Article

The Natal Giant Cycad (*Encephalartos natalensis*) associated nutrient-cycling microbes and enzymes contribute to soil nutrient inputs and persistence in nutrient-poor disturbed savanna woodland ecosystems.

Siphelele Ndlovu[1], Terence N Suinyuy[2], María A. Pérez-Fernández[3], Anathi Magadlela[1]*

[1] School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Westville Campus), Private Bag X54001, Durban 4000, South Africa.

[2] School of Biology and Environmental Sciences, University of Mpumalanga (Mbombela Campus), Private Bag X11283, Mbombela 1200, South Africa.

[3] Department of Physical, Chemical and Natural Systems, Universidad Pablo de Olavide, Carretera de Utrera Km 1, 41013 Seville, Spain

* Correspondence: Anathi Magadlela, Email: anathimagadlela@icloud.com; (+27) 31-260-2076

Abstract: *Encephalartos spp*. establish symbioses with nitrogen (N)-fixing bacteria that contribute to soil nutrition and improve plant growth. Despite the *Encephalartos* mutualistic symbioses with N-fixing bacteria, the identity of other bacteria and their contribution to soil fertility and ecosystem functioning are not well understood. This limited information presents a challenge in developing comprehensive conservation and management strategies for these cycad species. Therefore, this study identified the nutrient cycling bacteria in *Encephalartos natalensis* coralloid roots, rhizosphere, and non-rhizosphere soils. Additionally, the soil characteristics and soil enzyme activities of the rhizosphere and non-rhizosphere soils were assayed. The coralloid roots, rhizosphere, and non-rhizosphere soils of *E. natalensis* were collected from a population of >500 *E. natalensis* in a disturbed savanna woodland at Edendale in KwaZulu-Natal (South Africa) for nutrient analysis, bacterial identification, and enzyme activity assays. Nutrient cycling bacteria such as *Lysinibacillus xylanilyticus*; *Paraburkholderia sabiae,* and *Novosphingobium barchaimii* were identified in the coralloid roots, rhizosphere, and non-rhizosphere soils of *E. natalensis*. Phosphorus (P) cycling (alkaline and acid phosphatase) and N cycling (β-(D)-Glucosaminidase and nitrate reductase) enzyme activities showed a positive correlation with the P and N concentrations in the rhizosphere and non-rhizosphere soils of *E. natalensis*. Nutrient cycling bacteria identified in *E. natalensis* coralloid roots, rhizosphere, and non-rhizosphere soils and associated enzymes assayed may contribute to soil nutrient inputs of *E. natalensis* plants growing in acidic and nutrient-poor savanna woodland ecosystems.

Keywords: *Encephalartos natalensis*, microbe-symbiosis, soil nutrition, enzyme activities, soil nutrient cycling

 doi:10.20944/preprints202301.0366.v1

1. Introduction

Cycads are perennial dioecious gymnosperms regarded as "living fossils" as they possess intermediate morphological traits between angiosperms and gymnosperms [1]. Cycads originated (~265–290 Ma) in the late Palaeozoic period and were dominant during the Mesozoic era [2,3]. Cycad communities form significant vegetation in natural ecosystems as they provide critical ecosystem services such as biodiversity maintenance, carbon sequestration, and nutrient cycling [4,5,6]. Currently, cycads are distributed in the tropical and subtropical regions of America, Asia, Africa, and Oceania [7]. South Africa is classified as a centre of cycad diversity where thirty-seven of the sixty-six species of the African cycad *Encephalartos* occurs [8,9,10]. Cycads are regarded as the most threatened group of plants on earth, with over 60% of the 340 species threatened with a high risk of extinction [11]. Approximately 71% of South African cycad species are classified as threatened [12]. Cycads face many threats that cause a decline in their population, these threats include climate change, the presence of invasive species, and their illegal harvesting for medicinal purposes, overcollection, deforestation and habitat loss, disturbances like fires, floods, and droughts [13,14,15]. The observed decline in cycad populations is alarming as a decline in their abundance, and probable extinction would lead to the loss of the critical ecosystem services they provide.

Cycad species grow in grasslands, sand dunes, rocky outcrops, scarp and sclerophyll forests, and areas with recurrent fires [5], and their ability to grow and thrive in nutrient-poor and harsh environmental conditions is due to their ability to form symbioses with N-fixing, N cycling and P solubilizing bacteria [5,6]. Cycads establish mutualistic associations with various microorganisms such as fungi, bacteria, and cyanobacteria in their coralloid roots [16]. Cycads are the only gymnosperms known to establish a symbiotic relationship with N-fixing cyanobacteria (*Nostoc*, *Scytonema* and *Richelia*) and proteobacteria (*Bradyrhizobium* and *Burkholderia*) have been isolated in the coralloid roots of some cycad species [17,18,19,20]. The microbes that associate with cycads assist in plant growth by providing essential elements such as N compounds [21,22]. Additionally, soil

 doi:10.20944/preprints202301.0366.v1

microorganisms produce extracellular enzymes that hydrolyse and transform polymeric compounds into readily available nutrients for plant and microbe assimilation [23]. These extracellular enzymes regulate the mineralisation and cycling of terrestrial nutrients such as N, phosphate (P,) and carbon (C), and these enzymes can be grouped according to their function. However, some play a role in more than one cycle [24]. Enzymes such as β-(D)-glucosaminidase and asparaginase hydrolyse chito-oligosaccharides and convert asparagine into ammonia (NH_3) and aspartic acid [25,26]. This influences N bioavailability increasing N assimilation by plants [27]. Phosphatases hydrolyse phosphoric acid monoester into a phosphate anion [28]. Carbon cycling enzymes include dehydrogenase, β-D-cellobiohydrolase, and β-D-glucosidase. They release saccharides from glycosides and catalyse the degradation of cellotetraose and cellulose into cellobiose that will further be transformed into glucose [29].

South African *Encephalartos* spp. like other cycads develop coralloid roots, which host nitrogen-fixing bacteria [30] that improve soil fertility and enhance plant growth [31.32]. *Encephalartos natalensis* is widely distributed in KwaZulu-Natal savanna woodland nutrient-poor ecosystem soils, and it is near threatened in the wild[33, 34]. Similar to other cycads, *E. natalensis* may thrive in the nutrient poor soils because of their symbiosis with N-fixing and other nutrient cycling bacteria. However, the identity of the *E. natalensis* associated symbionts with nutrient cycling functions and their contribution to soil fertility is not well understood. Additionally, the available literature is limited to the associations of cycads with N-fixing bacteria and does not look at the associations of cycads with N cycling and P solubilising bacteria. Furthermore, no studies have assayed enzyme activities in *E. natalensis* rhizosphere and non-rhizosphere soils. This limited information presents a challenge in developing comprehensive conservation and management strategies for this cycad species. Moreover, elucidating *E. natalensis* microbe-symbiosis aligns with the KwaZulu-Natal Nature Conservation Management Act no. 9 of 1997 and the information generated from this study will feed into the knowledge of biodiversity and the enhancement of ecosystem services which aligns with South Africa's National Biodiversity Strategy and

 doi:10.20944/preprints202301.0366.v1

Action Plan (NBSAP) 2015-2025. Thus, the present study identified the bacteria in *E. natalensis* coralloid roots, rhizosphere, and non-rhizosphere savanna woodland soils. The rhizosphere and non-rhizosphere soil were assayed for nutrient characteristics and soil enzyme activities. The results from this study will increase our knowledge of *E. natalensis* cycad-microbe symbiosis and its contribution to soil fertility in savanna woodland nutrient-stressed ecosystem soils. Additionally, the results of this study will help in understanding the ecosystem services of endangered cycad species with evolutionary lineages that resemble *E. natalensis* but cannot be studied due to their critical conservational status. The scientific question was: is the survival of the South African *E. natalensis* cycad in nutrient-deficient ecosystem soils a result of its ability to form symbiotic associations with bacteria in its coralloid roots? We hypothesized that the bacteria in the coralloid roots, rhizosphere, and non-rhizosphere soils of the South African *E. natalensis* cycad contribute to soil nutrient inputs and *E. natalensis* persistence in nutrient-stressed and disturbed savanna woodland ecosystem soils.

2. Results

Soil characteristics

Soil nutrients

Rhizosphere soils had a higher concentration of primary nutrients, intermediate, and micronutrients compared to the non-rhizosphere soils. However, copper (Cu) and manganese (Mn) concentrations were higher in the non-rhizosphere soils than in the rhizosphere. Rhizosphere soils showed a higher P and Magnesium (Mg) concentration than the non-rhizosphere soils, although this was not significantly different statistically (Table 1). Nitrogen, Potassium (K), Calcium, (Ca) and Zinc (Zn) concentrations were significantly higher in the rhizosphere than in the non-rhizosphere soils (Table 1). Rhizosphere soil pH was 5.7 and significantly higher than the pH of the non-rhizosphere soils (5.14). Rhizosphere soils had a higher exchange acidity than non-rhizosphere soils, although this

 doi:10.20944/preprints202301.0366.v1

difference was not significant statistically (Table 1). Total cation exchange and exchange acidity were significantly higher in the rhizosphere than in the non-rhizosphere soils (Table 1). Organic C was higher in the rhizosphere than in the non-rhizosphere soils (Table 1). However, the rhizosphere had a lower C:N ratio than the non-rhizosphere soils (Table 1).

Table 1: Soil characteristics of *Encephalartos natalensis* rhizosphere and non-rhizosphere soils collected in a disturbed savanna woodland ecosystem at Edendale, Pietermaritzburg in the KwaZulu-Natal (KZN) province of South Africa. The results for: Primary nutrients, Intermediate nutrients, Micronutrients and Soil relative acidity are represented as mean ±SE. Differing uppercase letters show significant differences (independent sample t-test, $p \leq 0.05$, n=20).

	Rhizosphere	Non-rhizosphere
Primary nutrients (mg/kg)		
Nitrogen	4187.01±567.76[a]	3223.90±487.21[b]
Phosphorus	11.83±3.66[a]	9.24±0.94[a]
Potassium	537.76±50.27[a]	201.15±56.66[b]
Intermediate nutrients (mg/kg)		
Magnesium	620.96±44.48[a]	555.75±85.87[a]
Calcium	4644.63±829.93[a]	3542.67±1121.89[b]
Micronutrients (mg/kg)		
Zinc	5.54±0.54[a]	3.69±0.57[b]
Manganese	50.84±4.72[a]	75.94±7.82[a]
Copper	74.83±37.36[a]	89.77±27.50[b]
Soil relative acidity		
Ph	5.75±0.71[a]	5.14±0.42[b]
Exchange acidity (cmolc/kg)	0.08±0.07[a]	0.05±0.02[a]
Total cation exchange (cmolc/kg)	29.61±4.62[a]	22.62±6.37[b]
Parameter		
Organic Carbon (%)	5.24	4.21
Organic Nitrogen (%)	0.445	0.297

 doi:10.20944/preprints202301.0366.v1

Organic C:N ratio	12:1	14:1

Bacterial identification

Bacterial identification of *E. natalensis* coralloid roots

A total of 10 bacterial species from *E. natalensis* coralloid roots were isolated and identified (Table 2). Two of the bacterial species (*Lysinibacillus xylanilyticus* and *Paenibacillus peoriae*) are phosphate solubilizing bacteria (Table 2). Additionally, the isolated N-fixing bacteria were *Bacillus thuringiensis, Bacillus pumilus, Bacillus safensis, P. peoriae, Paenibacillus taichungensis, Paenibacillus kribbensis, Paenibacillus taichungensis, Beijerinckia fluminensis, Lysinibacillus macrolides, Lysinibacillus pakistanensis, and Lysinibacillus xylanilyticus* (Table 2).

Table 2: Molecular identification of the bacterial community isolated from the coralloid roots of *E. natalensis'* growing in a disturbed savanna woodland ecosystem at Edendale, Pietermaritzburg in the KwaZulu-Natal (KZN) province of South Africa.

Family	Scientific name	Accession number	Similarity (%)	Function
Bacillaceae	*Lysinibacillus xylanilyticus*	NR_116698.1	99.93	P solubilizing [82] N-fixing [82]
	Lysinibacillus macrolides	NR_114920.1	99.06	N-fixing [84]
	Lysinibacillus pakistanensis	NR_113166.1	99.02	N-fixing [85]
	Bacillus thuringiensis	MG470721.1	99.72	N-fixing [86]
	Bacillus pumilus	MN581190.1	99.93	N-fixing [87]
	Bacillus safensis	CPO43404.1	99.58	N-fixing [88]
Paenibacillaceae	*Paenibacillus peoriae*	NR_117742.1	100	P solubilizing [89] N-fixing [90]

 doi:10.20944/preprints202301.0366.v1

	Paenibacillus taichungensis	NR_044428.1	96.70	N-fixing [91]
	Paenibacillus kribbensis	NR_025169.1	99.04	N-fixing [91]
Beijerinckiaceae	*Beijerinckia fluminensis*	NR_116306.1	99.76	N-fixing [92]

Bacterial identification of *E. natalensis* rhizosphere.

A total of 13 bacterial species from *E. natalensis* rhizosphere were isolated and identified (Table 3). Phosphate solubilizing bacteria such as *Caballeronia fortuita* and *Paraburkholderia steyni* were found in the rhizosphere of *E. natalensis* (Table 3). The identified N-fixing bacteria were *Bacillus fungorum*, *Paraburkholderia steyni*, *Paraburkholderia sabiae*, *Paraburkholderia tuberum*, *Massilia agilis*, and *Rhizobium mesosinicum* (Table 3). Furthermore, the N cycling bacteria identified were *Caballeronia fortuita*, *P. steyni*, *Gottfriedia luciferensis*, *Bacillus pocheonensis*, *Bacillus ginsengisoli*, *Variovorax guangxiensis*, *Chitinophaga ginsengihumi*, and *Phyllobacterium brassicacearum* (Table 3).

Table 3: Molecular identification of the bacterial community isolated from the *E. natalensis* rhizosphere soils growing in a disturbed savanna woodland ecosystem at Edendale, Pietermaritzburg in the KwaZulu-Natal (KZN) province of South Africa.

Family	Scientific name	Accession number	Similarity (%)	Function
Burkholderiales	*Caballeronia fortuita*	NR_145600.1	99.09	P solubilizing [93] N cycling [94]
	Paraburkholderia steyni	NR_164972.1	99.19	P solubilizing [95] N-fixing [96] N cycling [97]
	Paraburkholderia sabiae	NR_115261.1	99.28	N-fixing [98]

 doi:10.20944/preprints202301.0366.v1

	Paraburkholderia tuberum	NR_118081.1	99.83	N-fixing [99]
	Gottfrieda luciferensis	NR_025511.1	98.41	N cycling [99]
	Bacillus pocheonensis	NR_041377.1	99.62	N cycling [100]
Bacillaceae	*Bacillus ginsengisoli*	NR_109068.1	98.93	N cycling [101]
	Bacillus fungorum	NR_170494.1	90.10	N-fixing [102]
Comamonadaceae	*Variovorax guangxiensis*	NR_134828.1	99.30	N cycling [103]
Chitinophagaceae	*Chitinophaga ginsengihumi*	NR_134000.1	99.19	N cycling [104]
Phyllobacteriaceae	*Phyllobacterium brassicacearum*	NR_043190.1	91.3	N cycling [105]
Oxalobacteraceae	*Massilia agilis*	NR_157770.1	98.51	N-fixing [106]
Rhizobiaceae	*Rhizobium mesosinicum*	NR_043548.1	99.26	N-fixing [107]

Bacterial identification of *E. natalensis* non-rhizosphere soils.

A total of 10 bacterial species from *E. natalensis* non-rhizosphere soils were isolated and identified (Table 4). The P solubilizing bacteria included *P. steyni, Pseudomonas plecoglossicida, Novosphingobium barchaimii,* and *Methylobacterium dankookense* (Table 4). N-fixing bacteria included *P. steyni*, *M. dankookense, and Olivibacter oleidegradans* were (Table 4). Additionally, *P. steyni*, *P. plecoglossicida*, *Neobacillus bataviensis*, *B. ginsengisoli*, *Sphingomonas jatrophae*, *Olivibacter jiluni*, and *Phyllobacterium brassicacearum* were identified as N cycling bacteria (Table 4).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 January 2023 doi:10.20944/preprints202301.0366.v1

Table 4: Molecular identification of the bacterial community isolated from the non-rhizosphere soils of *E. natalensis* growing in a disturbed savanna woodland ecosystem at Edendale, Pietermaritzburg in the KwaZulu-Natal (KZN) province of South Africa.

Family	Scientific name	Accession number	Similarity (%)	Function
Burkholderiales	*Paraburkholderia steyni*	NR_164972.1	98.41	P solubilizing [108] N-fixing [109] N cycling [110]
Pseudomonadaceae	*Pseudomonas plecoglossicida*	NR_114226.1	98.38	Psolubilizing [111] N cycling [112]
Erythrobacteraceae	*Novosphingobium barchaimii*	NR_118314.1	99.65	P solubilizing [113]
Methylobacteriaceae	*Methylobacterium dankookense*	NR_116545.1	99.77	P solubilizing [114] N-fixing [115]
Bacillaceae	*Neobacillus bataviensis*	NR_114093.1	99.51	N cycling [116]
	Bacillus ginsengisoli	NR_109068.1	83.02	N cycling [117]
Sphingomonadaceae	*Sphingomonas jatrophae*	NR_159248.1	85.41	N cycling [117]
Sphingobacteriaceae	*Olivibacter jilunii*	NR_109321.1	99.30	N cycling [118]
	Olivibacter oleidegradans	NR_108900.1	98.41	N-fixing [118]

 doi:10.20944/preprints202301.0366.v1

Phyllobacteri-aceae	*Phyllobacterium brassicacearum*	NR_043190.1	90.30	N cycling [119]

Soil enzyme activities

Acid and alkaline phosphatase activities were higher in the non-rhizosphere soils than in the rhizosphere, although the difference was not significant statistically (Table 5). β-(D)-Glucosaminidase and nitrate reductase enzymes showed higher activity in the rhizosphere soils than in the non-rhizosphere soils, although the activity levels were not significantly different ($P > 0.05$) (Table 5).

Table 5: Soil enzyme activities in the rhizosphere and non-rhizosphere soils of *Encephalartos natalensis* growing in a disturbed savanna woodland ecosystem at Edendale, Pietermaritzburg in the KwaZulu-Natal (KZN) province of South Africa. Results are represented as mean ±SE. Differing uppercase letters show significant differences (independent sample t-test, $p \leq 0.05$, n=20).

Enzyme activity	Rhizosphere	Non-rhizosphere soils
β-(D)-Glucosaminidase ($nmolh^{-1}g^{-1}$)	20.47 ± 0.95^{a}	20.44 ± 1.03^{a}
Nitrate reductase ($\mu molh^{-1}g^{-1}$)	4496 ± 2116.6^{a}	3178.87 ± 1408.48^{a}
Acid phosphatase ($nmolh^{-1}g^{-1}$)	13.13 ± 2.03^{a}	13.50 ± 3.98^{a}
Alkaline phosphatase ($nmolh^{-1}g^{-1}$)	15.88 ± 4.10^{a}	19.47 ± 6.81^{a}

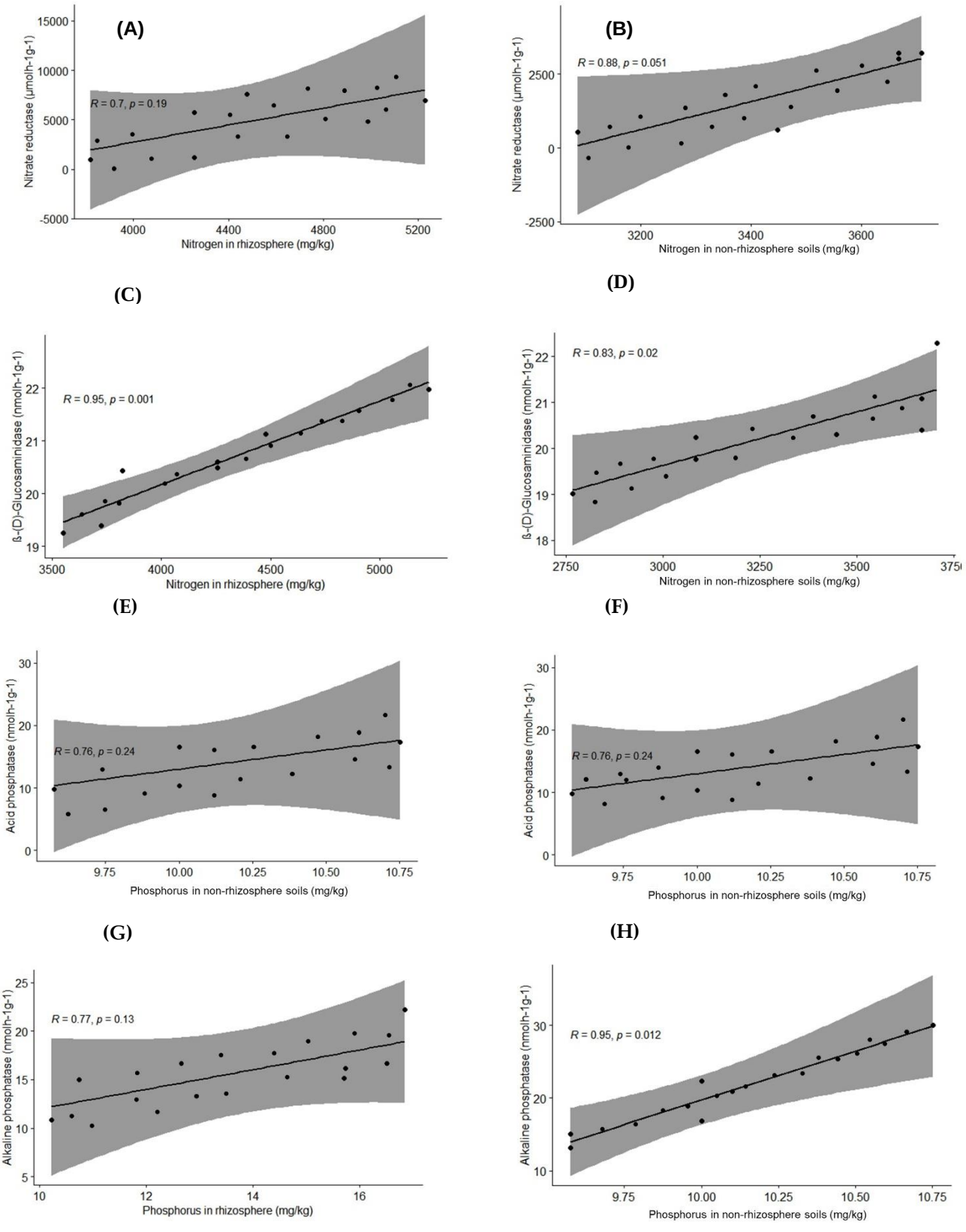
Linear correlation coefficients for nitrogen and phosphorus and their associated enzyme activities in *E. natalensis* rhizosphere and non-rhizosphere soils.

Figure 1A-B show that N concentration increased with increasing nitrate reductase activity in *E. natalensis* rhizosphere and non-rhizosphere soils. Similar trend was observed with

 doi:10.20944/preprints202301.0366.v1

N concentrations and β-(D)-Glucosaminidase activity in *E. natalensis* rhizosphere and non-rhizosphere soils (Figure 1C-D). Acid and alkaline phosphatase activities showed a positive correlation with P concentrations (Figure 1E-H).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 January 2023 doi:10.20944/preprints202301.0366.v1



 doi:10.20944/preprints202301.0366.v1

Figure 1: Linear correlation coefficient of nitrogen and phosphorus and their associated enzymes. A: nitrate reductase activity (μmolh^{-1}g^{-1}) vs nitrogen concentrations (mg/kg) in *E. natalensis's* rhizosphere. B: nitrate reductase activity (μmolh^{-1}g^{-1}) vs nitrogen concentrations (mg/kg) in *E. natalensis's* non-rhizosphere soils. C: β-(D)-Glucosaminidase activity (nmolh^{-1}g^{-1}) vs nitrogen concentrations (mg/kg) in *E. natalensis's* rhizosphere. D: β-(D)-Glucosaminidase activity (nmolh^{-1}g^{-1}) vs nitrogen concentrations (mg/kg) in *E. natalensis's* non-rhizosphere soils. E: acid phosphatase activity (nmolh^{-1}g^{-1}) activity vs phosphorus concentrations (mg/kg) in *E. natalensis's* rhizosphere. F: acid phosphatase activity (nmolh^{-1}g^{-1}) vs phosphorus concentrations (mg/kg) in *E. natalensis's* non-rhizosphere soils. G: alkaline phosphatase activity (nmolh^{-1}g^{-1}) activity vs phosphorus concentrations (mg/kg) in *E. natalensis's* rhizosphere. H: alkaline phosphatase activity (nmolh^{-1}g^{-1}) vs phosphorus concentrations (mg/kg) in *E. natalensis's* non-rhizosphere soils.

3. Discussion

Encephalartos natalensis-microbe symbionts and associated extracellular enzymes in soils are essential in enhancing soil nutrient inputs in savanna woodland disturbed ecosystem soils. Soil microbes are critical contributors to the mineralization and cycling of major soil nutrients and play an important role in promoting plant growth and development in natural ecosystems [40]. The most abundant group of microorganisms that occur in soils is bacteria and some of these culturable bacteria include the following genera: *Klebsiella*, *Paenibacillus*, *Lysinibacillus*, *Bacillus*, *Pseudomonas*, *Bradyrhizobium*, *Rhizobium*, and *Enterobacter* [41]. Bacteria play an important role in soil nutrient recycling, soil structure maintenance, and plant growth promotion [42,43,44]. Studies have demonstrated that indigenous growth-promoting bacteria contribute to P solubilization and N fixation, thus making P and N bioavailable for plant uptake in nutrient-deprived ecosystem soils [45]. In the coralloid roots, rhizosphere, and non-rhizosphere soils of *E. natalensis* growing at Edendale disturbed savanna woodland ecosystem, N cycling, N-fixing, and P solubilizing culturable bacteria

 doi:10.20944/preprints202301.0366.v1

strains were identified. The culturable bacteria strains identified included *Lysinibacillus*, *Bacillus*, *Paenibacillus*, *Beijerinckia*, *Caballeronia*, *Paraburkholderia*, *Gottfrieda*, *Variovorax*, *Chitinophaga*, *Phyllobacterium*, *Massilia*, *Rhizobium*, *Novosphingobium*, *Pseudomonas*, *Methylobacterium*, *Neobacillus*, and *Olivibacter* genera. [46] and [47] described bacteria in the *Bacillus*, *Pseudomonas*, *Paenibacillus*, and *Lysinibacillus* genera as plant growth-promoting rhizobacteria. *Bacillus* spp. secrete phytohormones such as (Indole-3-acetic acid) IAA, (Gibberellic acid) GA3, and kinetin and upregulate chlorophyll synthesis [48]. *Lysinibacillus macrolides* are N-fixing bacteria that enhance the total N content of soils and decompose organic matter [49]. *Paenibacillus polymyxa* strain ZM27 have Zn solubilizing properties, produces exopolysaccharides, indoles acetic acid, solubilizes phosphate, produces siderophores, and 1-aminocyclopropane-1-carboxylate ACC-deaminase catalase activity [50]. *Beijerinckia fluminensis* is a multifarious plant growth-promoting bacteria that produces exopolysaccharides (EPS), hydrogen cyanide (HCN), extracellular enzymes, indole-3-acetic acid, ammonia, siderophore, ACC-deaminase and plays a role in P solubilization [51]. *Paraburkholderia* is a bacterial genus that usually occurs in low pH agricultural and forest soil environments and contributes to several ecological processes, which include N_2 fixation, mineral weathering, and decomposition of plant litter (both lignin and cellulose) [52,53,54,55]. The *Massilia* bacteria are known to synthesize enzymes and various secondary metabolites and play a role in P dissolution [56]. *Paraburkholderia*, *Pseudomonas*, *Novosphingobium*, and *Methylobacterium* species play a role in P solubilization [56.57], producing phosphatase enzymes [57], and producing hormones promoting plant growth [58,59,60]. *Encephalartos natalensis* occurs in savannah and grassland ecosystems which are characterized by nutrient-poor and acidic soils [61,62]. The *E. natalensis* rhizosphere and non-rhizosphere soils richness in plant growth-promoting bacteria maybe linked to the species wide distribution in nutrient-deficient soils. The presence of these different bacterial species in *E. natalensis* rhizosphere and non-rhizosphere soils indicate that *E. natalensis* can form symbioses with a wide range of N-fixing, N cycling, and P solubilizing bacterial species. It is an indication that these bacteria play a crucial role in nutrient cycling, increasing the bioavailability of soil nutrition for *E. natalensis* and surrounding plants growing in savanna woodland ecosystems.

 doi:10.20944/preprints202301.0366.v1

Soil extracellular enzymes are important bioindicators of soil microflora metabolism and they provide information about soil quality, soil fertility, and the productivity status of soils [63]. Soil extracellular enzymes play a significant role in the conservation and recycling of key nutrients in nutrient-limited ecosystem soils [64]. Soil extracellular enzymes mineralize and recycle nutrients such as N, P, and C in soil increasing the bioavailability of the nutrients for uptake by plants [65]. Acetylglucosaminase, urease, and β-D-Glucosaminidase are enzymes involved in N cycling [66,67,68]. However, β-D-Glucosaminidase has also been reported to play a role in C-cycling and plays a significant role in the biological control of plant pathogens [69,70]. Phosphatases are responsible for P mineralization and cycling in soils [71]. Additionally, phosphatases produce P by hydrolysing phosphoric acid monoester to phosphate anions [72]. Acid and alkaline phosphatase enzymes are some examples of enzymes that have been studied extensively and their activity is strongly influenced by P availability and soil pH [73]. [74] observed increased β-Glucosaminidase, acid, and alkaline phosphatases activity in P deficient soils compared with P rich soils. These enzymes are linked to the bioavailability of nutrients in these nutrient-limited ecosystems through cycling N and P[75, 76]. Additionally, [77] and [78] suggested that increased enzyme activities in soils are regulated by soil P and N deficiency. Alkaline and acid phosphatase activities were positively correlated to the P concentrations in the rhizosphere and the non-rhizosphere soils of *E. natalensis* widely distributed in disturbed savanna woodland ecosystem at Edendale. [77] and [78] found that the P concentration of soils is directly proportional to phosphatase activities. This positive correlation between acid/alkaline phosphatase and total soil P concentration highlights that the bioavailability of soil P is positively correlated to the production of phosphatases in soils and that phosphatases contribute to soil P nutrition in this cycad's savanna woodland ecosystem soils. A study conducted by [79] showed that soil phosphatase activities were negatively correlated with the pool size of soil organic P fractions, suggesting that the bioavailability of P determines the activity of phosphatases. Furthermore, [79] reported that soil microbes increase phosphatases exudation in response to the chronic shortage of soil P, highlighting the functional role of microbes and associated enzymes in the efficient solubilization of P [79]. There was a positive correlation between β-(D)-Glucosaminidase activities and N concentration in the rhizosphere and the non-rhizosphere soils of

 doi:10.20944/preprints202301.0366.v1

E. natalensis. Studies conducted by [80] and [81] suggested that β-(D)-Glucosaminidase activities are positively related to soil N concentrations. Also, we observed a positive correlation between the nitrate reductase enzyme activity and soil N concentration in *E. natalensis* rhizosphere and the non-rhizosphere soils. [82] found that soil nitrate reductase was positively correlated with soil N concentration, highlighting the contribution of the enzyme nitrate reductase to soil N nutrition. Therefore, β-(D)-Glucosaminidase and nitrate reductase play a significant role in N mineralization in disturbed savanna woodland ecosystem at Edendale.

4. Materials and Methods

4.1.1. Study sites and target species:

This study was conducted in a disturbed savanna woodland ecosystem at Edendale, Pietermaritzburg in the KwaZulu-Natal (KZN) province of South Africa where the target species *E. natalensis* is widely distributed. *Encephalartos natalensis* rhizosphere and non-rhizosphere soils were collected from one large population (n>500) at Edendale (sampled plant co-ordinates not provided due to its red data listing[20] and conservation concerns as stated by sample collection permits issued by EzeMvelo KZN wildlife guided by The National Environmental Management Act (NEMA), Act 107 of 1998 and its amendments: National Environmental Management: Biodiversity Act (NEMBA), Act 10 of 2004, its amendments and regulations including the Threatened or Protected Species (TOPS) regulations of 2007). The Edendale savanna woodland is prone to overharvesting of *E. natalensis* bark for muthi/ traditional medicine, overgrazing by cattle and is dominated by invasive plants, such as, *Lantana camara* and *Isoglossa woodii*. Also, some *E. natalensis* plants are strangled by strangler figs.

4.1.2. Soil sampling and soil nutrition analysis

The sampled plants were between 15-30 meters apart from each other. From one savanna habitat 20 mature individuals of *E. natalensis* were randomly selected for the study. Soil samples were collected from 0-10 and 10-20 cm depths underneath the canopy of the cycad trees. The rhizosphere soils were collected at the four cardinal points at distances of about 30 cm from the stem and at the leaf canopy drip line. Similarly, the non-rhizosphere soils (similar depths) were collected from non-

 doi:10.20944/preprints202301.0366.v1

target sites defined by a radius of five meters from the base of each target plant as control. The direction of the control site was randomly selected using cardinal points of North, East, South, and West of the target plant. At each target plant, four soil samples for each depth were bulked to form a composite sample. For the control sample, 3-subsamples were collected within 1-m of each other. For any soil sample collected, the GPS coordinates were recorded as these serve as a reference for further investigations and can be utilized by other research projects of similar nature carried out within the area of study. Soil samples were sent to the KwaZulu-Natal Department of Agriculture and Rural Development's Agricultural Services Unit at Cedara College of Agriculture, South Africa for analysis of the following: total soil nutrition (primary, intermediate, and micronutrients) and cation concentrations; cation exchange acidity and pH.

4.1.3. Soil serial dilutions and bacterial DNA extraction

For bacterial extraction in the soil, serial dilutions were conducted for each soil sample, a spread plate technique was used. Once extracted, the bacteria were grown in sterile Petri dishes. Phosphate solubilizing bacteria were isolated and grown on Pikovskaya's agar plates which contained tricalcium phosphate (TCP) as the P source. The N cycling bacteria were grown on Simmons citrate agar plates which contained citrate as a carbon (C) source and inorganic ammonium salts as the only source of N and the N-fixing bacteria were grown on the Jensen's media agar (N-free media). Each selective media plate was replicated three times and incubated for 5 days at 30 ºC. Pure colonies were obtained by repeated streaking.

4.1.4. Coralloid roots surface sterilization and bacterial DNA extraction

The coralloid roots were collected from randomly selected adult plants of *E. natalensis* in the Edendale population (n>500). The coralloid roots were rinsed with distilled water and stored in ice. At the laboratory, 70% (v/v) ethanol was used to sterilize the coralloid roots for 30 seconds, the coralloid roots were then treated with 3.5% (v/v) sodium hypochlorite solution for 3 minutes. Thereafter, using distilled water, the coralloid roots were rinsed 10 times and stored in airtight vials that contained cotton wool and silica gel, then placed in the fridge at a temperature of 4 °C. Protocols as per [35] and [36] were used to conduct the bacterial isolation. For the bacterial extraction, the

 doi:10.20944/preprints202301.0366.v1

coralloid roots were subjected to 15% glycerol thereafter they were crushed using sterile tips. Once extracted, the bacteria were grown in sterile Petri dishes. Phosphate solubilizing bacteria were isolated and grown on Pikovskaya's agar plates which contained tricalcium phosphate (TCP) as the P source. The N cycling bacteria were grown on Simmons citrate agar plates which contained citrate as a C source and inorganic ammonium salts as the only source of N; and the N-fixing bacteria were grown on the Jensen's media agar (N-free media). Each selective media plate was replicated three times and incubated for 5 days at 30 ºC. Pure colonies were obtained by repeated streaking.

4.1.5. Coralloid roots, rhizosphere and non-rhizosphere soils bacterial amplification, sequencing, and identification.

Small portions of the 16S rDNA genes of pure bacterial colonies extracted from the coralloid roots, rhizosphere and non-rhizosphere soils were amplified using polymerase chain reaction (PCR). The specific primers used were 63F (5′- CAG GCCTAACACATGCAAGTC -3′) and 1387R (5′- GGGCGGTGTGTACAA GGC -3′) as per [37]. The PCR conditions were as follows: Initial denaturation: carried out at 94 ºC for 5 minutes, 30 cycles of denaturation at 94 ºC for 30 seconds; Annealing: carried out at 55 ºC for 30 seconds; Final elongation: carried out at 72 ºC for 10 minutes. Each 25 µl PCR reaction included the following: 11 µl sterile distilled water, 1 µl bacterial colony, 0.25 µl of the forward primer, 0.25 µl of the reverse primer and 12.5 µl Emerald AMP master mix (Takara Bio supplied by Separations, South Africa). Thereafter, the results were viewed using gel electrophoresis (1% agarose gel prepared using TAE buffer). The PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The resulting sequences were edited and subjected to BLASTN searches for bacterial identification (National Centre for Biotechnology Information, NCBI). https//: www.ncbi.nlm.nih.gov.

4.1.6. Soil enzymatic studies

Nitrogen cycling and P cycling enzyme activities (beta- glucosaminidase, acid phosphatase, and alkaline phosphatase) were conducted using the florescence-based method adapted from [38] and this was expressed in $nmolh^{-1}g^{-1}$. Five grams of each soil sample was homogenized at low speed in 50 mL ultrapure H_2O at 4 ºC for 2 hours. The resulting supernatants were transferred into 96-well

 doi:10.20944/preprints202301.0366.v1

microplates, thereafter the 4-MUB-phosphate substrate was added for P cycling enzymes and 4-MUB-N-acetyl-β-D-glucosaminide was added for the N cycling enzymes. Sample runs consisted of the following: 200 µl of soil samples of soil aliquots plus 50 µl of the substrate, these were incubated besides reference standards (200 µl buffer + 50 µl standard), quench standards (200 µl soil aliquots + 50 µl substrate), and blanks (250 µl buffer). After 2 hours of incubation at 30 ºC, 0.5 M of NaOH was used to stop the reaction. The fluorescent absorbance was measured at 450 nm using an Apex Scientific microplate reader (Durban, South Africa). It is important to note that before determining acid phosphate activity both the standards and buffer must have a pH of 5.

Nitrate reductase activities were conducted with an adapted method of [39]. Five grams of each soil sample was transferred in a solution that contained 4 mL of 0.9 mM 2.4-dinitrophenol, 1 mL of 25 mM KNO_3, and 5 mL of ultrapure H_2O in a sealed centrifuge tube (50 mL). The mixture was vigorously mixed before being incubated in the dark for 24-hours at a temperature of 30 ºC. After incubation, 10 mL of 4 M KCl solution was added to each sample and briefly mixed. Thereafter they were passed through Whatman number 1 filter paper. The enzymatic reaction was initiated by adding 2 ml of the filtrate to 1.2 ml of 0.19 M ammonium chloride buffer (pH 8.5) and 800 µl of the colour reagent (1% sulphanilamide in 1 N HCl and 0.2% N-(1-naphthyl) ethylenediamine di-hydrochloride (NEDD) before incubation for 30 mins in the dark at 30 º C. The absorbance was measured at 520 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA). The amount of nitrite (NO_2) released into the medium was expressed as 0.1 $\mu molh^{-1}g^{-1}$.

4.1.7. Statistical analysis

R studio (R version 4.1.2) was used for all analyses. An independent sample t-test was used to determine whether there is a significant difference between the soil characteristics and soil enzymatic activity of *Encephalartos natalensis* rhizosphere and non-rhizosphere soils. The assumption of normal distribution of data was tested using a 1-Kolmogorov-Smirnov test and the assumption of homogeneity of variance was tested using a Levene's test. Where these assumptions were not met a non-parametric (Wilcoxon signed-rank test) alternative was used. The independent sample t-test procedure was performed using R (4.1.2) using statistical package car, function Levene Test.

 doi:10.20944/preprints202301.0366.v1

5. Conclusions

Encephalartos natalensis species wide distribution in nutrient-poor and disturbed ecosystems such as acidic and nutrient-poor savanna woodland ecosystems may be linked to their established symbioses with nutrient-mineralizing microbes and associated extracellular enzymes in these ecosystems. Microbes identified in coralloid roots, rhizosphere, and non-rhizosphere soils and associated extracellular enzymes may contribute to soil nutrient inputs in savanna-woodland ecosystems.

Author Contributions: T.S. and S.N. were involved in field soil sample and data collection. S.N. conducted the study, analyzed results, and wrote the manuscript. A.M. is the study leader, planned and funded the experiments and analysis of this study as well as the manuscript write-up. M.P. was involved in writing and editing the manuscript. All authors read and approved the final copy of the submitted paper.

Funding: We appreciate the financial support from the National Research Foundation (Grant UID 129403 to TNS and 138091 to AM).

Data Availability Statement: All raw data and R scripts will be available upon request from Anathi Magadlela, email: MagadlelaA@ukzn.ac.za

Acknowledgments: We acknowledge the support of the University of KwaZulu-Natal (School of Life Sciences) and University of Mpumalanga (School of Biology and Environmental Sciences). Additionally, we acknowledge EzeMvelo KZN wildlife for issuing permits to work on cycads.

Conflicts of Interest: The authors declare no competing interests.

 doi:10.20944/preprints202301.0366.v1

References

1. Brenner, E. D., Stevenson, D. W., Twigg, R. W. 2003. Cycads: evolutionary innovations and the role of plant-derived neurotoxins. Trends in Plant Science 8, 446–452.
2. Mamay, S. H. 1969. Cycads: fossil evidence of late paleozoic origin. Science. 164, 295–296.
3. Donaldson, J. S. 2003. Regional overview: Africa. In: Donaldson JS, (ed), Status survey and conservation action plan, Cycad. IUCN/SSC Cycad Specialist Group. The World Conservation Union.
4. Ma, Y., Jiang, H., Wang, B., Zhou, G., Yu., S., Peng, S., Hao, Y., Wei, X., Liu, J., Yu., Z. 2009. Carbon Storage of cycad and other gymnosperm ecosystems in China: implications to evolutionary trends. Polish Journal of Ecology 57, 635–646.
5. Alvarez-Yepiz, J. C., Cueva, A., Dovciak, M., Teece, M., Enrico, A., Yepez, E. A. 2014. Ontogenic resource-use strategies in a rare long-lived cycad along environmental gradients. Conservation Physiology 2 7, 2–4.
6. Alvarez-Yepiz, J. C., Dovčiak, M. 2015. Enhancing ecosystem function through conservation: threatened plants increase local carbon storage in tropical dry forests. Tropical Conservation Science 8, 999–1008.
7. Berkelmann, D., Schneider, D., Engelhaupt, M., Heinemann, M., Christel, S., Wijayanti, M. 2018. How rainforest conversion to agricultural systems in Sumatra (Indonesia) affects active soil bacterial communities. Frontiers in Microbiology 9, 2381.
8. Donaldson, J. S. 2003. Regional overview: Africa. In: Donaldson JS, (ed), Status survey and conservation action plan, Cycad. IUCN/SSC Cycad Specialist Group. The World Conservation Union.
9. Hill, K. D., Chase, M. W., Stevenson, D. W., Hills, H. G., Schutzman, B. 2003. The families and genera of cycads: a molecular phylogenetic analysis of Cycadophyta based on nuclear and plastid DNA sequences. International Journal of Plant Science 164, 933–948.
10. Rousseau, P. 2012. A molecular systematics study of the African endemic cycads. MSc dissertation submitted in Department of Botany and Plant Biotechnology, University of Johannesburg, South Africa.
11. Mankga, L. T., Yessoufou, K. 2017. Factors driving the global decline of cycad diversity. AoB Plants 9, plx022.
12. Alvarez-Yepiz, J. C., Dovčiak, M. 2015. Enhancing ecosystem function through conservation: threatened plants increase local carbon storage in tropical dry forests. Tropical Conservation Science 8, 999–1008.
13. Donaldson, J. S. 2003. Regional overview: Africa. In: Donaldson JS, (ed), Status survey and conservation action plan, Cycad. IUCN/SSC Cycad Specialist Group. The World Conservation Union.

 doi:10.20944/preprints202301.0366.v1

14. Donaldson, J. S. 2008. South African *Encephalartos* species. Non-detrimental findings workshop case studies on succulents and cycads, study 4 on *Encephalartos*.
15. Bamigboye, S. O. 2013. Acceleration of cycads diversification towards the end of Pliocene in Africa. Journal of Ecosystem and Ecography 3, 4.
16. Alvarez-Yepiz, J. C., Cueva, A., Dovciak, M., Teece, M., Enrico, A., Yepez, E. A. 2014. Ontogenic resource-use strategies in a rare long-lived cycad along environmental gradients. Conservation Physiology 2 7, 2–4.
17. Alvarez-Yepiz, J. C., Dovčiak, M. 2015. Enhancing ecosystem function through conservation: threatened plants increase local carbon storage in tropical dry forests. Tropical Conservation Science 8, 999–1008.
18. Costa, J. L., Paulsrud, P., Lindblad, P. 1999. Cyanobiont diversity within coralloid roots of selected cycad species. FEMS Microbiology Ecology 28, 85–91.
19. Lobakova, E., Orazova, M. K., Dobrovolskaya, T. 2003. The structure of cyanobacterial communities formed during the degradation of apogeotropic roots of cycads. Microbiology 72, 634–637.
20. Gehringer, M. M., Pengelly, J. J., Cuddy, W. S., Fieker, C., Forster, P. I., Neilan, B. A. 2010. Host selection of symbiotic cyanobacteria in 31 species of the Australian cycad genus: *Macrozamia* (Zamiaceae). Molecular Plant-Microbe Interactions 23, 811–822.
21. Surez-Moo, P. D. J., Vovides, A. P., Griffith, M. P., Barona-Go´mez, F., Cibria´n-Jaramillo, A. 2019. Unlocking a high bacterial diversity in the coralloid root microbiome from the cycad genus *Dioon*. PLoS ONE 14(2), e0211271.
22. Sasse, J., Martinoia, E., Northern, T. 2018. Feed your friends: Do plant exudates shape the root microbiome? Trends in Plant Science 23(1), 35–41.
23. Warshan, D. 2017. Cyanobacteria in Symbiosis with Boreal Forest Feathermosses: From Genome Evolution and Gene Regulation to Impact on the Ecosystem. PhD Dissertation, Stockholm University.
24. Lucas, N., Bienaime, C., Belloy, C., Queneudec, M., Silvestre, F., Nava-Saucedo, J. E. 2008. Polymer biodegradation: mechanisms and estimation techniques–a review. Chemosphere, 73(4), 429–442.
25. Zungu, N. S., Egbewale, S. O., Olaniran, A. O., Pérez-Fernández, M., Magadlela, A. 2020. Soil nutrition, microbial composition, and associated soil enzyme activities in KwaZulu-Natal grasslands and savannah ecosystems soils. Applied Soil Ecology 155, 103663.
26. Hill, J. M., Roberts, J., Loeb, E., Khan, A., MacLellan, A., Hill, R. W. 1967. L-asparaginase therapy for leukemia and other malignant neoplasms: remission in human leukemia. Jama, 202(9), 882–888.

 doi:10.20944/preprints202301.0366.v1

27. Mega, T., Ikenaka, T., Matsushima, Y. 1972. Studies on N-acetyl-β-D-glucosaminidase of Aspergillus oryzae: II. Substrate specificity of the enzyme. Journal of Biochemistry, 71(1), 107−114.
28. Zungu, N. S., Egbewale, S. O., Olaniran, A. O., Pérez-Fernández, M., Magadlela, A. 2020. Soil nutrition, microbial composition, and associated soil enzyme activities in KwaZulu-Natal grasslands and savannah ecosystems soils. Applied Soil Ecology 155, 103663.
29. Turner, B. L., Baxter, R., Whitton, B. A. 2002. Seasonal phosphatase activity in three characteristic soils of the English uplands polluted by long-term atmospheric nitrogen deposition. Environmental Pollution 120(2), 313−317.
30. Henriksson, G., Sild, V., Szabó, I.G., Pettersson, G., Johansson, G. 1998. Substrate specificity of cellobiose dehydrogenase from *Phanerochaete chrysosporium*. Biochimica et Biophysica Acta Protein Structure and Molecular Enzymology 1383(1), 48−54.
31. Lindström, K., Mousavi, S. A. 2010. Rhizobium and Other N fixing Symbioses. In: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester.
32. Lindström, K., Mousavi, S. A. 2010. Rhizobium and Other N fixing Symbioses. In: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester.
33. Santi, C., Bogusz, D., Franche, C. 2013. Biological nitrogen fixation in non-legume plants. Annals of Botany 111, 743−767.
34. Raimondo, D., von Staden, L., Foden, W., Victor, J. E., Helme, N. A., Turner, R. C., Kamundi, D. A., Manyama, P. A. 2009. Red List of South African Plants. Strelitzia 25. South African National Biodiversity Institute, Pretoria.
35. Magadlela, A., Beukes, C., Venter, F., Steenkamp, E., Valentine, A. 2017. Does P deficiency affect nodule bacterial composition and N source utilization in a legume from nutrient-poor Mediterranean-type ecosystems? Soil Biology and Biochemistry 164−74.
36. Matiwane, S. E., Aremu, A. O., Valentine, A. J., Magadlela, A. 2019. Nutritional status of KwaZulu-Natal soils affects microbe symbiosis, nitrogen utilization and growth of *Vigna radiata* (L.) R. Walczak. South African Journal of Botany 15−20.
37. Magadlela, A., Beukes, C., Venter, F., Steenkamp, E., Valentine, A. 2017. Does P deficiency affect nodule bacterial composition and N source utilization in a legume from nutrient-poor Mediterranean-type ecosystems? Soil Biology and Biochemistry 164−74.

 doi:10.20944/preprints202301.0366.v1

38. Jackson, C. R., Tyler, H. L., Millar, J. J. 2013. Determination of microbial extracellular enzyme activity in waters, soils, and sediments using high throughput microplate assays. Journal of Visualized Experiments, e50399.
39. Bruckner, A., Wright, J., Kampichler, C., Bauer, R., Kandeler, E. 1995. A method of preparing mesocosms for assessing complex biotic processes in soils. Biology and fertility of soils 19(2), 257–262.
40. Hill, J. M., Roberts, J., Loeb, E., Khan, A., MacLellan, A., Hill, R. W. 1967. L-asparaginase therapy for leukemia and other malignant neoplasms: remission in human leukemia. Jama, 202(9), 882–888.
41. Matiwane, S. E., Aremu, A. O., Valentine, A. J., Magadlela, A. 2019. Nutritional status of KwaZulu-Natal soils affects microbe symbiosis, nitrogen utilization and growth of *Vigna radiata* (L.) R. Walczak. South African Journal of Botany 15–20.
42. Bell, T., Newman, J. A., Silverman, B. W., Turner, S. L., Lilley, A. K. 2005. The contribution of species richness and composition to bacterial services. Nature 436, 1157–1160.
43. Glick, B. R. 1995. The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology 41, 109–117.
44. Nacke, H., Thu¨rmer, A., Wollherr, A., Will, C., Hodac, L. 2011. Pyrosequencing-based assessment of bacterial community structure along different management types in German forest and grassland soils. PLoS One 6, e17000.
45. Gupta, S., Meena, M. K., Datta, S. 2014. Isolation, characterization of plant growth promoting bacteria from the plant *Chlorophytum borivilianum* and in-vitro screening for activity of nitrogen fixation, phosphate solubilization and IAA production. International Journal of Current Microbiology Applied Sciences 3 (7), 1082–1090.
46. Adeniyan, O. N., Ojo, A. O. O. A., Adediran, J. A. 2011. Comparative study of different organic manures and NPK fertilizer for improvement of soil chemical properties and dry matter yield of maize in two different soils. Journal of Soil Science and Environmental Management 2(1), 9–3.
47. Akinrinlola, R. J., Yuen, G.Y., Drijber, R. A., Adesemoye, A. O. 2018. Evaluation of *Bacillus* strains for plant growth promotion and predictability of efficacy by in vitro physiological traits. International journal of microbiology 5, 6–9.
48. Sunera, A., Saqib, S., Uddin, S., Zaman, W., Ullah, F., Ayaz, A., Asghar, M., Rehman, S., Munis, M. F. H. 2020. Characterization and phytostimulatory activity of bacteria isolated from tomato (*Lycopersicin esculentum* Mill.) rhizosphere. Microbial Pathogenesis 140, 103966.

49. Hameed, A., Yeh, M. W., Hsieh, Y. T., Chung, W. C., Lo, C. T., Young, L. S. 2015. Diversity and functional characterization of bacterial endophytes dwelling in various rice (*Oryza sativa* L.) tissues, and their seed-borne dissemination into rhizosphere under gnotobiotic P-stress. Plant and soil 394(1), 177–97.
50. Hussain, A., Ahmad, M., Nafees, M., Iqbal, Z., Luqman, M., Jamil, M., Maqsood, A., Mora-Poblete, F., Ahmar, S., Chen, J. T., Alyemeni, M. N. 2020. Plant-growth-promoting *Bacillus* and *Paenibacillus* species improve the nutritional status of *Triticum aestivum* L. PLoS One. 15(12), e0241130.
51. Al-Shwaiman, H. A., Shahid, M., Elgorban, A. M., Siddique, K. H., Syed, A. 2022. *Beijerinckia fluminensis* BFC-33, a novel multi-stress-tolerant soil bacterium: Deciphering the stress amelioration, phytopathogenic inhibition and growth promotion in *Triticum aestivum* (L.). Chemosphere 295, 133843.
52. Berkelmann, D., Schneider, D., Engelhaupt, M., Heinemann, M., Christel, S., Wijayanti, M. 2018. How rainforest conversion to agricultural systems in Sumatra (Indonesia) affects active soil bacterial communities. Frontiers in Microbiology 9, 2381.
53. Colin, Y., Nicolitch, O., Turpault, M. P., Uroz, S. 2017. Mineral types and tree species determine the functional and taxonomic structures of forest soil bacterial communities. Applied and Environmental Microbiology 83, e02684–e02616.
54. Nicolitch, O., Feucherolles, M., Churin, J. -L., Fauchery, L., Turpault, M. -P., Uroz, S. 2019. A microcosm approach highlights the response of soil mineral weathering bacterial communities to an increase of K and Mg availability. Scientific Reports 9, 14403.
55. Samuels, T., Bryce, C., Landenmark, H., Marie-Loudon, C., Nicholson, N., Stevens, A. H. 2020. Microbial weathering of minerals and rocks in natural environments in: Biogeochemical cycles: Ecological drivers and environmental impact. eds. K. Dontsova, B. Zsuzsanna, and G. Le Roux (New Jersey, NJ: John Wiley & Sons, Inc. and American Geophysical Union), pp 59–79.
56. Sithole, N., Pérez-Fernández M, Magadlela, A. 2019. Nutritional status of soils from KwaZulu-Natal modulates symbiotic interactions and plant performance in *Vigna unguiculata* L. (Walp). Applied Soil Ecology 142, 1–7.
57. Vyas, P., Gulati, A. 2009. Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. BMC microbiology 9(1), 1–5.
58. Kutschera, U. 2007. Plant-associated methylobacteria as co-evolved phytosymbionts: a hypothesis. Plant Signaling and Behaviour 2, 74–78.

 doi:10.20944/preprints202301.0366.v1

59. Omer, Z. S., Tombolini, R, Broberg, A., Gerhardson, B. 2004. Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. Plant Growth Regulation 43, 93−96.
60. Trotsenko, Y. A, Ivanova, E. G, Doronina, N. V. 2001. Aerobic methylotrophic bacteria as phytosymbionts. Mikrobiologiia 70, 623−632.
61. Gehringer, M. M., Pengelly, J. J., Cuddy, W. S., Fieker, C., Forster, P. I., Neilan, B. A. 2010. Host selection of symbiotic cyanobacteria in 31 species of the Australian cycad genus: *Macrozamia* (Zamiaceae). Molecular Plant-Microbe Interactions 23, 811−822.
62. Lobakova, E., Orazova, M. K., Dobrovolskaya, T. 2003. The structure of cyanobacterial communities formed during the degradation of apogeotropic roots of cycads. Microbiology 72, 634−637.
63. Lee, S. H., Kim, M. S., Kim, J. G., Kim, S. O. 2020. Use of soil enzymes as indicators for contaminated soil monitoring and sustainable management. Sustainability (Switzerland) 12, 1−14.
64. Magadlela, A., Lembede, Z., Egbewale, S. O., Olaniran, A. O. 2022. The metabolic potential of soil microorganisms and enzymes in phosphorus-deficient KwaZulu-Natal grassland ecosystem soils. Applied Soil Ecology 181, 104647.
65. Rao, M. A., Scelza, R., Gianfreda, L. 2014. Soil enzymes. Enzymes in Agricultural Sciences. Foster City: OMICS Group eBooks 2, 10−24.
66. Burns, R. G., DeForest, J. L., Marxsen, J., Sinsabaugh, R. L., Stromberger, M. E, Wallenstein, M. D, Weintraub, M. N, Zoppini, A. 2013. Soil enzymes in a changing environment: current knowledge and future directions. Soil Biology and Biochemistry 58, 216−234.
67. Fujita, K., Kunito, T., Matsushita, J., Nakamura, K., Moro, H., Yoshida, S., Toda, H., Otsuka, S., Nagaoka, K. 2018. The nitrogen supply rate regulates microbial resource allocation for the synthesis of nitrogen-acquiring enzymes. PloS One 13 (8), e0202086.
68. Ma, W., Liu, Y., Lv, X., Li, J., Du, G., Liu, L. 2019. Combinatorial pathway enzyme engineering and host engineering overcomes pyruvate overflow and enhances overproduction of N-acetylglucosamine in Bacillus subtilis. Microbial cell factories 18(1), 1−2.
69. Lorito, M., Hayes, C. K., Di Pietro, A., Woo, S. L., Harman, G. E. 1994. Purification, characterization, and synergistic activity of a glucan 1,3-b-glucosidase and and Nacetyl-b-glucosaminidase from *Trichoderma Harzianum*. Phytopathology 84, 398−405.

70. Parham, J. A., Deng, S. P. 2000. Detection, quantification, and characterization of β-glucosaminidase activity in soil. Soil Biology and Biochemistry 32 (8-9), 1183-1190.
71. Adetunji, A. T., Lewu, F. B., Mulidzi, R., Ncube, B. 2017. The biological activities of β-glucosidase, phosphatase and urease as soil quality indicators: a review. Journal of Soil Science and Plant Nutrition 17 (3), 794–807.
72. Kalsi, H. K., Singh, R., Dhaliwal, H. S., Kumar, V. 2016. Phytases from *Enterobacter* and *Serratia* species with desirable characteristics for food and feed applications. Biotechnology 6 (1), 1–13.
73. Touhami, D., McDowell, R. W., Condron, L. M. 2020. Role of organic anions and phosphatase enzymes in phosphorus acquisition in the rhizospheres of legumes and grasses grown in a low phosphorus pasture soil. Plants 9 (9), 1185
74. Magadlela, A., Lembede, Z., Egbewale, S. O., Olaniran, A. O. 2022. The metabolic potential of soil microorganisms and enzymes in phosphorus-deficient KwaZulu-Natal grassland ecosystem soils. Applied Soil Ecology 181, 104647.
75. Liu, Z., Rong, Q., Zhou, W., Liang, G. 2017. Effects of inorganic and organic amendment on soil chemical properties, enzyme activities, microbial community and soil quality in yellow clayey soil. PLoS ONE 12, 4–8.
76. Magadlela, A., Lembede, Z., Egbewale, S. O., Olaniran, A. O. 2022. The metabolic potential of soil microorganisms and enzymes in phosphorus-deficient KwaZulu-Natal grassland ecosystem soils. Applied Soil Ecology 181, 104647.
77. Gavrilova, A. N. Savchenka, N. I., Shimko, N. A. 1974. Forms of phosphorus and phosphatase activity of chief soil type in the BSSR. International Congress of Soil Science 4, 281–288.
78. Speir, T. W., Ross, D. J. 1978. Soil phosphatase and sulphatase. In: RG. Burns (ed.) soil enzymes. Academic Press New York.
79. Kitayama, K. 2013. The activities of soil and root acid phosphatase in the nine tropical rain forests that differ in phosphorus availability on Mount Kinabalu, Borneo. Plant and Soil 367(1), 215–224.
80. Cenini, V. L., Fornara, D. A, McMullan, G., Ternan, N., Carolan, R., Crawley, M. J, Clément, J. C., Lavorel, S. 2016. Linkages between extracellular enzyme activities and the carbon and nitrogen content of grassland soils. Soil Biology and Biochemistry 96, 198–206.
81. Ekenler, M., Tabatabai, M. 2002. β-Glucosaminidase activity of soils: effect of cropping systems and its relationship to nitrogen mineralization. Biology and Fertility of Soils 36(5), 367–376.

82. Cheng, Y., Elrys, A. S., Merwad, A. R., Zhang, H., Chen, Z., Zhang, J., Cai, Z., Müller, C. 2022. Global patterns and drivers of soil dissimilatory nitrate reduction to ammonium. Environmental Science and Technology 56(6), 3791−800.
83. Ahsan, N., Marian, M., Suga, H., Shimizu, M. 2021. *Lysinibacillus xylanilyticus* strain GIC41 as a potential plant biostimulant. Microbes and environments 36(4), ME21047.
84. Jyolsna, K. S., Bharathi, N., Ali, L. R., Paari, K. A. 2021. Impact of *Lysinibacillus macroides*, a potential plant growth promoting rhizobacteria on growth, yield and nutritional value of tomato Plant (*Solanum lycopersicum* L. f1 hybrid Sachriya). Plant Science Today 8(2), 365−372.
85. Rasheed, M., Naseer, T., Hassan, A., Hassan, F., Hayat, R., Jilani, G., Vaseer, S. G., Ali, M. B. 2020. Isolation of nodule associated bacteria for promotion of lentil growth. Pakistan Journal of Agricultural Research 33(1), 170−179.
86. Goswami, D., Thakker, J.N., Dhandhukia, P. C. 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food & Agriculture 2(1), 1127500.
87. Hernandez, J. P., de-Bashan, L. E., Rodriguez, D. J., Rodriguez, Y., Bashan, Y. 2009. Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. European Journal of Soil Biology 45(1), 88−93.
88. Hashem, A., Tabassum, B., Abd_Allah, E. F. 2019. *Bacillus subtilis*: A plant-growth promoting rhizobacterium that also impacts biotic al stress. Saudi Journal of Biological Science 26(6), 1291−1297.
89. Grady, E. N., MacDonald, J., Liu, L., Richman, A., Yuan, Z. C. 2016. Current knowledge and perspectives of *Paenibacillus*: a review. Microbial Cell Factories 15(1), 1−8.
90. von der Weid, I., Duarte, G. F., van Elsas, J. D., Seldin, L. 2002. *Paenibacillus brasilensis* sp. nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil. International Journal of Systematic and Evolutionary Microbiology 52(6), 2147−2153.
91. Mayer, E., Dörr de Quadros, P., Fulthorpe, R. 2019. *Plantibacter flavus*, *Curtobacterium herbarum*, *Paenibacillus taichungensis*, and *Rhizobium selenitireducens* endophytes provide host-specific growth promotion of Arabidopsis thaliana, basil, lettuce, and bok choy plants. Applied and environmental microbiology 85(19), e00383−89.
92. Oggerin, M., Arahal, D. R., Rubio, V., Marín, I. 2009. Identification of *Beijerinckia fluminensis* strains CIP 106281T and UQM 1685T as Rhizobium radiobacter strains, and proposal of *Beijerinckia doebereinerae* sp. nov. to

 doi:10.20944/preprints202301.0366.v1

accommodate *Beijerinckia fluminensis* LMG 2819. International Journal of Systematic and Evolutionary Microbiology 59(9), 2323–2328.

93. Uroz, S., Oger, P. 2017. *Caballeronia mineralivorans* sp. nov., isolated from oak-Scleroderma citrinum mycorrhizosphere. Systematic and Applied Microbiology 40(6), 345–351.
94. Hetz, S. A., Poehlein, A., Horn, M. A. 2020. Whole-genome sequences of two new *Caballeronia* strains isolated from cryoturbated peat circles of the permafrost-affected Eastern European Tundra. Microbiology Resource Announcements 9(31), e00731–20.
95. Hetz, S. A., Poehlein, A., Horn, M. A. 2020. Whole-genome sequences of two new *Caballeronia* strains isolated from cryoturbated peat circles of the permafrost-affected Eastern European Tundra. Microbiology Resource Announcements 9(31), e00731–20.
96. Guo, Y. Z., Ding, X. M., Yao, L., Xu, D. M., Zhao, Y. J., Feng, F. Y., Meng, J. Y. 2019. Isolation and Identification of *Massilia* sp. B260 and Its Effect on Seedling Raising. Biotechnology Bulletin 35(9), 144.
97. Tapia-García, E. Y., Arroyo-Herrera, I., Rojas-Rojas, F. U., Ibarra, J. A., Vásquez-Murrieta, M. S., Martínez-Aguilar, L., López-Lara, I. M., Whitman, W. B., De Los Santos, P. E. 2020. *Paraburkholderia lycopersici* sp. nov., a nitrogen-fixing species isolated from rhizoplane of *Lycopersicon esculentum* Mill. var. Saladette in Mexico. Systematic and Applied Microbiology 43(6), 126133.
98. Too, C. C., Ong, K. S., Yule, C. M., Keller, A. 2021. Putative roles of bacteria in the carbon and nitrogen cycles in a tropical peat swamp forest. Basic and Applied Ecology 52, 109–123.
99. Soares Neto, C. B., Ribeiro, P. R., Fernandes-Júnior, P. I., de Andrade, L. R., Zilli, J. E., Mendes, I. C., do Vale, H. M., James, E. K., dos Reis Junior, F. B. 2022. *Paraburkholderia atlantica* is the main rhizobial symbiont of Mimosa spp. in ultramafic soils in the Brazilian Cerrado biome. Plant and Soil 10, 1–5.
100. Turner, B. L., Baxter, R., Whitton, B. A. 2002. Seasonal phosphatase activity in three characteristic soils of the English uplands polluted by long-term atmospheric nitrogen deposition. Environmental Pollution 120(2), 313–317.
101. Beneduzi, A., Peres, D., Vargas, L. K., Bodanese-Zanettini, M. H., Passaglia, L. M. 2008. Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil. Applied Soil Ecology 39(3), 311–320.

 doi:10.20944/preprints202301.0366.v1

102. Sun, B., Bai, Z., Bao, L., Xue, L., Zhang, S., Wei, Y., Zhang, Z., Zhuang, G., Zhuang, X. 2020. *Bacillus subtilis* biofertilizer mitigating agricultural ammonia emission and shifting soil nitrogen cycling microbiomes. Environment International 144, 105989.

103. Seldin, L., Dubnau, D. 1985. Deoxyribonucleic acid homology among *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus azotofixans*, and other nitrogen-fixing *Bacillus* strains. International Journal of Systematic and Evolutionary Microbiology 35(2), 151–154.

104. Nguyen, T. M., Trinh, N. H., Kim, J. 2018. Proposal of three novel species of soil bacteria, *Variovorax ureilyticus*, *Variovorax rhizosphaerae*, and *Variovorax robiniae*, in the family Comamonadaceae. Journal of Microbiology 56(7), 485–492.

105. Jin, C. Z., Jin, L., Kang, D. H., Liu, M. J., Lee, J. M., Park, D. J., Kim, C. J. 2021. Description of desferrioxamine-producing bacterium *Chitinophaga agrisoli* sp. nov., isolated from soil. Antonie van Leeuwenhoek 114(6), 741–750.

106. Anderson, C. R., Hamonts, K., Clough, T. J., Condron, L. M. 2014. Biochar does not affect soil N-transformations or microbial community structure under ruminant urine patches but does alter relative proportions of nitrogen cycling bacteria. Agriculture, Ecosystems & Environment 191, 63–72.

107. Guo, Y. Z., Ding, X. M., Yao, L., Xu, D. M., Zhao, Y. J., Feng, F. Y., Meng, J. Y. 2019. Isolation and Identification of *Massilia* sp. B260 and Its Effect on Seedling Raising. Biotechnology Bulletin 35(9), 144.

108. Gao, Z. H., Ruan, S. L., Huang, Y. X., Lv, Y. Y., Qiu, L. H. 2019. *Paraburkholderia phosphatilytica* sp. nov., a phosphate-solubilizing bacterium isolated from forest soil. International Journal of Systematic and Evolutionary Microbiology 69(1), 196–202.

109. Tapia-García, E. Y., Arroyo-Herrera, I., Rojas-Rojas, F. U., Ibarra, J. A., Vásquez-Murrieta, M. S., Martínez-Aguilar, L., López-Lara, I. M., Whitman, W. B., De Los Santos, P. E. 2020. *Paraburkholderia lycopersici* sp. nov., a nitrogen-fixing species isolated from rhizoplane of *Lycopersicon esculentum* Mill. var. Saladette in Mexico. Systematic and Applied Microbiology 43(6), 126133.

110. Too, C. C., Ong, K. S., Yule, C. M., Keller, A. 2021. Putative roles of bacteria in the carbon and nitrogen cycles in a tropical peat swamp forest. Basic and Applied Ecology 52, 109–123.

 doi:10.20944/preprints202301.0366.v1

111. Pandey, A., Trivedi, P., Kumar, B., Palni, L. M. 2006. Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. Current microbiology 53(2), 102–107.

112. Toyofuku, M., Nomura, N., Kuno, E., Tashiro, Y., Nakajima, T., Uchiyama, H. 2008. Influence of the *Pseudomonas quinolone* signal on denitrification in *Pseudomonas aeruginosa*. Journal of bacteriology 190(24), 7947–7956.

113. Hernández-Hernández, L., Coutino-Megchun, J. T., Rincon-Molina, C. I., Ruíz-Valdiviezo, V. M., Culebro-Ricaldi, J. M., Cruz-Rodríguez, R. I., Palomeque-Dominguez, H. H., Rincón-Rosales, R. 2018. Endophytic bacteria from root nodules of *Ormosia macrocalyx* with potential as plant growth promoters and antifungal activity. Journal of Environmental Biology 39(6), 997–1005.

114. Dourado, M. N., Aparecida Camargo Neves, A., Santos, D. S., Araújo, W. L. 2015. Biotechnological and agronomic potential of endophytic, pink-pigmented methylotrophic *Methylobacterium* spp. BioMed Research International 4(1), 2–5.

115. Pascual, J. A., Ros, M., Martínez, J., Carmona, F., Bernabé, A., Torres, R., Lucena, T., Aznar, R., Arahal, D. R., Fernández, F. 2020. *Methylobacterium symbioticum* sp. nov., a new species isolated from spores of *Glomus iranicum* var. tenuihypharum. Current Microbiology 77(9), 2031–2041.

116. Jiang, L., Lee, M. H., Jeong, J. C., Kim, D. H., Kim, C. Y., Kim, S. W., Lee, J. 2021. *Neobacillus endophyticus* sp. nov., an endophytic bacterium isolated from Selaginella involvens roots. International Journal of Systematic and Evolutionary Microbiology 71(3), 004581.

117. Sun, B., Bai, Z., Bao, L., Xue, L., Zhang, S., Wei, Y., Zhang, Z., Zhuang, G., Zhuang, X. 2020. *Bacillus subtilis* biofertilizer mitigating agricultural ammonia emission and shifting soil nitrogen cycling microbiomes. Environment International 144, 105989.

118. Özdoğan, D. K., Akçelik, N., Akçelik, M. 2022. Genetic diversity and characterization of plant growth-promoting effects of bacteria isolated from rhizospheric soils. Current Microbiology 79(5), 1–3

119. Anderson, C. R., Hamonts, K., Clough, T. J., Condron, L. M. 2014. Biochar does not affect soil N-transformations or microbial community structure under ruminant urine patches but does alter relative proportions of nitrogen cycling bacteria. Agriculture, Ecosystems & Environment 191, 63–72.