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Evidence for the Role of Salicylic Acid in Thermoregulation in Sporophylls of Male Cones of Six Cycad Species

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The objective of this research was to provide a framework for the study of induction of thermogenesis by aspirin, salicylic acid, and 2,6-dihydroxybenzoic acid in detached sporophylls from male cones of six cycad species. The thermogenic species were: *Zamia elegantissima* Schutzman, Vovides & R. S. Adams; *Z. furfuracea* L.f.; *Z. pseudomonticola* L.D.Gómez ex D.W.Stev. & Sabato; *Z. skinneri* Warsz; *Encephalartos hildebrandtii* A. Braun & C. D. Bouché*; and Cycas siamensis* Miq. Temperature of single sporophylls was measured for 48 h using thermocouples in the absence and presence of inducers. Sporophylls treated with salicylic acid generated asymmetric temperature waves and square and sinusoidal temperature waves. Also a jump in temperature at a rate of ~ 1 ºC/h was detected in sporophylls treated with salicylic acid. A drop in temperature preceded the temperature jump to a new and higher steady-state level and it stayed unchanged. These unexpected thermal activities suggest cellular controls of mitochondrial thermoregulation.

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Keywords: Aspirin; cycads; 2,6-dihydroxybenzoic acid; salicylic acid; sporophyll.

ABBREVIATIONS

ANT :Adenine Nucleotide Translocator
AOX : Alternative Oxidase
ASA : Aspirin
CV : F_0F_1-ATP synthase
2,6-DHBA : 2,6-dihydroxybenzoic acid
OXPHOS : Oxidative phosphorylation
SA : Salicylic Acid
UCPs : Uncoupling Proteins

1. INTRODUCTION

Endogenous rises in temperature occur in the reproductive tissues of many plant families including aroids (Araceae), palms (Arecaceae), magnolias (Magnoliaceae) and cycads (Cycadales) [1-4]. In aroids, three phenolic compounds salicylic acid (SA), ASA, and 2,6-DHBA are capable of activating the process that leads to temperature rise in premature tissue slices of appendix of *Sauromatum venosum* (Aiton) Kunth and *Arum italicum* Mill inflorescences (Araceae) [5]. Three mitochondrial sources of heat were identified in the *Sauromatum* appendix: CV, ANT, AOX [6]. In thermogenic cycads our understanding is limited to the mitochondrial heat-generating proteins: AOX and UCPs [8-11]. AOX was detected in two forms in thermogenic male sporophylls of *Cycas revoluta Thunb.* [10] and UCP was also detected in several thermogenic male cones of cycads [9]. It has also been shown that SA is present is male cones of several cycad species at concentrations that can induce thermogenesis in a process similar to that detected in the *Sauromatum* appendix [7]. The effect of SA and the other inducers on thermogenesis in male cones of cycads have not been studied so far.

At maturity, the male cones of most cycad species exhibit a thermogenic state of multiple peaks over several days, with usually one high intensity temperature peak around noontime to early evening [2,12,13]. In a previous paper a rhythmic thermogenic activity in male sporophylls attached to the cones was detected in four species using thermocouples [14]. A microcalorimetric study provided more information on thermal energy in single sporophylls detached from their cones from three cycad species [8]. For example, in male sporophylls of *Z. furfuracea* the thermal energy oscillated at a frequency of 1 cycle per 10 min.

The data in the present paper provide evidence that application of the three inducers leads to the generation of square- wave temperature and temperature jump-like profiles in single sporophylls of male cones of *Zamia elegantissima*, *Z. furfuracea*, *Z. pseudomonticola*, *Z. skinneri*, *Encephalartos hildebrandtii, and Cycas siamensis*. The induction was carried out under constant light at ~ 21 ºC in an environmentally controlled chamber. Evidence for thermogenesis in three of the thermogenic species; *Z. furfuracea*, E. *hildebrandtii*, and *Z. skinneri* was provided [2]. *Zamia pseudomonticola* and *Z. elegantissima* are closely related to *Z. fairchildiana,* a known thermogenic species [2,14]. Thermogenesis in *Cycas siamensis* was also demonstrated [14].

2. MATERIALS AND METHODS

2.1 Plant Materials

Zamia elegantissima Schutzman, Vovides & R. S. Adams; *Z. furfuracea* L.f.; *Z. pseudomonticola* L. D. Gómez ex D. W. Stev. & Sabato; *Z. skinneri* Warsz; and *Encephalartos hildebrandtii* A. Braun & C.D.Bouché are member of the Zamiaceae family. *Cycas siamensis* Miq. Is a member of the Cycadaceae family. Male cones of these species were collected at pre-elongation, shortly before the pollen shedding stage at Montgomery Botanical Center (Coral Gables, FL) and Jones Landscape nursery (Davie, FL). Male cones were cut from cycad plants at the base of the peduncle in the afternoon and sent overnight to Seattle, WA. The detached male cones spent ~ 24 h in darkness (traveling time). Next morning, upon arrival, the cone peduncle was cut again and immediately placed in water. The cones were kept at ~ 21 ºC under 12 h light / 12 h dark for ~ 7 d [14].

Corms of the aroid *Sauromatum venosum* were kept at 4°C and the inflorescences were allowed to develop under a 15/9 day/night cycle at room temperature [5]. The inflorescence was cut 3 d prior to thermogenesis and placed in water for ~ 24 h in darkness for a comparison between the cycad species and *S. venosum* of the thermogenic response to inducers after a period of darkness.

2.2 Induction of a Thermogenic Response

Single sporophylls were placed in a 24-well plate (one sporophyll per well). The bottom of the

sporophylls was immersed in an appropriate solution and the top was exposed to air. The solution consisted of 0.5% Tween-20 and 20 mM HEPES buffer, pH 7.0 with or without one of the three inducers: SA, ASA, or 2,6-DHBA at increasing concentrations [5]. Sporophylls were immersed in the solution throughout the experimental period in an environmental chamber (SANYO) under constant light at ~ 21 ºC. Data collection started as soon as the sporophylls were placed in the solution. Each set of experiments was carried out with single sporophylls from one cone. Average size of single sporophylls: 1.5 cm for *Z. elegantissima*; 1 cm for *Z. furfuracea*; 0.8 cm for *Z. pseudomonticola*; and 0.7 cm for *Z. skinneri;* 3 cm for *E. hildebrandtii*; 0.5 cm for *C. siamensis*.

2.3 Temperature Measurement

Temperature was recorded using T-type thermocouples with an accuracy of ± 0.5 ºC (Omega Engineering, Stamford, CT, USA). Thermocouples were inserted into the part of individual sporophyll that was exposed to air. Temperature was recorded every 2 min with a data logger (Omega Engineering). There were 720 consecutive readings per 24 h. The temperature rise was calculated as the difference in temperature between an individual sporophyll and the environmental chamber [5]. An Excel spreadsheet was set up to calculate temperature rates, defined as ∆ºC/h. For example, the first data point was calculated by subtracting temperature value of the first reading (0 min from the beginning of the experiment) from the temperature value of the first h (60 min) and dividing by time. The data were smoothed using Excel's moving average function.

3. RESULTS

3.1 Induction of Spikes and Square Waves of Temperature by Thermogenic Inducers in Sporophylls of Male Cycads

Combination of sinusoidal temperature waves with spikes that resembled a sawtooth wave was detected in single male sporophylls of *C. siamensis* in the absence of any inducer (Fig. 1A1). Four clusters of wave temperature with varying amplitudes were detected at 2:42 h, 12:06 h, 19:06 h, and 26:12 h since the start of the experiment. A similar combination of temperature waves with spikes was detected in *Z. skinneri* sporophylls treated with 70 µM ASA at 38:34 h since the start of the experiment (Fig. 1D1).

A different temperature profile was detected in sporophylls of *E. hildebrandtii* treated with 20 µM SA. The temperature profile resembled a square wave (Fig. 1B1). The temperature jumped ~ 0.5 ºC in ~ 1 h to a higher temperature than the basal temperature at 17:00 h and stayed unchanged for ~ 3 h until 21: 00 h since the start of the experiment. Subsequently, the temperature dropped to a lower level until 24:12 h. The temperature dropped the second time to the basal temperature after 24:12 h. Two additional small square-like profiles were detected at 6:20 h and 34:40 h. A similar square-like temperature wave was detected in *Z. skinneri* sporophylls treated with 1 µM SA also generated (Fig. 1E1). The temperature jumped ~ 0.5 ºC in ~ 0.5 h at 27:08 h and stayed at this level for ~ 3 h. Subsequently, the temperature dropped to a lower level at 29:56 h and stayed unchanged for ~ 5 h. A square temperature wave with some modifications was detected in a sporophyll of *Z. furfuracea* treated with 60 µM SA (Fig. 1F1). The first jump of temperature was at 15:00 h since the start of the experiment. Subsequently, the temperature dropped to the basal temperature. Second temperature jump to a higher temperature level was detected at 22:54 h and stayed unchanged for 2 h. Subsequently, it dropped at 27:00 h. The direction of the two flat maxima is from a low to high maximum. It is in the opposite direction of that of *E. hildebrandtii* (Fig. 1B1) and *Z. skinneri* (Fig. 1E1). Two additional narrow square-like profiles were detected between 27:00 h and 31:00 h. The two flat maxima detected in these three cycad species may represent in tandem generation of heat by one or two heat sources.

Another temperature profile with multiple stair-steps down was detected in a second cone of *E. hildebrandtii* (Fig. 1C1) harvested at a later time than the first cone shown in Fig. 1B1. This temperature profile had a large upper plateau length relative to a lower small plateau length generating downward spikes. The drop in temperature may be associated with OXPHOS activity. This may generate a cooling effect in a thermogenic tissue. These various temperature profiles were not driven by changes in the environmental chamber temperature that stayed unchanged during the experiments (Fig. 1A2, B2, C2, D2, E2, and F2).

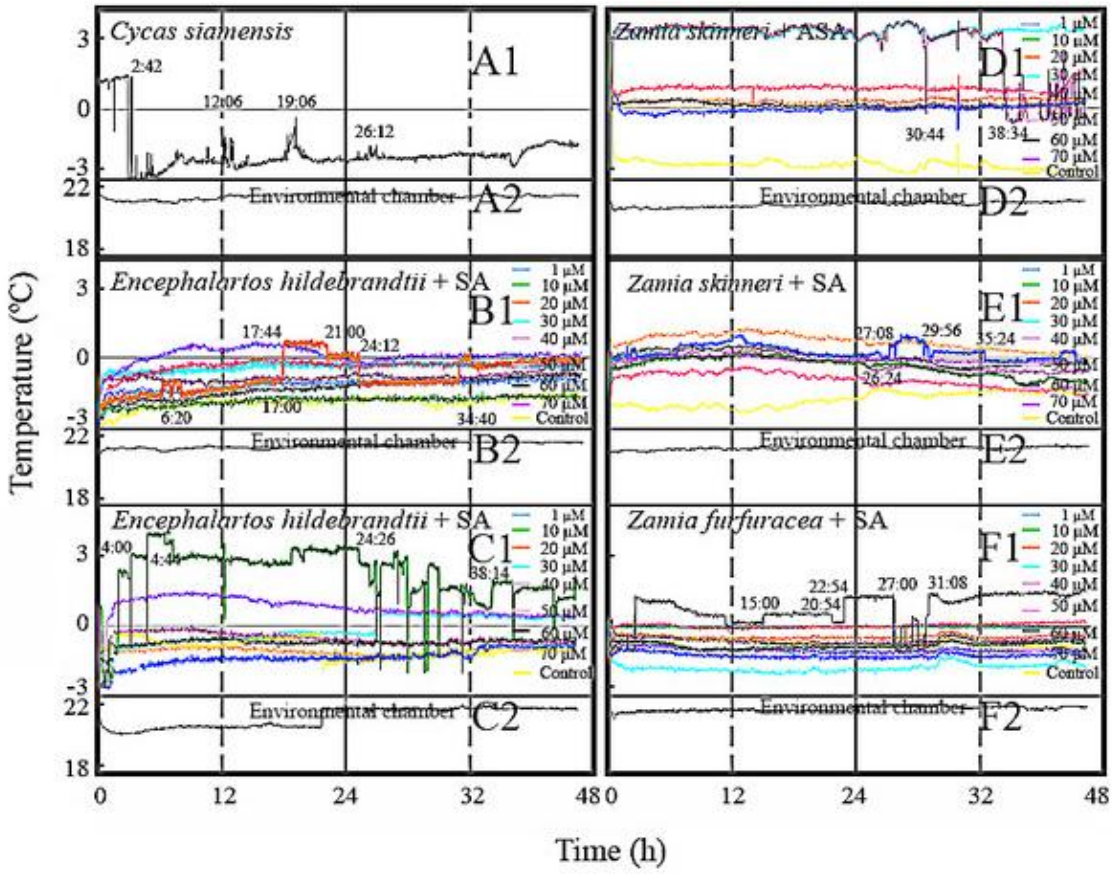

Fig. 1. Temperature profile of single male cone sporophylls of four cycad species

Temperature of single sporophylls was recorded every 2 min. Each line color in one set of experiments (A1, B1, C1, D1, E1, F1) represents the subtraction of the environmental chamber temperature (A2, B2, C2, D2, E2, F2) from the sporophyll temperature treated with SA at a given concentration. Negative temperature values mean that the sporophyll temperature was lower than the temperature in the environmental chamber. The increase in temperature at the first h is temperature equilibration in the environmental chamber. The time of changes in temperature since the start of the experiment is shown on the figure. Experimental data are based on 2-min measurement intervals. Male cones were cut at pre-elongation and elongation stages: C. siamensis in May (A1); E. hildebrandtii in November (B1 and C1); Z. skinneri in July (D1); and Z. furfuracea in August (E1 and F1)

3.2 Induction of Temperature Transition from a Dynamic Wave To a steady-state Plateau by Thermogenic Inducers in Sporophylls of Male Cycads

Salicylic acid induced an asymmetric sinusoidal wave followed by a temperature jump to a plateau (Fig. 2). Temperature rose when single *C. siamensis* sporophylls, at a mature state, were immersed in a buffer solution without any inducer (Fig. 2A1 and B1). The temperature jumped ~ 3 ºC in 3 h (green line in Fig. 2A1) and ~ 4 ºC in 2.5 h (black line in Fig. 2B1) in the absence of an inducer. When ASA (Fig. 2A1) and SA (Fig. 2B1) were added to the buffer solution at ~ 21:00 h since the start of the experiment the temperature profile changed. Treatment with ASA generated a slow decrease in temperature (Fig. 2A1) whereas treatment with 20µM and 60 µM SA generated a jump in temperature from the basal temperature to a higher level (Fig. 2B1). A drop in temperature preceded the jump in temperature seen in Fig. 2A1 and Fig. 2B1. A ~ 0.5 ºC decrease in temperature was detected in sporophylls treated with 20 µM and 40 µM SA at 31:36 h (Fig. 2C1) and another decrease was detected in the presence of 120 µM and 140 µM SA at 8:36 h and 14:26 h, respectively. The decrease in temperature was followed by a temperature jump of ~ 3 ºC in 2 h, at 8:36 h and of ~ 2 ºC in 2 h at 14:26, respectively. This behavior may suggest that the sporophyll tissue is sensitive and

response to changes in cellular temperature. Another temperature jump of ~ 1.5 ºC in ~ 3 h was detected in *Z. furfuracea* sporophylls treated with 10 µM and 50 µM 2,6-DHBA (Fig. 2D1).

In male sporophylls of *Z. elegantissima* the temperature jumped 2 ºC in 4 h *in the* presence of 20 µM SA (Fig. 2E1) and 50 µM SA (Fig. 2F1). A drop in temperature was detected prior to the transition to the new steady-state level. In this species a slight increase prior to the drop in temperature was also detected in sporophylls treated with 20 µM (Fig. 2E1) and 50 µM SA (Fig. 2F1). A moderate jump of ~ 0.5 ºC in ~ 2 h to a new steady-state temperature was detected in *Z. pseudomonticola* sporophylls treated with 1 µM, 10 µM, 30 µM, and 60 µM SA (Fig. 2G1).

The appendix of *Sauromatum* inflorescence were subjected to same environmental conditions as the cycads. When it was cut off and kept in the darkness for 24 h prior to the experiment, temperature did not rise in the appendix tissue slices treated with SA suggesting that cutting has a negative effect on SA induction in this species. ASA also did not induce temperature rise in the appendix tissue slices under these conditions (data not shown). However, 2,6-DHBA induced a modest temperature rise under these conditions (data not shown). Not all the sporophylls generated heat suggesting that either the heat sources are sensitive to cutting or that the sporophylls on one cone are not at the same stage of development. This jump in temperature was not driven by changes in the environmental chamber temperature that stayed unchanged during the experiments (Fig. 2A2, B2, C2, D2, E2, F2, G2, and H2).

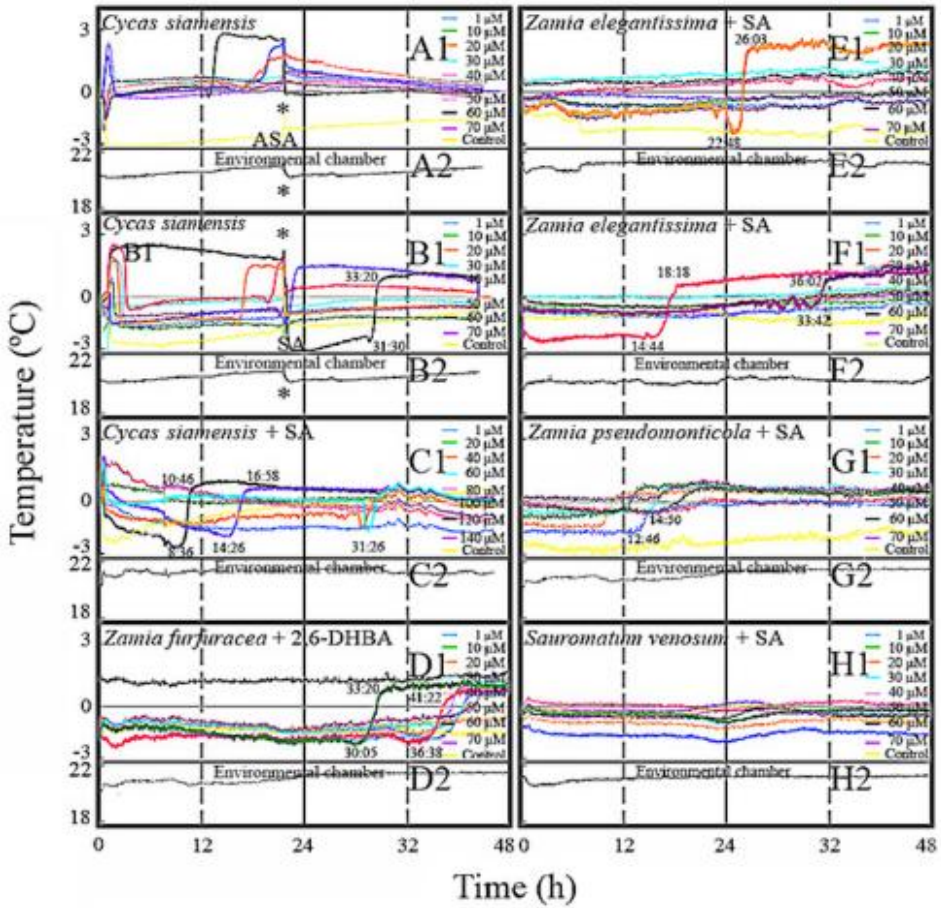

Fig. 2. Steady-state levels of temperature in single male cone sporophylls of four cycad species

Legend as described in Fig. 1. Star symbol depicts the change in temperature the environmental chamber because of door opening when an inducer was added to a solution. Male cones were cut at pre-elongation and elongation stages: C. siamensis in March (A1, B1, C1); Z. furfuracea in August (D1); Z. elegantissima in October (E1, F1); Z. pseudomonticola in November (G1); and S. venosum inflorescence 24 h prior the experiment (H1)

4. DISCUSSION

4.1 Salicylic Acid Induces a Square Wave of Temperature

In intact male cones of *C*. *miqueliana* as well as the tissue slices of *S. guttatum* and *A. italicum* appendices temperature rise has a sinusoidal profile [5,14]. A microcalorimetric analysis of heat generated by single male sporophylls of *Z. furfuracea* showed an oscillatory profile with different amplitudes at a frequency of 1 cycle per 10 min [8]. It is possible that the square temperature wave in this species (Fig. 1 F1) and in other species (Fig. 1 B1 and E1) was generated by one or two heat sources. Switching between two steady-state levels requires feedback loops. Square-wave oscillation may have either one positive feedback or one negative feedback loop, or mixtures of positive and negative feedback loops [15,16]. Square temperature waves and temperature jumps were not detected in thermogenic sporophylls attached to the central cone axis.

4.2 Salicylic Acid and 2,6-DHBA Induce Temperature Jump-Like Profiles

In the present study the rate of temperature jump of single sporophylls was ~ 1 ºC/h (Fig. 2). The highest temperature rate detected in *C*. *miqueliana* sporophylls attached to the central cone axis was ~ 0.09 and the lowest ~ 0.01 ºC/h [14] In the *S. venosum* appendix temperature rate was ~ 0.04 ºC/h [14]. This high jump in temperature appeared in the transition from a wave to a plateau was not detected in the appendix of the *Sauromatum* or *Arum* inflorescences so far.

In vitro studies showed that AOX activity increased upon reduction of its subunit disulfide bridge [17] and by α-keto acids [18]. However, some studies suggest that these two activation mechanisms may not play an important role in regulation of AOX activity *in vivo* [19]. Significant engagement of AOX occurred when the Q-pool reduction level reaches 35–40% and increased disproportionately on further reduction [20]. It is unclear how or whether these findings are involved in the transition from a dynamic sinusoidal wave to a plateau steady-state.

In a previous study, at least 3 successive temperature peaks with different amplitudes were detected in male sporophylls attached to the central cone axis of *Ceratozamia miqueliana* and *Z*. *fairchildiana* using T-type thermocouples [14]. The thermogenic activity of the major peaks of both species continued for 6-12 h. Single sporophylls of both species displayed ~ 3 waves with different temperature amplitudes with a frequency of about 1 cycle per 10 min when monitored every 30 s for ~ 1 h in a microcalorimeter [8]. Waves with such frequency are not resolvable using thermocouples and can appear as spikes. It has been shown that mitochondrial membrane potential and different mitochondrial activities can oscillate and generate spikes [21-24].

4.3 Is There a Cyclooxygenase Activity in Cycad Sporophylls?

A recent study demonstrated that different nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) suppress temperature rise induced by salicylates (ASA, SA, and 2,6-DHBA) in the appendix of the *Sauromatum* inflorescence [25]. NSAIDs (except for ASA) are reversible inhibitors of cyclooxygenase isomers (COX-1 and COX-2). They inhibit the conversion of arachidonic acid to prostaglandins that have a role in fever [26]. A cyclooxygenase (COX) enzyme, unrelated to mammalian COX isoenzymes (COX-1 and COX-2) was found in a red alga [27]. In plants, there are only a few reports on prostaglandin synthesis [28,29]. It is possible that the drop in temperature detected in sporophylls is the result of COX activity. Further studies ds are needed to determine the existence of a putative COX in cycads and its association with changes in mitochondrial thermogenesis.

4.4 Temperature and Circadian Clock

Temperature changes affect circadian clocks in many organisms including plants [30]. The amount of phase shift of a clock depends on the amplitude of the temperature change and its duration. Switching between a high and low temperature may lead to a stable phase and to a maximal amplitude of the circadian rhythm. Flowering in *Arabidopsis thaliana* is affected by temperature at a particular time of the day [31]. In *Neurospora crassa*, temperature shifts correspond to shifts in time of a circadian clock without immediate synthesis or turnover of components [32]. Asymmetry of square-like and sinusoidal waves in male cones of cycads may suggest temperature shifts that represent a clock time shift as is the case in *Neurospora*.

5. CONCLUSION

The differences in shape of the square and the sinusoidal waves seen in the male sporophylls treated with inducers may reflect differences in mitochondrial activity involved in a circadian clock mechanism [33]. Future efforts using mitochondrial respiration inhibitors and computational modeling should shed light on how the differences in waves relate to thermogenesis in cycads. Modeling studies could generate similar wave shape to provide information on the mechanism of induction by SA, ASA, and 2,6-DHBA. Study of thermogenesis in cycads is hindered by the scarcity of plant material and lack of sensitive instruments such as tiny thermometers embedded in living cells to detect short duration temperature changes [34].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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