Seed Ontogeny and Nonstructural Carbohydrates of *Cycas micronesica* Megagametophyte Tissue

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Additional index words. cycad, glucose, Guam, fructose, sucrose, starch

Abstract. The profile of nonstructural carbohydrates (NSC) was quantified to determine sugar and starch relationships of megagametophyte tissue during *Cycas micronesica* K.D. Hill seed ontogeny. Field work occurred in northern Guam where megastrobili were marked and dated as they emerged from stem apices of plants in a natural population. Seeds were harvested beginning 6 months after megastrobili emergence and continuing until 28 months, and gametophyte tissue was separated from the remainder of each seed. Carbohydrates within lyophilized gametophyte tissue were quantified by high-pressure liquid chromatography. The levels of glucose and fructose declined from a high at 6 months to a homeostasis at 11 months, and the levels of sucrose similarly declined from 6 months to a homeostasis at ≈ 14 months. Starch content exceeded sugar content and increased from 6 months to reach a homeostasis at ≈ 18 months. Maltose was not detected in any sample. Stoichiometric quotients changed dramatically until ≈ 14 months, when they became fairly stable until 28 months. Starch concentration was ≈ 5 -fold greater than sugar concentration at 6 months, and increased to ≈ 15 -fold greater than sugar concentration by 28 months. Total NSC in mature megagametophytes was almost 70% on a dry weight basis. Our results are in agreement with the biological function of this haploid tissue, as copious carbohydrate resources are readily available to support embryo and seedling growth.

Cycads are ancient gymnosperms that represent the most threatened plant group today (Brummitt et al., 2015; Fragniere et al., 2015). Their resilience and history provide ecologists and conservationists the unique opportunity to gain insight into various aspects of plant evolution and biology (Brenner et al., 2003). Cycad species are commonly represented in nursery and landscape horticulture within their native range. In fact, collecting plants from habitat for horticultural use has been the primary threat to many of the described species (Donaldson, 2003; Norstog and Nicholls, 1997). *Cycas revoluta* Thunb., marketed as sago palm, and *Zamia furfuracea* L. f. ex Aiton, marketed as cardboard palm, are the two species most commonly represented in the international horticulture trade. The study of cycad horticulture and physiology has been minimal relative to angiosperm species (Norstog and Nicholls, 1997) even though the International Union for Conservation of Nature and Natural Resources (IUCN) recommends more coordinated global emphasis to address knowledge gaps in the propagation and cultivation of

cycads (Donaldson, 2003). Gaining further knowledge regarding all aspects of cycad propagation is urgent to improve horticultural protocols.

Cycad plants are dioecious with pollen strobili on male plants exhibiting the appearance of a true cone. Megastrobili borne on most genera also exhibit the appearance of a cone, but within the *Cycas* genus female plants bear ovules and seeds on an assemblage of loose sporophylls. The cycad megagametophyte is a large seed structure that is composed of haploid tissue (Brenner et al., 2003; Norstog and Nichols, 1997). It serves a nutritive role for the cycad embryo in a manner that is analogous to the triploid endosperm of angiosperm seeds. Studies of cycad megagametophytes may increase our understanding of evolution of the angiosperm endosperm (Brenner et al., 2003). The starch content of cycad megagametophyte tissue has been exploited for human consumption in many cultures (Hayward and Kuwahara, 2012; Norstog and Nichols, 1997; Thieret, 1958; Vázquez-Torres et al., 1989; Whiting, 1963).

Non-structural carbohydrates have not been extensively studied for this threatened group of plants. A plant's NSC pool is composed of low molecular weight sugars (the most abundant free sugars in plants are the disaccharides sucrose and maltose, and the monosaccharides glucose and fructose) plus starch (Chapin et al., 1990). A general survey of a range of cycad species revealed that the four sugars vary among organs and among species (Marler and Lindström, 2014).

Our objective was to quantify NSC pools in megagametophyte tissue in *Cycas micronesica* seeds from 6 to 28 months in age. These results may be useful for defining developmental stages which may improve propagation protocols.

Materials and Methods

Field methods. The experimental site was located in northern Guam, centered at 13°38'43'' N, 144°51'30'' E, in secondary forests growing in a Ritidian-Rock outcrop complex soil series (clayey skeletal, gibbsitic, nonacid, isohyperthermic Lithic Ustorthents) (Young, 1988). The site was split into six 50 m $\times$ 200 m blocks. A total of 62 female *C. micronesica* plants were tagged and dated to record emergence of reproductive events from February to May 2002. Seeds were harvested beginning 6 months after emergence, and were harvested through 2004 in a manner that generated a maximum of 28 months in seed age. We elected to begin seed collections at 6 months because megagametophyte volume and bulk density were minimal prior this age and the number of seeds required for younger age categories was not justifiable.

Seeds were harvested exclusively from plants that had not been used for seed samples in any prior date, so each megastrobili from which seeds were obtained had not been altered by earlier seed removal. Eight seeds were harvested from each single-tree replication and were homogenized into one tissue sample. One replication was harvested from each block for each of seven seed age categories. Seeds were transported to the University of Guam for processing. General observations were made on various tissues, then gametophyte tissue was excised, and stored at -40°C until chemical analyses were conducted on 12 and 13 Aug. 2014.

Analytical methods. Tissue was lyophilized then soluble sugar extraction was conducted with 80 $^\circ\text{C}$ hot-water extraction with acetonitrile (Schloter et al., 2005). The concentrations of sucrose, fructose, glucose, and maltose were determined by Thermo Scientific HPLC-RI (SpectraLab Scientific Inc., Markham, Ontario, Canada). The RI detector was a TSP Spectra System RI-150, fitted with a 3.9 $\times$ 300 mm Waters Carbohydrate Analysis column (No. WAT084038), an AS3000 autosampler, and P2000 pump. The starch contents were determined as the glucose equivalents after being hydrolyzed enzymatically by amylase and amyloglucosidase.

Our direct response variables were concentration of the four sugars and starch. In addition, we evaluated the NSC stoichiometry of each age category by calculating the quotients among the three dominant sugars: glucose/fructose, glucose/sucrose, and fructose/sucrose. We determined the relative immediate carbohydrate availability with the quotient hexoses/disaccharides where the hexose content was the sum of glucose and fructose, and the disaccharide content was the sum of sucrose and maltose. We determined the available free sugar in relation to storage starch with

Received for publication 1 June 2016. Accepted for publication 8 July 2016.

We thank Gil Cruz for help with field work. Support for analytical work provided by the U.S. Department of the Navy through N40192-12-2-8000 and N40192-13-2-8003, administered by Naval Facilities Engineering Command Marianas, Guam.

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the quotient (glucose + fructose + maltose + sucrose)/starch.

Patterns of NSCs were plotted to observe the general trends. The younger seed age trends exhibited a linear response with considerable positive or negative slope. The older seed age trends were characterized by a flat plateau with no net change in NSC concentrations. The data in younger age categories were fitted for each block using linear regression performed with the Proc GLM function in SAS Version 9.1 (SAS Institute Inc., Cary, NC). Absolute slope was clearly disparate among the NSCs because the starch slope was positive and the sugar slopes were negative. Therefore, we determined the differences in the relative rate of change among the NSCs by disregarding whether direction was negative or positive. The numerical values of the slopes for each NSC were subjected to analysis of variance in a randomized complete block design with six replications. Means separation was performed by the least significant difference test.

Results

Megagametophyte tissue was translucent and wet in appearance at 6 months, and flotation tissue comprised a large proportion of the seed volume at this stage (Fig. 1B). This tissue is found within seeds of the members of the *Rumphiae* subsection of *Cycas* (Hill, 1994). The tissue provisions buoyancy and enables hydrochory in riparian settings and thalassochory (long-distance dispersal using oceanic currents). The megagametophyte tissue increased in volume to a greater extent than the other seed tissues, and by 11 months it was opaque but maintained a wet appearance (Fig. 1C). The dry, white megagametophyte tissue changed little in appearance from 14 months until seed maturity (Fig. 1D–F). Observable embryo development began before 20 months and was robust by 28 months (Fig. 1E and F).

NSC concentrations. Megagametophyte fructose and glucose concentrations were in the range of 20–25 mg·g⁻¹ at 6 months, and declined in a linear pattern until ≈11 months in age (Fig. 2). These hexoses remained stable at ≈11–13 mg·g⁻¹ from 11 to 28 months. The sucrose concentrations also exhibited an initial linear decline with seed age, beginning at ≈45 mg·g⁻¹ and reaching ≈20 mg·g⁻¹ by 14 months. Sucrose concentration was stable from 14 to 28 months. Starch content greatly exceeded sugar content throughout, and increased in concentration from ≈400 mg·g⁻¹ at 6 months to reach saturation of ≈650 mg·g⁻¹ at ≈18 months. The rank in concentration of these carbohydrates was glucose < fructose < sucrose < starch at 6 months, and fructose < glucose < sucrose < starch for seeds above 18 months in age. Maltose was not detected in any sample for the duration of the study.

The block effect was not significant for any of the linear regression slopes. The relative slopes were heterogeneous among the NSCs ($P < 0.0001$). The rate of change for starch was significantly and 10-fold greater than that for the sugars. This disparity is

Fig. 1. General appearance of *Cycas micronesica* seeds from emergence to maturity. (A) Megastrobili phenotype at 6 weeks following emergence. (B) Seed at 6 months. Gametophyte (g) and flotation (f) tissues are labeled. (C) Seed at 11 months. (D) Seed at 14 months. (E) Seed at 20 months. (F) Seed at 28 months.

Fig. 2. *Cycas micronesica* seed megagametophyte fructose, glucose, sucrose, and starch concentration as influenced by seed age. Markers are mean $\pm$ SE, $n = 6$. Fructose: If $x < 11.5$, then $y = 40.55 - 2.53x$; $r^2 = 0.99$; $P = 0.05$, glucose: If $x < 11$, then $y = 30.71 - 1.61x$; $r^2 = 0.99$; $P = 0.02$, sucrose: If $x < 14$, then $y = 61.98 - 3.07x$; $r^2 = 0.96$; $P = 0.02$, starch: If $x < 18$, then $y = 253.75 + 22.60x$; $r^2 = 0.99$; $P < 0.001$.

masked in Fig. 2 due to the differences in y axis scale for starch and the sugars.

Stoichiometric relations. The glucose/sucrose, fructose/sucrose, and hexose/disaccharide quotients began and ended at about the same values, and exhibited erratic interim patterns from 6 to 14 months (Fig. 3). The hexose concentration was similar to the disaccharide concentration such that a 1:1 relationship characterized the megagametophyte tissue at 6 and 28 months. The glucose/fructose quotient began at ≈0.8 at 6 months and

increased steadily until ≈1.2 at 28 months. The sugar/starch quotient began at 0.24 and declined to less than 0.10 at ≈18 months.

Discussion

To our knowledge, this look at the carbohydrate status of developing *C. micronesica* seeds provides a first direct measure of sugar and starch concentrations in seeds for any cycad species. The major changes in concentration of *C. micronesica* seed

Fig. 3. Stoichiometric relations of pairs of nonstructural carbohydrate pools for *Cycas micronesica* megagametophytes, defined as quotients and as influenced by seed age. Markers are mean values, $n = 6$.

megagametophyte monosaccharides ended by month 11, of disaccharides ended by month 14, and of starch ended by month 18. The sugars and starch were remarkably stable for the remainder of seed maturation. The major shifts in stoichiometric relations among the NSC components ended by month 14. These traits of the metabolites may indicate that seed harvest for propagation purposes or for starch extraction may occur at any time following the 18th month for this species. This is also the stage that external seed appearance changes from bronze to a dull brown color (Marler and Dongol, 2011).

Analytical work on cycad megagametophyte carbohydrates has been minimal. The medical literature has examples of starch content of washed megagametophyte flour after it has been processed for human consumption, but these data cannot be directly compared with intact gametophyte chemistry. Starch content of developing seeds has been studied for two cycad species (Sánchez-Tinoco et al., 2012); however, indirect methods were used as the authors quantified the number of starch grains rather than measuring starch directly. Pant (1973) reported that *Cycas* seeds contain an estimated starch content of 25% in discussions of the high-value food qualities of the megagametophyte tissue. Our results indicate that this may be a severe under estimation of *Cycas* megagametophyte starch content, as the starch content of *C. micronesica* megagametophyte was $\approx 65\%$ at maturity.

Marler and Lindström (2014) reported the sugar profile of an entire *Zamia muricata* Willd. megastrobilus was $\approx 65\%$ fructose. Our results indicated the sugar profile of *C. micronesica* megagametophyte tissue was only $\approx 25\%$ fructose. Marler and Lindström (2014) further reported the presence of maltose in the *Z. muricata* megastrobilus, yet herein we

report no detectable maltose in *C. micronesica* megagametophyte tissue. These differences underscore the need for more research to understand the roles of NSCs in reproductive structures of cycad species (Marler and Lindström, 2015).

The NSC traits of seeds are important to study and understand for numerous reasons. Content and stoichiometry of carbohydrates within seeds have been implicated in vigor, desiccation tolerance, germination ability, and storage (Leduc et al., 2012; Lehner et al., 2006; Lin and Huang, 1994; Vandecasteele et al., 2011). Clearly, more empirical research is needed to clarify the nutritive value of cycad megagametophyte tissue for embryo and seedling viability. The species of greatest importance for our conservation efforts are the members of the *Rumphiae* subsection of the *Cycas* genus (Hill, 1994). As a group, these species have evolved to exploit thalassochory traits for long-distance oceanic seed dispersal. In horticulture nursery settings, they are among the cycad species that benefit from seed storage to enable postharvest embryo development. These and other unique traits illuminate several issues that may contrast *Rumphiae* species from the other cycad species in relation to megagametophyte NSCs. First, the *Rumphiae* species must provision seeds with resources that are not water soluble, as these resources would be lost during the lengthy periods of oceanic dispersal. Therefore, greater pools of starch in relation to water-soluble sugars may characterize *Rumphiae* species seeds. Second, sustaining embryo viability during lengthy oceanic journeys may demand greater NSC pools for *Rumphiae* species seeds than for other species seeds. Third, *Cycas* species are distinct from species from the other nine cycad genera in that ovules and seeds are borne on loose assemblages of megasporophylls. Species within

the *Rumphiae* subsection display the developing seeds in a manner that enables direct access to light, generating the potential for direct photosynthesis by seeds. For *C. micronesica*, seeds remain green for more than 1 year (Fig. 1A–D), verifying the potential for photosynthesis. In contrast, megastrobilus design for the other cycad genera encloses ovules and seeds in a manner that causes megasporophyll tissues to obstruct light from the surface of seeds. Therefore, orchestration of the dynamics of NSC relations of seeds may be highly contrasting between cycad species with seeds that are exclusively sinks vs. *Cycas* species with seeds that may serve as sources and sinks. The extent of seed photosynthesis and how the fixed carbon contributes to primary metabolism of the *Cycas* seed is not known.

The Cycad Specialist Group of the IUCN recommends research toward improving propagation and cultivation of cycads (Donaldson, 2003). Yet to date, there have been only a few contemporary responses to this call for expanded research (Calonje et al., 2010, 2011; Murphy et al., 2013). Our results indicated that the cycad megagametophyte may serve as an ideal system for studying NSC interrelationships and how those relationships support embryo and seedling growth as one means of responding to the IUCN's call for more cycad horticulture research. For example, sucrose has been shown to control the expression of genes involved in starch synthesis (Cairns and Pollock, 1988; Müller-Röber et al., 1990). Studying expression of these genes during the younger seed age categories when stoichiometric relationships of sucrose with the other carbohydrates is erratic vs. after the transition to homeostatic sucrose concentration may shed light on the signaling pathways that regulate the molecular network involved in starch accumulation in this structure.

Two major developments have occurred since our field work was completed, rendering our results of greater value. First, this site was invaded by the armored scale *Aulacaspis yasumatsui* in 2005 (Marler and Lawrence, 2012). This nonnative scale insect is able to infest the epidermis of male and female reproductive structures, including the sporophylls, naked ovules, and seeds. At no time since this invasion has the in situ *C. micronesica* plant's reproductive events been completely devoid of this direct damage by the insect herbivory. Research is warranted to determine the influence of this direct herbivory of seed surfaces on carbohydrate relations of the developing seeds, and how that relates to subsequent seed germination and seedling performance. Second, *C. micronesica* was listed under the United States Endangered Species Act in Nov. 2015 (USFWS, 2015). Field research that involves collection of tissue has become more complicated as a result of the need for federal research permits, and experimental proposals that include harvest of seeds for purposes other than propagation, such as this study, may no longer be approved.

Efforts to conserve this species include three extensive ex situ collections located outside the native range of the species, and five in situ management plots established in northern Guam. Horticultural management decisions for these conservation efforts are difficult to make in the absence of empirical research. Our results indicate that 18 months after megastrobilus emergence may be an important transition period for seed development.

Conclusions

Understanding sugar and starch relations during cycad seed development is of scientific interest and conservation relevance. To our knowledge, this characterization of monosaccharide, disaccharide, and starch relations of seeds is the first for any cycad species. Hexoses and sucrose were in greatest relative quantities in young megagametophytes, but starch content greatly exceeded sugar content even in these younger age categories. The starch content exceeded past published estimations of cycad megagametophyte starch content. Total NSC in mature megagametophytes was almost 70% on a dry weight basis. Our results are in agreement with the biological function of this haploid tissue, as copious carbohydrate resources are readily available to support embryo and seedling growth.

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