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Illuminating Long-lived *Cycas* Cotyledons Reduces Sink Demands on Megagametophytes during Initial Seedling Growth

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Abstract. ***Cycas*** **megagametophyte resources are translocated to developing seedlings through the persistent green cotyledons. The source activities of cotyledons and megagametophytes following germination are not adequately understood. The decline in megagametophyte weight during seedling development was quantified until ultimate tissue desiccation for two *Cycas* species. In addition, the influence of illuminating the green cotyledons during germination and initial radicle growth on megagametophyte, root, and cotyledon dry weight (DW) was determined to evaluate the role of cotyledon photosynthesis on growth and megagametophyte source activity. *Cycas micronesica* megagametophytes declined 64% in DW during initial growth of the radicle and maturation of the first true leaf. The rate of DW decline slowed after this stage but continued for ≈18 months when the seedlings were supported by six leaves. *Cycas edentata* megagametophytes exhibited similar patterns with a 72% decline in DW when the first true leaf reached full expansion, and the resources were depleted by ≈14 months when the seedlings had five leaves. Illuminating cotyledons during initial seedling growth reduced the rate of megagametophyte DW decline by up to 20% and increased total seedling DW above that of dark-grown seedlings by up to 21%. The results provide direct evidence for the long-term source activity of the *Cycas* megagametophyte and provide indirect evidence that photosynthetic contributions of illuminated cotyledons reduce the reliance on gametophytes as a source of resources for initial *Cycas* seedling growth. Improved design of *Cycas* nursery germination microenvironments may be enabled by this new knowledge.**

Cycad seeds are supported by large, haploid megagametophytes (Brenner et al. 2003; Chamberlain 1919; Norstog and Nicholls 1997). The tissue acts as a sink during megastrobilus growth, with *C. micronesica* K.D. Hill megagametophytes accumulating almost 70% nonstructural carbohydrates by maturity (Marler and Cruz 2019; Marler and Dongol 2016). These stored reserves are exploited as a source of nutrition for seedling growth following germination (Norstog and Nicholls 1997).

Germination is initiated when the basal tips of the cotyledons grow out of the sclerotesta with the extending surface protected by a coleorhiza composed of suberized tissue (Chamberlain 1919; Norstog and Nicholls 1997). The marginal tips of the cotyledons remain embedded within the megagametophyte, and the stored resources are translocated through the protruding cotyledons to the developing seedling. The exposed tissues of the *Cycas* cotyledons become green when illuminated following germination. After several weeks to months of geotropic growth of the radicle, the first true leaf emerges from the space between the protruded portion of the two cotyledons. General characteristic of *Cycas* seed germination have been described (Chamberlain 1919; Dehgan and Schutzman 1989; Norstog and Nicholls 1997; Whitelock 2002).

The level of light during germination is appreciated as a factor that influences seed germination and early seedling growth, but very few studies have quantified light level and determined plant response for cycad species. *Zamia fairchildiana* L.D. Gómez seeds germinated more successfully in 10% sunlight transmission than in 70% transmission in a container nursery setting (Lopez-Gallego 2013). *Dioon edule* Lindl. seeds germinated more successfully under photosynthetic photon flux (PPF) of 17 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplied by commercial shadecloth than under 81 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplied by tree canopy cover (Yáñez-Espinosa and Flores 2016). *D. edule* seeds were reported to grow better in shade during germination, but the methods used various sowing depths instead of experimental imposition of different light treatments (López-Ovando and Treviño-Garza 2008). *D. edule* seeds were germinated in darkness, and produced more than five nonfunctional leaves per seedling over a 2-year period (Vovides 1990). Control seedlings grown in a traditional nursery produced fewer than one leaf per seedling during the same time period. The level of light in the nursery was not reported. These reports on the influence of light on cycad seed germination were not designed to study the full spectrum of light from darkness to full sun conditions.

The paucity of studies on this subject combined with the highly successful germination practices in commercial and research cycad nurseries indicate the precise level of light that cycad cotyledons initially experience may not be of crucial importance. However, a detailed look at a range of light levels on cycad seed germination is warranted considering the fact that cycads comprise the most threatened group of plants worldwide (Brummitt et al. 2015; Fragnière et al. 2015). Indeed, the threats to cycad persistence are consistent for most species, and among the list of threats is reproductive failure (Mankga and Yessoufou 2017). The broad concept of reproductive failure includes the full range of issues from production of ovules, success of pollination, success of fertilization, successful maturation of seeds, dispersal of seeds, germination, and recruitment to the sapling stage. For this study, a greater understanding of the source activities of green cotyledon photosynthesis would be of interest to conservationists to refine nursery protocols toward the goal of improving recruitment from the seedling to the sapling stage. Moreover, in situ seeds that germinate beneath the canopy of a conspecific adult tree exhibit more rapid seedling mortality than seeds that germinate away from a conspecific adult (Marler 2023). The level of shade may be a part of the causal agents that generate this response.

The objectives of this study were to determine the loss in weight of megagametophyte tissue during seed germination and seedling growth of two *Cycas* species to provide a measure of the role of the megagametophyte as a source for seedling growth. Second, the influence of illuminating the green portion of exposed cotyledons on seedling growth was determined to provide an indirect measure of the photosynthetic contributions of cotyledons as source organs.

Materials and Methods

Four experiments were conducted in Guam and the Philippines. *C. edentata* de Laub. and *C. micronesica* germplasm were used. The first two studies quantified the decline in megagametophyte fresh weight (FW) and DW until the tissue was no longer hydrated and presumably no longer capable of acting as a source of nutrition due to desiccation. The third and fourth studies determined the role of light availability following germination on DW components of seedlings following substantial radicle growth but immediately before emergence of the first true leaf. Termination of the studies at this stage ensured the

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true leaves were unable to act as source organs, and all quantified seedling growth was derived from megagametophyte resources and photosynthesis of cotyledons.

Megagametophyte source study. The initial *C. micronesica* study of this project was conducted at the University of Guam. Mature seeds were harvested in Dec 2013 within a protected plot located on the east coast of Guam. The plot had received imidacloprid applications to reduce *Aulacaspis yasumatsui* Takagi damage for 6 years at the time. A total of 288 seeds from four source trees were harvested and sarcotesta tissue was removed. Cleaned seeds were comingled and stored in ambient conditions for 9 months to enable embryo maturation.

Stored seeds were weighed individually to begin the study in Aug 2014. Seeds used for the experiment were restricted in FW to 25 to 28 g to ensure initial megagametophyte resource volume was homogeneous, and 100 seeds within this weight range were used to provide experimental units for the study. Six seeds were included for each of the growth stages defined hereinafter. The pre-germination stage (stage 0) was defined as fresh seeds before imbibition with six seeds selected before soaking. The remaining seeds were soaked for 24 h in municipal water, then sown in a perlite bed under 50% shaded conditions. The first growth stage was defined as seeds following imbibition with six seeds selected randomly (stage 1). The second growth stage was defined as seeds immediately before germination, which is unambiguously determined as the timing of opening of the platyspermic sclerotesta structure through which the coleorhiza extends (stage 2). The period between sowing of seeds and stage 2 can be lengthy, but the period between stage 2 and visible seedling growth is minimal. The third growth stage was defined as 6 weeks after seed germination at a time that radicles were 5 to 9 cm in length (stage 3). Beginning with this stage, and for every stage thereafter, the seed was removed from the growing seedling by slicing through the cotyledons at the edge of the sclerotesta. The fourth growth stage was the final growth stage in the perlite bed and was defined as the time period immediately before emergence of the first true leaf (stage 4; Fig. 1A). Radicles were 11 to 19 cm in length at this stage.

The remaining seedlings were removed from the perlite bed at stage 4 and planted individually in 2.6-L containers in medium that was equal parts #16 quartz sand, peat, and perlite. The containerized seedlings were grown on benches under 50% shade. Irrigation was supplied as needed, and fertilization was supplied using a stock solution composed of two parts water-soluble fertilizer (Scotts Miracle-Gro, Marysville, OH, USA; 24% nitrogen, 3.5% phosphorus, 13.2% potassium, 0.02% boron, 0.07% copper, 0.15% iron, 0.05% manganese, 0.0005% molybdenum, 0.06% zinc) to one part calcium nitrate such that the solution provided 7.5 mM nitrogen. The solution was applied every 2 weeks at 100 mL per container. In addition, supplemental iron, manganese, and sulfur were supplied as a stock solution of 15 g·L^{-1} of Fe-ethylenediamine-N, N′-bis (2-hydroxyphenylacetic acid) and 1 g·L^{-1} magnesium sulfate by monthly applications of 100 mL per container.

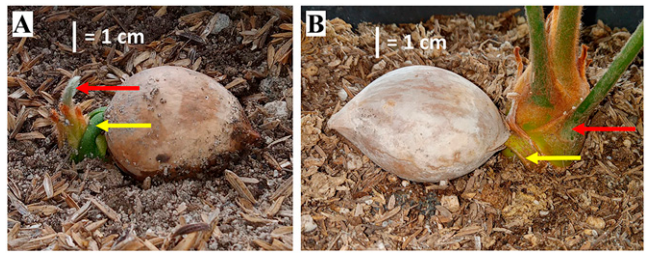

Fig. 1. Cotyledons (yellow arrows) and first true leaf (red arrows) of *Cycas* seedlings at two stages of development. (**A**) *Cycas micronesica* seedling following 2 months of root growth and when the first true leaf emerged from the space between the cotyledons. (**B**) Eighteen-month-old *Cycas micronesica* seedling with six leaves and persisting cotyledons.

The remainder of the growth stages were defined by the timing of each newly added leaf. Stage 5 was after one true leaf, stage 6 was after two true leaves, stage 7 was after three true leaves. The seed was harvested on the date that leaf expansion terminated, based on daily measurement of median leaflet length. The growing seedlings for subsequent stages were translocated to 5.1-L containers before the four-leaf stage. Irrigation and fertilization continued as previously described. Stage 8 was after four true leaves, stage 9 was after five true leaves, and stage 10 was after six true leaves (Fig. 1B). The study was terminated in Feb 2016 when the megagametophytes were desiccated. The mean daily maximum temperature was 30.7 °C, and the mean daily minimum temperature was 24.7 °C.

For each growth stage, there were three response variables. These were megagametophyte FW, DW following 48 h in a forced draft oven at 75 °C, and dry matter content defined as DW/FW.

The second study was conducted in a conservation nursery in Barangay Sapang Bato, Angeles City, Philippines. The Guam methods were employed using *C. edentata* seeds obtained from open-pollinated trees within a germplasm collection on the same site. Seeds were harvested in Oct 2017. For each seed, sarcotesta was cleaned from the sclerotesta, then seeds were stored in ambient conditions for 7 months. Seeds were weighed individually to begin the study in May 2018, and 100 seeds within a FW of 20 to 22 g were selected.

The sampling methods were as described for the Guam study. The bed for seed germination was composed of washed, locally quarried river sand. Radicle length was 4 to 8 cm at stage 3 and 10 to 19 cm at stage 4. The container medium following stage 4 was 50% river sand and 50% fresh rice husk. Irrigation and fertilization were as described for the Guam study. Sampling continued until the five-leaf stage when megagametophyte tissue was desiccated. The study was terminated in Jul 2019. Mean daily maximum temperature was 33.1 °C, and mean daily minimum temperature was 24.5 °C.

Data sets for each species were analyzed separately. For various reasons the data did not conform to requirements for parametric tests. The Kruskal-Wallis *H* test was used to determine level of significance among the growth stages for each of the three response variables. The data are reported as means ± standard deviation with six replications and 10 growth stages for *C. micronesica* and nine growth stages for *C. edentata*.

Cotyledon illumination study. Two experiments were conducted in the Philippine nursery to determine the influence of illuminating the *Cycas* cotyledons on initial seedling growth. *Cycas micronesica* seeds derived from Guam genotypes were harvested from the ex situ germplasm in Aug 2018. Megastrobili had been hand-pollinated, but due to timing of pollen availability, the pollen parents were not derived from the same area of occupancy on Guam as the ovulate parents. Therefore, the seedlings were of no value for future conservation efforts because of genetics issues. Sarcotesta tissue was cleaned, then the seeds were stored in ambient conditions until Apr 2019 for embryo maturation.

FWs and DWs of megagametophyte tissue were required for this study. Ending DW could be directly measured, but initial DW could only be estimated. To achieve this experimental requirement, a sample of 10 seeds with a range in FW of 19 to 32 g was destructively exploited to directly measure DW. This was accomplished by removing the sclerotesta, and subsequent drying of the gametophyte tissue at 75 °C for 48 h. Gametophyte DW was treated as the dependent variable to obtain the equation DW = (0.5262 × FW) + 0.6371 (r^2 = 0.94). Initial FWs of each seed were subjected to this regression to obtain estimated DW.

FW of each remaining seed was weighed and recorded. Seeds were soaked for 24 h in well water from the site, then sown in washed river sand beds. Germination began in May 2019, and 24 seeds with concurrent germination dates and similar weights (25 to 28 g) were removed from the germination bed on 4 Jun 2019. Germinated seeds were planted in individual 2.6-L containers filled with 25% rice husk and 75% #16 silica sand with the sclerotesta inserted half-way into the medium and with the cotyledons exposed. Four levels of illumination were used: 100% sunlight exposure, 47% sunlight exposure, 25% sunlight

exposure, and darkness. The 47% and 25% treatments were created using neutral density shade screen materials as determined by measurement of incident light by a quantum sensor (Skye SKP200; Skye Instruments, Llandrindod Wells, Powys, UK). This initial study was designed to determine the influence of cotyledon light exposure on seedling growth, so the exposed upper surface of every sclerotesta in the treatments receiving light was blocked from sunlight exposure with suspended cardboard panels. These cardboard covers were protected with a second layer of cardboard that blocked incoming solar radiation but enabled ventilation (Fig. 2A). These methods ensured radiative heat gain was not transferred directly to the sclerotesta, and removed the possibility that differences in heat stress of the gametophytes were causal of any seedling growth differences among the treatments. The dark treatment was created by completely covering the cotyledons and exposed sclerotesta surfaces with suspended cardboard panels. To verify homogeneity of surface temperatures, a type K thermocouple fitted to a portable data recorder (Model 54II; Fluke, Everett, WA, USA) was placed on the upper surface of every sclerotesta during midday on several days. The temperatures were 32.1 to 32.9 °C depending on the date, and surface temperature was similar for every light treatment. The emerging cotyledons were oriented south to ensure all morning, midday, and afternoon sunlight would impact the exposed cotyledon surfaces.

Seedlings were arranged in a randomized complete block with six replications. Irrigation with well water was supplied two times per week by adding enough water to create ≈10% leaching. Fertilizer was provided as a single dose on 10 Jul 2019 using the methods previously described, with each container receiving 100 mL of solution. An entire block was harvested when the first of the four seedlings began to initiate the first true leaf. The first block was harvested on 2 Jul 2019, and the final block was harvested on 13 Jul 2019 for a mean of 4.6 weeks of post-germination radicle growth before the emergence of the first true leaf. The mean daily maximum temperature was 33.8 °C, and the mean daily minimum temperature was 25.7 °C.

C. edentata seeds derived from germplasm from various Philippine islands were harvested from the ex situ plants in Mar 2023. Megastrobili had been open-pollinated by native pollinators, so the pollen parents within the ex situ garden were not known. Consequently, seedlings were of no value for future conservation efforts due to genetic mixtures. Sarcotesta tissue was cleaned, then the seeds were stored in ambient conditions until Dec 2023 for embryo maturation.

A sample of 10 seeds with a range in FW of 17 to 26 g was used to directly measure corresponding DW. This was accomplished by removing the sclerotesta, then drying the gametophyte tissue at 75 °C for 48 h. Gametophyte DW was treated as the dependent variable to obtain the equation DW = (0.2178 × FW) + 3.257 (r^2 = 0.98). Germination of the remaining seeds was initiated on 15 Dec 2023 using methods described for the *C. micronesica* study. The 24 seeds with concurrent germination and similar weights (21 to 23 g) were planted on 24 Jan 2024. Each block was harvested when the first of four seedlings initiated leaf growth. The first block was harvested on 29 Feb 2024, and the final block was harvested on 9 Mar 2024 for a mean duration of 5.6 weeks of post-germination radicle growth. The mean daily maximum temperature was 32.1 °C, and the mean daily minimum temperature was 22.3 °C.

For harvest procedures, the four seedlings within each block for each experiment were carefully bare-rooted to retrieve the intact seedlings (Fig. 2B). The roots were cut from the basal end of the cotyledons, then the sclerotesta was carefully removed from the gametophytes and discarded. The exposed green portion of the cotyledons was severed from at the edge of the gametophyte surface. The roots and gametophytes were sliced into sections and all tissue was dried at 75 °C for 48 h (Fig. 2C). Dry weights of the three tissue categories were recorded, then total ending seedling weight was derived by summing the three category weights. The marginal portions of the cotyledons embedded within the gametophytes (Fig. 2C) were included as part of the gametophyte weight. The estimated loss of gametophyte DW was determined individually for each seedling by estimating initial DW from the initial FW and the corresponding regression equations, then subtracting ending DW. The data were analyzed by analysis of variance for each species separately as a randomized complete block with six blocks and four illumination treatment levels (SAS Institute, Cary, NC, USA).

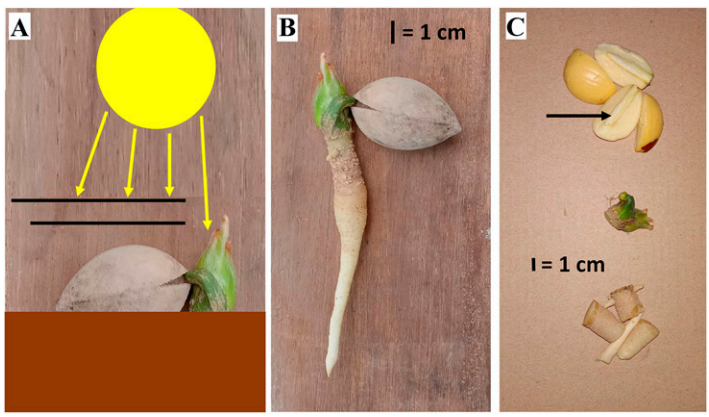

Fig. 2. Traits of *Cycas* seedlings during cotyledon illumination study. (**A**) Graphical depiction of the use of cardboard panels (black lines) to block direct sun exposure to sclerotesta surfaces. (**B**) Intact bare-rooted seedling after several weeks of radicle growth and immediately before growth of the first true leaf. (**C**) Tissue from megagametophyte, cotyledon, and root categories separated for tissue weight measurements. Black arrow points to marginal cotyledon tissue embedded within the megagametophyte.

Results

Megagametophyte source study. The appearance of the sclerotesta was unchanged for the duration of seedling growth that culminated in a desiccated megagametophyte. In contrast, the megagametophyte of a fresh seed appeared turgid and occupied the full volume within the sclerotesta (Fig. 3A) but was desiccated and shrunken at the final growth stage of the two studies (Fig. 3B).

FW of *C. micronesica* megagametophytes varied significantly among the stages of seedling growth (H = 60.216, P < 0.001). Initial FW ranged from 26 to 31 g, and remained remarkably stable until the first true leaf reached full expansion (Fig. 4A). Thereafter, FW gradually declined until the six-leaf stage. Megagametophyte DW also varied significantly (H = 63.317, P < 0.001), and initial DW was ≈18 g (Fig. 4A). DW was stable until germination ensued, then abruptly declined following emergence of radicles from the basal portion of the cotyledons. Gametophyte DW after several weeks of radicle growth but before emergence of the first true leaf (stage 4) was 54% of initial DW. The decline in DW continued at a considerable rate through the maturation of the first true leaf (stage 5), when gametophyte DW was 36% of initial DW. The rate of decline in DW decreased for the remainder of the study as each new leaf was constructed. The differences in rates of decline in FW vs. DW defined the pattern of dry matter content throughout the seedling growth, which varied significantly among the growth stages (H = 61.651, P < 0.001). Dry matter content of megagametophytes was ≈60% initially, and remained stable until emergence of the radicles (Fig. 4B). Thereafter, dry matter content declined considerably until maturation of the first true leaf (stage 5), when dry matter content was 43% of initial dry matter content. The loss in DW exceeded the loss in water weight as initial seedling growth relied exclusively on gametophyte resources. The dry matter content remained fairly stable from stage 5 until stage 9, indicating DW and water weight declined in synchrony. Dry matter content approached 100% at the 18-month stage of growth when six leaves had developed on the seedlings, indicating the tissue was no longer hydrated and could presumably no longer act as a source of nonstructural resources.

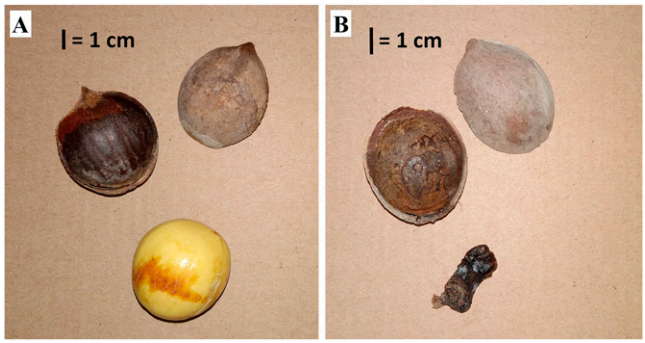

Fig. 3. Sclerotesta halves and megagametophyte of *Cycas edentata* seeds at two stages of development. (**A**) Fresh seed before germination with turgid gametophyte. (**B**) Seed following 14 months of seedling growth with desiccated gametophyte. Interior (left) and exterior (right) surfaces of each sclerotesta are shown.

FW of *C. edentata* megagametophytes changed significantly during the growth stages of the study ($H = 57.746$, $P < 0.001$). Initial FW ranged from 20 to 22 g, increased after imbibition, then began to decline as the radicle and leaf growth ensued (Fig. 5A). FW gradually declined until the five-leaf stage when the tissue was desiccated in appearance (stage 9). The DW changes were also significant ($H = 57.082$, $P < 0.001$). Initial DW of megagametophytes was about 12 g, and the decline in DW mirrored the pattern exhibited by the *C. micronesica* seedlings (Fig. 5A). Gametophyte DW after radicle growth but before emergence of the first true leaf was 50% of initial DW. Gametophyte DW after maturation of the first true leaf was 28% of initial DW. Dry matter content of the *C. edentata* seeds differed among the growth stages ($H = 52.903$, $P < 0.001$), and the changes were similar to those of the *C. micronesica* seeds except the final desiccated stage occurred at 14 months following the addition of only five leaves to the seedlings (Fig. 5B).

Cotyledon illumination study. The block effect was NS for all response variables for *C. micronesica* seedlings. Megagametophyte DW was similar for the three illumination treatments, and was 27% greater than megagametophyte DW of the dark treatment seedlings (Table 1). In contrast, the cotyledon and root DWs were similar among all four light treatments. The greater megagametophyte weight of the plants with illuminated cotyledons caused a 20% increase in DW of these seedlings above that of the dark seedlings. The direct measure of megagametophyte weight loss during the radicle growth stage was similar among the three illumination treatments, but weight loss was 22% greater for the dark-grown seedlings.

The block effect was NS for all response variables for *C. edentata* seedlings. The relative differences among the light treatments for each of the response variables was similar to those of *C. micronesica*. Megagametophyte DW was similar for the three illumination treatments, and was 14% greater than megagametophyte DW of the dark treatment seedlings (Table 1). Cotyledon and root DWs were similar among all four light treatments. Illuminating the cotyledons caused a 11% increase in total DW of the seedlings above that of the seedlings with cotyledons in darkness. Megagametophyte weight loss during the radicle growth stage was similar among the three illumination treatments, but weight loss was 21% greater for the dark seedlings.

Discussion

The influence of seed size on germination and early seedling growth rates has been heavily studied (Baraloto and Forget 2007; Metz et al. 2023; Zanne et al. 2005). Large seeds containing large gametophytes are well-known as defining characteristics of *Cycas* species (Brenner et al. 2003; Chamberlain 1919; Norstog and Nicholls 1997). With appropriate protocols, nurturing germination and initial seedling growth is also known to be relatively easy for most cycad horticulturists, with the abundant seed resources acknowledged as causal (Whitelock 2002). However, the source behavioral traits of the seed tissues have not been previously assessed experimentally. In response to the first objective, these findings have shown that the initial growth of the radicle before emergence of the first true leaf and the construction period of this first leaf is characterized by a short growth phase of less than 2 months that is highly dependent on megagametophyte resources. The gametophyte DW declined to only 30% of the initial gametophyte DW during these few weeks of *C. edentata* and *C. micronesica* seedling growth. The rate of gametophyte weight loss for both species was slower after maturation of the first true leaf, indirectly indicating that the added carbohydrate resources from leaf photosynthesis contributed as a source for meeting the sink needs of continued seedling growth. Despite the new availability of autotrophic photosynthates following the maturation of the first leaf, the results have also shown that the gametophytes remained mostly hydrated with continued gradual loss of dry matter until 14 to 18 months. These findings revealed a sustained long-term function of gametophyte source behavior that augmented autotrophic resources derived from concurrent photosynthesis of true leaves. The

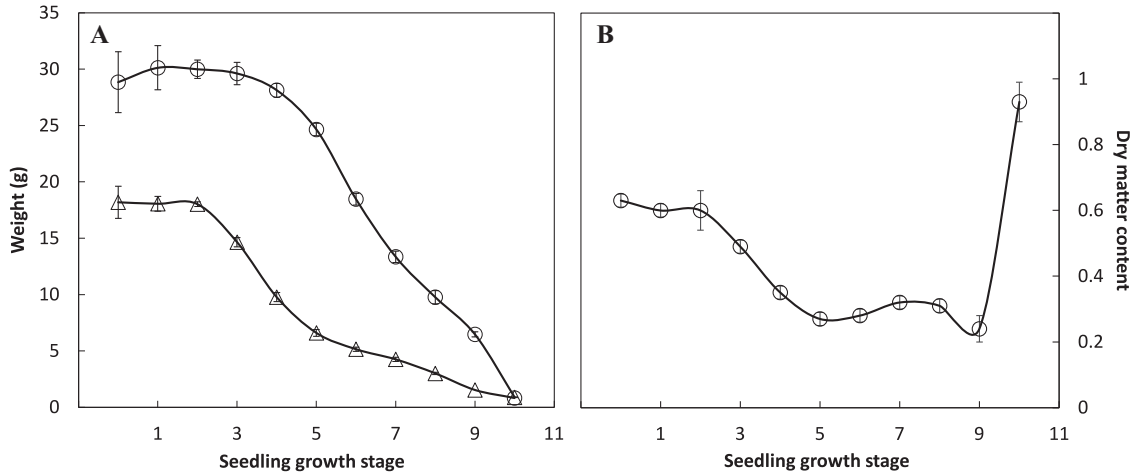

Fig. 4. *Cycas micronesica* megagametophyte fresh weight (circles), dry weight (triangles), and dry matter content as influenced by seedling growth. 0 = fresh seeds; 1 = seeds after imbibing for 24 h; 2 = incubated seeds before germination; 3 = seedlings after 6 weeks of radicle growth; 4 = seedlings immediately before leaf emergence; 5 = seedlings following one leaf; 6 = seedlings following two leaves; 7 = seedlings following three leaves; 8 = seedlings following four leaves; 9 = seedlings following five leaves; 10 = seedlings following six leaves. n = 6, mean ± standard deviation.

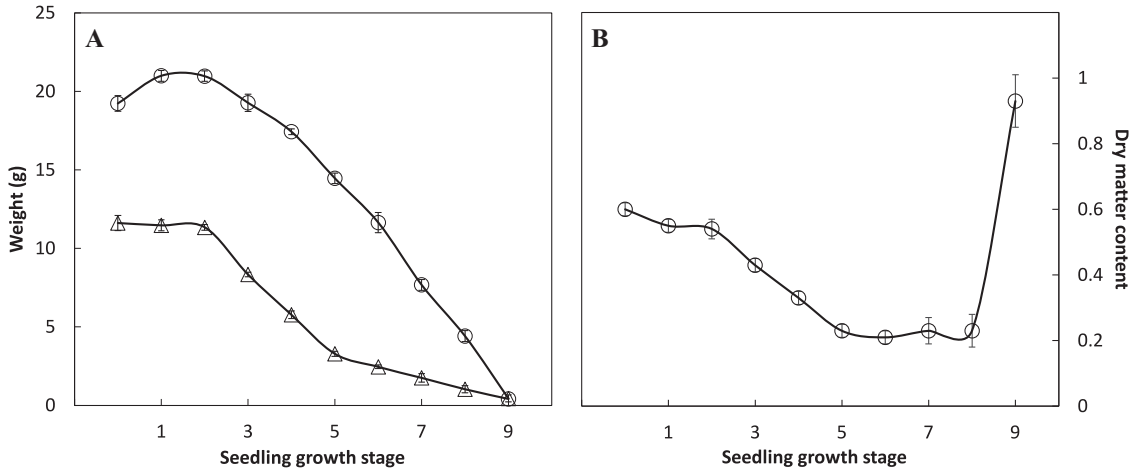

Fig. 5. *Cycas edentata* megagametophyte fresh weight (circles), dry weight (triangles), and dry matter content as influenced by seedling growth. 0 = fresh seeds; 1 = seeds after imbibing for 24 h; 2 = incubated seeds before germination; 3 = seedlings after 6 weeks of radicle growth; 4 = seedlings immediately before leaf emergence; 5 = seedlings following one leaf; 6 = seedlings following two leaves; 7 = seedlings following three leaves; 8 = seedlings following four leaves; 9 = seedlings following five leaves. n = 6, mean ± standard deviation.

results underscore the need for nursery managers to ensure the intact *Cycas* seeds and the long-lived cotyledons that connect the gametophytes to the growing seedling are protected from damage to sustain long-term exploitation of gametophyte resources.

Non-leaf green tissues such as seeds, fruits, and stems are abundant, and the study of their photosynthetic traits has received considerable interest in recent years (Lawson and Milliken 2023; Simkin et al. 2020). The contributions of photosynthates by these organs with chlorophyll-containing surfaces are often overlooked, primarily because the organs have other clearly understood functions (Aschan and Pfanz 2003). Although direct measurements of carbon dioxide gas exchange are important to reach a full understanding of non-leaf photosynthesis, indirect methods like shading the photosynthetic tissue surfaces are also important protocols for understanding how these organs influence overall plant carbon economy (Lawson and Milliken 2023). These were the methods used herein to address the second objective. The ability of green *Cycas* cotyledons to act as a carbon source has not been considered to date. The results herein provided direct evidence that initial sink activity of seedling growth was more reliant on megagametophytes as a source when the cotyledons were deprived of photosynthetically active radiation than when the cotyledons were illuminated. These findings revealed indirect evidence that providing light to *Cycas* cotyledons increased the ability of the cotyledons to act as source organs, as radicle DW did not differ among the three illumination treatments but total seedling DW was decreased by darkness. The conventional picture of *Cycas* cotyledons as vascular organs through which megagametophyte resources are translocated to developing radicle and true leaves is clearly too simple, and the function of chlorophyll-containing *Cycas* cotyledons is more complex and nuanced. The true nature of this multifunction *Cycas* cotyledon deserves further study.

The role of cotyledon strategy in seedling establishment and recruitment in competitive settings has received less attention than size of seed storage tissues (Baraloto and Forget 2007; Metz et al. 2023; Zanne et al. 2005). The range of taxa included in this research agenda indicate there is a tradeoff between seed size as one strategy and photosynthetic cotyledons as a second strategy for supporting early seedling establishment. The results herein indicate that cycad species should be added to this research agenda, as they combine the carbon reserves in relatively large storage tissues as one seed strategy with concurrent additions of photosynthates from long-lived cotyledons as an augmentation strategy to meet the needs for seedling establishment. Moreover, the durations of these disparate seed carbon source strategies are surprisingly lengthy after germination is initiated, as the cotyledons of most plant species are short-lived. *Cycas* belongs to the monogeneric family Cycadaceae, and the seed design of the members of the Zamiaceae family differs from *Cycas* in several respects. A greater understanding of cycad biology would develop by applying the methods used herein to germinating seeds of various Zamiaceae genera.

Cycad plants construct other non-leaf organs that contain chlorophyll in the surface tissues. For example, microsporophyll and megasporophyll blades are green for the strobili of many cycad species, and the naked ovules and developing young seeds are green for many *Cycas* species (Norstog and Nicholls 1997; Whitelock 2002). The types of plastids in cycad seed integuments are highly varied

Table 1. Dry weight (g) of *Cycas* seedling components after radicle growth but before leaf emergence as influenced by illumination of green cotyledon tissue. n = 6, mean ± standard error.

Sunlight transmission (%)	Gametophyte	Cotyledon	Root	Total seedling	Gametophyte wt loss
			Cycas micronesica		
0	6.642 ± 0.227	1.033 ± 0.027	1.113 ± 0.024	8.879 ± 0.235	9.333 ± 0.321
25	8.462 ± 0.299	1.116 ± 0.055	1.107 ± 0.030	10.685 ± 0.254	7.570 ± 0.555
47	8.513 ± 0.281	1.121 ± 0.056	1.099 ± 0.029	10.733 ± 0.238	7.543 ± 0.544
100	8.235 ± 0.300	1.120 ± 0.055	1.111 ± 0.024	10.465 ± 0.257	7.759 ± 0.578
$F_{3,15}$	9.866	0.646	0.126	16.625	3.511
P	<0.001	0.598	0.943	<0.001	0.042
			Cycas edentata		
0	5.350 ± 0.172	1.012 ± 0.030	0.565 ± 0.005	6.926 ± 0.147	5.169 ± 0.413
25	6.141 ± 0.191	1.030 ± 0.028	0.551 ± 0.007	7.722 ± 0.113	4.103 ± 0.138
47	6.168 ± 0.184	1.031 ± 0.023	0.573 ± 0.007	7.742 ± 0.171	4.290 ± 0.226
100	5.947 ± 0.178	1.115 ± 0.058	0.550 ± 0.009	7.612 ± 0.180	4.451 ± 0.272
$F_{3,15}$	8.448	0.867	0.307	8.215	3.899
P	<0.001	0.480	0.820	<0.001	0.030

among the species that have been studied (Whatley 1985). Similarly, the petiole and rachis of most cycad leaves are green and are constructed with considerable surface area. In addition to these organs that are green under typical growing conditions, numerous examples have been reported in which plant tissues such as roots or tubers do not contain chlorophyll when grown in darkness but develop chloroplasts when exposed to light (Henry et al. 2020). This phenomenon may occur with cycad roots that are exposed to light or megagametophytes whenever an opening of the sclerotesta allows direct illumination of the gametophyte surfaces (personal observations). Clearly, more anatomy, morphology, and physiology studies are warranted to fully understand how these chlorophyll-containing cycad organs may contribute photosynthates to influence the overall carbon economy of the cycad plants. For the long-lived cotyledons, the characteristics of chloroplast, stomata, and gas exchange behaviors have not been reported to date.

Conservation biology has become of paramount importance in today's imperiled world, and cycads are among the most threatened plants (Brummitt et al. 2015; Fragnière et al. 2015). Indeed, the two model species in this study are endangered as a result of various anthropogenic threats (Bösenberg 2022; Lindstrom 2023). As arborescent species, they are also among the 30% of the world's tree species that are threatened with extinction (Botanic Gardens Conservation International 2021). Long-standing recommendations have advocated for conservation nursery production of documented cycad seedlings as a means of reducing the threats of poaching in situ plants (Donaldson 2003; Vovides et al. 2010). Developing a greater understanding of how megagametophytes and cotyledons contribute to cycad seedling health and growth may enable improvements in these conservation nursery protocols. More manipulative germination studies would also generate the information that would enable more informed conservation within in situ cycad species recovery efforts. For example, seedlings that emerge beneath the canopy of adult conspecifics die more rapidly than seedlings that emerge away from adult conspecifics (Marler 2023). The added shade of the dense cycad leaf canopy and the resulting greater depletion of megagametophyte storage resources during germination may be part of the causal factors underlying this response.

The use of neutral density shadecloth to create the different levels of PPF did not allow the determination of light quality on *Cycas* cotyledon contributions to seedling growth. Many plant responses use light quality as the cue rather than light quantity (Han et al. 2024). Indeed, much confusion occurs when unclarified terms and light measurements are used in the literature (Zavafer et al. 2023). More studies are needed to determine the roles of light quality characteristics such as blue light (Karnachuk et al. 2008) and red:far red ratio (Sng et al. 2023) on the contributions of cycad cotyledon photosynthesis to seedling growth.

In summary, cycads are a highly threatened group of plants, command considerable appeal among plant enthusiasts, have been survivors for hundreds of millions of years, yet remain relatively understudied. This gametophyte-cotyledon study provides yet one more example of a consequential trait of cycad biology that has not been experimentally considered before now. The contributions of illuminated green cotyledons have been shown to influence the source-sink behaviors of cycad seedlings by decreasing the demands on the source function of gametophytes during the initial growth stages. With a greater understanding of cotyledon photosynthesis, horticulturists may become more skilled at manipulating the microenvironment of cycad conservation nurseries.

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