Encephalartos villosus and Vigna unguiculata L. (Walp) shared symbionts contribute to V. unguiculata plant nutrition and growth in nutrient-deficient ecosystems

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Short Report

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Abstract

Cycads are ancient plants that establish symbiotic associations with plant growth-promoting (PGP) microbes. These ancient associations are rarely contrasted with more recent associations involving PGP microbes and legumes. This study investigated if *V. unguiculata* growing in *Encephalartos villosus* acidic, and nutrient-deficient rhizosphere and surrounding soils may share similar symbionts. In addition, the biomass accumulation and plant nutrition in *V. unguiculata* growing in these soils was investigated. *Vigna unguiculata* seeds were grown in *E. villosus* rhizosphere and surrounding soils for 45 days. Thereafter, growth characteristics and plant nutrition were calculated. *Vigna unguiculata* plants grown in *E. villosus* rhizosphere and surrounding soils were nodulated by *Paenibacillus, Bacillus, Peribacillus, Brevibacillus, Alkalihalobacillus,* and *Lysinibacillus* species that were also identified in *E. villosus* coralloid roots. There were no significant differences in the total plant biomass, however, *V. unguiculata* plants in rhizosphere and surrounding soils invested more resources in belowground biomass. The findings of this study show that *V. unguiculata* and *E. villosus* growing in similar soil conditions may share the same symbionts promoting plant nutrient assimilation and growth.

Introduction

Cycads are the only gymnosperms with a symbiotic association with nitrogen (N) fixing bacteria (Grobbelaar et al., 1986; Zheng et al., 2002). Earlier studies have reported that cycads are predominantly associated with the *Nostoc* genus (Caiola, 1980; Grobbelaar et al., 1986). However, more recent studies have reported associations between cycads and species belonging to the *Rhizobium, Bradyrhizobium*, and *Burkholderia* genera (Barea et al., 2005; Gutièrrez-García et al., 2019). Sithole et al. (2019) and Makaure et al. (2022) reported that similar microbes were associated with *Vigna unguiculata* growing in nutrient-poor ecosystems. These microbes promote plant growth and development through the production of indole-3-acetic acid (IAA), auxin, and siderophores and play a role in N-fixation and P solubilisation (Weselowski et al., 2016; Passera et al., 2021).

According to Vessey et al. (2005), the survival of cycads through extinction events and harsh environments is attributed to cycad-microbe symbiosis. Although cycad-microbe symbiosis sheds light on ancient symbiotic associations, these associations are rarely contrasted with more recent associations, such as those involving legumes. Therefore, studying the growth physiology of *V. unguiculata* in *E. villosus* rhizosphere and surrounding soils will contribute information on the host specificity in cycads and legumes growing in soils with similar characteristics and the contribution of microbes on *V. unguiculata* growth and plant nutrition. This study investigates legume microbe symbiosis, biomass accumulation, and plant nutrition in *V. unguiculata* growing in *E. villosus* rhizosphere and surrounding soils.

Materials And Methods

Soil collection and soil characteristics

Encephalartos villosus rhizosphere and surrounding soils were collected from a population of ≥500 *E. villosus* plants growing in a disturbed woodlands ecosystem with invasive *Lantana camara* plants at Oceanview, Eastern cape. *Encephalartos villosus* populations in the Eastern Cape are threatened as they are being cleared for crop cultivation. In Oceanview, locals who clear the land for cultivation do not clear cycads. So, the crops are planted with cycads in the field, a practice similar to agro-forestry. Suggesting that these crops are benefiting from the cycads, but it requires investigation. Target soils were collected 50 cm from the stem and at the leaf canopy drip line of randomly selected *E. villosus* adult plants. Bulk soils were collected from non-target sites five meters from the base of each target plant as control. The rhizosphere and surrounding soils are relatively acidic, with pH of 5.4 and 5.0, respectively (Table S1). and did not vary significantly in the P and N concentrations (Table S1). *Encephalartos villosus* plants growing in the study site were associated with *Lysinibacillus, Brevibacterium, Paenibacillus*, and *Stenotrophomonas* species with PGP functions (Table S2).

Seed germination and growth conditions

This study used two treatments (rhizosphere and surrounding soils randomly collected from the ≥500 *E. villosus* population) with minimum 50 replicates in each treatment. *Vigna unguiculata* seedlings were planted at a depth of 1–2 cm in 18 cm diameter pots under greenhouse conditions (day temperatures 28–34˚C, night temperatures 13–16˚C). The plants were irrigated with an automated irrigation system (twice a day) and harvested 45 days after seedling emergence, with the initial harvest being 32 days after seedling emergence.

Plant nutrient analysis

Plants from each treatment were separated into leaves, stems, roots, and nodules (used for bacterial extraction). The leaves, stems, and roots were oven dried at 80˚C for five days, the dry weights were recorded, and the root-to-shoot ratio was calculated. The dried plant material was ground into a fine powder and sent for C, N, and P concentration analysis using Inductively Coupled Mass Spectrometry (ICP-MS) at the Central Analytical Facilities (CAF), Stellenbosch University, South Africa.

Bacterial extraction and identification

The nodules were rinsed with distilled water and surface sterilised with 70% (v/v) ethanol for 30 seconds and 3.5% (v/v) sodium hypochlorite for 3 minutes. After sterilisation, the nodules were crushed in sterile Eppendorf tubes containing 15% glycerol. The nodule solution was streaked in sterile Petri dishes containing yeast mannitol agar (YMA) and incubated at 30˚C. Pure colonies were obtained through repeated streaking. A small portion of the pure bacterial colonies was amplified through polymerase chain reaction (PCR) using 16S primers 63F (5’- CAGGCCTAACACATGCAAGTC – 3’) and 1387R (5’- GGGCGGTGTGTACAA – 3’). The PCR amplification was performed using an EmaraldAmp GT Master Mix (Takara Bio Inc, supplied by Separations, South Africa) with the following conditions: Initial denaturation at 94˚C (5 minutes), followed by 30 cycles of denaturation at 94˚C (30 seconds), annealing at 55˚C (30 seconds), and extension at 72˚C (2 minutes), with additional extension at 72˚C (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 and sent for sequencing (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). The DNA sequences were compared to the nucleotide sequences of some known bacteria in the GenBank database of the National Centre for Biotechnology Information (NCBI) by using the Local Basic Aligned Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov).

Growth calculations

Specific N/P absorption and utilization rate

The Specific N/P absorption rate (SN/PAR) and Specific N/P utilization rate (SN/PUR) was determined using the following equations:

$SNAR = (N2 - N1 / t2 - t1) * [(\log_e R2 - \log_e R1) / (R2 - R1)]$

$SPAR = (P2 - P1 / t2 - t1) * [(\log_e R2 - \log_e R1) / (R2 - R1)]$

$SNUR = (W2 - W1 / t2 - t1) * [(\log_e N2 - \log_e N1) / (N2 - N1)]$

$SPUR = (W2 - W1 / t2 - t1) * [(\log_e P2 - \log_e P1) / (P2 - P1)]$

Noteworthy, N and P represent the total nitrogen and phosphorus content in the plant, t is the time it took for the plant to grow, and R is the root dry weight, as described by Nielsen et al. (2001).

Relative growth rate

The relative growth rate was calculated using an equation derived from Ågren and Franklin (2003). The W represents the dry weights, and t is the time it took for the plant to grow.

$RGR = [(\ln W2 - \ln W1) / t2 - t1]$

Statistical analysis

The statistical software/program R (version 3.6.2) was used to compare the means of all *V. unguiculata* variables in *E. villosus* rhizosphere and surrounding soils using independent samples T-test. The Wilcoxon test, a non-parametric alternative, was used in cases where the assumptions of normality and homogeneity of variances were not met. A probability of $p \leq 0.05$ was considered significant.

Results And Discussion

Sequence comparison of 16S ribosomal RNA partial sequences revealed the presence of multiple bacterial species in *V. unguiculata* plants growing in rhizosphere and surrounding soils. The culturable strains isolated from the nodules of *V. unguiculata* growing in rhizosphere and surrounding soils belonged to the *Paenibacillus, Bacillus, Lysinibacillus, Brevibacillus, Alkalihalobacillus*, and *Peribacillus* genera (Table 1).

Table 1
Bacterial strains identified in root nodules of 45-day-old *Vigna unguiculata* grown in *Encephalartos villosus* rhizosphere and surrounding soils collected in Oceanview, Eastern Cape, South Africa

	Bacterial species	Accession number	Similarity index (%)
Rhizosphere	*Brevibacillus brevis*	NR_112204.1	86.92
	Bacillus aerius	NR_118439.1	98.08
	Lysinibacillus fusiformis	NR_042072.1	98.76
	Paenibacillus sabinae	NR_122054.1	84.96
	Paenibacillus oceanisediminis	NR_118441.1	98.84
Surrounding soils	*Alkalihalobacillus oceani*	NR_148621.1	87.59
	Bacillus altitudinis 41KF2b	NR_042337.1	99.87
	Paenibacillus tundrae	NR_044525.1	87.17
	Paenibacillus taichungensis	NR_044428.1	99.83
	Peribacillus simplex	NR_042136.1	92.22

Plant biomass and nutrition

Plants grown in rhizosphere soils had higher plant biomass than plants grown in surrounding soils (Table 2). The belowground biomass of plants grown in rhizosphere soils was significantly higher than plants grown in surrounding soils (Table 2). However, the root-to-shoot ratio was significantly higher for plants grown in surrounding soils (Table 2). Although the difference was insignificant, *V. unguiculata* plants grown in rhizosphere soils had a higher RGR (Table 2). Similarly, the total P concentration of plants in rhizosphere soils was higher than that of plants in the surrounding soil, but the difference was insignificant (Table 2). Plants in rhizosphere soils had a significantly higher total N concentration than plants in surrounding soils (Table 2). The SNAR, SNUR, and SPUR were significantly higher in plants grown in rhizosphere soils.

Table 2
Growth kinetics of 45 days old *Vigna unguiculata* grown in *Encephalartos villosus* rhizosphere and surrounding soils, Oceanview, Eastern Cape. Values are means ± SE, n = 60). In each row, different letters denote statistical differences ($p \leq 0.05$) after an independent samples t-test.

Parameter	**Treatments**	
	Rhizosphere	**Surrounding soils**
Biomass	**0.967 ± 0.270^{a}**	**0.807 ± 0.128^{a}**
Total plant (g)		
Leaves (g)	0.284 ± 0.112^{a}	0.250 ± 0.066^{a}
Shoots (g)	0.269 ± 0.080^{a}	0.257 ± 0.053^{a}
Roots (g)	0.457 ± 0.105^{a}	0.329 ± 0.096^{b}
Root: Shoot ratio	1.269 ± 0.116^{c}	1.460 ± 0.160^{d}
Growth kinetics		
Relative growth rate (g day^{-1})	0.020 ± 0.007^{a}	0.016 ± 0.004^{a}
SPUR (g plant DW mg^{-1} P day^{-1})	0.008 ± 0.002^{p}	0.006 ± 0.0004^{q}
SPAR (g root DW mg^{-1} P day^{-1})	103.85 ± 5.42^{a}	117.65 ± 11.57^{a}
SNAR (mg N g^{-1} root DW day^{-1})	21.21 ± 3.98^{g}	16.07 ± 1.11^{h}
SNUR (mg N g^{-1} plant DW day^{-1})	4.19 ± 1.21^{j}	2.02 ± 0.95^{k}
Carbon costs (mmol C g^{-1})	0.004 ± 0.001^{a}	0.005 ± 0.002^{a}
Plant Nutrition		
Phosphorus (µmol g^{-1})	20.17 ± 2.05^{a}	19.84 ± 2.30^{a}
Nitrogen (mmol g^{-1})	4.51 ± 1.05^{a}	3.19 ± 0.53^{b}

Vigna unguiculata plants grown in *E. villosus* rhizosphere and surrounding soils were nodulated by bacterial species belonging to the *Paenibacillus, Bacillus, Brevibacillus, Lysinibacillus, Alkalihalobacillus*, and *Bacillus* genera. Some of these bacterial species are similar to those associated with *E. villosus* plants growing in Oceanview soils. In the current study, bacterial species belonging to the *Bradyrhizobium* genera were not isolated even though *Bradyrhizobium* has been reported to be associated with *V. unguiculata* (Sithole et al., 2019; Makaure et al., 2022). In a study on the competitive nature of *Bradyrhizobium* in pigeon peas, Chalasani et al. (2021) reported that *Bradyrhizobium* is a poor competitor against non-nodulating bacteria. The *Bacillus, Brevibacillus*, and *Paenibacillus* associated with *V.*

unguiculata are non-nodulating bacteria (Rajendran et al., 2008) that may have had a competitive advantage over *Bradyrhizobium. Vigna unguiculata* plants growing in the rhizosphere and surrounding soils were predominantly nodulated by bacterial species belonging to the *Paenibacillus* genera. The *Paenibacillus* genera promote plant growth through P solubilisation, IAA production, siderophore secretion, and N-fixation (Grady et al., 2016; Weselowski et al., 2016). Bacterial strains such as *Lysinibacillus fusiformis* were identified in the coralloid roots of *E. villosus* growing in Oceanview. The same bacterial strain was isolated in *V. unguiculata* plants grown in rhizosphere soils, indicating the potential similarities in symbiotic associations involving cycads and legumes.

Encephalartos villosus is a forest understorey plant known to grow in acidic soil environments. The sampled *E. villosus* rhizosphere and surrounding soils showed a pH range between 5 and 5.4 and low P levels (3.86–6.1 mg/kg). Under these conditions, P is unavailable for plant uptake due to the formation of insoluble complexes with cations such as aluminum and iron (Karyotis et al., 2005). Thus, the association of *V. unguiculata* plants growing in *E. villosus* rhizosphere and surrounding soils with phosphobacteria such as *Bacillus* could have enhanced P uptake. White et al. (2008) reported high root biomass in plants growing in P-deficient soils, which may explain the higher root biomass than shoot biomass in *V. unguiculata* plants in rhizosphere and surrounding soils. According to Iqbal *et al.* (2020), elevated root biomass is attributed to high P utilisation rather than P availability. Therefore, the higher root biomass of *V. unguiculata* plants grown in rhizosphere soils may be attributed to a higher SPUR.

According to Marschner et al. (1991), nodulation is an energetically costly process affected by soil acidity. In this study, soil acidity did not affect nodulation in *V. unguiculata* roots, as evidenced by nodules in all plants grown in the rhizosphere and surrounding soils. The *Paenibacillus sabinae* and *Bacillus altitudinis* were isolated from the nodules of *V. unguiculata* roots, and these species are reported to be N-fixing (Lu et al., 2014; Zhang et al., 2021). Though this study did not assess the N preference of *V. unguiculata* plants grown in the rhizosphere and surrounding soils. The presence of nodules infected with culturable N-fixing bacteria and the ability of the plants to grow without being supplemented with N nutrients support the hypothesis that the plants may have partaken in BNF.

Vigna unguiculata plants grown in *E. villosus* rhizosphere and surrounding soils established symbioses with multiple bacterial species, some of which have been isolated from *E. villosus* coralloid roots, indicating similarities between cycads and legume symbiotic associations. The contributions of these microbes enabled *V. unguiculata* plants to grow in acidic and nutrient-deficient soils by enhancing nutrient bioavailability and uptake. Future studies can compare the symbionts and growth of *V. unguiculata* in contrasting ecosystem soils, e.g., heavily used agricultural soils and cycad rhizosphere soils from different localities. Also, the microbial community composition in the nodules can be assessed using techniques such as Illumina sequencing.

Declarations

Conflict of interest

We declare no known competing interests with regards to the current research.

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Authors' contributions

Experimental work and draft manuscript were done by NM, TS and AM. NM, TS and AM assisted with experimental design. NM and AM reviewed and edited the manuscript. All the authors have read the manuscript and agreed to submit it in its current form for publication in the Journal.

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