

Encephalartos villosus relies on atmospheric nitrogen than soil derived nitrogen to maintain growth in nutrient-deficient and acidic soils

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Research Article

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Abstract

Background

Encephalartos villosus is a forest understory dwarf cycad growing in nutrient-deficient and acidic soils due to their association with plant growth promoting bacteria. This study investigated the effects of soil characteristics (pH, nutrition, total cation, and exchange acidity) on cycad-microbe symbiosis and the nitrogen (N) source preference of *E. villosus* growing in Rhebu and Oceanview, Eastern Cape, South Africa.

Methods

Coralloid roots and leaf material from targeted *E. villosus* individuals in both locations were analysed for N isotope, total plant N, and total plant phosphorus (P).

Results

Plant growth promoting bacteria were associated with *E. villosus* growing in Rhebu and Oceanview. The culturable bacteria strains isolated from the coralloid roots of *E. villosus* growing in Rhebu and Oceanview with N-fixing traits were *Lysinibacillus fusiformis*, *Rhizobium huautlense*, *Stenotrophomonas* sp., *Enterobacter cloacae*, and *Paenibacillus polymyxa*. *Encephalartos villosus* growing in Rhebu and Oceanview effectively fixed more than 70% of its total N from the atmosphere. Less than 25% of the total N utilized by *E. villosus* was derived from the soil. There were no significant variations in the leaf number, length, total N, and P in *E. villosus* growing in Rhebu and Oceanview.

Conclusions

The findings of this study revealed that *E. villosus* maintains growth and plant nutrition in nutrient-deficient and acidic soils by establishing symbiotic associations with plant growth-promoting bacteria and relying mostly in atmospheric N.

Introduction

Cycads are ancient gymnosperms that have persisted through many extinction events, survived competition with fast-growing angiosperms and environmental stresses such as drought and nutrient deficiencies (Brenner et al., 2003; Marler and Lindström, 2018; Erdei et al., 2019). The Zamiaceae and Stangeriaceae are the most diverse cycad families in South Africa (Cousins et al., 2013). The *Encephalartos* genus belongs to the Zamiaceae family and has 37 species that are indigenous to South Africa (Raimondo et al., 2009; Condamine et al., 2015). Thus, making South Africa an important centre for cycad diversity (Donaldson, 2003). In South Africa, cycads are used for horticultural purposes and

traditional medicine (Raimondo et al., 2009; Cousins et al., 2012). *Encephalartos villosus* is a forest understory dwarf cycad believed to repel evil spirits and lightning by African traditional people (Hutching, 1996; Ravele and Makhado, 2009). Being among the most diverse and widely distributed species in the *Encephalartos* genus makes *E. villosus* easily assessable and more prone to be harvested (Yessoufou et al., 2014; Cousins et al., 2012). Using cycads for traditional medicine leads to unsustainable harvesting practices that threaten cycad diversity (Donaldson, 2008; Cousins et al., 2012). Cycads are slow-growing plants, existing in isolated populations with low recruitment rates and species-specific pollination, which makes it hard for populations to recover, thus making cycads vulnerable to extinction (Dehgan, 1983; Golding and Hurter, 2003; Raimondo and Donaldson, 2003; Laidlaw and Forster, 2012; Roodt et al., 2017; Mankga et al., 2020).

The continual use of cycads for traditional medicine increases unsustainable harvesting practices leading to population declines (Bamigboye and Tshisikhawe, 2020) and what we now know as the “cycad extinction crisis.” According to Donaldson (2003), 82% of the world’s cycads were listed as threatened by the 1997 IUCN red list of threatened plants. Also, Raimondo et al. (2009) reported that all species belonging to the *Encephalartos* genus are listed on the red list of South African plants. Consequently, cycads are the most threatened plant group, are of conservation concern, and have been identified as flagship species for conservation (Keppel, 2002; Donaldson, 2003; Golding and Hurter, 2003). Cycad conservation is of utmost importance because of their role in carbon (C) sequestration (Ma et al., 2009) and nutrient cycling (Nackley et al., 2018; Marler and Calonje, 2020; Pecundo et al., 2021). Cycads are the only gymnosperms that can partake in a symbiotic association with nitrogen (N) fixing bacteria (Chang et al., 2019; Zheng et al., 2002). This association enables cycads to contribute to soil nutrient inputs. Marler and Calonje (2020) reported higher N, C and phosphorus (P) concentrations in *Cycas micronesica* and *Zamia integrifolia* rhizosphere soils compared to bulk soil, highlighting the soil nutrient contributions by cycads.

Cycads are reportedly associated with cyanobacteria belonging to the *Nostoc*, *Scytonema* and *Richelia* genera and proteobacteria belonging to the *Bradyrhizobium* and *Burkholderia* genera (Grobbelaar et al., 1986; Barea et al., 2005; Gutiérrez-García et al., 2019). Although cycads have been reported to be associated with cyanobacteria and proteobacteria, this study focuses on the N-fixing role of proteobacteria associated with cycads. The proteobacteria are housed in the coralloid roots and play a role in N-fixation through biological N fixation (BNF) (Ahern and Staff, 1994; Costa et al., 1999; Lindblad, 2009). Atmospheric N is chemically inert and cannot be assimilated by plants, microbes use nitrogenase to break down the triple bond between N atoms, and the N is converted to a form (NH_4) that can be easily assimilated by plants (Zheng et al., 2002; Chang et al., 2009). Biological N fixation is an energetically costly process, increasing the energy and P demands of plants that partake in BNF (Magadlela et al., 2016; Liu et al., 2018). Phosphorus is an essential element in the production of ATP and improves the efficiency of BNF (Míguez-Montero et al., 2020). Low P concentrations reduce BNF in leguminous trees *Virgilia divaricata* and *Virgilia oroboides* (Magadlela et al., 2014;2016). Being a forest understory plant, *E. villosus* grows in acidic environments (Matzner, 1992; Raimondo and Donaldson, 2003). In acid

environments, P forms insoluble complexes with aluminium and iron cations that cannot be assimilated by plants thus, acidic soils are prone to P deficiency (Vance, 2001). Fisher and Vovides (2004) reported that arbuscular mycorrhizal fungi (AMF) in the coralloid roots improve P and water uptake.

Vessey et al. (2004) reported that although the N source preference of plants associated with N-fixing bacteria has been extensively studied in plants such as legumes, the proportion of N derived from the atmosphere and soils in cycads is not studied. Also, studies such as those conducted by Kipp et al. (2019) determine the composition of the ^{15}N isotope in cycad foliage to show that cycads partake in BNF, but the percentage N derived from the atmosphere is not quantified. In this study, we investigated the effects of soil characteristics (pH, nutrition, total cation, and exchange acidity) on cycad-microbe symbiosis and N preference of *E. villosus* growing in Rhebu and Oceanview. Conducting a study on the role of cycad-associated microbes on *E. villosus* growth and nutrition is essential because *E. villosus* has a life history similar to cycads that cannot be studied due to their conservation status (Raimondo et al., 2009). Therefore, studying the role of microbes on *E. villosus* growth will not only shed light on how microbial interactions in *E. villosus* contribute to growth and plant nutrition but will also give insights into other cycad species with similar life history. We hypothesized that *E. villosus* growing in acidic and nutrient-deficient ecosystem soils will switch N sources, relying mostly on atmospheric reduced N driven by the symbionts.

Methods And Materials

Study site and species

Adult *E. villosus* individuals growing in Oceanview Farm in East London and Rhebu village in Port St Johns, Eastern Cape, South Africa (Coordinates not included due to cycads conservation concerns) were studied. These plants grew in acidic soils with pH range of 4.28–5.43 in Oceanview and Rhebu, respectively (Table S6). A geochemical analysis revealed that Oceanview and Rhebu soils were N and P deficient (Table S5). Using pure bacterial colonies amplified through polymerase chain reaction (PCR) using 16S ribosomal RNA gene primers, P-solubilizing, N-cycling, and N-fixing bacteria isolated from Rhebu and Oceanview soils belonged to the *Pseudomonas* and *Paraburkholderia* genera (Table S1, Table S2, Table S3). These microbes produced extracellular enzymes (Fig S1) with N (N-acetylglucosaminidase and nitrate reductase) and P (acid and alkaline phosphatase) cycling traits. The P and N cycling enzymes were positively correlated with soil P and N, respectively (Fig S2, Fig S3, Fig S4, Fig S5).

Bacterial extraction and identification in *E. villosus* coralloid roots

Coralloid roots were harvested from randomly selected *E. villosus* adult plants in Oceanview and Rhebu, respectively. 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 distilled water and stored at 4°C in sterile Petri plates (90mm). The harvested coralloid roots were

immersed in 100µl of 15% glycerol in sterile Eppendorf tubes and crushed using sterile pipette tips for bacterial extraction. Ten µL of the root suspension was grown in sterile Petri plates containing selective media for N-fixing bacteria using Jensen's media agar, replicated three times, and incubated at 30°C for five days. Pure bacterial colonies were obtained by repeated streaking/subculturing. A small portion of the pure bacterial colonies was amplified through polymerase chain reaction (PCR) using the 16S ribosomal RNA gene primers: 485R (5' TACCTTGTTACGACTTCACCCCA 3') and 27F (5' AGATTTGATCCTGGCTCAG 3'). The PCR amplification was performed using an EmeraldAmp GT Master Mix with 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 additional extension at 72°C for 10 minutes. The PCR products were separated by electrophoresis on 1% (w/v) agarose gel and visualized under UV light to determine amplification of the correct product size. The amplicons were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The DNA sequences were edited and compared to the nucleotide sequences of known bacteria in the GenBank database of the National Centre for Biotechnology Information (NCBI) by using Basic Local Aligned Search Tool (BLAST) (<https://www.ncbi.nlm.nih.gov>).

Plant nutrition analysis

Coralloid roots and leaves were collected from selected adult *E. villosus* individuals growing in Rhebu and Oceanview and oven dried at 80°C for approximately seven days. The dry plant matter was ground and sent to the Central Analytic Facilities at the University of Stellenbosch (South Africa) for P and N analysis through Inductively Coupled Mass Spectrometry (ICP-MS) and the Archeometry Department at the University of Cape Town (South Africa) for N isotope analysis.

Plant biomass

The number of leaves and the leaf length were measured for selected adult *E. villosus* individuals in Rhebu and Oceanview, respectively. Thereafter, the vegetation coverage of each individual was determined.

Percentage N Derived from the atmosphere (%NDFA)

The University of Cape Town Archeometry Department conducted the N isotope analysis. The isotopic ratio of N was calculated as $\delta = 1000 (R_{\text{sample}}/R_{\text{standard}})$, where R is the molar ratio of the heavier to the lighter isotope of the samples and standards. Between 2.10 and 2.20 mg of each milled sample were weighed into 8 mm × 5 mm tin capsules (Elemental Micro-analysis, Devon, UK) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The nitrogen isotope values for the N gas released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Five standards were used to correct the samples for machine drift, namely, two in-house standards (Merck Gel and Nasturtium) and the IAEA (International Atomic Energy Agency) standard $(\text{NH}_4)_2\text{SO}_4$.

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

Where NDFA is the N derived from the atmosphere. The B value represents the $\delta^{15}N$ natural abundance of the N derived from biological N₂ fixation.

Statistical analysis

The statistical software/program R (version 3.6.2) was used to compare the means of all *E. villosus* variables in Oceanview and Rhebu using independent samples T-test. Values were considered significant when $p \leq 0.05$. Normality and homogeneity of variances were tested using a one-sample Kolmogorov-Smirnov normality test and Levene’s test ($p > 0.05$), respectively. The Wilcoxon test, a non-parametric alternative, was used in cases where the assumptions were not met. A probability of $p \leq 0.05$ was considered significant. Relations between soil characteristics, biomass, plant nutrition, and N preference were determined using principal component analysis (PCA) on R version 3.6.2 using statistical package gg plot, function pr comp.

Results

Nitrogen fixing bacteria isolated from *E. villosus* coralloid roots

The N-fixing bacteria isolated from the coralloid roots of *E. villosus* growing in Rhebu and Oceanview were *Lysinibacillus fusiformis*, *Rhizobium huautlense*, *Stenotrophomonas* sp., *Enterobacter cloacae*, and *Paenibacillus polymyxa* (Table 1).

Table 1 Molecular identification of culturable N-fixing bacteria isolated from the coralloid roots of <i>E. villosus</i> growing in Rhebu and Oceanview, Eastern Cape, South Africa.					
Rhebu			Oceanview		
Strain	Accession number	Similarity (%)	Strain	Accession number	Similarity (%)
<i>Lysinibacillus fusiformis</i>	CP041696.1	99.9	<i>Enterobacter cloacae</i>	MK116463.1	98.92
<i>Rhizobium huautlense</i>	KC355318.1	98.41	<i>Paenibacillus polymyxa</i>	KT783525.1	99.15
<i>Stenotrophomonas</i> sp.	MG725655.1	97.38			

Plant nutrition and N preference

Plant biomass

Encephalartos villosus plants growing in Rhebu had a higher number of leaves and vegetation coverage than Oceanview plants however, the differences were not significant (Table 1). Oceanview plants were recorded to have longer leaves compared to plants in Rhebu, however, the difference was not significant (Table 1).

Table 2
Number of leaves, leaf length (cm), and vegetation cover (%) of *Encephalartos villosus* plants growing in Rhebu and Oceanview, Eastern Cape. Values are means $\pm$ SE, n = 24. In each row, different letters denote statistical differences ($p \leq 0.05$) after an independent samples t-test.

Parameter	Oceanview	Rhebu
Number of leaves	10.00 $\pm$ 4.21 ^a	11.38 $\pm$ 5.29 ^a
Leaf length (cm)	223.63 $\pm$ 28.50 ^a	186.50 $\pm$ 49.60 ^a
Vegetation cover (%)	76.00 $\pm$ 0.20 ^a	80.00 $\pm$ 0.19 ^a

Correlations between soil characteristics, biomass, and nitrogen preference

The first PCA explained 43.8% of the total variation, and the second PCA explained 19.9% of the total variation (Fig. 2). The clusters showed that *E. villosus* plants respond similarly in the Oceanview and Rhebu (Fig. 2). Variations in *E. villosus* plants growing in Rhebu were mainly due to soil P concentration (represented as C) and total exchange acidity (represented as H). In Oceanview, *E. villosus* plant variations were due to pH (represented as F) and cation concentration (represented as G) (Fig. 2). The PCA plot also showed relationships between soil characteristics, biomass, and N preference, groups closer to each other represented a high correlation (Fig. 2). Soil pH was highly correlated with cation concentration, soil P concentration was correlated with exchange acidity, and the number of leaves was correlated with leaf length (Fig. 2).

Discussion

Encephalartos villosus is a forest understory dwarf cycad growing in acidic and nutrient-deficient soils. Its association with plant growth-promoting bacteria (PGP) enable *E. villosus* to persist in these soil conditions. Culturable plant growth-promoting bacteria isolated in the coralloid roots of *E. villosus* growing in Rhebu and Oceanview belonged to the *Lysinibacillus*, *Paenibacillus*, *Brevibacterium*, *Stenotrophomonas*, *Rhizobium* and *Enterobacter* genera. These microbes have been reported to be associated with legumes (Makaure et al., 2022; Sithole et al., 2019) and other cycad species (e.g., *Dioon edule*) (Gutiérrez-García et al., 2019). The PGP traits of these microbes include indole-3-acetic acid (IAA) and siderophore production (Haas and Dèfago, 2005; Gupta et al., 2015), N-fixation (Kong et al., 2017; Yousuf et al., 2017; Ahsan et al., 2021; Li et al., 2022) and solubilisation (Verma et al., 2016; De Mandal et al., 2018). Gutiérrez-García et al. (2019) reported that cycads are predominantly associated with rhizobial

species however, in this study *E. villosus* was associated with rhizobial and non-rhizobial bacteria. According to Peix et al. (2015) the coexistence of rhizobial and non-rhizobial bacteria improves N fixation and nodulation in legume hosts. Also, Lu et al. (2017) and Martínez-Hidalgo and Hirsch (2017) reported that non-rhizobial bacteria enhance atmospheric N fixation because of their increased atmospheric N fixing efficiency. Thus, based on the findings of this study, association with rhizobial and non-rhizobial bacteria enabled *E. villosus* to use atmospheric N as the primary source of N nutrition.

Nitrogen supply depends on environmental conditions such as nutrient availability (Kipp et al., 2019). According to Shay et al. (2015), nutrient deficiencies favour BNF, which may be why atmospheric N was the preferred N source in *E. villosus* growing in nutrient-deficient soils in Rhebu and Oceanview. The N-fixing traits of the isolated *Stenotrophomonas*, *Paenibacillus*, *Rhizobium*, *Enterobacter*, and *Lysinibacillus* genera enabled *E. villosus* to partake in BNF. According to Sims et al. (2002), plants partake in BNF when there are no other N sources other than atmospheric N, further elucidating how nutrient deficiency was the primary reason why *E. villosus* relied more on atmospheric N. Biological N fixation is energetically costly and requires sixteen adenosine triphosphate (ATP) molecules to break down one N₂ molecule (Buscot and Varma, 2005). Additionally, twelve more ATP molecules are required to assimilate and transport ammonium (Buscot and Varma, 2005). Consequently, plants that partake in BNF require more ATP (Valentine et al., 2011; Magadlela et al., 2016). Phosphorus plays an essential role in ATP production; therefore, BNF relies on P supply (Taylor et al., 1991). In a study on the leguminous tree *Virgilia divaricata*, Magadlela et al. (2016) reported declines in BNF in P deficient soil, illustrating the effects of P deficiency on BNF. Rhebu and Oceanview soils were acidic (pH 4.28–5.43) and thus P deficient.

In acidic soils, P forms insoluble complexes with aluminium (Al) and iron (Fe), making P unavailable for plant uptake (Adnan et al., 2003). Thus, explaining the correlation between soil P and pH in the PCA. Considering that Rhebu and Oceanview soils were acidic and P deficient, *E. villosus* may have utilised soil N to reduce the energy demands of BNF. Fisher and Vovides (2004) reported that cycads might require arbuscular mycorrhizal fungi (AMF) for successful N fixation in P-deficient soils thus we can assume that based on the high reliance on atmospheric N *E. villosus* associated with AMF to enhance P uptake (Jeffries et al., 2003). Rhebu and Oceanview soil microbial communities consisted of P-solubilizing bacteria such as *Pseudomonas*, *Paraburkholderia*, *Variovorax*, *Lysobacter*, and *Hymenobacter*. These microbes produced acid and alkaline phosphatases that solubilise soil organic matter, thus making P available for plant uptake (Merino et al., 2016; Adetunji et al., 2017). The resource allocation model for extracellular enzyme activities states that soil microbes exude extracellular enzymes to mineralise and cycle deficient nutrients (Sinsabaugh and Moorhead, 1994). Thus, the positive correlations between N and P cycling enzymes and soil N and P, respectively, may have been because Rhebu and Oceanview soils were severely N and P deficient.

Positive correlations between acid and alkaline phosphatases and soil P and the presence of phosphobacteria such as *Bacillus* and *Paenibacillus* in the coralloid roots may have increased P bioavailability, thus enabling *E. villosus* to rely on atmospheric N. According to Marler and Lindström (2021), symbiotic associations might help increase N and P concentrations in cycads. In a study on the

leaf nutrient relations in cycads, Marler and Lindström (2021) reported insignificant variations in the N and P concentrations of cycad leaves, and this was attributed to the benefits of associations with PGP bacteria. Thus, the lack of significant variations in the plant P and N concentrations in *E. villosus* growing in Rhebu and Oceanview may also be due to the influence of PGP bacteria on the plants. The *Paenibacillus* and *Bacillus* genera also play a role in potassium (K) solubilisation (Verma et al., 2017). Potassium plays a significant role in N metabolism, photosynthesis, cell growth, and elongation and significantly impacts total leaf area (Hepler et al., 2001; Xu et al., 2020). In a study on the effects of K deficiency on cotton leaves, Hu et al. (2017) reported significantly lower leaf K content, leaf number and leaf area in K deficiency. Thus, showing the impact of K on leaf growth and development. Although Rhebu soils had a significantly higher K concentration than Oceanview, the leaf length and number of leaves did not vary significantly, and this may have been due to K solubilizing bacteria present improving K bioavailability and uptake.

Conclusions

Encephalartos villosus association with plant growth-promoting bacteria with nutrient cycling and N-fixing traits enabled *E. villosus* to rely heavily on atmospheric N, thus maintaining growth and plant nutrition in nutrient-deficient and acidic soils.

Declarations

Acknowledgements

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Conflicts of interests

We declare no known competing financial and non-financial interests with regards to the current research. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Availability of data and material

The data and material can be made available upon request.

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Figures

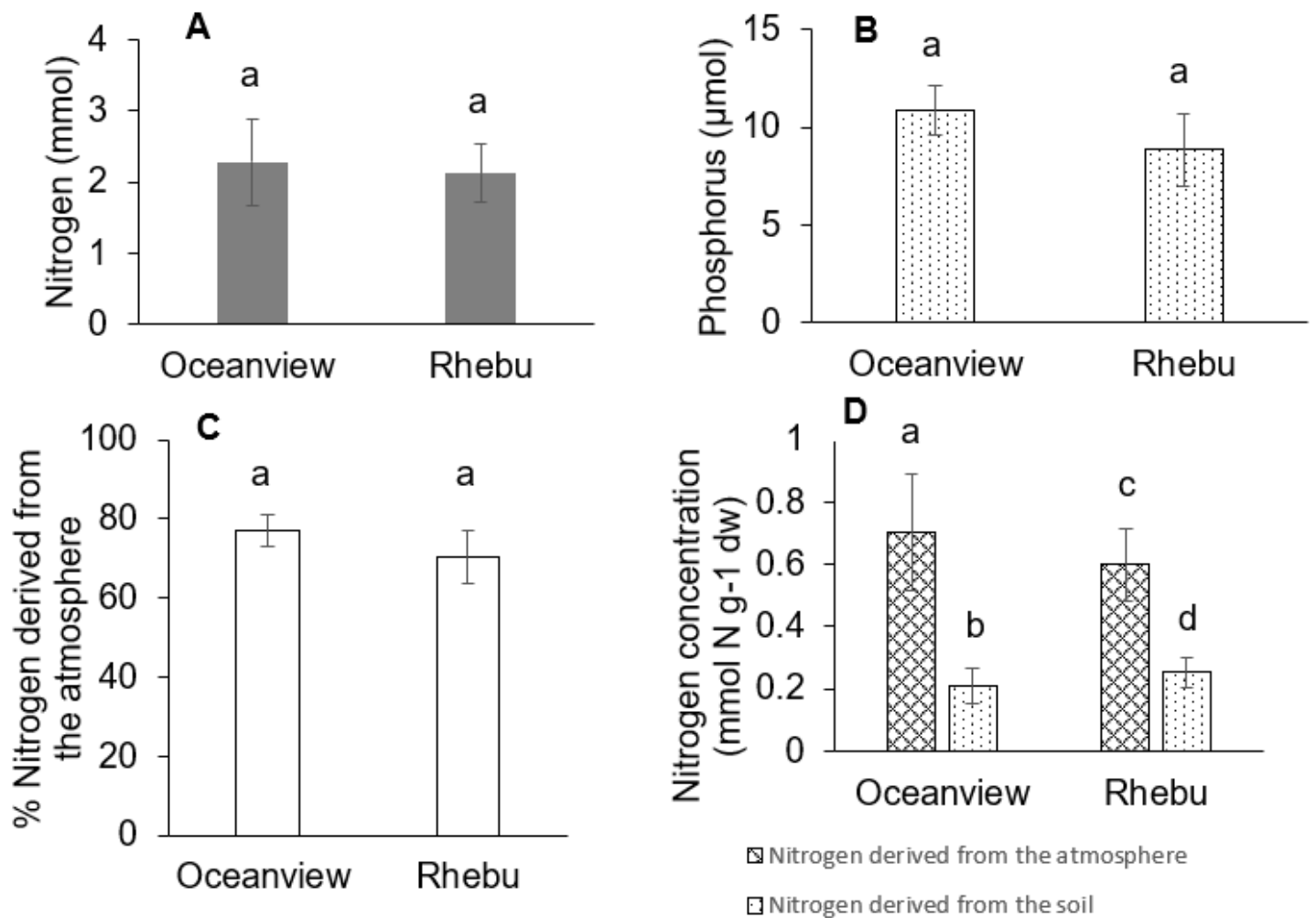


Figure 1

Mineral nutrition and nitrogen preference of *Encephalartos villosus* plants growing in Rhebu and Oceanview, Eastern Cape. A: Total plant nitrogen, B: Total plant phosphorus, C: Percentage nitrogen derived from the atmosphere, D: Nitrogen preference. The values represent the means $\pm$ SE, $n=24$. Different letters represent statistical differences after an independent samples T-test where $p \leq 0.05$ is considered significant.

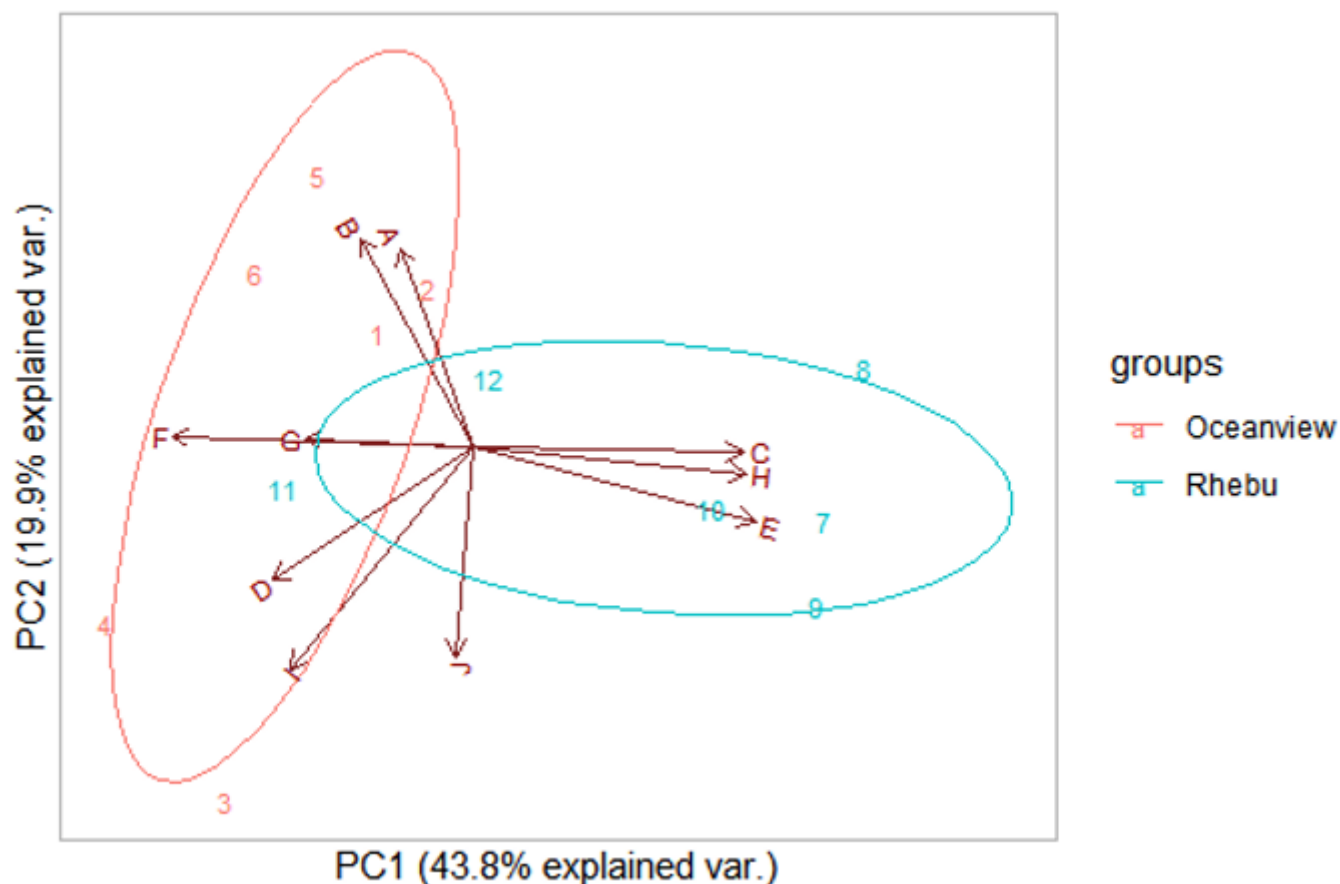


Figure 2

Principal component analysis showing correlation between soil characteristics, biomass, and nitrogen preference of *Encephalartos villosus* plants growing in Rhebu and Oceanview, Eastern Cape. Soil characteristics are represented as follows: C= Phosphorus concentration, D= potassium concentration, E= nitrogen concentration, F= pH, G= total cations, H= exchange acidity. Plant parameters were represented as follows: A= number of leaves, B= leaf length, K= vegetation coverage. Nitrogen preference was represented as I= nitrogen derived from the atmosphere, J= nitrogen derived from the soil.

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