincon-Rosales *et al*., 2013) P-solubilizing (Gen-Jiménez *et al*., 2023)
	Rhizobium multihospitium	AB908088.1	99.80	N-fixing (Mir *et al*., 2021) P-solubilizing (Mir *et al*., 2021)
	Rhizobium lusitanum	MN594803.1	100	N-fixing (Valverde *et al*., 2011)
	Rhizobium rhizogenes	MN181336.1	100	N-fixing (Velázquez *et al*., 2005)

Table 3. 7: Soil microorganisms from *E. ghellinckii* non-rhizosphere soils in Cathedral Peak

Family	Scientific name	Accession number	Similarity %	Function
Caulobacteraceae	*Caulobacter rhizosphaerae*	MK138628.1	100	N-fixing (Sithole *et al*., 2021)
Burkholderiaceae	*Paraburkholderia terrae*	MN150518.1		N-fixing (Yang *et al*., 2006) P-solubilizing (Yang *et al*., 2006)
	Paraburkholderia fungorum	CP157347.1	100	N-fixing (Raihan *et al*., 2022) P-solubilizing (Napitupulu *et al*., 2023)
	Trinickia sp.	ON738789.1	99.58	N-fixing (De Lajudie *et al*., 2019)
Enterobacteriaceae	*Pantoea agglomerans*	MK883113.1	98.73	N- fixing (Asis and Adachi, 2004) P-solubilizing (Son *et al*., 2006)
	Enterobacter chuandaensis	CP162195.1	100	N-fixing (Tham *et al*., 2024) P-solubilizing (Tham *et al*., 2024)
	Cedecea davisae	PQ097725.1	100	N-fixing (Kumar *et al*., 2023)
	Pantoea ananatis	PQ097728.1	100	P-solubilizing (Da Silva *et al*., 2015)
Pseudomonadacea	*Pseudomonas vancouverensis*	MT322943.1	99.85	P-solubilizing (Samaddar *et al*., 2019)
	Pseudomonas marginalis	LC409071.1	100	P-solubilizing (Germán *et al*., 2024; Shahid, 2013)
	Pseudomonas palleroniana	PQ113825.1	100	N-fixing (Suyal *et al*., 2018) P-solubilizing (Adhikari *et al*., 2021)

	Pseudomonas fluorescens	CP133209.1	100	N-fixing (Wu *et al*., 2023) P-solubilizing (Branch, 2012)
	Pseudomonas monteilii	PQ115154.1	100	N-fixing (Li *et al*., 2024) P-solubilizing (Li *et al*., 2024)
	Pseudomonas moraviensis	PQ013180.1	99.42	P-solubilizing (Wang *et al*., 2022)
	Pseudomonas tolaasii	PP723431.1	98.89	N-fixing (Mbonambi *et al*., 2024) P-solubilizing (Mbonambu *et al*., 2024)
	Pseudomonas veronii	PQ097730.1	98	N-fixing (Haque *et al*., 2020) P-solubilizing (Haque *et al*., 2020)

Table 3. 8: Soil microorganisms from *E. ghellinckii* rhizosphere soils in Thabankulu

Family	Scientific name	Accession number	Similarity %	Function
Enterobacteriaceae	*Raoultella planticola*	MH669168.1	98.95	N-fixing (Shabayev, 2010) N- cycling (Huang *et al*., 2022) P-solubilizing (Rosas *et al*., 2006)
Micrococcaceae	*Arthrobacter humicola*	MG571731.1	100	N-fixing (Wang *et al*., 2024) P-solubilizing (Wang *et al*., 2024)
	Paenarthrobacter nicotinovorans	MT533904.1	100	P-solubilizing (Yadav *et al*., 2022)
Rhodospirillaceae	*Inquilinus ginsengisoli*	MK696985.2	100	N-fixing (Man *et al*., 2021) P-solubilizing (Man *et al*., 2021)
	Inquilinus limosus	KY924326.1	100	N-fixing (Afzal *et al*., 2017) P-solubilizing (Afzal *et al*., 2017)
Pseudomonadacea	*Pseudomonas putida*	LC507959.1	99.69	N-fixing (Mehnaz *et al*., 2006) P-solubilizing (Mehnaz *et al*., 2006)
	Pseudomonas baetica	CP054880.1	100	P-solubilizing (Nesha *et al*., 2023)
	Pseudomonas chlororaphis	PQ002078.1	100	N-fixing (Bertani *et al*., 2021) P-solubilizing (Bertani *et al*., 2021; Wei *et al*., 2024)
	Pseudomonas orientalis	CP027724.1	99.34	P-solubilizing (Samaddar *et al*., 2019)

	Pseudomonas vancouverensis	MT322943.1	100	P-solubilizing (Germán *et al*., 2024; Shahid, 2013)
	Pseudomonas marginalis	LC409076.1	99.82	P-solubilizing (Samaddar *et al*., 2019)
	Pseudomonas helvetica	PQ037652.1	100	N-cycling (Poli *et al*., 2024)
	Pseudomonas bijieensis	CP048810.1	100	P-solubilizing (Chaudhary *et al*., 2023)
	Pseudomonas veronii	PQ097982.1	99.82	N-fixing (Haque *et al*., 2020) P-solubilizing (Haque *et al*., 2020)
	Pseudomonas koreensis	MT501807.1	99.08	N-fixing (Li *et al*., 2017)
	Pseudomonas viridiflava	MT386130.1	99.78	N-fixing (Hassen *et al*., 2018) P-solubilizing (Hassen *et al*., 2018)
	Pseudomonas rhizophila	PQ056001.1	99.61	N-fixing (Hassen *et al*., 2018) P-solubilizing (Hassen *et al*., 2018)

Table 3. 9: Soil microorganisms from *E. ghellinckii* non-rhizosphere soils in Thabankulu

Family	Scientific name	Accession number	Similarity %	Function
Pectobacteriaceae	*Pectobacterium peruviense*	CP161826.1	97.10	N-fixing (Lurthy *et al*., 2018) P-solubilizing (Kim *et al*., 1998)
Caulobacteraceae	*Caulobacter rhizosphaerae*	MK138628.1	100	N-fixing (Sithole *et al*., 2021)
Pseudomonadaceae	*Pseudomonas brassicacearum*	MN006386.1	97.85	N-fixing (Kong *et al*., 2017) P-solubilizing (Kong *et al*., 2017)
	Pseudomonas caspiana	MN203731.1	97.18	N-fixing (Hassen *et al*., 2018) P-solubilizing (Hassen *et al*., 2018)
	Pseudomonas rhizophila	PQ056001.1	99.73	N-fixing (Hassan *et al*., 2018) P-solubilizing (Hassan *et al*., 2018)
	Pseudomonas silesiensis	MT611297.1	99.66	N-fixing (Chumpitaz-Segovia *et al*., 2020) P-solubilizing (Chumpitaz-Segovia *et al*., 2020)
	Pseudomonas purpurea	PQ037651.1	99.81	P-solubilizing (Gupta *et al*., 2016)
	Pseudomonas veronii	PQ097982.1	100	N-fixing (Haque *et al*., 2020) P-solubilizing (Haque *et al*., 2020)
	Pseudomonas mediterranea	MN712327.1	100	N-fixing (Gu *et al*., 2020) P-solubilizing (Gu *et al*., 2020)
	Pseudomonas viridiflava	MT386130.1	99.78	N-fixing (Li *et al*., 2024) P-solubilizing (Li *et al*., 2024)
	Pseudomonas monteilii	PQ115154.1	100	N-fixing (Shabayev, 2010)

				P-solubilizing (Rosas *et al*., 2006)
	Pseudomonas putida	LC507959.1	99.77	N-fixing (Shabayev, 2010) N-cycling (Huang *et al*., 2022) P-solubilizing (Rosas *et al*., 2006)

Table 3. 10: Soil microorganisms from *E. ghellinckii* rhizosphere soils in Oribi Gorge

Family	Scientific name	Accession number	Similarity %	Function
Burkholderiaceae	*Burkholderia stabilis*	PP900092.1	99.12	N-fixing (Kim *et al*., 2020) P-solubilizing (Kim *et al*., 2020)
	Burkholderia cepacia	CP163501.1	100	N-fixation (Li *et al*., 2022) P-solubilizing (Song *et al*., 2008)
Enterobacteriaceae	*Proteus mirabilis*	PP535554.1	91.07	N-fixing (Verma *et al*., 2021)
	Enterobacter cloacae	EF446900.1	90.55	N-fixing (Ji *et al*., 2020) P-solubilizing (Borham *et al*., 2017)
	Klebsiella oxytoca	PQ097709.1	99.34	N-fixing (Yoshidome *et al*., 2024) P-solubilizing (Walpola *et al*., 2014)
Xanthomonadaceae	*Luteibacter rhizovicinus*	MN589775.1	100	P-solubilizing (Guglielmetti *et al*., 2013)
	Dyella marensis	MN181225.1	100	N-cycling (Lee and Lee, 2009)
Pseudomonadaceae	*Pseudomonas gessardii*	PQ097242.1	100	N-fixing (Devi *et al*., 2022) P-solubilizing (Devi *et al*., 2022)
	Pseudomonas putida	PQ097763.1	100	N-fixing (Shabayev, 2010) N- cycling (Huang *et al*., 2022) P-solubilizing (Rosas *et al*., 2006)
	Pseudomonas moraviensis	PQ038303.1	100	P-solubilizing (Wang *et al*., 2022)
	Pseudomonas veronii	PQ097730.1	99.52	N-fixing (Haque *et al*., 2020) P-solubilizing (Haque *et al*., 2020)

	Pseudomonas chlororaphis	KT877595.1	91.41	N-fixation (Bertani *et al*., 2021) P-solubilizing (Bertani *et al*., 2021; Wei *et al*., 2024)
	Pseudomonas lutea	MN956535.1	94.21	P-solubilizing (Kwak *et al*., 2016)

Table 3. 11: Soil microorganisms from *E. ghellinckii* non-rhizosphere soils in Oribi Gorge

Family	Scientific name	Accession number	Similarity %	Function
Flavobacteriaceae	*Flavobacterium johnsoniae*	CP031763.1	99.11	N-fixing (Fang *et al*., 2019)
Xanthomonadaceae	*Stenotrophomonas maltophilia*	OR831908.1	99.44	N-fixing (Alexander *et al*., 2019) P-solubilizing (Shama *et al*., 2024)
	Stenotrophomonas pavanii	OQ024084.1	99.32	N-fixing (Ghosh *et al*., 2020)
Burkholderiaceae	*Burkholderia cepacia*	CP163501.1	100	N-fixation (Li *et al*., 2022) P-solubilizing (Song *et al*., 2008)
	Burkholderia seminalis	PQ001816.1	99.04	P-solubilizing (Hwang *et al*., 2021)
	Burkholderia cenocepacia	PQ008563.1	98.10	N-fixing (Li *et al*., 2022)
	Burkholderia stabilis	MT039445.1	98.10	N-fixating (Kim *et al*., 2020) P-solubilizing (Kim *et al*., 2020)
Pseudomonadaceae	*Pseudomonas veronii*	PQ097982.1	99.82	N-fixing (Haque *et al*., 2020) P-solubilizing (Haque *et al*., 2020)
	Pseudomonas frederiksbergensis	MT534275.1	99.56	N-fixing (Bashizi *et al*., 2023) P-solubilizing (Zeng *et al*., 2016,2019)
	Pseudomonas gessardii	PQ097242.1	98.84	N-fixing (Devi *et al*., 2022) P-solubilizing (Devi *et al*., 2022)
	Pseudomonas syringae	MT579859.1	99.82	N-fixing (Parangan-Smith and Lind, 2013)
	Pseudomonas marginalis	PP725249.1	100	P-solubilizing (Germán *et al*., 2024; Shahid, 2013)

Table 3. 12: Soil microorganisms from *E. ghellinckii* rhizosphere soils in Umgano

Family	Scientific name	Accession number	Similarity %	Function
Enterobacteriaceae	*Pantoea agglomerans*	MG733944.1	93.88	N-fixing (Loiret *et al*., 2004) P-solubilizing (Son *et al*., 2006)
Erythrobacteraceae	*Novosphingobium resinovorum*	JQ073780.1	78.57	P-solubilizing (Zhang *et al*., 2024)
Bacillaceae	*Bacillus cereus*	MN243659.1	81.33	N-fixation (Narayanan *et al*., 2022) P-solubilizing (Narayanan *et al*., 2022)
Burkholderiaceae	*Burkholderia stabilis*	PP815020.1	100	N-fixating (Kim *et al*., 2020) P-solubilizing (Kim *et al*., 2020)
	Burkholderia pyrrocinia	CP150851.1	99.02	N-fixation (Lopes *et al*., 2018) P- solubilizing (Madhaiyan *et al*., 2010)
	Burkholderia cepacia	OQ048294.1	92.06	N-fixing (Li *et al*., 2022) P-solubilizing (Song *et al*., 2008)
Lysobacteraceae	*Stenotrophomonas maltophilia*	MZ414198.1	79.22	N-fixation (Alexander *et al*., 2019) P-solubilization (Sharma *et al*., 2024)
	Stenotrophomonas geniculata	OM761072.1	78.11	N-fixing (Ercole *et al*., 2024) P-solubilizing (Ercole *et al*., 2024)
	Stenotrophomonas rhizophila	MT828550.1	77.25	N-fixing (Vijayalakshmi and Senthilkumar, 2023) N-cycling (Vijayalakshmi and Senthilkumar, 2023) P-solubilizing (Vijayalakshmi and Senthilkumar, 2023)

Pseudomonadaceae	*Pseudomonas mohnii*	MT631988.1	100	P-solubilizing (Amora-Lazcano *et al*., 2022)
	Pseudomonas putida	PQ097695.1	100	N-fixing (Shabayev, 2010) N-cycling (Huang *et al*., 2022) P-solubilizing (Rosas *et al*., 2006)
	Pseudomonas parafulva	MT367815.1	100	N-fixing (Shabayev, 2010) N- cycling (Huang *et al*., 2022) P-solubilizing (Rosas *et al*., 2006)
	Pseudomonas frederiksbergensis	MT078678.1	100	N-fixing (Bashizi *et al*., 2023) P-solubilizng (Zeng *et al*., 2016,2019)
	Pseudomonas rhodesiae	MG571697.1	100	P- solubilizing (Rolón-Cárdenas *et al*., 2021)

Table 3. 13: Soil microorganisms from *E. ghellinckii* non-rhizosphere soils in Umgano

Family	Scientific name	Accession number	Similarity %	Function
Yersiniaceae	*Serratia marcescens*	MH424891.1	87.85	N-fixing (Dong *et al*., 2024) P-solubilizing (Dong *et al*., 2024)
Bacillaceae	*Bacillus wiedmannii*	PQ057037.1	99.59	N-fixing (de Araújo *et al*., 2020) P-solubilizing (Torres *et al*., 2024)
	Bacillus cereus	PQ047533.1	100	N-fixing (Narayanan *et al*., 2022) P-solubilizing (Narayanan *et al*., 2022)
Burkholderiaceae	*Paraburkholderia caribensis*	MK905509.1	99.49	P-solubilizing (Leite *et al*., 2021)
	Paraburkholderia terrae	MG457692.1	99.72	N-fixing (Yang *et al*., 2006) P-solubilizing (Yang *et al*., 2006)
	Paraburkholderia caribensis	MK905509.1	99.60	P-solubilizing (Leite *et al*., 2021)
	Burkholderia stabilis	PP900092.1	100	N-fixing (Kim *et al*., 2020) P-solubilizing (Kim *et al*., 2020)
	Burkholderia pyrrocinia	CP024904.1	99.07	N-fixing (Lopes *et al*., 2018; Lu *et al*., 2017) P- solubilizing (Madhaiyan *et al*., 2010)
Enterobacteriaceae	*Morganella morganii*	MK354010.1	87.85	N-fixing (Naqqash *et al*., 2024) P-solubilizing (Naqqash *et al*., 2024)
	Kluyvera intermedia	CP162029.1	93.70	P-solubilizing (Pallavi and Gupta, 2013; Pratiwi *et al*., 2020)

	Citrobacter freundii	PP989673.1	93.70	N-fixing (French *et al*., 1976)
	Lelliottia amnigena	OR821474.1	92.78	N-fixing (Thuakur *et al*., 2024) P-solubilizing (Menéndez *et al*., 2016)
	Klebsiella michiganensis	OR831380.1	92.78	N-fixing (Mitra *et al*., 2018) P-solubilizing (Mitra *et al*., 2018)
Pseudomonadaceae	*Pseudomonas marginalis*	LC409071.1	99.80	P-solubilizing (Germán *et al*., 2024; Shahid, 2013)
	Pseudomonas vancouverensis	MT322943.1	100	P-solubilizing (Samaddar *et al*., 2019)
	Pseudomonas rhizophila	PQ056001.1	99.49	N-fixing (Hassen *et al*., 2018) P-solubilizing (Hassen *et al*., 2018)
	Pseudomonas monteilii	PQ115154.1	100	N-fixing (Li *et al*., 2024) P-solubilizing (Li *et al*., 2024)
	Pseudomonas lini	MK920122.1	99.34	N-fixing (Quadriya *et al*., 2024) P-solubilizing (Barbaccia *et al*., 2022)
	Pseudomonas putida	PQ097695.1	97.17	N-fixing (Shabayev, 2010) N- cycling (Huang *et al*., 2022) P-solubilizing (Rosas *et al*., 2006)
	Pseudomonas prosekii	MG571651.1	100	N-fixing (Puri *et al*., 2020) P-solubilizing (Yu *et al*., 2019)
	Pseudomonas helvetica	PQ037652.1	98.80	N-cycling (Poli *et al*., 2024)

	Pseudomonas tolaasii	PP386802.1	83.73	N-fixing (Mbonambi *et al*., 2024) P-solubilizing (Mbonambu *et al*., 2024)
	Pseudomonas fluorescens	KC191582.1	87.01	N-fixing (Wu *et al*., 2023) P-solubilizing (Branch, 2012)
	Pseudomonas azotoformans	PP843790.1	83.03	N-fixing (Castanheira *et al*., 2014) P-solubilizing (Castanheira *et al*., 2014)

3.3.4 Extracellular enzyme activities from *E.ghellinckii* rhizosphere and non-rhizosphere soils from Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano

Nitrate reductase activity at Cathedral Peak was higher in non-rhizosphere soil (11831.1 ± 2903.7) than in rhizosphere soil (8664.4 ± 3099.0), although the difference was not statistically significant ($p = 0.999$; Table 3.14). At Thabankulu, rhizosphere soils exhibited greater activity (14135.0 ± 1454.8) than non-rhizosphere soils (10230.0 ± 6012.2), but the difference was also not significant ($p = 0.999$; Table 3.14). At Oribi Gorge, activity was slightly higher in rhizosphere soil (14612.7 ± 4745.7) than in non-rhizosphere soil (14093.3 ± 5029.3), with no significant difference ($p = 1.000$; Table 3.14). Similarly, at Umgano, non-rhizosphere soil showed slightly higher activity (17896.7 ± 8745.4) than rhizosphere soil (17358.9 ± 6225.2), but the difference was not statistically significant ($p = 1.000$; Table 3.14). No significant differences were observed among the study sites (all $p > 0.05$; Table 3.14).

For β-glucosidase activity, significant differences were observed between the rhizosphere soils of Oribi Gorge and those from Cathedral Peak non-rhizosphere ($p = 0.033$) and Thabankulu rhizosphere ($p = 0.039$; Table 3.14). At Oribi Gorge, rhizosphere soil exhibited higher activity (4.0 ± 0.6) compared to non-rhizosphere soil (1.7 ± 0.4), although this difference was not statistically significant ($p = 0.150$; Table 3.14). At Cathedral Peak and Thabankulu, β-glucosidase activity did not differ significantly between rhizosphere and non-rhizosphere soils (all $p > 0.05$). Similarly, at Umgano, both soil types showed comparable activity levels (rhizosphere: 3.0 ± 0.6; non-rhizosphere: 2.1 ± 0.6), with no significant difference ($p = 0.918$; Table 3.14).

Alkaline phosphatase activity was higher in non-rhizosphere soil than in rhizosphere soil at Cathedral Peak (3.2 ± 0.6 vs. 1.2 ± 0.4), although the difference was not statistically significant ($p = 0.170$; Table 3.14). A similar pattern was observed at Oribi Gorge, where non-rhizosphere soils also showed elevated activity (2.7 ± 0.5) compared to rhizosphere soils (1.7 ± 0.7), with no significant difference ($p = 0.963$; Table 3.14). When comparing rhizosphere soils across all study sites, no statistically significant variation was found (all $p > 0.05$; Table 3.14).

Acid phosphatase activity followed a similar trend of non-significant differences. At Cathedral Peak, rhizosphere soil exhibited slightly higher activity (2.6 ± 0.6) than non-rhizosphere soil (2.0 ± 0.4; $p = 0.994$; Table 3.14). At Thabankulu, rhizosphere and non-rhizosphere soils displayed similar activities (2.6 ± 0.7 vs. 2.5 ± 0.5; $p = 1.000$). At Umgano, non-rhizosphere

soil showed higher activity (3.0 ± 0.8) compared to rhizosphere soil (2.5 ± 0.5), although the difference was not significant ($p = 0.999$; Table 3.14). At Oribi Gorge, rhizosphere soil exhibited greater activity (2.5 ± 0.6) than non-rhizosphere soil (1.6 ± 0.3), but this was also not statistically significant ($p = 0.980$). No significant differences were detected among sites (all $p > 0.05$; Table 3.14).

Table 3.14: Extracellular enzyme activities (mean ± SE) in *Encephalartos ghellinckii* rhizosphere and non-rhizosphere soils collected from Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), and Umgano (UM), Eastern Cape and KwaZulu-Natal. Different uppercase letters indicate significant differences between sites ($p < 0.05$); difference lowercase letters indicate significant differences between rhizosphere and non-rhizosphere soils within a site ($p < 0.05$).

Enzyme activity (µmol·g⁻¹·h⁻¹)	**Cathedral Peak**		**Thabankulu**		**Oribi Gorge**		**Umgano**	
	Rhizosphere	Non-rhizosphere	Rhizosphere	Non-rhizosphere	Rhizosphere	Non-rhizosphere	Rhizosphere	Non-rhizosphere
Nitrate reductase (mean ± SE)	8664.4±3099.0 ^{Aa}	11831.1±2903.7^{Aa}	14135.0±1454.8 ^{Aa}	10230.0±6012.23 ^{Aa}	14612.67±4745.66 ^{Aa}	14093.3±5029.3 ^{Aa}	17358.9±6225.2 ^{Aa}	17896.7±8745.4 ^{Aa}
Glucosidase (mean ± SE)	1.8±0.3^{ABa}	1.4±0.5 ^{Ba}	1.7± 0.5^{Ba}	2.5±0.7^{ABa}	4.02±0.60^{Aa}	1.7±0.4 ^{ABa}	3.1±0.6 ^{ABa}	2.1±0.6^{ABa}
Alkaline phosphatase (mean ± SE)	1.2±0.4^{Aa}	3.2±0.6^{Aa}	2.4±0.6^{Aa}	1.8±0.5 ^{Aa}	1.7±0.7 ^{Aa}	2.7±0.5 ^{Aa}	1.5±0.5 ^{Aa}	1.6±0.4 ^{Aa}
Acid phosphatase (mean ± SE)	2.6±0.6 ^{Aa}	2.0±0.4 ^{Aa}	2.6±0.7 ^{Aa}	2.5±0.5 ^{Aa}	2.5±0.6^{Aa}	1.6±0.3 ^{Aa}	2.5±0.5^{Aa}	3.0±0.6 ^{Aa}

3.3.5 Linear correlations between soil N and P concentrations and the activities of nitrate reductase, acid phosphatase, and alkaline phosphatase in Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano

For Cathedral Peak soils, nitrate reductase activity showed positive correlations with soil N concentrations in both rhizosphere (Figure 3.5A) and non-rhizosphere soils (Figure 3.5B). Additionally, acid phosphatase activity exhibited a negative correlation with soil P concentrations in both rhizosphere (Figure 3.6A) and non-rhizosphere soils (Figure 3.6B). Alkaline phosphatase activity indicated a negative correlation with P concentrations in rhizosphere soils (Figure 3.7A) and a positive correlation in non-rhizosphere soils (Figure 3.7B).

For Thabankulu soils, positive correlations were observed between nitrate reductase activity and soil N concentrations in both rhizosphere (Figure 3.5C) and non-rhizosphere soils (Figure 3.5D). The correlation in the rhizosphere was weak, while in the non-rhizosphere soils, it was stronger. Acid phosphatase activity showed a negative correlation with soil P concentrations, which was stronger in rhizosphere soils (Figure 3.6C) compared to non-rhizosphere soils (Figure 3.6D). Similarly, a negative correlation was found between alkaline phosphatase activity and soil P concentrations in both rhizosphere (Figure 3.7C) and non-rhizosphere soils (Figure 3.7D).

For Oribi Gorge soils, nitrate reductase activity showed strong positive correlations with soil N concentrations in both rhizosphere (Figure 3.5E) and non-rhizosphere soils (Figure 3.5F). Acid phosphatase activity exhibited a negative correlation with soil P concentrations in both rhizosphere (Figure 3.6E) and non-rhizosphere soils (Figure 3.6F). Similarly, alkaline phosphatase activity showed a negative correlation with soil P concentrations in the rhizosphere (Figure 3.7E) and a positive correlation in non-rhizosphere soils (Figure 3.7D).

For Umgano soils, nitrate reductase activity exhibited positive correlations with soil N concentrations in both rhizosphere (Figure 3.5G) and non-rhizosphere soils (Figure 3.5H). Acid phosphatase activity showed a negative correlation with soil P concentrations in both rhizosphere (Figure 3.6G) and non-rhizosphere soils (Figure 3.6H). Alkaline phosphatase activity displayed a positive correlation with soil P concentrations in both rhizosphere (Figure 3.7G) and non-rhizosphere soils (Figure 3.7H).

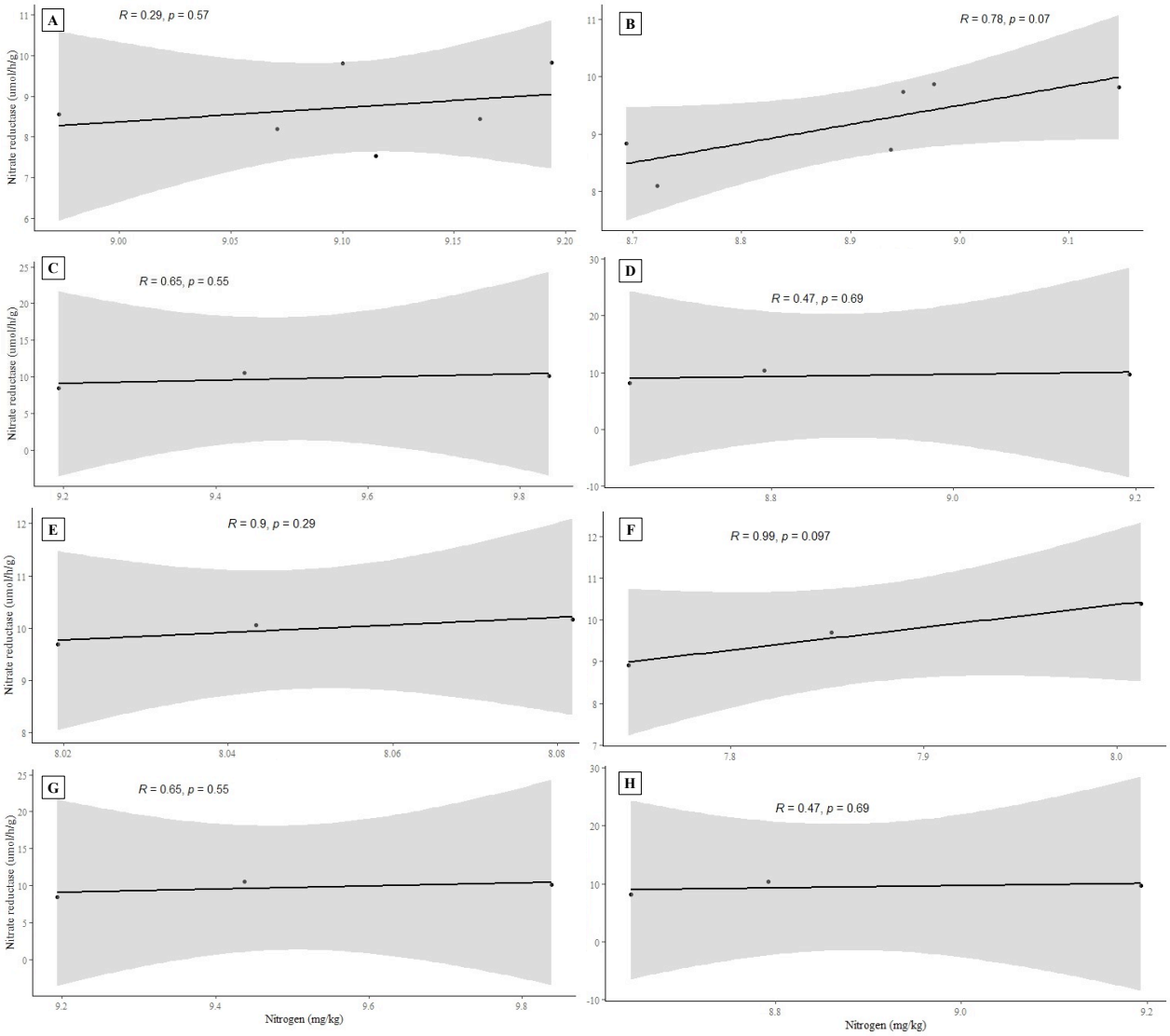

Figure 3. 5: A linear correlation graph showing the relationship between nitrate reductase activity and soil N concentrations in Cathedral Peak: (A) rhizosphere soil and (B) non-rhizosphere soil; Thabankulu: (C) rhizosphere soil and (D) non-rhizosphere soil; Oribi Gorge: (E) rhizosphere soil and (F) non-rhizosphere soil; Umgano: (G) rhizosphere soil and (H) non-rhizosphere soil.

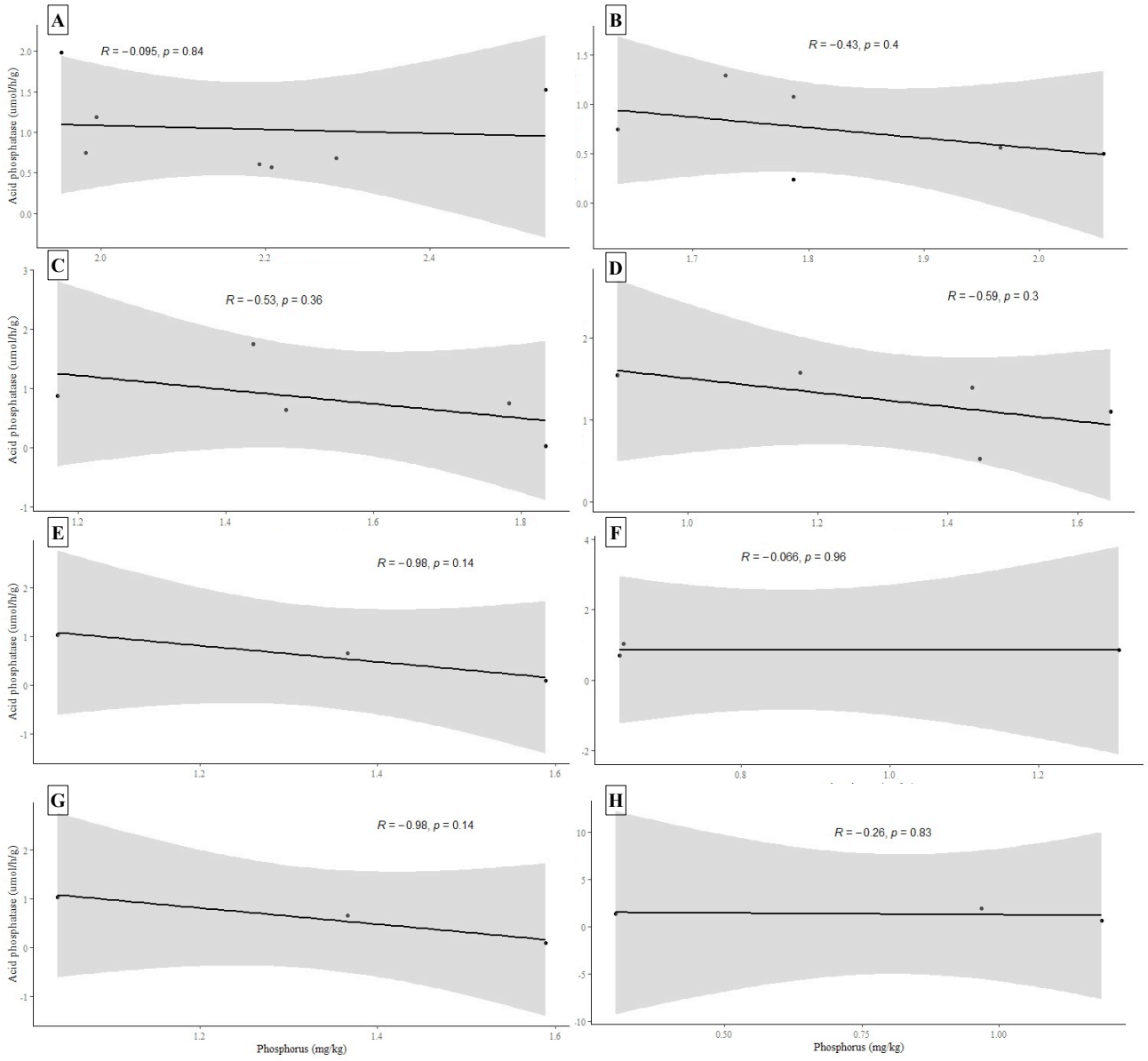

Figure 3. 6: A linear correlation graph showing the relationship between acid phosphatase activity and soil P concentrations at Cathedral Peak: (A) rhizosphere soil and (B) non-rhizosphere soil, Thabankulu: (C) rhizosphere soil and (D) non-rhizosphere soil, Oribi Gorge: (E) rhizosphere soil and (F) non-rhizosphere soil, Umgano: (G) rhizosphere soil and (H) non-rhizosphere soil.

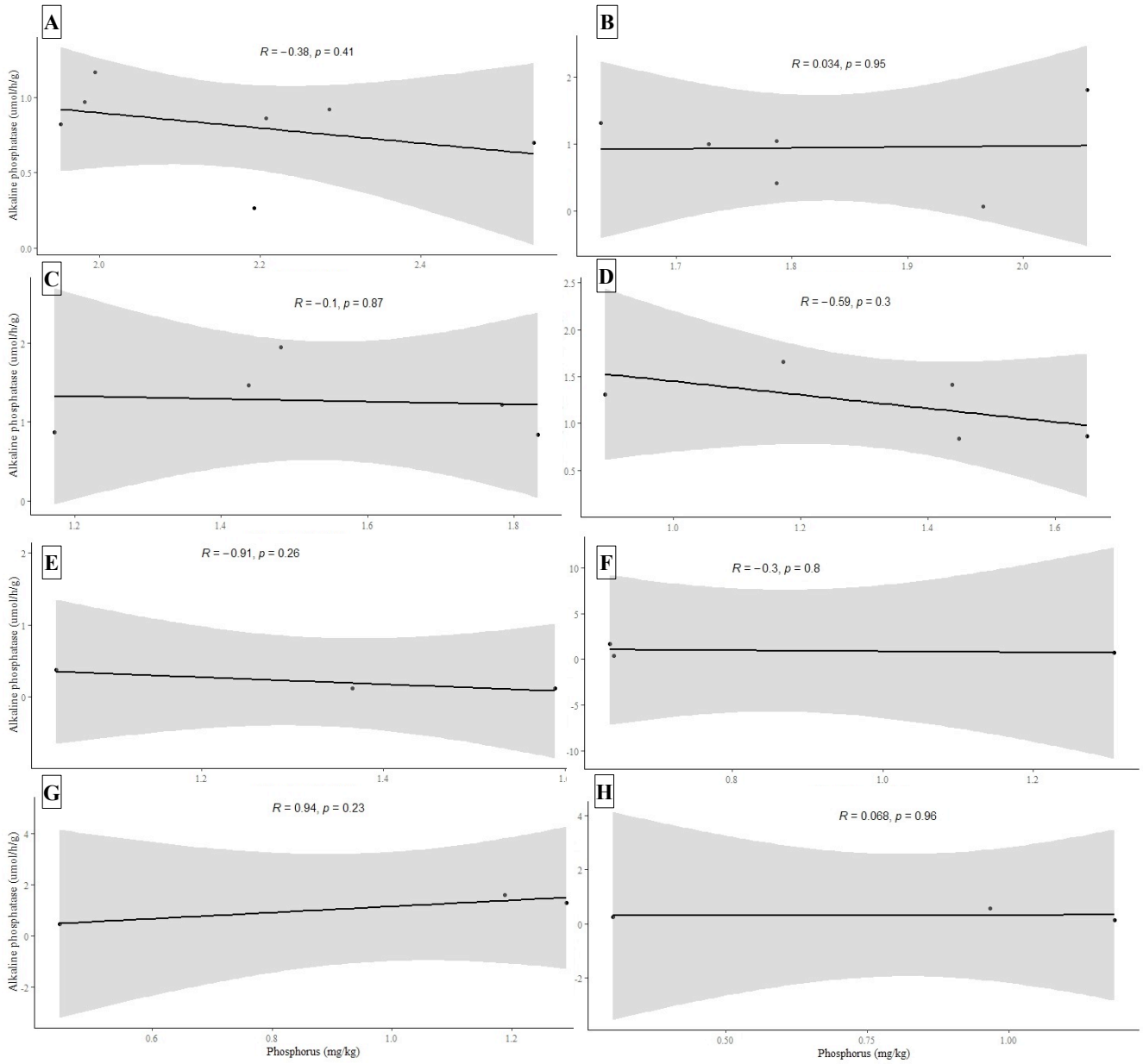

Figure 3. 7: A linear correlation graph showing the relationship between alkaline phosphatase activity and soil P concentrations at Cathedral Peak: (A) rhizosphere soil and (B) non-rhizosphere soil, Thabankulu: (C) rhizosphere soil and (D) non-rhizosphere soil, Oribi Gorge: (E) rhizosphere soil and (F) non-rhizosphere soil, Umgano: (G) rhizosphere soil and (H) non-rhizosphere soil.

3.4 Discussion

This study investigated the role of cycad-microbe symbiosis, soil microorganisms, and associated extracellular enzyme activities in making nutrients available for *E. ghellinckii* in nutrient-deprived grassland soils. The findings of this study revealed that the coralloid roots of

E. ghellinckii across the four study sites harboured predominantly N-fixing and P-solubilizing bacteria. The culturable bacterial strains identified from the coralloid roots of *E. ghellinckii* growing in Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano belonged to the genera *Acinetobacter*, *Brevibacillus*, *Burkholderia*, *Paraburkholderia, Cereibacter, Caballeronia*, *Trinickia*, and *Enterobacter*. These microbes have previously been associated with cycad species (Gutiérrez-García *et al*., 2019; Zheng *et al*., 2021) and legumes (Martínez-Hidalgo and Hirsch, 2017). They are known to have plant growth-promoting (PGP) traits (Rai *et al*., 2019; Zheng *et al*., 2021; Napitupulu *et al*., 2023), which are essential for plants such as *E. ghellinckii* to survive in nutrient-deprived acidic soils under harsh climatic conditions.

Microbes associated with *E. ghellinckii* exhibit a diverse range of plant growth-promoting traits that are essential for the plant's survival in nutrient-deprived grassland ecosystems. These microbes not only fix N and solubilize P, but also play a key role in nutrient cycling and enhancing stress tolerance, thereby supporting plant growth in nutrient-deficient and harsh environmental conditions. For instance, *Paraburkholderia fungorum*, *Klebsiella variicola*, and *Kosakonia oryzae*, isolated in the coralloid roots and rhizospheric soil of *E. ghellinckii* are known to solubilize potassium (K) and produce phytohormones such as indole-3-acetic acid (IAA), which enhance nutrient acquisition and promote plant growth (Rahman et al., 2018; Yang et al., 2020). Similarly, other species such as *Enterobacter ludwigii* produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase and gibberellic acid, which reduce stress-induced ethylene levels and enhance plant resilience in nutrient-poor environments (Lee et al., 2019). Other species, such as *Brevibacillus reuszeri*, produce enzymes like catalase, which protect plants from oxidative damage under environmental stress (Yildirim *et al*., 2011).

The multifunctional roles of these microbes underscore their broader ecological importance. Beyond their contribution to nutrient cycling, these microbial traits enable *E. ghellinckii* to thrive in its native grassland ecosystem, where nutrient availability is limited, and environmental stressors are common. Through the solubilization of potassium and silicate weathering, these microbes facilitate access to otherwise unavailable nutrients in acidic soils (Singh *et al*., 2018). Their ability to produce stress-mitigating enzymes and phytohormones further enhances plant growth and resilience, supporting *E. ghellinckii*'s survival and overall fitness in these marginal environments. This aligns with previous studies that highlight the essential, synergistic roles of cycad-associated microbes in promoting nutrient cycling, plant growth, and stress tolerance in nutrient-limited ecosystems (Gutiérrez-García *et al*., 2019).

Interestingly, cyanobacteria were not identified among the bacterial strains isolated from the coralloid roots of *E. ghellinckii* across all four study sites. This finding contrasts with previous studies reporting cyanobacteria as the dominant group in coralloid roots (Gutiérrez-García *et al*. 2019; Liu *et al*. 2023; Zheng *et al*. 2018). However, Zheng *et al*. (2021) reported that in the coralloid roots of *Cycas dolichophylla*, cyanobacteria co-dominated with Proteobacteria. Similarly, Suarez-Moo *et al*. (2019) reported that N-fixing bacteria from various phyla, including Actinobacteria, Bacteroidetes, Cyanobacteria, and Proteobacteria, were evenly distributed in four species of *Dioon*, while cyanobacteria dominated in three other *Dioon* species. Suarez-Moo *et al*. (2019) partially contested earlier studies that suggested cyanobacteria were the dominant endophytes in coralloid roots due to their ability to inhibit the growth of other microbes through secondary metabolites. They proposed that the age of coralloid roots might significantly influence cyanobacterial dominance, with older roots supporting larger populations of cyanobacteria. Their findings imply that the absence of cyanobacteria in the coralloid roots of *E. ghellinckii* in our study could likely be related to the collection of younger roots, as root age was not considered during sampling.

Furthermore, the microbial composition of the surrounding soil plays a crucial role in shaping the root microbiome (Adeleke *et al*. 2021, 2024; Shi *et al*. 2016). The absence of cyanobacteria in the root endosphere of *E. ghellinckii* may thus reflect their absence in the surrounding soil. Jacoby *et al*. (2017) and Zhang *et al*. (2019) noted that plant roots release exudates that attract a range of microorganisms to the rhizosphere; however, only specific microbes, selected based on their functional traits, are able to colonize the root endosphere (Suarez-Moo *et al*. 2019; Adeleke *et al*. 2024). Consequently, while cyanobacteria were absent in the soils around *E. ghellinckii* at all four study sites, the plant selectively attracted other N-fixing microorganisms into its endosphere. This highlights the unique adaptations and symbiotic relationship between the coralloid roots and beneficial N-fixing bacteria, a phenomenon also observed in other cycad species.

Microbial diversity in both rhizosphere and non-rhizosphere soils showed minimal variation across the four study sites, likely due to similar environmental conditions. Environmental factors and root exudates shape microbial communities (Coyne and Mikkelsen, 2017; Jacoby *et al*., 2017), which may explain the observed similarities in microbial composition. Since all *E. ghellinckii* plants were located in grassland areas, they likely release comparable exudates in response to similar environmental stimuli. Soil samples from Cathedral Peak, Thabankulu,

Oribi Gorge, and Umgano were all acidic, with pH values ranging from 4.09 to 5.61. Acidic soils typically exhibit low cation-exchange capacities, limiting the availability of essential nutrients such as calcium (Ca^{2+}), potassium (K^{+}), and ammonium (NH_4^{+}) (Aprile and Lorandi, 2012; Zungu *et al*., 2020). Under these nutrient-limited conditions, soil microorganisms play a critical role in promoting nutrient cycling and mineralization, enhancing nutrient availability for plant uptake (Coyne and Mikkelsen, 2015; Martínez-Hidalgo and Hirsch, 2017).

Microorganisms isolated from both the *E. ghellinckii* rhizosphere and non-rhizosphere soils across the four study sites included symbiotic and free-living species with known plant growth-promoting traits (Hayat *et al*., 2010). These microorganisms support plant survival through a range of mechanisms, such as solubilizing P and K, N fixing and cycling, producing phytohormones, improving soil structure and health, generating siderophores to combat pathogens, and secreting extracellular enzymes (Hayat *et al*., 2010; Lapsansky *et al*., 2016; Jacoby *et al*., 2017; Esmaeel *et al*., 2018; Chialva *et al*., 2018; Hubbard *et al*., 2019; Berendsen *et al*., 2021; Adeleke *et al*., 2024).

Among these mechanisms, the secretion and activity of extracellular enzymes are crucial for nutrient cycling and availability in soils. The production of extracellular enzymes by microbes is a significant investment and serves as an important indicator of nutrient availability through soil cycling processes (Dick, 1994; DeForest *et al*., 2012; De Almeida *et al*., 2015; Zheng *et al*., 2015). These enzymes break down complex organic compounds into simpler molecules, making them accessible for plant absorption and usable as energy sources by microorganisms (Allison *et al*., 2007; Lucas *et al*., 2008; De Almeida *et al*., 2015). Since extracellular enzymes are typically present in low concentrations, evaluating their activity, rather than their quantity, is crucial for assessing their role in nutrient cycling (De Almeida *et al*., 2015).

Beta glucosidase activity highlights the ongoing cycling of organic matter, signaling carbon availability in both the rhizosphere and non-rhizosphere soils of *E. ghellinckii* across the study sites (Table 3.1). Cycads, known for their role in carbon sequestration, contribute to the persistence of plant species in nutrient-poor soils by capturing atmospheric carbon (Ma *et al*., 2009; Álvarez-Yépiz and Dovčiak, 2015). This process not only enhances soil carbon levels but also supports symbiotic microbial associations by increasing carbon availability, which can attract beneficial microbes to the rhizosphere.

Phosphatase enzyme activities are among the most studied soil enzymes due to their essential role in hydrolyzing ester-phosphate bonds, thereby releasing inorganic phosphate that plants can readily absorb (Nannipieri *et al*., 2011). These enzymes are particularly important for plants like *E. ghellinckii*, which thrive in acidic soils, often characterized by high concentrations of cations such as aluminum (Al) and iron (Fe) (Oburger *et al*., 2011; Magadlela *et al*., 2022). These cations can form insoluble complexes with P, reducing their availability for plant uptake (Penn and Camberato, 2019; Lacave *et al*., 2021). Since P is a crucial nutrient for plant growth and development (Fisher and Jayachandran, 2008), soil microorganisms secrete both acid and alkaline phosphatases to solubilize bound P, making it available for plant assimilation (Dick *et al*., 2000; Pant and Warman, 2000; Margalef *et al*., 2021).

Acid and alkaline phosphatase activities showed no significant variation between rhizosphere and non-rhizosphere soils across all four study sites, indicating that similar microbial communities may drive enzyme activity in both environments. The negative correlation observed between acid phosphatase activity and soil P concentrations across all sites indicates that enzyme activity increases when P availability is low. This pattern aligns with the findings of Burns *et al*. (2013), who suggested that enhanced enzyme activity is a physiological response to nutrient deficiency. Similarly, Olander and Vitousek (2000) and Treseder and Vitousek (2001) reported negative correlations between phosphatase activity and inorganic P availability, reinforcing the notion that enzyme activity is upregulated in response to P scarcity.

In contrast, Margalef et al. (2017) and Sekaran et al. (2019) documented positive correlations between soil phosphorus concentrations and acid phosphatase activity, as well as between alkaline phosphatase activity and available phosphorus levels in the soil. This discrepancy highlights the complex relationship between enzyme activity and nutrient availability. In our study, the correlations for alkaline phosphatase activity varied across sites, despite similarities in microbial communities. At Cathedral Peak, a negative correlation was observed in rhizosphere soil, whereas a positive correlation was found in non-rhizosphere soil. Both Thabankulu and Oribi Gorge showed negative correlations in both soil types, whil-e Umgano exhibited positive correlations.

These contrasting patterns suggest that site-specific factors, such as soil composition and plant-root interactions, may significantly influence alkaline phosphatase responses to P availability (Hunter *et al*. 2014). Increased enzyme activity could indicate either enhanced nutrient availability under certain conditions or a response to nutrient deficiency. While some studies

report positive correlations with nutrient availability, our findings highlight that enzyme activity is a multifaceted response to soil P dynamics, underscoring the complex relationship between enzyme activity and nutrient availability in ecosystems.

Among the extracellular enzyme activities measured, nitrate reductase activity was the highest across all sites, corresponding to the elevated total N concentrations compared to other nutrients. Nitrate reductase plays a crucial role in converting nitrate (NO_3^-) to nitrite (NO_2^-), marking the initial step in incorporating nitrate into organic N compounds, such as amino acids and proteins, which are essential for plant growth and development (Abdelmagid and Tabatabai 1987; Campbell 1988; Singh and Kumar 2008). A positive correlation between nitrate reductase activity and soil N levels was observed at all study sites. This correlation indicates that increased nitrate availability stimulates enzyme activity, reflecting its role in facilitating the conversion of nitrate into plant-usable N forms, critical for the growth of *E. ghellinckii*.

3.5 Conclusion

This study demonstrated that *E. ghellinckii* thrives in acidic, nutrient-poor grassland soils by forming symbiotic relationships with beneficial microbes in its coralloid roots and surrounding soils, both in the rhizosphere and non-rhizosphere. These microbial communities drive essential processes such as P solubilization, N fixation, and N cycling, enhancing nutrient availability under challenging environmental conditions. Additionally, extracellular enzymes secreted by soil microbes, including acid phosphatase, alkaline phosphatase, nitrate reductase, and β-glucosidase, played a critical role in nutrient mineralization, converting unavailable forms into accessible nutrients for plant uptake. The increased nutrient bioavailability facilitated by these microbial processes supported the growth and survival of *E. ghellinckii* and contributed to improved soil health and ecosystem function in grasslands. Moreover, as highlighted by Halliday and Pate (1976), N fixation in cycads benefit both the plant and the surrounding vegetation, underscoring the ecological significance of these symbiotic relationships. Consequently, *E. ghellinckii* play a vital ecological role, reinforcing the need for its conservation to maintain biodiversity and ecosystem function in its natural habitat.

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Appendix

Table 3.15: Pairwise p-values from ANOVA for nitrate reductase activity between rhizosphere (R) and non-rhizosphere (Non-R) soils across sites. Site abbreviations: CP = Cathedral Peak, TB = Thabankulu, UM = Umgano, OR = Oribi Gorge.

Site	CP R	CP Non-R	TB R	TB Non-R	OR R	OR Non-R	UM R	UM Non-R
CP R	–	0.99	0.99	0.99	0.97	0.98	0.92	0.89
CP Non-R	0.99	–	0.99	0.99	0.99	0.99	0.99	0.98
TB R	0.98	0.10	–	0.10	1.00	1.00	0.10	0.10
TB Non-R	0.10	0.10	0.10	–	0.10	0.10	0.98	0.96
OR R	0.97	0.10	1.00	0.10	–	1.00	0.10	0.10
OR Non-R	0.98	0.10	1.00	0.10	1.00	–	0.10	0.10
UM R	0.92	0.10	0.10	0.98	0.10	0.10	–	1.00
UM Non-R	0.89	0.99	0.10	0.96	0.10	0.10	1.00	–

Table 3.16: Pairwise p-values from ANOVA for acid phosphatase activity between rhizosphere (R) and non-rhizosphere (Non-R) soils across sites. Site abbreviations: CP = Cathedral Peak, TB = Thabankulu, UM = Umgano, OR = Oribi Gorge.

Site	CP R	CP Non-R	TB R	TB Non-R	UM R	UM Non-R	OR R	OR Non-R
CP R	–	0.99	1.00	1.00	1.00	1.00	1.00	0.95
CP Non-R	0.99	–	1.00	1.00	1.00	0.87	1.00	1.00
TB R	1.00	1.00	–	1.00	1.00	1.00	1.00	0.96
TB Non-R	1.00	1.00	1.00	–	1.00	1.00	1.00	0.98
UM R	1.00	1.00	1.00	1.00	–	1.00	1.00	0.96
UM Non-R	1.00	0.87	1.00	1.00	1.00	–	1.00	0.75
OR R	1.00	1.00	1.00	1.00	1.00	1.00	–	0.98
OR Non-R	0.95	1.00	0.96	0.98	0.96	0.75	0.98	–

Table 3.17: Pairwise ANOVA p-values from Tukey's HSD post hoc test for β-glucosidase activity in rhizosphere (R) and non-rhizosphere (Non-R) soils across study sites. Site abbreviations: CP = Cathedral Peak, TB = Thabankulu, UM = Umgano, OR = Oribi Gorge.

Site	CP R	CP Non-R	TB R	TB Non-R	UM R	UM Non-R	OR R	OR Non-R
CP R	–	1.00	1.00	0.98	0.73	1.00	0.12	1.00
CP Non-R	1.00	–	1.00	0.82	0.39	0.98	0.03	1.00
TB R	1.00	1.00	–	0.92	0.50	1.00	0.04	1.00
TB Non-R	0.98	0.82	0.92	–	1.00	1.00	0.55	0.97
UM R	0.73	0.39	0.50	1.00	–	0.92	0.91	0.74
UM Non-R	1.00	0.98	1.00	1.00	0.92	–	0.24	1.00
OR R	0.12	0.03	0.04	0.55	0.91	0.24	–	0.15
OR Non-R	1.00	1.00	1.00	0.97	0.74	1.00	0.15	–

Table 3.18: Pairwise ANOVA p-values from Tukey's HSD post hoc test for P alkaline activity in rhizosphere (R) and non-rhizosphere (Non-R) soils across study sites. Site abbreviations: CP = Cathedral Peak, TB = Thabankulu, UM = Umgano, OR = Oribi Gorge.

Site	CP R	CP Non-R	TB R	TB Non-R	UM R	UM Non-R	OR R	OR Non-R
CP R	–	0.17	0.76	0.99	1.00	1.00	1.00	0.64
CP Non-R	0.17	–	0.93	0.50	0.25	0.29	0.68	1.00
TB R	0.76	0.93	–	0.99	0.90	0.93	0.99	1.00
TB Non-R	0.99	0.50	0.99	–	1.00	1.00	1.00	0.95
UM R	1.00	0.25	0.90	1.00	–	1.00	1.00	0.79
UM Non-R	1.00	0.29	0.93	1.00	1.00	–	1.00	0.84
OR R	1.00	0.68	0.99	1.00	1.00	1.00	–	0.96
OR Non-R	0.64	1.00	1.00	0.95	0.79	0.84	0.96	–

Table 3.19: Pairwise p-values from Tukey's test for phosphorus (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
Cathedral Peak R	-	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Cathedral Peak NR	0.01	-	0.67	0.10	0.25	0.01	0.02	0.01
Thabankulu R	0.00	0.67	-	0.95	0.98	0.29	0.47	0.25
Thabankulu NR	0.00	0.10	0.95	-	1.00	0.84	0.96	0.80
Oribi Gorge R	0.00	0.25	0.98	1.00	-	0.90	0.97	0.87
Oribi Gorge NR	0.00	0.01	0.29	0.84	0.90	-	1.00	1.00
Umgano R	0.00	0.02	0.47	0.96	0.97	1.00	-	1.00
Umgano NR	0.00	0.01	0.25	0.80	0.87	1.00	1.00	-

Table 3.20: Pairwise p-values from Tukey's test for Potassoum (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
Cathedral Peak R	-	0.00	0.00	0.00	0.03	0.00	0.24	0.01
Cathedral Peak NR	0.00	-	0.74	0.18	1.00	0.73	0.86	1.00
Thabankulu R	0.00	0.74	-	0.98	0.75	1.00	0.20	0.98
Thabankulu NR	0.00	0.18	0.98	-	0.27	1.00	0.04	0.62
Oribi Gorge R	0.03	1.00	0.75	0.27	-	0.72	0.98	1.00
Oribi Gorge NR	0.00	0.73	1.00	1.00	0.72	-	0.22	0.95
Umgano R	0.24	0.86	0.20	0.04	0.98	0.22	-	0.83
Umgano NR	0.01	1.00	0.98	0.62	1.00	0.95	0.83	-

Table 3.21: Pairwise p-values from Tukey's test for Calcium (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
Cathedral Peak R	-	0.96	0.27	0.44	0.44	0.82	0.99	1.00
Cathedral Peak NR	0.96	-	0.04	0.08	0.11	0.35	0.75	1.00
Thabankulu R	0.27	0.04	-	1.00	1.00	1.00	0.92	0.30
Thabankulu NR	0.44	0.08	1.00	-	1.00	1.00	0.98	0.45
Oribi Gorge R	0.44	0.11	1.00	1.00	-	1.00	0.95	0.42
Oribi Gorge NR	0.82	0.35	1.00	1.00	1.00	-	1.00	0.75
Umgano R	0.99	0.75	0.92	0.98	0.95	1.00	-	0.97
Umgano NR	1.00	1.00	0.30	0.45	0.42	0.75	0.97	-

Table 3.22: Pairwise p-values from Tukey's test for Magnesium (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	1.00	0.00	0.00	0.85	0.95	1.00	1.00
CP NR	1.00	-	0.00	0.00	0.75	0.89	1.00	1.00
TB R	0.00	0.00	-	0.95	0.00	0.00	0.00	0.00
TB NR	0.00	0.00	0.95	-	0.00	0.00	0.00	0.00
OR R	0.85	0.75	0.00	0.00	-	1.00	0.99	0.90
OR NR	0.95	0.89	0.00	0.00	1.00	-	1.00	0.97
UM R	1.00	1.00	0.00	0.00	0.99	1.00	-	1.00
UM NR	1.00	1.00	0.00	0.00	0.90	0.97	1.00	-

Table 3.23: Pairwise p-values from Tukey's test for zinc (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	0.95	0.79	0.99	0.94	0.48	1.00	0.99
CP NR	0.95	-	1.00	1.00	1.00	0.93	0.85	0.71
TB R	0.79	1.00	-	1.00	1.00	1.00	0.67	0.51
TB NR	0.99	1.00	1.00	-	1.00	0.92	0.92	0.81
OR R	0.94	1.00	1.00	1.00	-	0.99	0.84	0.71
OR NR	0.48	0.93	1.00	0.92	0.99	-	0.40	0.28
UM R	1.00	0.85	0.67	0.92	0.84	0.40	-	1.00
UM NR	0.99	0.71	0.51	0.81	0.71	0.28	1.00	-

Table 3.24: Pairwise p-values from Tukey's test for Magnanese (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	1.00	0.00	0.00	0.09	0.34	1.00	1.00
CP NR	1.00	-	0.00	0.00	0.06	0.25	1.00	1.00
TB R	0.00	0.00	-	1.00	0.00	0.00	0.00	0.00
TB NR	0.00	0.00	1.00	-	0.00	0.00	0.00	0.00
OR R	0.09	0.06	0.00	0.00	-	1.00	0.15	0.08
OR NR	0.34	0.25	0.00	0.00	1.00	-	0.43	0.27
UM R	1.00	1.00	0.00	0.00	0.15	0.43	-	1.00
UM NR	1.00	1.00	0.00	0.00	0.08	0.27	1.00	-

Table 3.25: Pairwise p-values from Tukey's test for Copper (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	1.00	0.40	0.32	0.88	1.00	0.21	0.35
CP NR	1.00	-	0.17	0.13	0.65	0.97	0.09	0.16
TB R	0.40	0.17	-	1.00	1.00	0.93	1.00	1.00
TB NR	0.32	0.13	1.00	-	1.00	0.89	1.00	1.00
OR R	0.88	0.65	1.00	1.00	-	1.00	0.96	0.99
OR NR	1.00	0.97	0.93	0.89	1.00	-	0.70	0.84
UM R	0.21	0.09	1.00	1.00	0.96	0.70	-	1.00
UM NR	0.35	0.16	1.00	1.00	0.99	0.84	1.00	-

Table 3.26: Pairwise p-values from Tukey's test for pH concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	1.00	0.21	0.15	0.81	0.94	1.00	1.00
CP NR	1.00	-	0.22	0.15	0.82	0.95	1.00	1.00
TB R	0.21	0.22	-	1.00	1.00	0.97	0.81	0.76
TB NR	0.15	0.15	1.00	-	0.99	0.94	0.73	0.68
OR R	0.81	0.82	1.00	0.99	-	1.00	1.00	0.99
OR NR	0.94	0.95	0.97	0.94	1.00	-	1.00	1.00
UM R	1.00	1.00	0.81	0.73	1.00	1.00	-	1.00
UM NR	1.00	1.00	0.76	0.68	0.99	1.00	1.00	-

Table 3.27: Pairwise p-values from Tukey's test for Exchange acidity (cmol/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	1.00	0.05	0.04	0.13	0.14	0.23	0.74
CP NR	1.00	-	0.08	0.06	0.17	0.18	0.18	0.65
TB R	0.05	0.08	-	1.00	1.00	1.00	0.00	0.01
TB NR	0.04	0.06	1.00	-	1.00	1.00	0.00	0.01
OR R	0.13	0.17	1.00	1.00	-	1.00	0.00	0.02
OR NR	0.14	0.18	1.00	1.00	1.00	-	0.00	0.02
UM R	0.23	0.18	0.00	0.00	0.00	0.00	-	0.99
UM NR	0.74	0.65	0.01	0.01	0.02	0.02	0.99	-

Table 3.28: Pairwise p-values from Tukey's test for Total cations (cmol/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	1.00	0.00	0.00	0.28	0.67	0.84	1.00
CP NR	1.00	-	0.00	0.00	0.19	0.53	0.71	1.00
TB R	0.00	0.00	-	0.99	0.01	0.00	0.00	0.00
TB NR	0.00	0.00	0.99	-	0.00	0.00	0.00	0.00
OR R	0.28	0.19	0.01	0.00	-	1.00	0.99	0.61
OR NR	0.67	0.53	0.00	0.00	1.00	-	1.00	0.90
UM R	0.84	0.71	0.00	0.00	0.99	1.00	-	0.97
UM NR	1.00	1.00	0.00	0.00	0.61	0.90	0.97	-

Table 3.29: Pairwise p-values from Tukey's test for Nitrogen (mg/kg) concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	0.03	0.01	0.00	0.00	0.00	0.88	0.25
CP NR	0.03	-	0.99	0.45	0.00	0.00	0.01	1.00
TB R	0.01	0.99	-	0.91	0.00	0.00	0.00	0.99
TB NR	0.00	0.45	0.91	-	0.00	0.00	0.00	0.57
OR R	0.00	0.00	0.00	0.00	-	1.00	0.00	0.00
OR NR	0.00	0.00	0.00	0.00	1.00	-	0.00	0.00
UM R	0.88	0.01	0.00	0.00	0.00	0.00	-	0.06
UM NR	0.25	1.00	0.99	0.57	0.00	0.00	0.06	-

Table 3.30: Pairwise p-values from Tukey's test for Organic Carbon concentrations between rhizosphere and non-rhizosphere soils across sites.Sites: Cathedral Peak (CP), Thabankulu (TB), Oribi Gorge (OR), Umgano (UM). Soil types: R = Rhizosphere, NR = Non-rhizosphere.

Site	CP R	CP NR	TB R	TB NR	OR R	OR NR	UM R	UM NR
CP R	-	1.00	1.00	1.00	0.00	0.00	1.00	1.00
CP NR	1.00	-	1.00	1.00	0.00	0.00	1.00	1.00
TB R	1.00	1.00	-	1.00	0.00	0.00	1.00	1.00
TB NR	1.00	1.00	1.00	-	0.00	0.00	1.00	1.00
OR R	0.00	0.00	0.00	0.00	-	1.00	0.00	0.00
OR NR	0.00	0.00	0.00	0.00	1.00	-	0.00	0.00
UM R	1.00	1.00	1.00	1.00	0.00	0.00	-	1.00
UM NR	1.00	1.00	1.00	1.00	0.00	0.00	1.00	-

CHAPTER 4: PRESENCE OF NITROGEN-FIXING BACTERIA PROMOTES THE DEPENDENCE OF *ENCEPHALARTOS GHELLINCKII* ON ATMOSPHERIC NITROGEN IN NUTRIENT-POOR GRASSLANDS

Abstract

Encephalartos ghellinckii thrives in nutrient-deficient, acidic grassland soils through symbiotic associations with N-fixing bacteria housed in its coralloid roots. The acquisition of atmospheric N is beneficial not only to the host plant but also to the surrounding vegetation. However, the specific N acquisition strategies of *E. ghellinckii*, and the N source reliance in nutrient-deficient grassland soils, remain poorly understood. The aim of this study was to investigate N source (atmospheric or soil) preference of *E. ghellinckii* growing in nutrient deficient grasslands ecosystems. Leaf material, soil samples, and coralloid roots were collected from four study sites: Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano. Nitrogen isotopic composition, total plant N, and P concentrations were analyzed in leaf material, while N utilization from both atmospheric and soil sources was assessed using $\delta^{15}N$ values. Soil characteristics were determined by analyzing soil nutrients, pH, relative acidity and cation exchange. Nitrogen-fixing and P-solubilizing microbes isolated from the coralloid roots of *E. ghellinckii* belonged to genera such as *Acinetobacter, Burkholderia, Klebsiella, Trinickia, Enterobacter,* and *Brevibacillus*. The $\delta^{15}N$ results indicated that more than 80% of the N in *E. ghellinckii* leaves was derived from atmospheric N. A positive correlation between atmospheric N fixation and soil P concentrations was observed across all sites, suggesting that P availability may enhance biological N fixation (BNF). The findings of this study highlight the critical role of *E. ghellinckii*'s symbiotic relationship with N-fixing bacteria in enabling its survival in nutrient-poor soils, emphasizing its adaptive strategy of relying on atmospheric N due to the limited availability of soil-derived N.

Keywords: *Encephalartos ghellinckii*, symbiosis, nitrogen-fixing, grasslands, atmospheric nitrogen

4.1 Introduction

Cycads are ancient seed plants, with fossil records dating back approximately 300 million years, making them one of the most primitive extant gymnosperm lineages (Mamay, 1969; Norstog and Nicholls, 1997). As the closest living relatives of all other seed plants, cycads provide crucial insights into the evolutionary history of terrestrial flora (Brenner *et al*., 2003; Hill *et al*., 2003; Nixon *et al*., 1994). Over millions of years, they have developed remarkable adaptations that enable their survival in diverse and often challenging climatic and geological conditions (Brenner *et al*., 2003; Kipp *et al*., 2024). These adaptations have allowed cycads to persist in nutrient-deficient ecosystems, where their ability to cycle nutrients, sequestrate carbon, and fix atmospheric nitrogen (N) highlights their significant ecological roles (Ma *et al*., 2009; Chang *et al*., 2019; Raven, 2002; Zheng *et al*., 2018).

Despite their crucial ecological functions, cycads are among the most threatened plant groups globally, with over 70% of the more than 300 known species classified as endangered (Mankga and Yessoufou, 2017; Yessoufou *et al*., 2017; International Union for Conservation of Nature (IUCN), 2022). Habitat destruction is considered one of the primary drivers of cycad population decline (Mankga and Yessoufou, 2017) and has been shown to negatively impact soil fertility and plant assemblages (Donaldson, 2003; Mayfield and Daily, 2005; Fischer and Lindenmayer, 2007; Ohsowski *et al*., 2012). This has raised significant conservation concerns, as the essential ecosystem services provided by cycads may be lost without effective conservation efforts.

Among them is *Encephalartos ghellinckii*, a South African species uniquely adapted to the harsh conditions of acidic, nutrient-deficient grasslands, where low soil fertility, high acidity, and periodic disturbances such as fire are common (Giddy, 1974; Nandwa, 2001; Donaldson, 2003, 2008). It thrives on steep, rocky outcrops and withstands climatic extremes, from heavy snowfall and frost and snow in winter, to mild and hot summers (Giddy, 1974; Donaldson, 2008). The species is frequently exposed to fire, a management tool widely used in grasslands, which is also believed to stimulate its cone and leaf development (Giddy, 1974; Whitelock, 2002; Carbutt and Kirkman, 2022). However, repeated fires can contribute to soil erosion and leaching, further depleting the already limited nutrient availability in these ecosystems. However, the mechanisms enabling *E. ghellinckii* to survive and persist under such challenging conditions remain poorly understood. Investigating these adaptations is crucial for developing targeted conservation strategies that ensure the survival of this species and the ecosystems it supports.

Nitrogen (N) and phosphorus (P) are essential nutrients for the development and growth of plants (Kiba and Krapp, 2016; Sinha and Tandon, 2020). However, Morgan and Connolly (2013) and Kiba and Krapp (2016), reported that in natural soils these essential nutrients are often present in low concentrations or forms that plants cannot readily utilize. Phosphorus availability is restricted, particularly in acidic soils, where it tends to form insoluble complexes with iron (Fe^{3+}), calcium (Ca^{2+}), and aluminum (Al^{3+}) cations (Vance, 2001; Chen and Liao, 2016). Nitrogen is a vital element required for protein and nucleic acid synthesis but is often in limited supply (Kiba and Krapp, 2016)). While plants primarily absorb N in inorganic forms such as nitrate and ammonium, these compounds are usually present in natural soils at concentrations too low to meet their needs (Kiba and Krapp, 2016).

To overcome nutrient constraints, many plants, including cycads, establish symbiotic relationships with N-fixing bacteria (NFB) (Brenner 2003; Zheng *et al*., 2018; Chang *et al*., 2019). These bacteria enable plants to access atmospheric N by converting it into usable forms, such as ammonium (NH_4^+). This process is facilitated by the bacterial enzyme nitrogenase, which breaks the strong triple bond between nitrogen atoms, allowing atmospheric nitrogen to be transformed into ammonium (Zheng *et al*., 2002; Chang *et al*., 2019). In exchange, the bacteria benefit from carbon compounds supplied by the plant, as well as a protective habitat within the plant's tissues (Black *et al*., 2002; Ekman *et al*., 2006; Bano and Iqbal, 2016). These mutualistic interactions are especially critical in nutrient-poor environments, where they support plant survival and productivity despite challenging soil conditions.

Cycads are the only gymnosperm known to engage in biological nitrogen fixation (BNF) through symbiotic associations with specialized microbes housed in their coralloid roots (Lobakova *et al*., 2003; Zheng *et al*., 2018). Early research identified *Nostoc* as an N-fixing genus in cycad coralloid roots (Caiola, 1980; Grobbelaar *et al*., 1986). However, recent studies have revealed diverse microbial communities in cycad coralloid roots belonging to the different genera such as *Bacillus*, *Burkholderia, Rhizobium* and *Bradyrhizobium* that enhances nutrient acquisition and resilience to environmental stress (Gutiérrez-García *et al*., 2019; Suárez-Moo *et al*., 2019; Pecundo *et al*., 2021; Ndlovu *et al*., 2023; Wang *et al*., 2023).

Biological N fixation is an energy-intensive process, with its efficiency closely linked to P availability, as P is essential for adenosine triphosphate (ATP) production during N fixation (Bano and Iqbal, 2016; Rousk *et al*., 2016). For instance, Magadlela *et al*. (2016), found that low soil P concentrations reduced BNF efficiency in leguminous trees such as *Virgilia*

oroboides and *V. divarcata*. Similarly, Zheng *et al*. (2019), reported that P addition in the soil had varying effects on BNF, reducing free-living N fixation by 7.5 % while increasing symbiotic N fixation by 85.5 % in specific ecosystems. These findings highlight the critical role of P in enhancing BNF, particularly in nutrient-deprived environments. In acidic soils like those inhabited by *E. ghellinckii*, P availability is limited due to the formation of insoluble complexes with metals (Vance, 2001; Chen and Liao, 2016), potentially constraining BNF efficiency. To mitigate this challenge, cycads have evolved symbiotic associations with arbuscular mycorrhizal fungi (AMF), which play a vital role in P acquisition in P-limited soils (Fisher and Vovides, 2004).

These adaptive mechanisms are thought to have been conserved in both ancient and modern cycads, underpinning their survival in nutrient-poor conditions (Brenner, 2003; Zheng *et al*., 2018; Chang *et al*., 2019). While N fixation is extensively studied in plant groups like legumes, N dynamics in cycads remain poorly understood (Vessey *et al*., 2005; Britto and Kronzucker, 2013; Kipp *et al*., 2024). Although cycads engage in BNF, the relative contributions of atmospheric and soil-derived N to their N economy are not well-documented (Kipp *et al*., 2024). For instance, Kipp *et al*. (2020), suggested that plants with N-fixing symbioses can bypass soil-derived N entirely, relying instead on atmospheric N fixed by microbial symbionts. However, such findings are not yet well-documented for cycads.

Ndlovu *et al*. (2024), in a recent study documented the reliance of the African cycad *Encephalartos natalensis* on atmospheric N for its nutrient supplies. Ndlovu *et al*. (2023), demonstrated that *E. natalensis* play a critical role in nutrient availability in acidic and nutrient-poor savanna woodland soils. The ability of cycads to improve soil nutrient may be attributed to the occurrence of NFB that are present in their coralloid roots (Zheng *et al*., 2002; Chang *et al*., 2019). The NFB has been shown to enhance and support the growth of *E. natalensis* in acidic, nutrient-deficient soils by relying on N derived from the atmosphere (Ndlovu *et al*., 2024). It is uncertain if this is perculiar to *E. natalensis* or is common in other *Encephalartos* species that grow in nutrient-poor soils.

Similar to *E. natalensis*, *E. ghellinckii* grows in acidic, nutrient-poor soils in the montane grasslands of South Africa, and occurs predominantly in KwaZulu-Natal and the Eastern Cape provinces (Giddy, 1974; Donaldson, 2003). In addition to N fixation and P solubilisation, microbes present in the coralloid roots and rhizosphere soils of *E. ghellinckii* contribute to nutrient cycling and stress tolerance, hence supporting plant growth under harsh environmental

conditions (Chapter 3). However, this study did not investigate and quantify the proportion of N derived from the atmosphere and soils in *E. ghellinckii*. Studying the N and general nutrient acquisition dynamics in *E. ghellinckii* is of ecological significance, because as a keystone species in grassland plant community, *E. ghellinckii* contributes to the overall biodiversity of the grassland ecosystems. Understading the ecological interaction and nutrient acquisition in *E. ghellinckii* is critical for the conservation and management of the plant species and its habitat, the acidic and nutrient-deficient grassland ecosystems that are threatened from biophysical factors.

Thus, the present study investigates the influence of low nutrient availability and soil acidity on the N source reliance of *E. ghellinckii*, comparing atmospheric versus soil-derived N in grassland habitats across KwaZulu-Natal and the Eastern Cape in South Africa. We hypothesized that N fixing bacteria in the coralloid roots will support the growth of *E. ghellinckii* in acidic and nutrient-poor grassland ecosystem soils by increasing its reliance on atmospheric N.

4.2 Materials and Methods

4.2.1 Species description and study sites

Encephalartos ghellinckii is a cycad species native to the grasslands of KwaZulu-Natal and the Eastern Cape in South Africa, with an estimated population of 5000 to 10000 mature individuals (Donaldson, 2003). This study focused on adult *E. ghellinckii* plants located at Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano (Figure 4.1), which occur across varying elevations and climatic conditions. At Cathedral Peak, the plants are found at an average elevation of 2085.57 m above sea level (asl), followed by Umgano at 1473.75 m asl, Thabankulu at 1258 m asl, and Oribi Gorge at 497 m asl.

Encephalartos ghellinckii displays two distinct morphological forms: the highland or giant form, which grows at elevations between 1500 and 2700 meters, and the lowland or dwarf form, found at elevations between 500 and 1400 m asl (Jones 1993; Suinyuy and Johnson 2021). The highland form can grow up to three meters tall with stem diameters of 0.5 to 0.9 meters, while the lowland form typically reaches about one meter in height, with stem diameters of 0.4 to 0.7 meters (Suinyuy and Johnson, 2021). The International Union for Conservation of Nature

(IUCN) has classified *E. ghellinckii* as vulnerable, emphasizing its risk of extinction in the wild (IUCN, 2022).

The Cathedral Peak research catchments are located in South Africa's summer rainfall region, characterized by wet, humid summers and cold, dry winters with frost and occasional snow (Everson *et al*., 1998). The mean annual precipitation (MAP) is approximately 1400 mm, with 84 % of this rainfall occurring between October and March (Morris *et al*., 2016). In contrast, the climate at Oribi Gorge is subtropical, with temperatures always exceeding 10 °C and an average annual rainfall of 1176 mm. Rainfall peaks in summer, particularly in November and February–March, while winters (May to August) experience relatively low rainfall (Glen, 1996; Bleher *et al*., 2003).

Tabankulu, located in the Eastern Cape, experiences seasonal variations in rainfall and temperature. The region receives its highest rainfall during summer, with monthly averages peaking at 153.6 mm in March. Temperatures in Tabankulu range from average minimums of 1.9 °C in winter to 20.6 °C in summer, while maximum temperatures vary between 21.2 °C and 31.6 °C (Aveling *et al*., 2020). Similarly, the Umgano region in KwaZulu-Natal experiences seasonal rainfall, with an average annual precipitation of approximately 1100 mm. Temperatures in Umgano range from 10 °C in July, the coldest month, to 19 °C in January, the warmest month (Mucina and Rutherford, 2006; Fisher, 2024).

Figure 4. 1: (A) *E. ghellinckii* in Cathedral, (B) *E. ghellinckii* in Thabankulu, (C) *E. ghellickii* in Oribi Gorge, and (D) *E. ghellickii* in Umgano.

4.2.2 Soil sample collection and nutrient analysis

The selection of *E. ghellinckii* individuals was guided by the conditions outlined in the collection permit, while also aiming to capture spatial variation within each site. Sampled plants were positioned approximately 20–30 meters apart, with seven individuals selected at Cathedral Peak, five at Thabankulu, and three each at Oribi Gorge and Umgano. Rhizosphere soils were obtained beneath the canopy of each plant using a hand auger at two depth intervals (0–10 cm and 10–20 cm). For each plant, a composite sample was prepared by pooling four subsamples collected from the north, south, east, and west sides of the canopy.

Soil samples were air-dried and passed through a 2 mm sieve before analysis. Laboratory analyses were conducted at the Soil Analytical Services Unit, Cedara College of Agriculture (KwaZulu-Natal Department of Agriculture and Rural Development, South Africa). The

parameters assessed included total soil nutrients concetrations, total cation exchange, exchange acidity, and pH. Analytical procedures followed the methods described by Manson and Roberts (2000). Total nitrogen content was determined using the Automated Dumas dry combustion technique with a LECO CNS 2000 analyzer (LECO Corporation, USA). Phosphorus and potassium concentrations were assessed using atomic absorption spectroscopy following extraction with 25 ml of ambic-2 solution (pH 8) mixed with 2.5 ml soil, stirred at 400 rpm for 10 minutes, and filtered using Whatman No. 1 paper. Calcium and magnesium concentrations were determined using the same procedure, except 1 M KCl was used as the extractant. Soil pH was measured in a mixture of 10 ml soil and 25 ml 1 M KCl solution, stirred at 400 rpm for 5 minutes and recorded using a gel-filled combination glass electrode.

The experimental design considered site (Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano) and sample type (rhizosphere, non-rhizosphere, and coralloid roots) as the main factors. This allowed for comparisons of soil nutrients, plant biomass, and microbial diversity between sites and sample types. Although soil samples were collected at two depths (0–10 cm and 10–20 cm), these were combined to produce composite samples for each plant, and depth was therefore not included as a factor in the analysis.

4.2.3 Coralloid root bacterial isolation

Coralloid roots were obtained from *E. ghellinckii* individuals at each study location alongside soil sampling. In total, seven root samples were collected from Cathedral Peak, five from Thabankulu, and three from each of the Oribi Gorge and Umgano sites. After collection, the roots were gently rinsed with distilled water to remove adhering soil and transported on ice to the laboratory for further processing.

In the lab, surface sterilization was carried out to eliminate external contaminants. Roots were first treated with 70% ethanol for 30 seconds, followed by immersion in 3.5% sodium hypochlorite for 3 minutes. Afterward, they were rinsed thoroughly ten times with sterile distilled water. The sterilized roots were transferred into sterile Eppendorf tubes containing 100 µl of 15% glycerol and kept at 4 °C until analysis.

To isolate the endophytic bacteria, roots were crushed inside the glycerol solution using sterile pipette tips. The resulting root suspensions were inoculated onto different types of selective agar media depending on the target bacterial group: Jensen’s medium for nitrogen-fixing

bacteria, Pikovskaya's agar for phosphate-solubilizers, and Simmons citrate agar for nitrogen cycling bacteria. Plates were incubated at 30 °C for 3 to 5 days. To ensure proper isolation and accurate identification, bacterial cultures were repeatedly streaked on fresh agar plates until pure colonies were obtained.

Polymerase chain reaction (PCR) was conducted using 16S ribosomal RNA gene primers, 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGTGTGTACAA-3'), for the amplification of pure bacterial DNA. The amplification was performed with EmeraldAmp GT Master Mix under the following conditions: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes. Polymerase chain reaction products were separated on a 1% (w/v) agarose gel by electrophoresis and visualized under ultraviolet (UV) light to confirm the amplification of the expected product size. The amplicons were subsequently sent to Inqaba Biotechnical Industries (Pty) Ltd, South Africa, for sequencing. The resulting DNA sequences were then processed and compared against bacterial nucleotide sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov, accessed on 13 August 2024).

4.2.4 Plant biomass and nutrient analysis

For each targeted *E. ghellinckii* plant at Cathedral Peak, Oribi Gorge, Umgano, and Thabankulu, the number of leaves and their lengths were measured. The leaf samples of *Encephalartos ghellinckii* in the study sites were collected from beneath the canopy of each target plant and stored in paper bags. The samples were then oven-dried at 70 °C for 72 hours, following the procedure outlined by Donohue *et al*. (1992). After drying, the material was ground into a fine powder using a Summit Pro blender and stored in 2 ml airtight plastic vials. The powdered plant material was sent to the Central Analytical Facilities (CAF) at Stellenbosch University for P concentration analysis, conducted using the inductively coupled plasma mass spectrometry (ICP-MS). Nitrogen isotope analysis was performed at the Archaeometry Department of the University of Cape Town using a LECO nitrogen analyzer. The isotopic ratio of nitrogen was calculated as $\delta = 1000 \times (R_{sample}/R_{standard})$, where R represents the molar ratio of the heavier isotope (^{15}N) to the lighter isotope (^{14}N) in both the samples and standards.

The following formula was used to calculate the percentage of nitrogen-derived from atmosphere (%NDFA):

%NDFA = 100 (($\delta^{15}N$ reference plant – $\delta^{15}N$ cycad)/ (δ^{15} N reference plant − β))

Where NFDA is the N derived from the atmosphere and β is the δ^{15}N natural abundance of N derived exclusively from the biological N fixation of *Pisum sativum* grown in N-free culture. The β value of *M. pruriens* for this study was -2.58%.

4.2.3 Statistical analysis

The statistical analyses were performed using STATISTICA 7 software. One-way ANOVA was employed to compare the means of soil nutrient concentrations, plant biomass, and nitrogen (N) and phosphorus (P) concentrations in *E. ghellinckii* samples collected from the study sites. Significant differences between means ($p \leq 0.05$) were evaluated using Tukey's HSD test. A simple linear regression analysis was conducted to assess the relationship between two quantitative variables: nitrogen derived from the atmosphere and soil nitrogen concentration. This regression analysis tested the strength of the relationship between atmospheric nitrogen uptake and soil nitrogen levels.

4.3 Results

4.3.1. Soil nutrient concentration and soil relative acidity

Among the primary nutrients, total N had the highest concentrations across all study sites (Table 4.1). Nitrogen concentrations were significantly higher in Umgano and Cathedral Peak, with no statistically significant difference between these two sites ($p = 0.09$; Table 4.1). In contrast, total N concentrations in Thabankulu ($p = 0.0002$) and Oribi Gorge ($p = 0.0002$) were significantly lower compared to Umgano and Cathedral Peak (Table 4.1). Phosphorus concentrations were generally low across the study sites (Table 4.1). However, Cathedral Peak soils had significantly higher P concentrations compared to Thabankulu ($p = 0.003$), Oribi Gorge ($p = 0.002$), and Umgano ($p = 0.0005$). No statistically significant differences in potassium concentrations were observed among Thabankulu, Oribi Gorge, and Umgano ($p > 0.05$; Table 4.1). Potassium exhibited the lowest concentrations among the primary nutrients (Table 4.1). No significant difference was observed between Cathedral Peak and Umgano ($p = 0.21$). However, K concentrations in Thabankulu ($p = 0.001$) and Oribi Gorge ($p = 0.04$) were significantly lower than those in Cathedral Peak (Table 4.1).

Among the intermediate nutrients, calcium concentrations did not differ significantly across any of the study sites, with all p-values above 0.19 (Table 4.1). Magnesium concentrations were significantly higher in Thabankulu compared to Cathedral Peak ($p = 0.0006$), Oribi Gorge ($p = 0.0212$), and Umgano ($p = 0.0055$), while no significant differences were found among the other sites (Table 4.1). A similar trend was observed for zinc, with Thabankulu showing significantly higher concentrations than Cathedral Peak ($p = 0.0118$) and Umgano ($p = 0.0046$), and Oribi Gorge also differing significantly from Umgano ($p = 0.0179$; Table 4.1). Copper concentrations in Cathedral Peak were significantly higher than those in Thabankulu ($p = 0.0002$), Oribi Gorge ($p = 0.0019$), and Umgano ($p = 0.0002$), with an additional significant difference between Oribi Gorge and Umgano ($p = 0.0112$; Table 4.1).

Soils across all study sites were acidic, with pH values ranging from 4.09 to 5.61 (Table 4.1).Exchange acidity differed significantly between Cathedral Peak and Thabankulu ($p = 0.0265$), Thabankulu and Umgano ($p = 0.0011$), and Oribi Gorge and Umgano ($p = 0.0025$; Table 4.1). For organic carbon, Thabankulu had significantly higher concentrations than all other sites ($p = 0.0002$), while no significant differences were observed among Cathedral Peak, Oribi Gorge, and Umgano (Table 4.1).

Table 4. 1: Soil nutrient concentrations and relative acidity (mean ± SE) in *E. ghellinckii* rhizosphere soils from Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano, in the Eastern Cape and KwaZulu-Natal. In each row, letters indicate significant variations in soil nutrient concentrations and relative acidity between different sites (One-Way ANOVA, $p \leq 0.05$; CP: n = 7; TB: n = 5; OR: n = 3; UM: n = 3).

Nutrients	**Cathedral Peak**	**Thabankulu**	**Oribi Gorge**	**Umgano**
Primary nutrients (mg/kg)				
P	8.89 ± 0.76^{a}	4.81 ± 0.57^{b}	3.88 ± 0.60^{b}	2.83 ± 0.64^{b}
K	0.72 ± 0.08^{a}	0.23 ± 0.02^{b}	0.39 ± 0.11^{b}	0.50 ± 0.04^{ab}
Total N	8973.64 ± 235.98^{a}	6800.77 ± 224.26^{b}	3129.24 ± 57.17^{c}	9836.07 ± 0.00^{a}
Intermediate nutrients (mg/kg)				
Ca	3.44 ± 1.01^{a}	5.92 ± 0.53^{a}	5.97 ± 0.17^{a}	4.39 ± 1.06^{a}
Mg	1.63 ± 0.25^{b}	15.11 ± 3.53^{a}	5.19 ± 0.38^{b}	2.93 ± 0.59^{b}
Micronutrients (mg/kg)				
Zn	1.09 ± 0.22^{bc}	2.74 ± 0.50^{a}	2.56 ± 0.45^{ab}	0.40 ± 0.12^{c}
Mn	9.01 ± 0.99^{c}	88.70 ± 6.82^{a}	39.01 ± 3.49^{b}	6.80 ± 1.86^{c}
Cu	3.65 ± 0.18^{a}	1.63 ± 0.21^{bc}	2.19 ± 0.31^{b}	0.822 ± 0.14^{c}
Soil relative acidity				
pH	4.09 ± 0.54^{a}	5.51 ± 0.10^{a}	5.06 ± 0.07^{a}	4.50 ± 0.10^{a}
Exchange acidity (cmol/kg)	0.82 ± 0.15^{ab}	0.02 ± 0.01^{c}	0.06 ± 0.01^{bc}	1.51 ± 0.44^{a}
Total cation exchange (cmol/kg)	5.35 ± 0.42^{b}	21.34 ± 3.65^{a}	11.05 ± 0.54^{b}	8.75 ± 1.15^{b}
Parameter				
OC (%)	6.00 ± 0.00^{a}	6.00 ± 0.00^{a}	3.77 ± 0.9^{b}	6.00 ± 0.00^{a}

In which, P = Phosphorus, K= Potassium, Total N = Total Nitrogen, Ca = Calcium, Mg = Magnesium, Zn = Zinc, Mn = Manganese, Cu = Copper, OC = Organic Carbon.

4.3.2. Microbes identified from the coralloid roots of *E.ghellinckii*

A total of 11 bacterial strains were isolated from Cathedral Peak coralloid roots, with most assigned to the *Burkholderiaceae* family (Table 4.2). Additional families represented included *Moraxellaceae*, *Alcaligenaceae*, and *Rhodobacteraceae* (Table 4.2).The isolates showed functional capabilities related to nitrogen fixation and phosphorus solubilization, with several strains associated with both (Table 4.2).

In Thabankulu samples, 11 bacterial isolates were recorded. These belonged to a broader range of taxonomic groups, including *Burkholderiaceae*, *Moraxellaceae*, *Enterobacteriaceae*, and *Paenibacillaceae* (Table 4.3).Functional screening indicated nitrogen-fixing and phosphorus-solubilizing potential among these strains (Table 4.3).

Five bacterial isolates were obtained from Umgano, comprising species within *Paraburkholderia* and *Acinetobacter* (Table 4.4). All showed at least one functional trait linked to nutrient cycling, and some strains had dual roles in nitrogen fixation and phosphorus solubilisation (Table 4.4).

At Oribi Gorge, three bacterial strains were identified, all belonging to *Enterobacteriaceae* (Table 4.5). These included *Enterobacter*, *Kosakonia*, and *Klebsiella* species, with nitrogen fixation observed in all isolates (Table 4.5). Phosphorus-solubilizing activity was also noted in *E. cloacae* (Table 4.2).

Table 4. 2: Microbial diversity in *E. ghellinckii* coralloid roots from Cathedral Peak

	Scientific name	Accession number	Similarity %	Functions
Moraxellaceae	*Acinetobacter septicus*	MN725744.1	99.62	P-solubilization (Mujumdar *et al*., 2023)
Burkholderiaceae	*Paraburkholderia fungorum*	MT611735.1	99.61	N-fixing (Raihan *et al*., 2022) P-solubilizing (Napitupulu *et al*., 2023)
	Burkholderia multivorans	OQ926016.1	92.31	P- solubilizing (Li *et al*., 2013)
	Paraburkholderia graminis	OR831931.1	92.45	N-cycling (Viallard *et al*., 1998)
	Paraburkholderia phytofirmans	OR430493.1	100	N- fixing (Puri *et al*., 2020) P- solubilizing (Aziz *et al*., 2020)
	Paraburkholderia sediminicola	OQ076265.1	100	P- solubilizing (Vanwijnsberghe *et al*., 2021)
	Paraburkholderia nemoris	OR922244.1	100	N- fixing (Vanwijnsberghe *et al*., 2021) P- solubilizing (Vanwijnsberghe *et al*., 2021)
	Paraburkholderia madseniana	OR922229.1	100	P-solubilization (Wilhelm *et al*., 2020)
	Paraburkholderia aromaticivorans	OQ076254.1	96.86	N- fixing (Vio *et al*., 2020)
Alcaligenaceae	*Achromobacter xylosoxidans*	MT040694.1	80.8	N- fixing (Jha and Kumar, 2009) P- solubilizing (Jha and Kumar, 2009)
Rhodobacteraceae	*Cereibacter sphaeroides*	EF633263.1	80.9	N-fixing (Llamas *et al*., 2023) P- solubilizing (Dat *et al*., 2024)

Table 4. 3: Microbial diversity in *E. ghellinckii* coralloid roots from Thabankulu

Family	Scientific name	Accession number	Similarity %	Function
Moraxellaceae	*Acinetobacter septicus*	MN725744.1	99.67	P-solubilizing (Mujumdar *et al*., 2023)
	Acinetobacter guillouiae	OR821296.1	89.1	N-fixing (Kour *et al*., 2023)
Burkholderiaceae	*Paraburkholderia fungorum*	MH972166.1	99.83	N-fixing (Raihan *et al*., 2022) P-solubilizing (Napitupulu *et al*., 2023)
	Paraburkholderia phytofirmans	CP157347.1	100	N- fixing (Puri *et al*., 2020) P- solubilizing (Aziz *et al*., 2020; Puri *et al*., 2020)
	Paraburkholderia sediminicola	PP905144.1	98..08	P- solubilizing (Vanwijnsberghe *et al*., 2021)
	Paraburkholderia graminis	OR831931.1	98.00	N-fixing (Viallard *et al*., 1998)
	Caballeronia mineralivorans	PQ034603.1	98.00	N-fixing (Uroz and Oger, 2017) P-solubilizing (Uroz and Oger, 2017)
	Trinickia caryophylli	CP131478.1	86.9	N-fixing (Estrada-de Los Santos *et al*., 2019)
Enterobacteriaceae	*Enterobacter cloacae*	OR084780.1	100	N-fixing (Ji *et al*., 2020) P-solubilizing (Macedo-Raygoza *et al*., 2019)
	Enterobacter ludwigii	OR079894.1	100	P-solubilizing (Lee *et al*., 2019)
Paenibacillaceae	*Brevibacillus reuszeri*	MT071700.1	100	N-fixing (Çakmakçı, 2019) P-solubilizing (Yildirim *et al*., 2011)

Table 4. 4: Microbial diversity in *E. ghellinckii* coralloid roots from Umgano

Family	Scientific name	Accession number	Similarity %	Function
Moraxellaceae	*Acinetobacter septicus*	MN725744.1	99.81	Solubilize P (Mujumdar *et al*., 2023)
Burkholderiaceae	*Paraburkholderia fungorum*	CP157347.1	100	N-fixing (Raihan *et al*., 2022)
				P- solubilizing (Napitupulu *et al*., 2023)
	Paraburkholderia phytofirmans	OR430493.1	100	N- fixing (Puri *et al*., 2020)
				P- solubilizing (Aziz *et al*., 2020)
	Paraburkholderia aromaticivorans	OQ076254.1	100	N- fixing (Vio *et al*., 2020)
	Paraburkholderia ginsengisoli	CP066075.1	100	N-fixing (Vio *et al*., 2020; Kim *et al*., 2020)
				P- solubilizing (Kim *et al*., 2020)

Table 4. 5: Microbial diversity in *E. ghellinckii* coralloid roots from Oribi Gorge

Family	Scientific name	Accession number	Similarity %	Function
Enterobacteriaceae	*Enterobacter cloacae*	OR084780.1	100	N-fixing (Ji *et al*., 2020) P- solubilizing (Macedo-Raygoza *et al*., 2019).
	Kosakonia oryzae	OM909321.1	99.35	N-fixing (Peng *et al*., 2009)
	Klebsiella vaiicola	MN725749.1	98.61	N-fixing (Li *et al*., 2018)

4.3.3 Plant nitrogen and phosphorus concentrations *E.ghellinckii* leaves

The percentage of nitrogen derived from the atmosphere (%NDFA) exceeded 80% across all study sites, with no statistically significant differences detected among any of the site comparisons (all $p > 0.05$; Figure 4.2A). In contrast, total plant P concentrations differed significantly across sites ($p < 0.05$), with Thabankulu exhibiting significantly lower values compared to Cathedral Peak ($p = 0.007$) and Umgano ($p = 0.001$). No significant differences were observed between Cathedral Peak and Oribi Gorge ($p = 0.880$), Cathedral Peak and Umgano ($p = 0.206$), Thabankulu and Oribi Gorge ($p = 0.115$), or Oribi Gorge and Umgano ($p = 0.126$; Figure 4.2B).

A similar trend was observed for NDFA concentrations, where Thabankulu recorded significantly lower values than Cathedral Peak ($p = 0.038$) and Umgano ($p = 0.009$), while all other comparisons were not statistically significant (all $p > 0.05$; Figure 4.2C). For nitrogen derived from the soil (NDFS), a significant difference was detected only between Cathedral Peak and Thabankulu ($p = 0.045$), with no significant variation found in the remaining site comparisons (all $p>0.05$; Figure 4.2C). Additionally, NDFS was significantly lower that NDFA across all sites (all $p < 0.05$; Figure 4.2C).

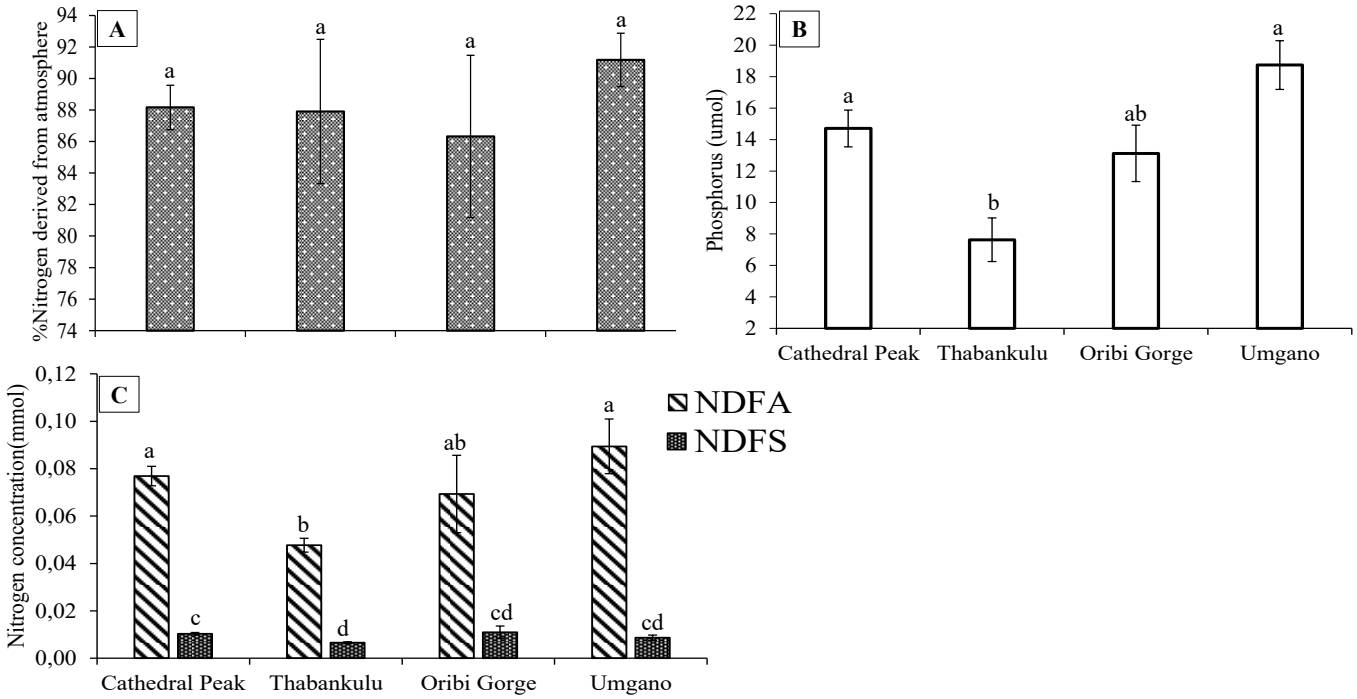

Figure 4. 2: Nitrogen and phosphorus dynamics in leaf material of *Encephalartos ghellinckii* across study sites: (A) percentage of nitrogen derived from the atmosphere; (B) total

phosphorus content; and (C) concentrations of atmospheric- and soil-derived nitrogen in plants from Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano.

4.3.4 Linear correlations between NDFA and soil N concentrations

The correlation between N derived from the atmosphere (NDFA) and soil N concentrations varied across the study sites, demonstrating different strengths and directions. In Cathedral Peak, a moderate positive correlation was observed, but it was not statistically significant ($p > 0.05$; Figure 4.3A). Similarly, Thabankulu exhibited a weak positive correlation, with a high p-value indicating no significant relationship (Figure 4.3B). By contrast, Oribi Gorge showed a very strong negative correlation that approached significance ($p = 0.069$; Figure 4.3C). In Umgano, a strong positive correlation was observed, though it too was not statistically significant ($p > 0.05$; Figure 4.3D).

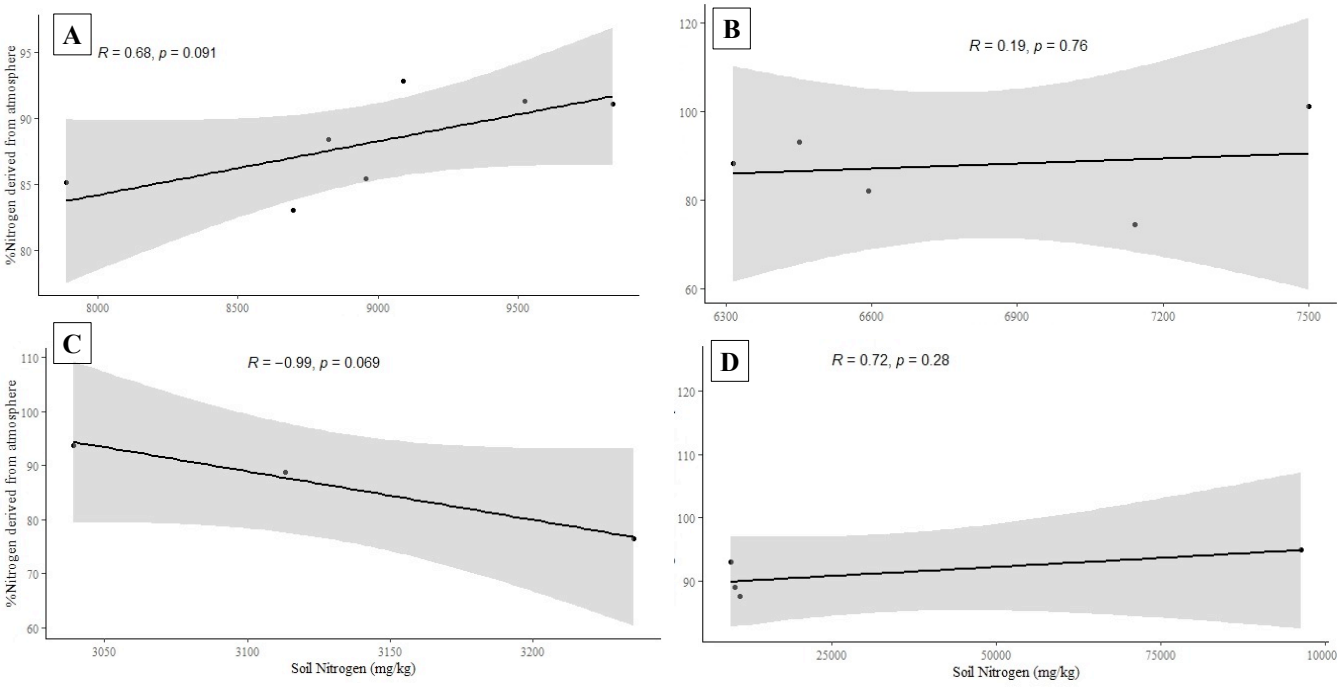

Figure 4. 3: Linear correlations between the percentage of NDFA in plant leaves and soil N concentration at A: Cathedral Peak, B: Thabankulu, C: Oribi Gorge, and D: Umgano.

4.3.5 Linear correlations between the concentrations of the NDFA and soil P

The results indicated a positive correlation between NDFA concentrations in *E. ghellinckii* leaf materials and soil P concentrations from the rhizosphere across the four study sites: Cathedral Peak, Thabankulu, Oribi Gorge, and Umgano (Figure 4.4A to 4.4D). The strength and significance of these correlations varied among the sites. Thabankulu exhibited the strongest and most significant relationship (Figure 4.4B), while Cathedral Peak and Umgano showed moderate to strong correlations that were not statistically significant (Figure 4.4A and D). In contrast, Oribi Gorge displayed a weaker positive correlation, which was also not significant (Figure 4.4C). These findings emphasize the site-specific nature of the relationship between NDFA and soil P concentrations.

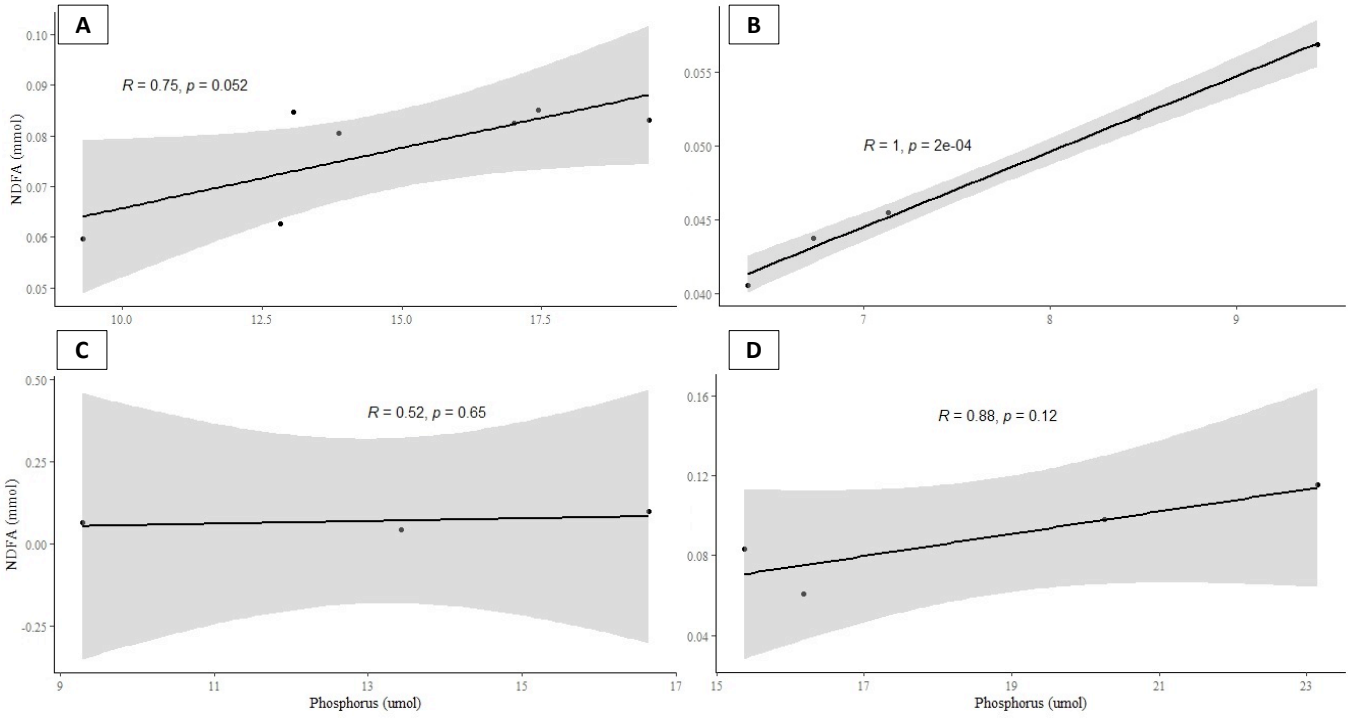

Figure 4. 4: Linear correlations between the concentration of nitrogen derived from the atmosphere (NDFA) and phosphorus (P) in *E. ghellinckii* plant leaves. A: Cathedral Peak, B: Thabankulu, C: Oribi Gorge, and D: Umgano.

4.3.6 Plant biomass measurements

Encephalartos ghellinckii plants from Umgano exhibited the largest crown cover, followed by those from Cathedral Peak, Thabankulu, and Oribi Gorge, respectively. Regarding leaf

production, the number of leaves was significantly higher at Cathedral Peak (60.3 ± 2.7) compared to Thabankulu ($p = 0.0002$), Oribi Gorge ($p = 0.0002$), and Umgano ($p = 0.0018$). Plants at Umgano (40.5 ± 4.4) also had significantly more leaves than those at Thabankulu ($p = 0.0098$) and Oribi Gorge ($p = 0.0002$). Additionally, Thabankulu (23.2 ± 4.4) had significantly more leaves than Oribi Gorge (5.5 ± 0.3; $p = 0.0035$).

For leaf length, plants from Umgano had the longest leaves (79.0 ± 2.1 cm), which were significantly longer than those from Thabankulu ($p = 0.0002$), but not significantly different from Cathedral Peak ($p = 0.0515$) or Oribi Gorge ($p = 0.0525$). Cathedral Peak (72.7 ± 1.8 cm) and Oribi Gorge (71.0 ± 2.7 cm) had similar leaf lengths ($p = 0.95$), both significantly longer than Thabankulu (60.3 ± 1.8 cm), with p-values of 0.0003 and 0.0127, respectively.

4.4 Discussion

The results from this study confirm the hypothesis that *E. ghellinckii* relies more on NDFA than NDFS. This is evident across all study sites, where the presence of N-fixing and P-solubilizing bacteria in the coralloid roots of *E. ghellinckii* likely contributes to the conversion of atmospheric N into usable nitrates, thereby enhancing the species' persistence in nutrient-poor environments. The results further show a positive correlation between NDFA and soil nitrogen and phosphorus, suggesting that phosphorus may play a role in BNF.

Encephalartos ghellinckii is distributed in the grasslands of KwaZulu-Natal and the Eastern Cape, South Africa (Giddy, 1974; Donaldson, 2003, 2008), where soils are typically acidic and nutrient-poor (Nandwa, 2001). This study observed *E. ghellinckii* populations at altitudes ranging from 2085 meters in the highlands to 497 meters in the lowlands above sea level. Notably, some individuals were found on cliffside slopes at sites such as Cathedral Peak, Thabankulu, and Umgano, highlighting the species' remarkable ability to thrive in these challenging environments.

The findings indicate that the coralloid roots of *E. ghellinckii* harbor diverse N-fixing and P-solubilizing bacteria, which may enhance the species' survival in nutrient-limited ecosystems. The 16S rRNA gene sequence analysis across all study sites identified various bacterial genera, including *Acinetobacter*, *Burkholderia*, *Paraburkholderia*, *Cereibacter*, *Achromobacter*, *Enterobacter*, *Klebsiella*, and *Kosakonia*. Microbes from the families Moraxellaceae and Burkholderiaceae dominated in Cathedral Peak, Thabankulu, and Umgano, while Oribi Gorge hosted microbes exclusively from the Enterobacteriaceae family. All identified microbes

exhibited PGP capabilities, which likely contribute to the survival of *E. ghellinckii* in nutrient-deficient soils.

These capabilities reflect a functionally diverse microbiome, with specific microbial traits that play integral roles in facilitating nutrient acquisition and enhancing plant resilience under environmental stress. These traits include N fixation, P solubilization, phytohormone production, siderophore-mediated iron acquisition, modulation of ethylene levels, and enhancement of plant tolerance to abiotic stress (Coyne and Mikkelsen, 2015; Jacoby et al., 2017; Martínez-Hidalgo and Hirsch, 2017; Basu et al., 2021). Many of these microbes also further facilitate nutrient cycling through extracellular enzyme production and protect plants against pathogens through antagonistic interactions or induced systemic resistance (Yildirim et al., 2011; Kamli et al., 2022; Raihan et al., 2022; Vio et al., 2020). Collectively, these functions suggest that *E. ghellinckii* benefits from a functionally diverse microbiome that enhances nutrient availability and supports persistence in the grassland environments it inhabits.

Although *E. ghellinckii* is associated with N-fixing microbes, the efficiency of BNF is closely linked to P availability, as P is essential for ATP production, which fuels N fixation (Bano and Iqbal, 2016; Magadlela et al., 2016). Soils at all study sites were acidic, with pH values ranging from 4.09 to 5.61, leading to P immobilization due to the formation of insoluble complexes with Fe^{3+}, Ca^{2+}, and Al^{3+} (Vance, 2001; Chen and Liao, 2016). Despite these constraints, *E. ghellinckii* appears to mitigate nutrient limitations through symbiosis with a microbial community capable of N fixation and P solubilization. Identified P-solubilizing bacteria, including *Enterobacter ludwigii*, *Acinetobacter septicus*, *Paraburkholderia sediminicola*, and *Burkholderia multivorans*, enhance phosphorus availability by solubilizing cation-bound P, making it more accessible for plant uptake.

In addition to bacterial symbionts, cycads form associations with arbuscular mycorrhizal fungi (AMF), which play a crucial role in P-limited conditions (Muthukumar and Udaiyan, 2002; Fisher and Vovides, 2004; Soka and Ritchie, 2015). Arbuscular mycorrhizal fungi improve P acquisition by extending the effective root surface area through their hyphal networks, enhancing nutrient uptake (Fisher and Vovides, 2004; Soka and Ritchie, 2015). The combined activity of P-solubilizing bacteria and AMF contributes to P availability, which may, in turn, improve N fixation efficiency. This is supported by the observed positive correlation between atmospheric N fixation and soil P concentrations across study sites, aligning with previous

studies that linked BNF efficiency to phosphorus availability (Bano and Iqbal, 2016; Magadlela et al., 2016; Rousk et al., 2016).

The conversion of atmospheric N into ammonia by bacteria is a key process for plant productivity, especially in nitrogen-deficient soils (Martínez-Hidalgo and Hirsch, 2017). This adaptation enables nitrogen-fixing plants to meet their nutritional requirements in environments where N is scarce. *Encephalartos ghellinckii*, which thrives in South African grasslands characterized by low N and P availability (Giddy, 1974; Nandwa, 2002; Craine et al., 2008), appears to rely heavily on atmospheric N for survival. Over 80% of the total nitrogen in *E. ghellinckii* is derived from the atmosphere, with less than 20% sourced from the soil. This finding suggests that the species is well adapted to nutrient-deficient environments, relying predominantly on BNF through its symbiotic association with PGP bacteria in its coralloid roots.

At Cathedral Peak, Umgano, and Thabankulu, a positive correlation was observed between soil N concentration and the percentage of N fixed from the atmosphere by *E. ghellinckii*. These findings suggest that while the species predominantly relies on atmospheric N fixation, soil N availability may still play a role in modulating this process. In contrast, a negative correlation at Oribi Gorge, where soil nitrogen concentrations are lower and leaf litter is reduced, may reflect limited N inputs into the soil.

Despite forming symbiotic relationships with N-fixing microbes in its coralloid roots, *E. ghellinckii*'s reliance on atmospheric N is not entirely determined by soil nitrogen levels. Previous studies by Kipp et al. (2020) have shown that certain N-fixing plants continue to fix atmospheric N, regardless of soil nitrogen abundance. This suggests that N fixation in *E. ghellinckii* could be influenced by factors beyond soil N content. According to Vitousek et al. (2013) and Kipp et al. (2020), various factors such as soil moisture, temperature, and plant age or sex can also influence N fixation. Furthermore, Vessey et al. (2005) observed that N fixation may decrease when soil N levels are high, as plants downregulate the process to conserve energy. These findings highlight the complexity of N dynamics in *E. ghellinckii*, demonstrating that while soil N content can influence nitrogen fixation, other environmental and physiological factors play a critical role. This underscores the importance of site-specific conditions in shaping the species' N acquisition strategies.

4.5 Conclusion

The symbiotic association between *E. ghellinckii* N-fixing bacteria is crucial for the survival of this species in acidic, nutrient-deprived grasslands. The microbes associated with *E. ghellinckii* are multifunctional, however, N fixation may be their primary role. The significant presence of N derived from the atmosphere in *E. ghellinckii* leaves strongly indicates that this plant relies more on atmospheric N than soil-derived N. Biological N fixation not only supports the nutritional needs of *E. ghellinckii* but also contributes to soil fertility by enriching the N pool through N-rich plant litter, which decomposes over time. Additionally, N-fixing plants enhance N availability for surrounding vegetation, making them indispensable to the overall health of their ecosystems (Halliday and Pate, 1976). These findings underscore the importance of incorporating the role of N-fixing plants in ecosystem management and conservation strategies. As *E. ghellinckii* and other cycads face growing environmental threats, understanding their ecological functions will be critical for ensuring their long-term preservation in the wild.

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Appendix

Table 4.6: Plant nitrogen and phosphorus concentrations at four study sites, showing nitrogen derived from the soil (NDFS), nitrogen derived from the atmosphere (NDFA), percentage of nitrogen derived from the atmosphere (NDFA%), and soil phosphorus concentration. Values are presented as mean ± standard error.

Site	NDFS	NDFA	NDFA (%)	Phosphorus (µmol)
Cathedral Peak	0.01032 ± 0.00055	0.07692 ± 0.00412	88.17 ± 1.41	14.71 ± 1.17
Thabankulu	0.00657 ± 0.00040	0.04773 ± 0.00294	87.90 ± 4.58	7.63 ± 1.39
Oribi Gorge	0.01099 ± 0.00259	0.06935 ± 0.01633	86.32 ± 5.15	13.12 ± 1.79
Umgano	0.00865 ± 0.00112	0.08943 ± 0.01152	91.18 ± 1.70	18.74 ± 1.55

Table 4.7: Pairwise Tukey's HSD post hoc p-values for phosphorus concentration between sites.

Site	Cathedral Peak	Thabankulu	Oribi Gorge	Umgano
Cathedral Peak	–	0.01	0.88	0.21
Thabankulu	0.01	–	0.11	0.00
Oribi Gorge	0.88	0.11	–	0.13
Umgano	0.21	0.00	0.13	–

Table 4.8: Pairwise Tukey's HSD post hoc p-values for nitrogen derived from the atmosphere (NDFA) between sites.

Site	Cathedral Peak	Thabankulu	Oribi Gorge	Umgano
Cathedral Peak	–	0.04	0.91	0.63
Thabankulu	0.04	–	0.31	0.01
Oribi Gorge	0.91	0.31	–	0.41
Umgano	0.63	0.01	0.41	–

Table 4.9: Pairwise Tukey's HSD post hoc p-values for nitrogen derived from the soil (NDFS) between sites.

Site	Cathedral Peak	Thabankulu	Oribi Gorge	Umgano
Cathedral Peak	–	0.04	0.97	0.62
Thabankulu	0.04	–	0.06	0.51
Oribi Gorge	0.97	0.06	–	0.51
Umgano	0.62	0.51	0.51	–

Table 4.10: Pairwise Tukey's HSD post hoc p-values for percentage of nitrogen derived from the atmosphere (%NDFA) between sites.

Site	Cathedral Peak	Thabankulu	Oribi Gorge	Umgano
Cathedral Peak	–	0.04	0.97	0.62
Thabankulu	0.04	–	0.06	0.51
Oribi Gorge	0.97	0.06	–	0.51
Umgano	0.62	0.51	0.51	–

Table 4.11: Plant parameters of *Encephalartos ghellinckii* across study sites (mean ± standard error).

Parameter	Cathedral Peak	Thabankulu	Oribi Gorge	Umgano
Number of leaves	60.29 ± 2.65	23.20 ± 4.40	5.50 ± 0.34	40.50 ± 4.37
Leaf length (cm)	72.67 ± 1.84	60.33 ± 1.83	71.00 ± 2.72	79.00 ± 2.08
Altitude (m)	2085.57 ± 2.72	1258.00 ± 6.79	497.00 ± 6.81	1473.75 ± 4.55

CHAPTER 5: GENERAL CONCLUSION

5.1. Conclusion

Encephalartos ghellinckii is a cycad species endemic to South Africa, predominantly found in the acidic, nutrient-deficient grassland ecosystems in KwaZulu-Natal and the Eastern Cape (Giddy, 1974; Donaldson, 2003). Surviving in such soil environments requires specialized adaptations, particularly mechanisms that enhance nutrient acquisition. This study examined microbial diversity in coralloid roots, rhizosphere, and non-rhizosphere soils, as well as soil nutrient composition, enzyme activities, and nitrogen (N) isotope ratios in leaf material to assess the role of microbial symbiosis in *E. ghellinckii*'s survival.

The findings confirm that *E. ghellinckii* relies significantly on microbial interactions for nutrient acquisition in nutrient-poor environments. Soil nutrient analysis revealed that the species thrives in highly acidic, nutrient-deprived soils, with both rhizosphere and non-rhizosphere soils harboring diverse plant growth-promoting (PGP) microbes. These microbes facilitate N cycling, N fixation, and phosphorus (P) solubilization, thereby enhancing nutrient availability. Additionally, soil microbes secrete extracellular enzymes that contribute to nutrient cycling, supporting soil fertility and plant growth. Although enzyme activities did not differ significantly across sites, their correlations with soil nutrients highlights the dynamic microbial interactions that regulate nutrient availability.

Beyond the rhizosphere, *E. ghellinckii*'s coralloid roots supported a diverse community of PGP bacteria, further enhancing its persistence in harsh conditions. Nitrogen isotope analysis revealed that *E. ghellinckii* primarily assimilates atmospheric nitrogen, highlighting the critical role of N-fixing bacteria in its nutrient acquisition strategy. The presence of P-solubilizing bacteria suggests that microbial activity enhances P availability, which, in turn, influences biological N fixation. The positive correlation between soil P levels and the proportion of atmospheric N fixed suggests that P availability enhances N fixation. This finding aligns with previous research indicating that P plays a vital role in biological N fixation, as N-fixing is an energy-intensive processes and is reliant on the availability of P as ATP (Bano and Iqbal, 2016; Magadlela *et al*., 2016). These results emphasize the interconnected nature of microbial functions, where P solubilization and N fixation work together to improve nutrient availability in the soil.

These findings support the broader understanding that cycads have persisted in nutrient-deficient environments largely due to their symbiotic associations with N-fixing bacteria (Chang *et al*., 2019). While N fixation remains the primary function of coralloid roots, the multifunctional roles of associated microbes further enhance *E. ghellinckii*'s ability to survive in challenging ecosystems. Understanding these microbial interactions not only provides insights into cycad adaptation but also has implications for conservation strategies and ecological restoration efforts in nutrient-poor grasslands.

5.2. Suggestions for future research

This study provides insights into how the symbiotic association between *E. ghellinckii* and plant growth-promoting microbes supports its survival in acidic, nutrient-deprived soils. These findings also contribute to understanding plant-microbe dynamics in closely related, highly threatened cycad species that are difficult to access and study.

Previous studies (Zheng *et al*., 2002; Suárez-Moo *et al*., 2019) suggest that the age of coralloid roots may significantly influence the composition and diversity of associated microbial communities. Future research should investigate how the age of *E. ghellinckii* coralloid roots affects microbial diversity, functionality, and symbiotic efficiency. Understanding whether older or younger roots harbor distinct microbial profiles could provide deeper insights into plant-microbe interactions and their implications for nutrient acquisition.

While this study provides valuable information on microbial diversity associated with *E. ghellinckii*, seasonal variations in microbial community composition and function remain largely unexplored. Future research could focus on the temporal dynamics of microbial communities associated with *E. ghellinckii*, examining how changes in temperature, moisture, and nutrient availability influence nutrient cycling and biological N fixation throughout the year. Such research would offer valuable insights into the resilience of these associations under varying environmental conditions.

Fisher and Vovides (2004) and Ibrahim (2021) reported that cycads form symbiotic associations with arbuscular mycorrhizal fungi (AMF), which play a crucial role in P acquisition and, in turn, support the N fixation process. Investigating potential interactions between *E. ghellinckii* and AMF could provide further insight into its P uptake strategies and this can be assessed through spore counts and colonization percentage analysis.

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