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Abstract

Background and Aims: Research required to clarify leaf nutrient relations of cycad species has been inadequate. Common garden studies are useful for determining the influence of genetics on leaf traits because of the homogeneous environment among experimental units. To date, there have been no common garden studies which included all ten genera of cycads. The full phylogenetic breadth has, therefore, not been included in this important area of study.

Methods: We examined macronutrient and micronutrient content of leaves from one representative species from each of the ten cycad genera at Nong Nooch Tropical Botanical Garden in Thailand. Nitrogen content was determined by dry combustion, and the remaining nutrients were quantified by spectrometry.

Results: The least variable elements were nitrogen and phosphorus, and the most variable elements were boron and sodium. Nutrient content based on leaflet area was more variable than based on leaflet mass, reflecting species differences in specific leaf area. There were no universal macronutrient or micronutrient signals indicating clear phylogenetic distinctions.

Implications for Conservation: Active management of threatened cycad taxa requires research to develop the knowledge to enable evidence-based decisions. This common garden study inclusive of all 10 cycad genera creates a foundation to determine leaf nutrient sufficiency ranges to inform management decisions.

Keywords

Cycadaceae, Cycadales, Zamiaceae, stoichiometry

Introduction

Cycads are a lineage of ancient dioecious gymnosperms that have persisted for hundreds of millions of years (Norstog & Nicholls, 1997). The contemporary cycads are phylogenetically separated into 10 genera (Calonje et al., 2013). Much of the recent cycad research has focused on plant exploration and resulting changes in taxonomy, and research in more applied sciences such as horticulture has been lacking (Cascasan & Marler, 2016). *Ex situ* conservation of cycads is considerable, and some species are heavily used in the international horticulture industry (Norstog & Nicholls, 1997). The lack of cycad-specific research in the applied sciences constrains the knowledge needed to make information-based decisions such as plant nutrition management. For example, tissue analysis is widely used in agronomy, horticulture, and silviculture to determine nutrient availability and to guide decisions for fertilizer management (Bould, 1963; Robinson et al., 1997). For this approach to function, nutrient sufficiency ranges for various plant tissues must be clarified for each species.

The status of knowledge on cycad leaf nutrient relations was recently reviewed (Deloso et al., 2020). Several gaps in knowledge were identified from the literature, including the absence of the genus *Microcycas* from the worldwide cycad data set. Some plant traits and soil characteristics which influence cycad leaf nutrition have been identified to refine research methods (Marler & Ferreras, 2015; Marler & Krishnapillai, 2018a, 2018b, 2019a, 2019b). Many of the published cycad reports did not account for these influential factors, compromising

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the usefulness of the data for improving the status of knowledge on the subject (Deloso et al., 2020).

Our objectives were to use a common garden setting to determine the range in leaflet nutrient concentrations of representatives from all ten cycad genera. In achieving this objective, we report the first leaflet nutrient concentrations for the genus *Microcycas*. We also aimed to determine which elements exhibited the greatest phenotypic plasticity to inform future research designed to more fully understand ecology, horticulture, and physiology of the Cycadales.

Methods

Species and Study Site

The study was conducted in a common garden setting at Nong Nooch Tropical Botanical Garden in Chonburi, Thailand. The experimental site was centered at 12.762°N, 100.936°E at 52–72 meters above sea level with $\approx 275^\circ$ aspect. Climate at this location is classified as Type Am on the Köppen-Geiger climate scale. Mean daily maximum temperature is 31.3°C and minimum is 25.0°C. Annual precipitation is 111 cm, and a distinct rainy season extends from May to November.

Homogeneity of edaphic conditions among the sampled garden plants was ensured through the use of constructed mineral soil medium placed in raised beds. The plants were grown under shade cloth with $\approx 50\%$ sunlight transmission and received irrigation as needed during the dry season, but no fertilization. They were positioned in single species groups, ensuring no interspecific competition for any of the sampled plants. Six mature plants of each species were treated as single-plant replications.

The research beds were constructed and planted in 2005 and all of our species were planted at the same time. The plants were all grown from seed, and were produced in a container nursery under homogeneous growing conditions. They ranged from one to two years in age in 2005. The plants were growing under the homogeneous research production conditions for 13 years prior to initiation of our study.

We selected one representative species for each of the 10 described cycad genera (Table 1). Plant size influences the leaf nutrient concentration (Marler & Krishnapillai, 2018b), so we measured the stem height of each replication before leaflet sampling. All measurements were conducted on 18 January 2018.

Soil Nutrients

Cycad plants may alter the edaphic chemical characteristics of the soils within the root and litterfall zone (Marler & Krishnapillai, 2018a). Moreover, soil nutrient

availability may exert a direct effect on cycad leaflet nutrient concentration (Marler & Ferreras, 2015). Therefore, we determined soil chemistry from the soils directly beneath the sampled plants.

A soil core was sampled half way between each plant for each species and the five cores were combined into a composite sample for each species ($n = 10$). Leaf length was greater than half of the distance between adjacent plants, so the cores were beneath the leaf zone. The soil cores were 15 cm in depth. Total carbon (C) and nitrogen (N) content was determined by dry combustion (FLASH EA1112 CHN Analyzer, Thermo Fisher, Waltham, Mass, U.S.A.) (Dumas, 1831). Available phosphorus (P) was determined by the Olsen method (Olsen et al., 1954). Extractable essential nutrients other than P were quantified following digestion with diethylenetriaminepentaacetic acid (Berghage et al., 1987). Analysis was by inductively coupled plasma optical emission spectrometry (ICP-OES; Spectro Genesis; SPECTRO Analytical Instruments, Kleve, Germany) (Hou & Jones, 2000).

Leaf Nutrients

Leaf age influences cycad leaf nutrient concentrations (Marler & Krishnapillai, 2019b), so leaflets from the youngest leaves on plants with no visible active leaf growth were sampled. Position of leaflets along a cycad compound leaf rachis influences nutrient concentrations (Marler & Krishnapillai, 2019a), so leaflets were collected from basal, midpoint, and apical locations on each leaf. One leaf from each cardinal direction was sampled per plant. This protocol enabled leaflet collection from 12 locations for each single-plant replication. All leaflets were homogenized into one sample per replication.

The tissue was dried at 75°C and milled to pass through 20-mesh screen. Total N was determined by dry combustion. Milled leaflet samples were also digested by a microwave system with nitric acid and peroxide, then P, potassium (K), calcium (Ca), manganese (Mn), magnesium (Mg), sulfur (S), iron (Fe), zinc (Zn), boron (B), copper (Cu), and sodium (Na) were quantified by ICP-OES.

Leaflet sections were collected from each plant to determine specific leaf area. Two leaflets per plant were harvested and the base and apical portions were excised to retain the mid-section. Diameter and length of each leaflet section were measured to the nearest millimeter, then all sections were combined, dried at 75°C, then weighed. The relationship of area to dry weight was used to calculate leaflet nutrient content as mass per leaflet area.

Table 1. Characteristics of the Ten Cycad Species Used to Determine Leaf Nutrient Relations in a Common Garden Setting at Nong Nooch Tropical Botanical Garden.

| Species | Distribution | Height (cm) | Growth form and habitat notes |
|---|-----------------------------|--------------|---|
| <i>Bowenia serrulata</i> (W.Bull) Chamb. | Australia | 10.1 ± 0.9 | Subterranean, wet tropical rainforest, understory habit. |
| <i>Ceratozamia hildae</i> G.P.Landry & M.C.Wilson | Mexico | 28.1 ± 2.3 | Subterranean, dry deciduous oak forest, understory habit. |
| <i>Cycas revoluta</i> Thunb. | Japan | 64.2 ± 10.7 | Arborescent, seasonal dry forest, open habit. |
| <i>Dioon spinulosum</i> Dyer ex Eichler | Mexico | 104.9 ± 8.3 | Arborescent, wet rainforest, understory habit. |
| <i>Encephalartos ferox</i> G.Bertol | Southeast African continent | 21.3 ± 0.9 | Subterranean, littoral wet forest, open habit. |
| <i>Lepidozamia hopei</i> Regel | Australia | 145.4 ± 9.3 | Arborescent, wet tropical rainforest, understory habit. |
| <i>Macrozamia moorei</i> F.Muell. | Australia | 66.3 ± 2.9 | Arborescent, open moist grassland, open habit. |
| <i>Microcycas calocoma</i> (Miq.) A.DC. | Cuba | 161.9 ± 17.8 | Arborescent, dry deciduous forest, understory habit. |
| <i>Stangeria eriopus</i> (Kunze) Baill. | Australia | 14.1 ± 1.6 | Subterranean, dry deciduous forest, understory habit. |
| <i>Zamia muricata</i> Willd. | Venezuela | 14.5 ± 1.1 | Subterranean, wet cloud forest, understory habit. |

Data Analyses

The concentration and mass per area data were subjected to ANOVA using the PROC GLM model (SAS Institute, Cary, NC, U.S.A.). We also calculated a phenotypic plasticity index (PPI) using the formula ((maximum – minimum)/maximum). This PPI ranges from 0 indicating no plasticity to 1 indicating maximal plasticity. In addition, the quotients nitrogen/phosphorus, nitrogen/potassium, and potassium/phosphorus were calculated from the concentration data. Log transformation was required prior to subjecting these quotients to ANOVA. Means separation for significant nutrients was conducted by Tukey's HSD test. The general ranking of each genus within the range in concentration for each of the 11 macro- and micronutrients was calculated to more fully understand the leaflet nutrient relations for each of the genera. A standardized ranking was created by designating the minimum concentration for each element as 0 and the maximum concentration as 1, then the position of each genus mean was calculated based on the range in concentrations.

Results

Soil Nutrients

The alkaline substrate in which the garden's cycad plants were growing did not contain substantial resources to support plant nutrition (Table 2). As no fertilization was provided, the plants relied exclusively on these edaphic pools of nutrients with the exception of C and N, which were also derived from atmospheric sources through photosynthesis and biological N-fixation by cyanobacteria endosymbionts.

Leaf Nutrients

The differences of every macronutrient among the 10 cycad genera were significant when expressed on a dry mass basis (Table 3). Nitrogen means separated into four overlapping groups with the subterranean *Bowenia*, *Stangeria*, and *Zamia* species exhibiting the greatest concentration and *Macrozamia* exhibiting the least. Phosphorus means separated into five overlapping groups with the *Zamia* species exhibiting the greatest concentration. Potassium means also separated into five groups with the *Zamia* species exhibiting the greatest and *Microcycas* exhibiting the least concentration. Calcium means separated into five groups with *Encephalartos* species exhibiting the greatest and the *Bowenia* species exhibiting the least concentration. Magnesium means separated into four groups with the *Dioon* and *Stangeria* species exhibiting the greatest concentration and the *Microcycas* species exhibiting the least. The sulfur concentrations were similar for most genera, with *Cycas* and *Macrozamia* species means separating below those of the other species. The PPI of macronutrient concentration exhibited a 2.6-fold range and was least for N and greatest for Ca (Table 3).

The differences of every micronutrient among the 10 cycad genera were significant (Table 3). Manganese concentrations were variable, with the *Stangeria* leaflets containing the greatest concentration. Iron concentrations were greatest in the *Stangeria*, *Microcycas*, and *Lepidozamia* species and least in *Dioon*, *Cycas*, and *Ceratozamia* species. Zinc concentrations exhibited several overlapping groups with the *Zamia* leaflets containing the greatest concentration and the *Encephalartos* leaflets containing the least concentration. The B concentrations were more similar for many of the taxa at the low end of the range. However, the *Stangeria* leaflets

Table 2. Edaphic Characteristics of Substrates Subtending Cycad Plants That Were Tested for Leaflet Nutrient Content.

| Characteristic | | Characteristic | |
|--|-----------------|---|----------------|
| pH | 7.42 ± 0.03 | Magnesium ($\mu\text{g}\cdot\text{g}^{-1}$) | 142.17 ± 13.66 |
| Carbon ($\text{mg}\cdot\text{g}^{-1}$) | 115.89 ± 5.01 | Manganese ($\mu\text{g}\cdot\text{g}^{-1}$) | 18.49 ± 1.14 |
| Nitrogen ($\text{mg}\cdot\text{g}^{-1}$) | 4.69 ± 0.78 | Iron ($\mu\text{g}\cdot\text{g}^{-1}$) | 10.89 ± 0.57 |
| Phosphorus ($\mu\text{g}\cdot\text{g}^{-1}$) | 9.08 ± 0.59 | Copper ($\mu\text{g}\cdot\text{g}^{-1}$) | 1.66 ± 0.28 |
| Potassium ($\mu\text{g}\cdot\text{g}^{-1}$) | 64.71 ± 8.56 | Zinc ($\mu\text{g}\cdot\text{g}^{-1}$) | 4.92 ± 0.82 |
| Calcium ($\mu\text{g}\cdot\text{g}^{-1}$) | 986.38 ± 100.18 | | |

Mean ± standard error, n = 10.

Table 3. Macronutrient Concentrations ($\text{mg}\cdot\text{g}^{-1}$) in 10 Cycad Genera Growing in a Common Garden Setting at Nong Nooch Tropical Botanical Garden.

| Species | Nitrogen | Phosphorus | Potassium | Calcium | Magnesium | Sulfur |
|----------------------------|----------------|---------------|----------------|----------------|---------------|---------------|
| <i>Bowenia serrulata</i> | 28.59 ± 0.65a | 1.07 ± 0.05ef | 10.17 ± 0.29c | 2.87 ± 0.23e | 1.72 ± 0.09c | 0.91 ± 0.14a |
| <i>Ceratozamia hildae</i> | 25.11 ± 0.61 b | 1.22 ± 0.03d | 10.53 ± 0.25c | 9.09 ± 0.49 b | 2.58 ± 0.06 b | 0.85 ± 0.08a |
| <i>Cycas revoluta</i> | 22.31 ± 0.43c | 1.51 ± 0.06c | 10.17 ± 0.30c | 3.57 ± 0.32d | 1.73 ± 0.06c | 0.43 ± 0.04c |
| <i>Dioon spinulosum</i> | 22.53 ± 0.52c | 0.98 ± 0.04f | 10.37 ± 0.32c | 8.17 ± 0.47 b | 3.68 ± 0.13a | 1.03 ± 0.10a |
| <i>Encephalartos ferox</i> | 23.42 ± 0.59c | 0.98 ± 0.06f | 9.17 ± 0.41 d | 11.65 ± 0.86a | 2.51 ± 0.06 b | 1.02 ± 0.14a |
| <i>Lepidozamia hopei</i> | 25.13 ± 0.61 b | 1.02 ± 0.03ef | 15.42 ± 0.55 b | 4.02 ± 0.37d | 1.67 ± 0.06c | 0.63 ± 0.04 b |
| <i>Macrozamia moorei</i> | 20.39 ± 0.59d | 1.41 ± 0.06c | 8.55 ± 0.52d | 3.41 ± 0.29d | 1.43 ± 0.08d | 0.51 ± 0.06bc |
| <i>Microcycas calocoma</i> | 25.65 ± 0.59 b | 1.15 ± 0.07de | 7.23 ± 0.41e | 3.93 ± 0.38d | 1.33 ± 0.05d | 1.07 ± 0.15a |
| <i>Stangeria eriopus</i> | 27.95 ± 0.56a | 1.88 ± 0.06 b | 10.63 ± 0.36c | 10.07 ± 0.81ab | 3.38 ± 0.12a | 1.18 ± 0.14a |
| <i>Zamia muricata</i> | 27.08 ± 0.41a | 2.17 ± 0.04a | 18.08 ± 0.49a | 6.32 ± 0.17c | 1.69 ± 0.05c | 1.12 ± 0.13a |
| Significance | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| PPI | 0.29 | 0.55 | 0.6 | 0.75 | 0.64 | 0.64 |

Mean ± standard error, n = 6. Means within columns with same letters do not differ according to Tukey's test. PPI = ((maximum – minimum)/maximum) within columns.

contained much more B than any of the other genera. Copper concentrations were similar for several of the taxa in the upper end of the range, but the *Lepidozamia* species was separated from the remainder of the taxa at the lowest end of the range in concentration. The beneficial element Na accumulated to greater concentrations in the *Encephalartos*, *Lepidozamia*, and *Zamia* leaflets.

Total macronutrient concentration (sum of all six macronutrients) exhibited greater differences among the genera than individual macronutrient concentrations. The greatest total macronutrient content was measured in the *Zamia* leaflets ($57.3\text{ mg}\cdot\text{g}^{-1}$), and the least total macronutrient content was measured in the *Macrozamia* leaflets ($36.1\text{ mg}\cdot\text{g}^{-1}$). Total macronutrient cation concentration (Ca + K + Mg) was also highly variable among the genera. The *Microcycas* leaflets contained the least ($12.5\text{ mg}\cdot\text{g}^{-1}$) and the *Zamia* leaflets contained the greatest ($26.1\text{ mg}\cdot\text{g}^{-1}$) total cation concentration. Total micronutrient concentration (sum of the five measured micronutrients) also exhibited greater differences among the genera than individual micronutrient concentrations. The greatest total micronutrient content was measured in the *Stangeria* leaflets ($570.2\text{ }\mu\text{g}\cdot\text{g}^{-1}$),

and the least total micronutrient content was measured in the *Ceratozamia* leaflets ($216.1\text{ }\mu\text{g}\cdot\text{g}^{-1}$). The PPI of micronutrient concentration exhibited a 1.4-fold range and was least for Fe and greatest for B (Table 4). The PPI for Na was greater than that for the micronutrients.

The leaflet nutrient contents based on leaflet area were more variable among the 10 cycad genera than were leaflet contents based on mass. The differences in N content based on leaflet area exhibited seven significantly different groups (Table 5), with the *Encephalartos* leaflets containing the greatest and the *Bowenia* and *Zamia* leaflets containing the least N. The differences in P content based on leaflet area also exhibited seven significantly different groups, with the *Cycas* leaflets containing the greatest and the *Bowenia* leaflets containing the least P. The differences in K content based on leaflet area exhibited eight overlapping different groups, with the *Lepidozamia* leaflets containing the greatest and the *Bowenia* leaflets containing the least K. The difference in Ca content based on leaflet area exhibited the greatest amplitude, but the means separation categories were similar with seven different groups among the 10 genera. The ranking was similar to that of N, with the *Encephalartos* leaflets containing the greatest and the

Table 4. Micronutrient and Beneficial Element Concentrations ($\mu\text{g}\cdot\text{g}^{-1}$) in 10 Cycad Genera Growing in a Common Garden Setting at Nong Nooch Tropical Botanical Garden.

| Species | Manganese | Iron | Zinc | Boron | Copper | Sodium |
|----------------------------|---------------|-----------------|---------------|--------------|--------------|-----------------|
| <i>Bowenia serrulata</i> | 81 \pm 6 b | 244 \pm 20c | 14 \pm 2bc | 22 \pm 1 b | 13 \pm 2a | 209 \pm 37de |
| <i>Ceratozamia hildae</i> | 29 \pm 2d | 149 \pm 6f | 12 \pm 1 cd | 16 \pm 1c | 10 \pm 2ab | 155 \pm 19e |
| <i>Cycas revoluta</i> | 41 \pm 3c | 169 \pm 6e | 20 \pm 4ab | 15 \pm 1c | 9 \pm 2bc | 101 \pm 19f |
| <i>Dioon spinulosum</i> | 68 \pm 4 b | 171 \pm 6e | 12 \pm 1 cd | 14 \pm 1c | 9 \pm 2bc | 283 \pm 40 cd |
| <i>Encephalartos ferox</i> | 75 \pm 5 b | 211 \pm 12d | 10 \pm 1d | 13 \pm 1 | 11 \pm 2ab | 708 \pm 57a |
| <i>Lepidozamia hopei</i> | 41 \pm 4c | 304 \pm 31ab | 22 \pm 5ab | 24 \pm 1 b | 5 \pm 1d | 795 \pm 62a |
| <i>Macrozamia moorei</i> | 37 \pm 5 cd | 275 \pm 23bc | 15 \pm 2 b | 12 \pm 1c | 9 \pm 2bc | 335 \pm 42bc |
| <i>Microcycas calocoma</i> | 33 \pm 2d | 345 \pm 39a | 12 \pm 1 cd | 14 \pm 1c | 7 \pm 2c | 398 \pm 37 b |
| <i>Stangeria eriopus</i> | 107 \pm 6a | 363 \pm 27a | 19 \pm 3ab | 65 \pm 5a | 11 \pm 2ab | 733 \pm 61a |
| <i>Zamia muricata</i> | 33 \pm 2d | 236 \pm 23 cd | 26 \pm 6a | 13 \pm 1c | 11 \pm 2 | 817 \pm 79a |
| Significance | <0.0001 | <0.0001 | 0.0006 | <0.0001 | 0.0018 | <0.0001 |
| PPI | 0.73 | 0.59 | 0.62 | 0.82 | 0.62 | 0.87 |

Mean \pm standard error; n = 6. Means within columns with same letters do not differ according to Tukey's test. PPI = ((maximum – minimum)/maximum) within columns.

Table 5. Macronutrient Mass Per Area ($\text{g}\cdot\text{m}^{-2}$) in 10 Cycad Genera Growing in a Common Garden Setting at Nong Nooch Tropical Botanical Garden.

| Species | Nitrogen | Phosphorus | Potassium | Calcium | Magnesium | Sulfur |
|----------------------------|-------------------|-------------------|-------------------|--------------------|-------------------|--------------------|
| <i>Bowenia serrulata</i> | 1.98 \pm 0.05 g | 0.07 \pm 0.01 g | 0.71 \pm 0.01 h | 0.20 \pm 0.01 g | 0.12 \pm 0.01f | 0.06 \pm 0.01e |
| <i>Ceratozamia hildae</i> | 2.86 \pm 0.06f | 0.14 \pm 0.01f | 1.20 \pm 0.03fg | 1.04 \pm 0.06c | 0.29 \pm 0.03e | 0.10 \pm 0.01 cd |
| <i>Cycas revoluta</i> | 6.42 \pm 0.12 b | 0.43 \pm 0.03a | 2.92 \pm 0.12ab | 1.03 \pm 0.05 cd | 0.50 \pm 0.03c | 0.12 \pm 0.02c |
| <i>Dioon spinulosum</i> | 5.76 \pm 0.14c | 0.25 \pm 0.02d | 2.65 \pm 0.12c | 2.09 \pm 0.12 b | 0.94 \pm 0.03a | 0.26 \pm 0.02ab |
| <i>Encephalartos ferox</i> | 7.25 \pm 0.12a | 0.31 \pm 0.02c | 2.84 \pm 0.13bc | 3.61 \pm 0.27a | 0.77 \pm 0.02 b | 0.31 \pm 0.03a |
| <i>Lepidozamia hopei</i> | 5.21 \pm 0.11de | 0.21 \pm 0.02e | 3.19 \pm 0.11a | 0.83 \pm 0.07de | 0.35 \pm 0.01d | 0.13 \pm 0.02c |
| <i>Macrozamia moorei</i> | 5.38 \pm 0.14d | 0.37 \pm 0.03 b | 2.26 \pm 0.11d | 0.89 \pm 0.05d | 0.41 \pm 0.02d | 0.13 \pm 0.02c |
| <i>Microcycas calocoma</i> | 4.97 \pm 0.11e | 0.22 \pm 0.02de | 1.41 \pm 0.08e | 0.76 \pm 0.03e | 0.26 \pm 0.02e | 0.21 \pm 0.03 b |
| <i>Stangeria eriopus</i> | 3.09 \pm 0.06f | 0.21 \pm 0.02e | 1.17 \pm 0.04 g | 1.11 \pm 0.09c | 0.37 \pm 0.02d | 0.12 \pm 0.02c |
| <i>Zamia muricata</i> | 1.91 \pm 0.03 g | 0.15 \pm 0.01f | 1.28 \pm 0.04ef | 0.45 \pm 0.01f | 0.12 \pm 0.01f | 0.08 \pm 0.01de |
| Significance | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| PPI | 0.74 | 0.84 | 0.78 | 0.94 | 0.87 | 0.81 |

Mean \pm standard error; n = 6. Means within columns with same letters do not differ according to Tukey's test. PPI = ((maximum – minimum)/maximum) within columns.

Bowenia leaflets containing the least Ca. The Mg relations were similar to the other macronutrients, but the means only separated into six significant groups. The ranking of S followed a pattern that was similar to that of N and Ca, with the *Encephalartos* leaflets containing the greatest and the *Bowenia* leaflets containing the least S.

The PPI for macronutrient content based on leaflet area was less than for content based on mass, with a 1.3-fold difference among the taxa (Table 5). Nitrogen exhibited the least PPI and Ca exhibited the greatest PPI, corroborating the PPI based on leaflet dry mass. The taxa which exhibited the greatest or least macronutrient content based on leaflet area were similar among the six macronutrients. These similarities ensured that the *Encephalartos* species contained the most total

macronutrients ($15.1\text{ g}\cdot\text{m}^{-2}$) and the *Bowenia* species contained the least total macronutrients ($3.1\text{ g}\cdot\text{m}^{-2}$).

The micronutrient content based on leaflet area was variable among the genera (Table 6). Leaflet Mn for the 10 genera means separating into eight significantly different groups. The *Encephalartos* leaflets contained the most Mn, and the *Ceratozamia* leaflets contained the least Mn. Leaflet Fe was much less variable, with only four groups of means. The *Encephalartos*, *Lepidozamia*, *Macrozamia*, and *Microcycas* leaflets contained more Fe than the other genera, whereas the *Bowenia*, *Ceratozamia*, and *Zamia* leaflet Fe was less than the other genera. Leaflet Zn was variable among the genera, with *Cycas* leaflets containing the greatest and *Bowenia* leaflets containing the least Zn. Leaflet B exhibited the greatest difference with the 10 genera separating

Table 6. Micronutrient and Beneficial Element Mass Per Area ($\text{mg}\cdot\text{m}^{-2}$) in 10 Cycad Genera Growing in a Common Garden Setting at Nong Nooch Tropical Botanical Garden.

| Species | Manganese | Iron | Zinc | Boron | Copper | Sodium |
|----------------------------|---------------------|---------------------|--------------------|--------------------|-------------------|----------------------|
| <i>Bowenia serrulata</i> | 5.52 ± 0.39 g | 16.89 ± 1.69 d | 0.95 ± 0.04 f | 1.54 ± 0.04 h | 0.88 ± 0.08 d | 13.87 ± 2.53 g |
| <i>Ceratozamia hildae</i> | 3.32 ± 0.12 h | 17.04 ± 1.69 d | 1.33 ± 0.06 e | 1.82 ± 0.08 g | 1.09 ± 0.08 c | 17.35 ± 2.15 g |
| <i>Cycas revoluta</i> | 11.65 ± 0.85 cd | 48.75 ± 2.74 b | 5.85 ± 1.05 a | 4.36 ± 0.29 bc | 2.49 ± 0.39 b | 28.76 ± 5.25 f |
| <i>Dioon spinulosum</i> | 17.44 ± 0.92 b | 43.74 ± 2.55 bc | 2.98 ± 0.41 bc | 3.51 ± 0.19 d | 2.17 ± 0.17 b | 72.48 ± 10.26 de |
| <i>Encephalartos ferox</i> | 23.32 ± 1.39 a | 65.22 ± 5.69 a | 2.17 ± 0.36 c | 3.92 ± 0.13 c | 3.51 ± 0.55 a | 216.71 ± 21.29 a |
| <i>Lepidozamia hopei</i> | 8.19 ± 0.73 e | 63.07 ± 6.34 a | 4.46 ± 1.06 ab | 5.04 ± 0.32 b | 1.21 ± 0.18 c | 165.79 ± 15.35 b |
| <i>Macrozamia moorei</i> | 9.76 ± 1.01 de | 72.59 ± 6.11 a | 3.87 ± 0.55 b | 3.12 ± 0.11 e | 2.46 ± 0.26 b | 87.94 ± 11.12 c |
| <i>Microcycas calocoma</i> | 6.44 ± 0.23 f | 67.11 ± 7.53 a | 2.41 ± 0.35 c | 2.79 ± 0.08 f | 1.33 ± 0.19 c | 77.72 ± 7.11 d |
| <i>Stangeria eriopus</i> | 12.31 ± 0.86 c | 40.11 ± 1.97 c | 2.13 ± 0.25 cd | 7.21 ± 0.55 a | 1.18 ± 0.21 c | 80.96 ± 10.79 d |
| <i>Zamia muricata</i> | 2.29 ± 0.15 c | 16.65 ± 1.59 d | 1.86 ± 0.12 d | 0.94 ± 0.01 i | 0.75 ± 0.07 d | 57.58 ± 5.59 e |
| Significance | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| PPI | 0.9 | 0.77 | 0.77 | 0.87 | 0.79 | 0.94 |

Mean \pm standard error; n = 6. Means within columns with same letters do not differ according to Tukey's test. PPI = ((maximum – minimum)/maximum) within columns.

into nine significant groups. The *Stangeria* leaflets contained the most and the *Zamia* leaflets contained the least amounts of B. The range in Cu was similar to the other micronutrients, with *Encephalartos* leaflets containing the greatest and *Zamia* leaflets containing the least Cu. The beneficial element Na was also variable among the genera. The Na content was greatest in the *Encephalartos* leaflets and least in the *Bowenia* leaflets.

The PPI based on leaflet area was greater for all five micronutrients and Na than the PPI based on leaflet dry mass (Table 6). The variation of micronutrient PPI among the genera was also less than PPI for macronutrients, with a 1.2-fold difference among the taxa.

The N:P:K stoichiometry was calculated for leaflet concentration data (Figure 1). The N:P exhibited a 2.2-fold difference among the genera, with *Zamia* and *Bowenia* positioned at the low and high end. The N:K was more plastic than N:P with a 2.4-fold difference with *Zamia* at the low end and *Microcycas* at the high end. The K:P was even more plastic than the other stoichiometry traits, with a 2.8-fold range from *Stangeria* to *Lepidozamia*.

The general ranking of each genus within the range in concentration for each of the 11 macro- and micronutrients was determined (Figure 2). *Bowenia*, *Dioon*, *Encephalartos*, and *Zamia* leaflets exhibited the full range with some elements at the minimum position and some at the maximum position. In contrast, *Cycas* and *Macrozamia* leaflets were centered in the low end of the range for all 11 elements. Finally, *Stangeria* leaflets exhibited the greatest overall concentrations with the means restricted to the upper end of the spectrum.

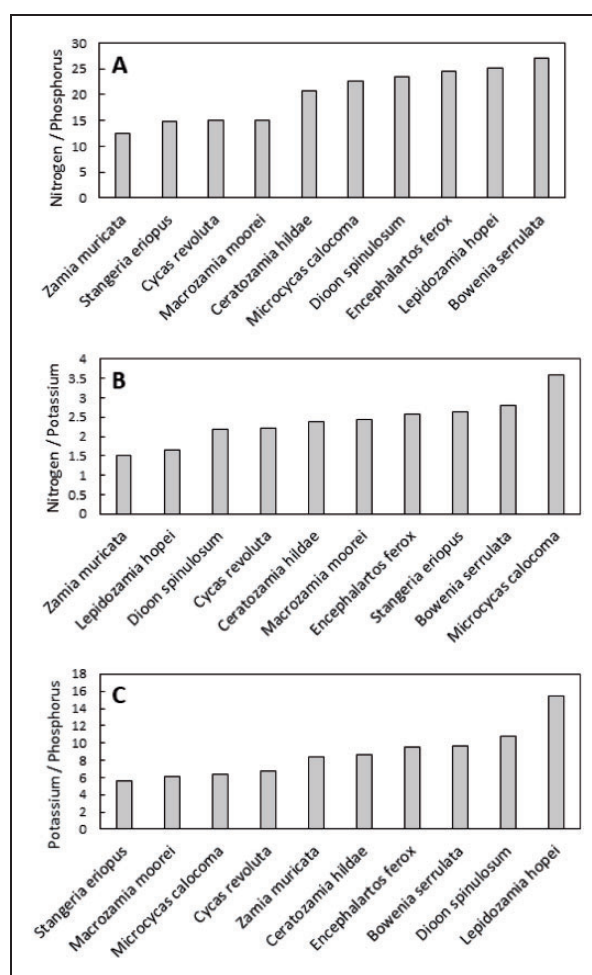


Figure 1. Stoichiometry of Cycad Leaflet Macronutrient Concentration as Influenced by Genera. A: Nitrogen/phosphorus. B: Nitrogen/potassium. C: Potassium/phosphorus.

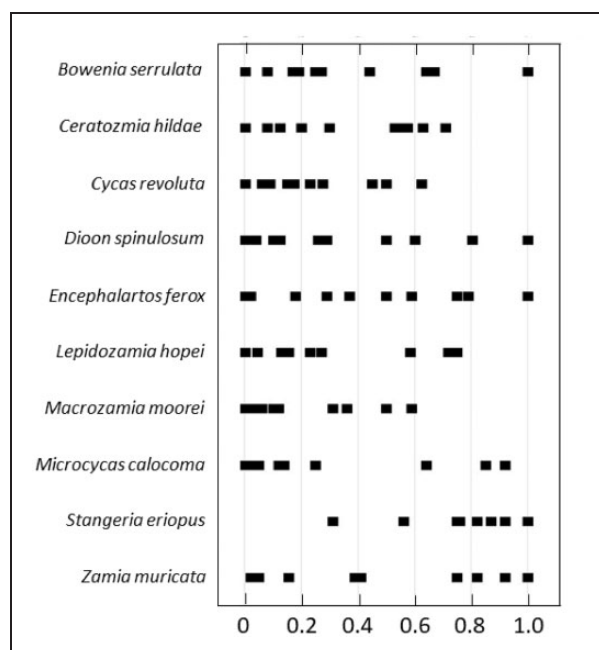


Figure 2. Ranking Among 10 Cycad Genera Within the Range in Concentration for 11 Macro- and Micro-Nutrients With the Lowest Concentration Set at 0 and the Greatest Concentration Set at 1.

Discussion

The continued accumulation of published leaflet elemental concentration ranges for members of the Cycadales is important for conservationists and horticulturists to begin to use leaflet sufficiency ranges to inform management decisions. We have addressed this with individuals from different cycad species within the first common garden study that used protocols which accounted for the plant traits and soil characteristics which are known to influence cycad leaf element concentrations.

Global efforts to understand leaf traits and phylogenetic relations that covary with elemental concentrations have greatly improved our understanding of leaf nutrient relations (Watanabe et al., 2007; Wright et al., 2004). Unfortunately, cycads were not represented well in these global data sets (Marler & Lindström, 2018). Several reports attempted to place leaf nutrient concentrations of the Cycadales within the range of the global data, and revealed the cycad concentrations were well within the full range for all spermatophytes (Zhang et al., 2015; see Deloso et al., 2020 for review). The results herein corroborated these findings.

Phylogenetic divergences of the cycad genera were ancient evolutionary events, with *Cycas* diverging from the remainder of the Cycadales as one of the earliest divergence events (Norstog & Nicholls, 1997). However, contemporary molecular tools have revealed that most living species have evolved in more recent

times (Nagalingum et al., 2011). Therefore, convergent evolution phenomena among the genera may have removed many of the phylogenetic signals concerning leaflet nutrient relations. In our study, *Cycas* did not exhibit any overt behaviors in leaflet nutrient relations that separated the phenotypes of the genus from the other nine genera. Most of our reported *Cycas* response variables were within the range of the other nine genera, although none of the elements were in the upper end of the range.

The previous cycad leaf element reports did not include some representative cycad taxa. For example, the genus *Microcycas* was missing from the previous publications. Our data indicate that exclusion of *Microcycas* from the representative cycad species may have caused inaccuracies. For example, *Microcycas* exhibited the least K, Mg, and total macronutrient cation concentrations, and the greatest N/K quotient. Moreover, the global data (Wright et al., 2004) were obtained from natural settings rather than botanic gardens to ensure the data have ecological relevance. Most of the published cycad leaf element data have been generated in botanic gardens (Deloso et al., 2020), where leaflet concentrations of nutrients are generally greater than from competitive forests (Marler & Lindström, 2020). The common garden approach is important to ensure all phylogenetic comparisons are based on data obtained from the same edaphic and climatic conditions, but the historical focus on cycad garden studies must be augmented with more data from natural settings. Therefore, the positioning of Cycadales within the leaf economics spectrum and the global leaf element data range remains to be accurately pursued with further research.

The mass-based PPI exhibited a 3-fold range among the elements, indicating plasticity of each element was not homogeneous among the 12 measured elements. Interestingly, the PPI was least for N and P. The ability of cycad plants to accumulate these two elements is benefited by associating with N-fixing cyanobacteria (Chang et al., 2019; Gutiérrez-García et al., 2019; Suárez-Moo et al., 2019; Zheng et al., 2018) and mycorrhizae (Fisher & Jayachandran, 2008; Fisher & Vovides, 2004; Muthukumar & Udaiyan, 2002). We believe these mutualisms have reduced the variability and increased the homeostasis of N and P leaflet concentrations among cycad plants with highly diverse phylogenetic relationships.

The mass-based PPI for P was almost double that for N. This greater range of P concentrations among the genera may reflect the fealty of the relationship of plant-cyanobacteria versus plant-mycorrhizal pairs. More is known about the plant-cyanobacteria relationship (Chang et al., 2019; Gutiérrez-García et al., 2019; Norstog & Nicholls, 1997; Suárez-Moo et al., 2019;

Zheng et al., 2018) and this relationship is characterized by facultative features. For example, the cyanobacteria genotypes found in cycad coralloid roots may live as autotrophs, then switch to heterotrophs when carbohydrates from the host plant become available. Moreover, the range in genotype of cyanobacteria may be extensive for a single cycad plant root system, indicating a generalist approach by the plant concerning recruitment of the bacteria mutualist. Much less is known about the plant-mycorrhizal relationship (Fisher & Jayachandran, 2008; Fisher & Vovides, 2004; Muthukumar & Udaiyan, 2002). Mycorrhizal fungi are obligate heterotrophs, but the level of dependence by the cycad plant on the fungus is not known. Moreover, the level of specificity required by the plant for recruitment of the fungal mutualist is not known. In our study, the mycorrhizae genotypes that were fortuitously available to the cycad plants may align more closely with genotypes from native habitat of our *Zamia* species, and this may account for the greater relative leaf P concentrations. In contrast, these fungal genotypes may contrast sharply with genotypes from native habitat of our *Encephalartos* species, and this may account for the lower relative P concentrations.

Our *Zamia* species contained the greatest concentrations of total macronutrients. The indigenous tribes in Mexico consider *Zamia* to be more toxic than *Ceratozamia* or *Dioon* (personal communications, A.J. L.). The direct relationship between tissue elements and tissue secondary metabolites that serve as mammal toxins may deserve further study. Indeed, plant tissue mineral balance is one plant trait that may mediate herbivory (Phelan et al., 1996). Identification of which nutrients enhance anti-herbivore compounds in cycad plants may enable the development of fertilizers that enhance or decrease these compounds.

The influence of SLA on the nutrient relations based on leaflet area can be profound (Zhang et al., 2015, 2017), and the results herein revealed some interesting comparisons. For example, the *Zamia* species exhibited the greatest N content based on mass and the least N content based on leaflet area. The *Encephalartos* species exhibited the greatest N content based on area, but ranked in the middle of the range of the genera when N content was based on mass. The range in SLA among the 364 described cycad taxa (Calonje et al., 2013) is likely more variable by orders of magnitude than the range in mass-based elemental concentration. More research is needed to determine the full range in SLA for the Cycadales, as some species such as *Zamia vazquezii* D.W. Stev., Sabato & De Luca produce thin leaflets and other species such as *Encephalartos horridus* (Jacq.) Lehm. produce thick leaflets.

A full understanding of lamina nutrient relations for a single species or for comparing various taxa in a phylogenetic study cannot be achieved unless the study

methods account for the plant and soil factors which are known to influence leaf nutrients, such as the methods herein. Most of the studies on cycad leaf nutrient relations did not report soil nutrient availability within the root zone of the sampled plants (Deloso et al., 2020). We addressed these ambiguities by quantifying the nutrient content of the soils directly beneath the sampled plants. Plant height (Marler & Krishnapillai, 2018b), leaflet position along the rachis (Marler & Krishnapillai, 2019a), leaf age (Marler & Krishnapillai, 2019b), and level of shade (Marler & Krishnapillai, 2019b) are among the plant traits that are known to influence cycad leaflet nutrient concentrations.

Many plant species use root exclusion or root sequestration of Na to protect shoot and leaf tissues from toxicities (Munns, 1993; Tester & Davenport, 2003). Under our growing conditions of minimal soil Na availability, the leaflet Na concentration of 10 cycad genera varied greatly. For example, the PPI for Na was 0.87 based on leaflet mass and 0.94 based on leaflet area. Tolerance to salinity often involves Ca signaling and therefore Ca relations may be linked to salinity tolerance (Hadi & Karimi, 2012; Seifikalhor et al., 2019). The PPI for Ca was also substantial among our 10 taxa. Our data indicate there may be substantial variation in salinity tolerance among the Cycadales taxa. A preliminary study is needed to compare cycad taxa with native range that includes littoral habitats, where the plants evolved with saline groundwater and aerosol salt deposits, and other taxa with native range that is far removed from saline conditions. The tolerance mechanisms of cycad plants to other abiotic stresses have not been adequately studied.

Implications for Conservation

Ex situ conservation and use of cycad specimens in commercial and residential communities requires knowledge to inform management decisions. No commercial fertilizers have been tailored for use on cycad plants, and development of a cycad fertilizer formulation continues to be hindered by the lack of data on this ancient plant group. Continued research on nutrient sufficiency levels is needed to reach the goal of developing a cycad-specific fertilizer that can be used to improve the otherwise comparable slow growth in cycads. Reaching this goal would then be a direct link to provide more and larger plants to curb the illegal wild collecting of large specimens.

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