



Contents lists available at ScienceDirect

Scientia Horticulturae

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## Distribution of free and glycosylated sterols within *Cycas micronesica* plants

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### ARTICLE INFO

#### Article history:

Received 21 March 2009

Received in revised form 29 October 2009

Accepted 8 November 2009

#### Keywords:

*Cycas micronesica*

Phytosterol

Sterol

Steryl glucoside

### ABSTRACT

Flour derived from *Cycas micronesica* seeds was once the dominant source of starch for Guam's residents. Cycad consumption has been linked to high incidence of human neurodegenerative diseases. We determined the distribution of the sterols stigmasterol and  $\beta$ -sitosterol and their derived glucosides stigmasterol  $\beta$ -D-glucoside and  $\beta$ -sitosterol  $\beta$ -D-glucoside among various plant parts because they have been identified in cycad flour and have been shown to elicit neurodegenerative outcomes. All four compounds were common in seeds, sporophylls, pollen, leaves, stems, and roots. Roots contained the greatest concentration of both free sterols, and photosynthetic leaflet tissue contained the greatest concentration of both steryl glucosides. Concentration within the three stem tissue categories was low compared to other organs. Reproductive sporophyll tissue contained free sterols similar to seeds, but greater concentration of steryl glucosides than seeds. One of the glucosides was absent from pollen. Concentration in young seeds was higher than old seeds as reported earlier, but concentration did not differ among age categories of leaf, sporophyll, or vascular tissue. The profile differences among the various tissues within these organs may help clarify the physiological role of these compounds.

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### 1. Introduction

Guam's cycad (*Cycas micronesica* K.D. Hill) was once one of the most abundant horticultural species tended in home gardens on Guam, and foods prepared from flour derived from cycad seeds historically supplied a majority of starch in the diet of Guam's residents (Barratt, 2003). The use of cycad tissue for flour preparation is no longer a practice. One reason this historically important source of starch is no longer a part of the diet on Guam is the implication of cycad consumption to the island's high incidence of amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) (Borenstein et al., 2007).

Neurodegenerative properties of free and glycosylated sterols in mammalian systems have been reported (Kim et al., 2008; Shaw et al., 2007; Tabata et al., 2008a). We have been studying this group of phytochemicals in Guam's *C. micronesica* seeds as probable causal neurotoxins in relation to the island's prevalence of ALS-PDC because they appear in the water-insoluble lipid fraction. Cycad flour preparation on Guam has always employed a lengthy water-leaching phase, so water-soluble compounds do not emerge as viable candidate toxins.

The function of secondary compounds identified in cycad plants has remained relatively unstudied to date (Brenner et al., 2003). Our recent focus has been restricted to seed chemistry because this was the tissue used for flour preparation. Our attempts to more fully understand the role of the sterols in seed relations began by determining distribution of the compounds among seed tissues to reveal that the sclerotesta and sarcotesta tissues contained greater concentrations than the gametophyte tissue that was historically used for flour production (Marler et al., 2005b). We have also begun determining distribution of cycad seed sterols at larger spatial scales to reveal that concentration differences among seeds within a plant is less variable than among plants within a location or among locations throughout Guam (Marler et al., 2005b, 2007). Individual seed size, total seed load per plant, developmental stage of the embryo, and plant size were not significant predictors of megagametophyte sterol concentration (Marler and Shaw, 2009b). In contrast, seed age strongly influenced sterol relations, with a non-linear decline in concentration (Marler et al., 2006) accompanied by a linear increase in bioaccumulation (Marler and Shaw, 2009a).

To date, variation in concentration of sterols among organs has not been addressed for any cycad species. Similarly, the influence of age on sterol relations of organs (other than seeds) has not been determined for our model species. Thus, the purpose of this work was to determine the concentration of two sterols and their glucosides among various plant organs and tissues of the Guam

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cycad. A second objective was to determine if tissue age influenced tissue concentration of various organs.

## 2. Materials and methods

Quantifying physiological variables of perennial plants requires an understanding of relevant growth and ontogenetic characteristics of the model species. Mature individuals of *Cycas* species typically display a discontinuous primary growth habit (Norstog and Nicholls, 1997). Periods of rapid expansion of leaves or reproductive structures occur until mature size is attained, and are separated by extended periods during which no apparent primary growth occurs. The timing of these growth pulses and the age of the tissue following the flush may influence physiological responses (e.g. Marler, 2007). Secondary growth of *Cycas* pachycaulis stems is also unique. Vascular tissue is located in cylinders between parenchymatous pith and cortex tissue, and distinct cylinders are added from the stem base as plants increase in bulk and age (Fisher et al., 2009). At any given height on the stem, therefore, the inner cylinder is the oldest and the outer cylinder is the youngest.

Plants were intensively sampled on 19 December 2003 to quantify variation in sterols across three age classes of two types of foliage tissue, two age classes of seed and sporophyll tissue, two age classes of vascular cylinder tissue and three other types of stem tissue, one type of root tissue, and pollen. In order to achieve this goal, we marked a large set of reproductive female *C. micronesica* trees in north-west Guam during a synchronized reproductive event in October 2002. Each of the marked plants was visited monthly to record phenological events in preparation for eventual seed harvests. By the time we harvested tissue, we were able to identify 6 individuals from among the original group of marked trees that exhibited fully synchronized phenology. This time span included two synchronized reproductive flushes and three synchronized vegetative flushes.

**Leaf tissue:** Leaflet tissue was collected from each of the six replicate plants by sampling four leaves for each of the 2-, 6-, or 11-month-age classes positioned on N, S, E, W orientations from stem apex. Leaflets were collected from each leaf from tip, median, and base positions on the rachis. Thus, the samples for each replicate plant within each age class were comprised of a composite of 12 leaflets: three sampling positions among four leaves. We also collected rachis tissue from the same age classes, with each sample comprised of tissue collected from the tip, median, and base sections of the rachis.

**Reproductive tissue:** Seeds and sporophyll tissues were collected from each of the N, S, E, W directions for one composite sample for each of the 8 or 14-month-age classes. Tissue from the tip, median, and base of each of the four sporophylls was combined into a composite sample for each replicate plant. Seeds are able to germinate following harvest as young as 12 months in age. However, seeds were harvested for historical flour production only when brown mature, which typically occurs any time from 17 to 21 months in age (Marler et al., 2005a).

**Stem tissue:** We harvested four cores from each plant by boring 1-cm cores positioned on the N, S, E, W orientations of the stem surface. The plants were  $225.2 \pm 3.3$  cm in height, and we positioned the cores at 140–150 cm height. Each core extended into the stem until 1 cm of pith tissue was evident. The four cores were separated into five tissue categories: outer cortex, inner cortex, the outermost (youngest) vascular cylinder, the innermost (oldest) vascular cylinder, and pith. The cortex tissue was  $26.1 \pm 0.9$  mm wide, vascular tissue was  $25.7 \pm 1.3$  mm wide, and pith exhibited a diameter of  $69.8 \pm 3.6$  mm. Tissue from the four cores was combined into one sample for each tissue category for each replicate plant.

**Root tissue:** We had one root category comprised of coralloid roots. These were harvested from each of the N, S, E, W orientations from the stem base and combined into one sample for each replicate plant.

**Pollen tissue:** For comparative purposes only, we added another tissue category of pollen. Since cycad plants are dioecious, this tissue category could not come from the same female plants that supplied the other tissue categories. Therefore, we harvested mature cones from six male plants in the same habitat in December 2003. Pollen was collected by placing each cone on newspaper until pollen had dehisced. These data are presented but not included in the ANOVA.

These methods employing a single day of harvest from a set of plants with uniform developmental history reduced experimental artifacts due to plant-to-plant variation, disparity of timing of phenological events, and disparity among variables like season or geography. These sources of variation may influence phenotypic expression of these secondary compounds (see Marler et al., 2005a, 2007).

All samples were frozen at  $-40$  °C, lyophilized at the University of Guam, then stored at  $-80$  °C until we had time to conduct the assays. Reversed-phase HPLC analysis was conducted with an Agilent HP1050 HPLC. Details of analytical procedures were previously described (Marler et al., 2005b). The response variables were concentration of the free sterols stigmasterol (SS) and  $\beta$ -sitosterol (BSS) and the derived steryl glucosides stigmasterol  $\beta$ -D-glucoside (SG) and  $\beta$ -sitosterol  $\beta$ -D-glucoside (BSSG).

Our statistical analysis began by looking at the influence of age on sterol concentration with the leaf, sporophyll, and seed tissue categories. We used PROC GLIMMIX procedure (SAS software version 9.13) to perform repeated measures ANOVA and adjust for the correlation between the tissue category. The first analysis was a factorial with three age categories and two tissue types for leaf tissue. Then seeds, sporophyll, cortex, and vascular tissue data were analyzed as a one-way ANOVA with two classes of age (seeds, sporophylls) or radial sections (cortex, vascular tissue). When age or spatial categories were not significant in each of these initial ANOVAs, those tissue types were included in the final ANOVA which compared all tissue types except pollen. The individual plants were used as experimental units and the following SAS GLIMMIX syntax (random \_residual\_/subject=plantid type=ar(1);) correctly assigned error DF and performed appropriate ANOVA. Mean comparisons were performed using LSD method and the results of the mean comparisons were presented using the alphabet notation in Table 2.

## 3. Results

### 3.1. Sterols within tissue categories

The interaction between age and tissue type was not significant within leaves for any of the compounds. The main factor age was also not significant for leaf tissue, indicating the concentration of sterols and steryl glucosides did not differ among leaflet or rachis

**Table 1**

The influence of age on concentration (mg/g dry weight) of stigmasterol (SS),  $\beta$ -sitosterol (BSS), stigmasterol  $\beta$ -D-glucoside (SG), and  $\beta$ -sitosterol  $\beta$ -D-glucoside (BSSG) in *Cycas micronesica* seeds. SE shown in parentheses.  $N=6$ .

Age	Compound			
	SS	BSS	SG	BSSG
8 month	0.667 (0.062)	0.625 (0.049)	0.790 (0.051)	0.722 (0.025)
14 month	0.413 (0.025)	0.378 (0.019)	0.539 (0.036)	0.513 (0.058)
Significance	$P \leq 0.0035$	$P \leq 0.0009$	$P \leq 0.0025$	$P \leq 0.0083$

**Table 2**

The influence of tissue type on concentration (mg/g dry weight) of stigmasterol (SS),  $\beta$ -sitosterol (BSS), stigmasterol  $\beta$ -D-glucoside (SG), and  $\beta$ -sitosterol  $\beta$ -D-glucoside (BSSG) in *Cycas micronesica* plants. ANOVA was performed at the plant level (N) using SAS GLIMMIX procedure. SE shown in parentheses. Means within each column followed by the same letter are not significantly different using LSD.

Tissue category	Compound			
	SS	BSS	SG	BSSG
Root <sup>a</sup>	0.867A (0.081)	1.381A (0.112)	1.425B (0.251)	0.452C (0.253)
Stem cortex <sup>b</sup>	0.086C (0.057)	0.237C (0.088)	0.331D (0.208)	0.120C (0.208)
Stem vascular tissue <sup>b</sup>	0.100C (0.057)	0.338C (0.088)	0.499D (0.206)	0.282C (0.207)
Stem pith <sup>b</sup>	0.070C (0.081)	0.228C (0.110)	0.284D (0.243)	0.136C (0.247)
Leaf rachis <sup>c</sup>	0.205C (0.047)	0.411C (0.076)	1.040BC (0.183)	0.310C (0.182)
Leaf leaflet <sup>c</sup>	0.347B (0.047)	0.823B (0.076)	2.994A (0.184)	2.221A (0.182)
Sporophyll <sup>d</sup>	0.225BC (0.057)	0.600B (0.088)	1.006BC (0.203)	1.289B (0.204)
Significance	$P \leq 0.0001$	$P \leq 0.0001$	$P \leq 0.0001$	$P \leq 0.0001$
Pollen <sup>e</sup>	0.591 (0.003)	0.300 (0.016)	0.864 (0.764)	Not detected

<sup>a</sup> N=6, 4 samples per N.

<sup>b</sup> Two sampling zones were NS, and were combined for analysis. N=12, 4 samples per N.

<sup>c</sup> 2, 6, or 11 month age categories were NS, and were combined for analysis. N=18, 12 samples per N.

<sup>d</sup> 8 or 14 month age categories were NS, and were combined. N=12, 12 samples per N, 12 samples per N.

<sup>e</sup> Pollen from distinct plants and not included in ANOVA. N=6.

tissue for 2, 6, or 11-month-old leaves. Concentration of all four compounds differed between leaflet and rachis tissue, therefore these two leaf tissue categories were added to the final ANOVA. Age of sporophyll tissue and radial sections of cortex and vascular tissue did not influence concentration of any compound, so these tissue categories were also added for the final ANOVA. Age influenced concentration of all four compounds within seed tissue. Concentration of all four compounds of 8-month-old seeds exceeded that of 14-month-old seeds (Table 1).

### 3.2. Sterols among tissue categories

The comparison of organ differences included every tissue category except seeds (because the two age categories differed and could not be combined) and pollen (because pollen was harvested from a distinct set of plants). Differences were highly significant for all four compounds (Table 2). Overarching trends in the sterol profile are not evident, as each tissue category exhibited a distinct concentration and ranking of the four compounds. However, several specific points are noteworthy. Coralloid roots contained the greatest concentration of both free sterols. Photosynthetic leaflet tissue contained the greatest concentration of both sterol glucosides. Concentration of all four compounds was less in the structural leaf rachis than in the functional leaflets. Stem vascular tissue contained greater concentration of all four compounds than stem cortex or stem pith tissue. Concentrations of these compounds within the three stem tissue categories were low compared to other organs. Reproductive sporophyll tissue on which seeds are borne contained free sterols in the general range of that found in seeds, but greater concentration of sterol glucosides than seeds.

We added pollen for comparative purposes because inhalation of airborne pollen is one proposed unstudied avenue of exposure to cycad toxins (Kurland, personal communication). Concentration of SS, BSS, and SG in pollen was within the range of the other tissue categories. However, BSSG was absent from the pollen samples.

### 3.3. Sterol profile relations

We calculated two derived variables to more fully understand the relationships among these metabolites. First, the relationship between the pool of free versus glycosylated sterols was defined as  $(SS + BSS)/(SG + BSSG)$ . This ratio exhibited more similarity among tissue categories within each organ than did the individual

compounds (Fig. 1). The two seed, three stem, and two leaf tissue categories each exhibited similar values. Root and pollen tissue contained more free sterols than sterol glucosides ( $<1$ ), and the remainder of the tissue categories contained more glucosides than free sterols (Fig. 1). Our second derived variable was the relationship between the  $\beta$ -sitosterol compounds versus stigmasterol compounds throughout the plant parts. We defined this variable as  $(BSS + BSSG)/(SS + SG)$ . The majority of our tissue categories exhibited values close to 1, indicating the total pool was fairly evenly distributed between these two sterols. The absence of BSSG in pollen caused this tissue to exhibit the lowest values for  $\beta$ -sitosterol/stigmasterol.

## 4. Discussion

### 4.1. Organ age effect

Our methods allowed the designation of accurate age for the sporophyll, seed, and leaf harvests because we recorded the emergence dates for each flush of primary growth that led to the addition of these sampled organs. Additionally, the initial and innermost vascular cylinder of *Cycas* stems is continuous from the root zone through the stem apex (Stevenson, 1980; Terrazas, 1991). Successive concentric vascular cylinders are added from the stem base as radial growth progresses. Therefore, at any specified height of the pachycaulis *Cycas* stem, the innermost vascular cylinder is the oldest, and the outermost cylinder is the youngest.

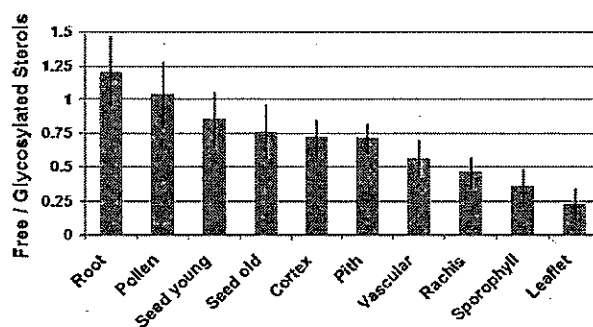


Fig. 1. The general relationship of free sterols versus sterol glucosides as defined by  $(\text{stigmasterol} + \beta\text{-sitosterol})/(\text{stigmasterol } \beta\text{-D-glucoside} + \beta\text{-sitosterol } \beta\text{-D-glucoside})$  for various *Cycas micronesica* tissue categories. Bars are mean  $\pm$  SE. N for each tissue as reported in Tables 1 and 2.

We have now confirmed with three distinct studies that concentration of the sterols and steryl glucosides in *C. micronesica* seeds decline in concentration with age. We used a robust sampling of discrete age categories (Marler et al., 2005b, Table 1) or a continuous sampling over 30+ months of seed development (Marler et al., 2006) in studies that spanned several years. Therefore, we are confident that these seed sterol changes can be attributed to ontogeny (genetically programmed developmental changes).

In contrast to seeds, age did not influence concentration of the free or glycosylated sterols in leaves within the range of 2–11 months or sporophylls from 8 to 14 months. Similarly, concentration of these compounds in the oldest vascular cylinder did not differ from that in the youngest vascular cylinder. Therefore, a general decline in sterol concentration with tissue age is not a universal trait among all *C. micronesica* organs, rather it appears to be a trait that is unique to seeds.

Several reports include the influence of organ age on concentration of secondary compounds in other cycad species, although their sample size renders the results ambiguous. Cycasin concentration of young leaves exceeded that of older leaves for *Zamia* (Rothschild et al., 1986) and *Encephalartos hopei* (Yagi, 2004). Yagi et al. (1983) reported *Cycas revoluta* leaf cycasin concentration increased until about 2 months, then gradually declined with age. Macrozamin concentration in the same leaves increased until about 1 month, but declined to the point it could not be detected after about 2 months. Banack and Cox (2003) reported greater BMAA concentration in immature male cone tissue than in mature cone tissue, but glutamic acid was similar for both age categories.

#### 4.2. Sterol profile

When comparing the ratio free/glycosylated sterol among organs (Fig. 1), the two extreme values were from tissue categories with unique plant function. The function of coralloid roots at our current level of understanding is to attract and house cyanobacteria, supply the symbiont with plant-derived compounds, and acquire nitrogenous and possibly other compounds from the symbiont. These roots exhibited the greatest concentration of free sterols relative to glycosylated sterols. Free sterols exhibit structural, regulatory, and gene-expression roles in membrane relations (He et al., 2003; Schaller, 2003). Therefore, the free sterols may fulfill a unique role in membrane relations within this exceptional structure where plant and cyanobacteria tissues intimately co-exist.

Leaflet tissue also has a unique role from among our tissue categories, in that it is the main source of photoassimilates for primary metabolism. Leaflets exhibited the greatest concentration of steryl glucoside concentration relative to free sterols (Fig. 1). Steryl glucosides are carriers or donors of substrates for biosynthesis of structural or functional end-products (Cantatore et al., 2000; Peng et al., 2002). Steryl glucosides are also important in thermal relations (Murakami-Murofushi et al., 1997) and exposed leaves are among the most sensitive organs to high temperature inhibitions (Nilsen and Orcutt, 1996). Therefore, the glucosides may fulfill a role in the biogenesis of membrane structure and carrier relations or thermo-tolerance mechanisms that are particularly needed in the leaflet photosynthetic tissue.

The ranking of  $\beta$ -sitosterol compounds versus stigmasterol compounds among organs (Fig. 2) exhibited no clear patterns in relation to organ function. In fact, the two extremes in ranking of this ratio were both reproductive tissues, with sporophyll tissue containing more  $\beta$ -sitosterol variants and pollen containing more stigmasterol variants. The extreme ranking for pollen was not entirely due to the absence of BSSG, as the BSS concentration was also relatively low compared to the female tissue categories.

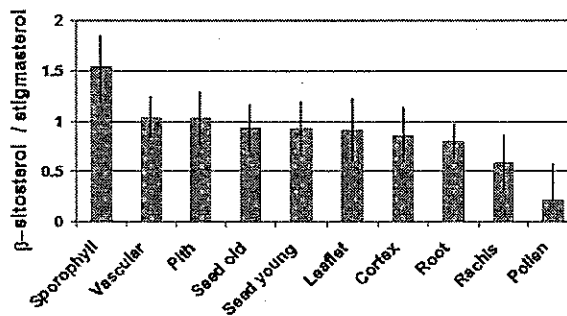


Fig. 2. The general relationship of  $\beta$ -sitosterol versus stigmasterol as defined by  $(\beta$ -sitosterol +  $\beta$ -sitosterol  $\beta$ -D-glucoside)/( $\beta$ -sitosterol +  $\beta$ -sitosterol  $\beta$ -D-glucoside) for various *Cycas micronesica* tissue categories. Bars are mean  $\pm$  SE. *N* for each tissue as reported in Tables 1 and 2.

#### 4.3. Distribution of sterols among tissues in non-cycads

The sterol content of horticultural products that are consumed by humans has been widely studied (e.g. Piironen et al., 2003). In contrast, the profile of sterols among structural tissues has been little studied. Jeong and Lachance (2001) determined the sterols and their distribution among fruits and stems of *Ficus carica*. Stigmasterol and sitosterol content in stem tissue, especially stem pith tissue, greatly exceeded that in reproductive fruit tissue. In contrast, these sterols were present in higher concentration in reproductive seed tissue of *C. micronesica* than in all of our stem categories (Tables 1 and 2). Sterol concentration and accumulation in soybean (*Glycine max*) shoots greatly exceeded that in roots (Fenner et al., 1986). In contrast, concentration of both free sterols in *C. micronesica* coralloid roots exceeded that for all other plant parts, and their glucosides exhibited moderate concentrations (Table 2). Sterol concentration in fenugreek (*Trigonella foenum-graecum*) maternal pod tissue was less than half of that in seed tissue (Brenac and Sauvaire, 1996). In contrast, concentration of free sterols in the maternal sporophyll tissue of *C. micronesica* was comparable to that in seeds, and concentration of steryl glucosides in sporophylls greatly exceeded that in seeds (Tables 1 and 2).

#### 4.4. Distribution of non-sterol metabolites among cycad tissues

Several reports include the concentration of secondary compounds in various cycad tissues. Relevance of their results is difficult to resolve due to limitation of sample size, harvest of tissue categories from distinct plants that disallowed within-replication comparisons, comparison of various organs from plants in distinct geographic regions rather than from plants in the same location, and vague descriptions of sampling and reporting methods. Despite these limitations, we report their findings.

Yagi (2004) compared the seed and leaf content of the azoxyglycosides cycasin and macrozamin in *Encephalartos villosus*. Both compounds were more abundant in seed tissue than in leaf tissue. Whether the concentration was based on fresh or dry tissue basis was not reported. Cycasin concentration was greatest in leaves and similar for seeds and pith of *C. revoluta* (Yagi and Tadera, 1987). Macrozamin was absent from seeds and present in low concentration in leaves and pith tissue. Unfortunately, age of the tissue which may have affected results was not reported, so unambiguous assignment of these results to organ differences is inappropriate.

Charlton et al. (1992) compared 2-amino-3-(methylamino)-propanoic acid (BMAA) concentration in seeds and leaves of five *Bowenia*, *Cycas*, and *Dioon* species. With the exception of one *Cycas* species, the leaves of all species had greater tissue concentration on a fresh weight basis than did the seed tissue. Banack and Cox

(2003) reported the opposite for *C. micronesica*, with BMAA concentration of seed tissue greatly exceeding that of leaf tissue. Further, male cones and outer integument of seeds had the greatest concentration of BMAA from among many root, stem, and reproductive structures. In contrast, glutamic acid was greatest in stem cortex tissue. These authors also failed to report if results were based on fresh or dry weight of tissue.

#### 4.5. Cycad phytosterols in the human diet

Phytosterol variants elicit neurodegenerative outcomes in various mammalian models (Kim et al., 2008; Shaw et al., 2007; Tabata et al., 2008a). Our most recent work has revealed that synthetic SG fed to mice recapitulates many of the neurodegenerative features we have previously observed after feeding mice washed cycad flour (Tabata et al., 2008b) and cycad flour fed to rats appeared to generate a frank parkinsonism phenotype with no motor neuron losses (Shen et al., 2008). These findings of previous studies strongly suggest that metabolic and/or transport variations amongst rodent species determine, in part, the region of the CNS impacted by toxic steryl glucosides.

Such data generated from independent laboratories signify that dietary intake of phytosterols from horticultural products is contraindicated, at least for a fraction of the population. In contrast, purported health benefits of phytosterols in the diet include cholesterol-lowering and anti-cancer properties (Bradford and Awad, 2007; Moreau et al., 2002), and treatment of maladies such as rheumatoid arthritis and allergies (Oomah and Mazza, 1999). These outcomes indicate phytosterols in fruits and vegetables may be useful as functional foods. These conflicting results and their recommendations for the inclusion or exclusion of sterols from the human diet indicate resolution of the health versus risk factors with sterol consumption is urgent. Undoubtedly, further clarification of phytosterol biosynthetic and allocation relations in horticultural plant parts may prove useful for interpretation of hydrolysis and fate of these compounds in mammalian digestive systems.

Various animal herbivores of Guam's cycad plants have been previously reported. These herbivores include several arthropods that feed on male cones, leaves, and stems; feral pigs that consume seeds and toppled stems; fruit bats that consume outer seed integument; and feral deer that consume leaves and young sporophylls (Conry, 1989; Marler and Muniappan, 2006; Wiles, 1987; Wiles et al., 1999). We have also observed toppled stem herbivory by terrestrial crabs and consumption by fresh water shrimp of outer integument on fallen seeds in Guam's waterways (Marler, personal observation). To date, no reports have been made on direct sequestration measurements of any cycad metabolite in the common herbivores of the Guam cycad in habitat. Rothschild et al. (1986) reported an accumulation of cycasin from cycad tissue within the body of *Eumaeus atala* butterflies as they aged from larva to pupa to adult. This butterfly is an obligate consumer of Florida's native *Zamia* cycad, and provides an example that sequestration of some cycad toxins within herbivores of cycads may be a common phenomenon. Human exposure to sequestered cycad toxins is the core of a hypothesis that fruit bats may accumulate BMAA from consumed cycad tissue (Cox and Sacks, 2002). This hypothesis has eluded validation, and Borenstein et al. (2007) report the hypothesis is without merit.

This deficiency in determining the fate of consumed cycad compounds should be corrected because five of the cycad herbivores (crab, deer, fruit bat, pig, shrimp) enter the historical or contemporary human diet on Guam. Understanding the sequestration potential of all plant neurotoxins within these animals may improve our understanding of how Guam's residents have been exposed to the comprehensive suite of environmental

toxins as we attempt to further understand the etiology of these human diseases.

## 5. Conclusions

The sterols stigmasterol and  $\beta$ -sitosterol and their derived glucosides stigmasterol  $\beta$ -D-glucoside and  $\beta$ -sitosterol  $\beta$ -D-glucoside were present throughout *C. micronesica* seed, sporophyll, pollen, leaf, stem, and root tissue. One caveat to our approach is that we have focused on four compounds from among the suite of free and conjugated sterols. This was by design as they are the relevant potential neurotoxins identified in our animal studies, and it is their role in plant function and human disease in which we are interested. A decline in sterol concentrations with seed ontogeny was confirmed, but age of sporophyll, leaf, and stem tissues did not correlate with sterol concentrations. The profile differences among the various tissue categories within these organs may help clarify the physiological role of these compounds in horticultural species.

## Acknowledgments

We thank V. Lee, D. Kwok, and N. Dongol for lab and field technical assistance. We thank George Fernandez, UNR Center for Research Design and Analysis for statistical analyses. Support provided by USDA CSREES (Project No. 2003-05495) to TEM and U.S. Army Medical and Materiel Command (DAMD17-02-1-0678), NSERC Canada, and Scottish Rite Charitable Foundation of Canada to CAS.

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