Evidence for the role of salicylic acidin thermoregulation in sporophylls of male cones of sixcycad species

Abstract

The present study describespreliminary experiments that provide a framework for the study of induction of thermogenesisby aspirin, salicylic acid, and 2,6-dihydroxybenzoic acid in cycads. Temperature of single sporophylls of six cycad specieswas measured for 48 h using thermocouples in the absence and presence of inducers. The 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, *andCycas siamensis* Miq. Temperature pattern was used to distinguish between different waves. Male sporophyll tissue of six species treated with salicylic acid generatedwaves, asymmetric square and sinusoidal waves. The sporophylls also generated a jump in temperature at a rate of ~ 1ºC/hwhen treated with salicylic acid.A drop in temperature preceded the temperature jump to a new and higher steady state and stayed unchanged for at least 48 h after induction. These unexpected thermal activities suggest cellular controls of mitochondrial thermoregulation.

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Key words:Aspirin, cycads, 2,6-dihydroxybenzoic acid, salicylic acid, sporophyll.

Introduction

Endogenous rises in temperatures occur in the reproductive tissues of diverse plant groups including aroids (Araceae), palms (Arecaceae), magnolias (Magnoliaceae) and cycads (Cycadales; Meeuse 1975, Tang 1987, Ervik and Barfod 1999, Seymour *et al.* 2010). In aroids, three phenolic compounds salicylic acid (SA), aspirin (ASA), and 2,6-dihydroxybenzoic acid (2,6-DHBA) are capable of activating the process that leads to temperature rise in premature tissue slices of appendix of *Sauromatumvenosum*(Aiton) Kunthand *Arum italicum*Mill inflorescences (Araceae; Skubatz 2014). Three mitochondrial sources of heat were identified in the *Sauromatum* appendix: F_0F_1-ATP synthase (CV), adenine nucleotide translocator (ANT), and alternative oxidase (AOX). It has been shown that SA is present is male cones of several cycad species at concentration that could induce thermogenesis (Raskin *et al.* 1990). The effect of SA and other inducers on thermogenesis and the involvement of the mitochondrial heat sources in thermogenesis in male cones of cycads have not been studied so far. Our current understanding of thermogenesis in cycads is limited to the mitochondrialheat-generating proteins;AOX and uncouple protein (UCP; Skubatz *et al.* 1993,Zhu *et al.* 2011,Ito-Inaba*et al.* 2019, Johnson 2019).AOX is present in two forms in male sporophylls of thermogenic *Cycas revoluta*Thunb. (Ito-Inaba et al. 2019) andUCPisalso likely to be present in cycads (Zhu *et al.* 2011).

At maturity, the male conesof most cycad species exhibit a thermogenic state of multiple peaksover several days, with usually one high intensity temperature peakaround noontime to early evening(Tang 1987, Roemer *et al.* 2008, Suinyu *et al.* 2013).In a previous paper a rhythmic thermogenic activityin male sporophylls attachedto the cones was detected infour speciesusing thermocouples (Skubatz *et al.* 2019). Amicrocalorimetric studyprovided more information onthermal energyinsingle sporophylls detached from their cones from three cycad species(Skubatz *et al.* 1993). For example, in male sporophyllsof *Z. furfuracea*the thermal energy oscillated at a frequency of 1 cycle per 10 min.

The data in the present paper provide evidence that the three inducersinduced squarewaves andtemperature jump-like patterns in single sporophylls of known thermogenic male cones of *Zamia elegantissima*, *Z. furfuracea*,*Z. pseudomonticola*,*Z.skinneri*,*Encephalartos hildebrandtii*, *and**Cycas siamensis*under constant light at ~21ºC in an environmentally controlled chamber. Tang (1987) provided evidence for thermogenesis in three of the thermogenic species; Z. furfuracea, E. hildebrandtii, and Z. skinneri. Thermogenesis in *Cycas siamensis*was demonstrated by Skubatz *et al.* 2019,*Zamia pseudomonticola*and *Z. elegantissima*are closely related to *Z. fairchildiana*, a known thermogenic species (Tang 1987, Skubatz *et al.* 2019).

Materials and Methods.

PLANT MATERIALS.

*Zamia elegantissima*Schutzman, Vovides & R.S.Adams, *Z. furfuracea*,*Z*.L.f,*Z.pseudomonticola*L.D.Gómez ex D.W.Stev.& Sabato, *Z.skinneri*Warsz,and *Encephalartos hildebrandtii*A.Braun & C.D.Bouchéare member of the Zamiaceae family and *Cycas siamensis* Miq.of the Cycadaceae family. Male cones of these species were collected at pre-elongation, shortly before the pollen shedding stagesat the cycad collection at Montgomery Botanical Center(Coral Gables, FL) and Jones Landscape nursery (Davie, FL).Several 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 coneswere kept at ~21ºC under 12 h light/ 12 h dark for ~ 7 d(Skubatz 2019).

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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(Skubatz 2019). The inflorescence was cut 3 d prior to heat-production and placed in water for ~24 h in darknessfor a comparison between the cycad species and *S. venosum*of the thermogenic response to inducers.

INDUCTION OF A THERMOGENIC RESPONSE.

Upon arrival, single sporophylls were placed in 24-well plates (one sporophyll per well). The bottom of the sporophylls wasimmersed in an appropriate solution and the top was exposed to oxygen in the air.The solution consisted of 0.5% Tween-20 and 20 mM

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HEPES buffer, pH 7.0 with or without one of the three inducersat increasing concentrations; SA, ASA, or 2,6-DHBA(Skubatz 2014). Sporophylls were immersed in the solution throughout the experimental period in an environmental chamber (SANYO) under constant light regime at ~ 21ºC. 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 sizesof 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*.

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TEMPERATURE MEASUREMENT.

Temperature was recorded using T-type thermocouples with an accuracy of± 0.5ºC(Omega Engineering, Stamford, CT, USA). Thermocouples were inserted into the exposed part of individual sporophyll that got oxygen directly from the 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 (Skubatz 2014). An Excel spreadsheet was set up to calculate temperature rates, defined as ΔºC/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.

Results.

Salicylic acid induces spikes and square waves of temperaturein sporophylls of male cycads.

Clusters of square wave temperature (a cycle with a steepincrease in amplitude and a steepdecrease in amplitude) were observed in a single sporophyll of *C. siamensis* in the absence of any inducer (Fig. 1A1). Four clusters of square wave temperature were detected at 2:42 h, 12:06 h, 19:06 h, and 26:12 h with varying amplitudes. In sporophylls of *E. hildebrandtii*treated with 20 μMSAthe temperature pattern also followed a square wave (Fig. 1B1). The temperature jumped ~ 0.5ºC in ~1 h to a higher temperature than the basal temperature at 17:00h 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 (thermogenic state). The temperature dropped the second time to the basal temperature after 24:12h. The two flat maxima may representone complete cyclewith2stair-steps down and its direction was from a high to low maximum.Two additionalsmall square-like patternswith complete cycleswere observed at 6:20 h and 34:40 h witha 0stair-step. Each square wavemay arise from a sum of spikes with similar amplitude in the presence of SA.The 2 stair-steps down maybea result of drops in temperature amplitude of one cycle.

Male sporophylls of *Z. skinneri* treated with ASA generated an asymmetricsquare-like pattern(the first half cycle is widerthan the second half)with a 2 stair-steepstemperature drop(Fig. 1D1). Thedropswere generated at 30:44 h and 38:34 h. Subsequently, after the second dropspikes in temperature appeared. Thesesteep drops of short duration generated an asymmetric square wave suggesting two complete cycles of heat.Salicylic acidtreated

*Z. skinneri*sporophylls also generated a square-like pattern(Fig. 1E1).The temperature jumped ~ 0.5ºC in ~0.5 h and stayed at this level for ~ 3h.Subsequently, the temperature dropped to a lower level and stayed unchanged for ~ 5 h. As the case of *E. hildebrandtii*,the two flat maxima may represent one complete cycle with 2 stair-steps and its direction was from a high to low maximum.

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A male sporophyll of *Z. furfuracea*(Fig. 1F1) generated a squarewavewith some modifications. The first jump of temperature wasat 15:00 h. Subsequently, the temperature dropped to the basal temperature. Second temperature jump was to a higher temperature levelwas detected at 22:54 h and it stayed unchanged for 2 h.Subsequently,it dropped at 27:00 h.The direction of the two flat maxima isfrom a low to high maximum. It isin the opposite direction of that of *E. hildebrandtii*(Fig. 1B1) and *Z. skinneri* (Fig. 1E1).Two additional narrow square-like patterns were detected between 27:00 h and 31:00 h. Another temperature square-like pattern with 3stair-steps upand multiple stair-stepsdown (more than 5) were observed in another coneof *E. hildebrandtii*(Fig. 1C1).These asymmetric cycles have large upper plateau lengthrelative to the lower small plateau lengthand thus,the downwardspikes can generate a cooling effectin the thermogenicmitochondria. Temperature asymmetry was not driven by changes in the environmental chamber temperature that stayed unchanged during the experiments (Fig. 1A2, B2, C2, D2, E2, and F2).

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Salicylic acid induces temperature transition from an asymmetric wave to a constate plateau.

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Salicylic acidinduced an asymmetric sinusoidalwavefollowed bya temperature jump to a plateau (Fig.2). Temperature rose whensingle *C. siamensis* sporophylls,at the right stage of development, were immersed in a buffer solution without any inducer the (Fig. 2A1 and B1). The temperature jumped~3ºC in 3 h (green line in Fig. 2A1) and ~ 4ºC 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 solutionat ~ 21:00 h the temperature pattern changed. Treatment with ASA generated a slow decrease in temperature(Fig. 2A1) whereas treatment with SA generated a jump in temperature from the basal temperature to a higher level at20 µM and 60 µM, respectively (Fig. 2B1).A drop in temperature preceded the jump in temperaturein (Fig. 2A1) and (Fig. 2B1). A ~ 0.5ºC decrease in temperature was observed in sporophyll tissue treated with 20 µM and 40 µM SA at 31:36 h (Fig. 2C1) and another decrease with120 µM and 140 µM SA at 8:36 h and 14:26 h. The temperature of sporophyll tissue jumpedat 8:36 h ~ 3ºC in 2 hand at 14:26~2ºC in 2 h.This behavior can be attributed to a new form of an asymmetric sinusoidal wavefollowed by an increase and prolonged flat temperature level.

In male sporophylls of *Z. elegantissima*the temperature jumped 2ºC in 4 h(Fig. 2E1and F1). A drop in temperature was also observed prior to the transition to a new steady state level. In this species a slight increase prior to the drop in temperature was also detected in sporophyll tissue treated with 20 µM (Fig. 2E1) and 50 µM SA (Fig. 2F1). A moderate jump to a new steady state temperature was observed in male sporophyll tissue of *Z. pseudomonticola*treated with 1 µM, 10 µM, 30 µM, and 60 µM SA, ~ 0.5ºC in ~2 h(Fig. 2G1) and in male sporophyll tissue of*Z. furfuracea*treated with 10 µM and 50 µM 2,6-

DHBA, ~1.5ºC in ~ 3 h(Fig. 2D1).The drop in temperature may be associated with OXPHOS activity.

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 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 cycad sporophylls generated heat suggesting that either the heat sources are sensitive to cutting or that the sporophyllson 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 (Fig. 2A2, B2, C2, D2, E2, F2, G2, and H2).

Discussion.

Salicylic acid and 2,6-DHBA induce temperature jump-like patterns.

In a previous study, at least 3 successive temperature peaks with different amplitudes were detected in male sporophylls attached to central cone axis of *Ceratozamiamiqueliana* H.Wendl.and*Z.fairchildiana*using T-type thermocouples (Skubatz *et al.* 2019).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 (Skubatz *et al.*

1993).Waveswithsuch frequency are not resolvable usingthermocouples and can appear as spikes.Spike clusterscan form asymmetric square and sinusoidal waves.It has been shown that mitochondrial membrane potential and differentmitochondrial activities can oscillate (Gooch and Packer 1974, Feng *et al.* 2019, Hattori *et al.* 2005, Aon *et al.* 2007).

In the present study the rate of temperature jump of single sporophylls was ~1 ºC/h. In *C. miqueliana* sporophylls attached to central cone axis the higher rate was ~0.09 and the lowest ~0.01 ºC/h (Skubatz *et al.* 2019).In the *S. venosum* appendix temperature rate was ~0.04 ºC/h (Skubatz 2014).This high jump in temperature in the transition from a wave to a plateau seems to be unique to cycads and was not observed in the appendix of the *Sauromatum* or *Arum*inflorescences.

*In vitro*studies showed that AOX activity increased upon reduction of its subunit disulfide bridge (Umbach and Siedow 1993) and by α-keto acids (Umbach and Siedow 1996). However, some studies suggest that these two activation mechanisms may not play an important role in regulation of AOX activity *in vivo* (Millenaar and Lambers 2003). Significant engagement of AOX occurred when the Q-pool reduction level reaches 35–40% and increased disproportionately on further reduction (Dry *et al.* 1989). It is unclear how or whether these findings are involved in the regulation ofthermogenesis.

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 *Arum italicum* appendices temperature rise has a sinusoidal pattern (Skubatz, 2014,Skubatz *et al.* 2019). A microcalorimetric analysis of heat generated by single male sporophylls of *Z. furfuracea*showedan oscillatory pattern with different amplitudes at a frequency of 1 cycle per 10 min (Skubatz *et al.* 1993). It is possible that the square wave in this species and in other male sporophylls of cycads is a series of sine waves with similar frequency added together. It strongly suggests that the square wave seen in sporophylls of *Z. furfuracea* (Fig. 2) can be the sum of sinusoidal waves.Initially the heat maybe absorbed by the sporophyll tissue and the solution but the temperature rose when it reached a critical temperature. Oscillatory sources have either one positive feedback or one negative feedback, or mixtures of positive and negative feedback (Brandman and Meyer 2008,Ananthasubramaniam and Herzel 2014).

The square waves and temperature jumps were not detected in thermogenic activities of sporophylls detached to the central cone axis. This tissue contained very few mitochondria (Ito-Inaba *et al.* 2019). It is conceivable that the cone axis suppressed the formation of a square waves and temperature jumps.

Is there a cyclooxygenase activity in cycads?

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 venosum* inflorescence (Skubatz 2022). 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 (Milton 1998).Acyclooxygenase (COX) enzyme, unrelated to mammalian COX isoenzymes (COX-1 and COX-2) was found in a red alga (Kanamoto *et al.* 2011). In plants, there are only a few reports on prostaglandin synthesis (Ali *et al.* 1990;Groenewald&van der Westhuizen 1997). It is possible that the drop in temperature observed in the cycad sporophylls is the result of COX activity. Further studies in cycads are needed to determine the existence of a putative COX activity and its association with changes in mitochondrial thermogenesis.

Temperature and circadian clocks.

Temperature changes affect circadian clocks in many organisms including plants (Rensing and Ruoff 2002). The amount of phase shift of a clock depends on the amplitude of the temperature change andits 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*(L.) Heynhis affected by temperature and is dependent transcription factors by an unknown mechanism (Thines *et al.* 2014). In *Neurosporacrassa*Shear & B.O. Dodge,temperature shifts correspond to shifts in time of a circadian clock without immediate synthesis or turnover of components(Liu *et al.* 1998). Asymmetry of square-like and sinusoidal waves in cycads may suggest temperature shifts that represent a clock time shift as is the case in *Neurospora*.

Conclusions.

The differences in shape of the square and the sinusoidal wavesseen in the male sporophyll tissue treated with inducers may reflect differences in mitochondrial activity involved in a circadian clock mechanism (Schmitt *et al.* 2018).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 couldgenerate 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 instrumentssuch as tiny thermometers embedded in living cellsto detect short duration temperature changes (Sokolov 2013).

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Figures

Fig. 1. Temperature patternofsingle 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 temperaturetreated 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 experimentis 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 (E1and F1).

Fig.2. Temperature steady state levels 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).

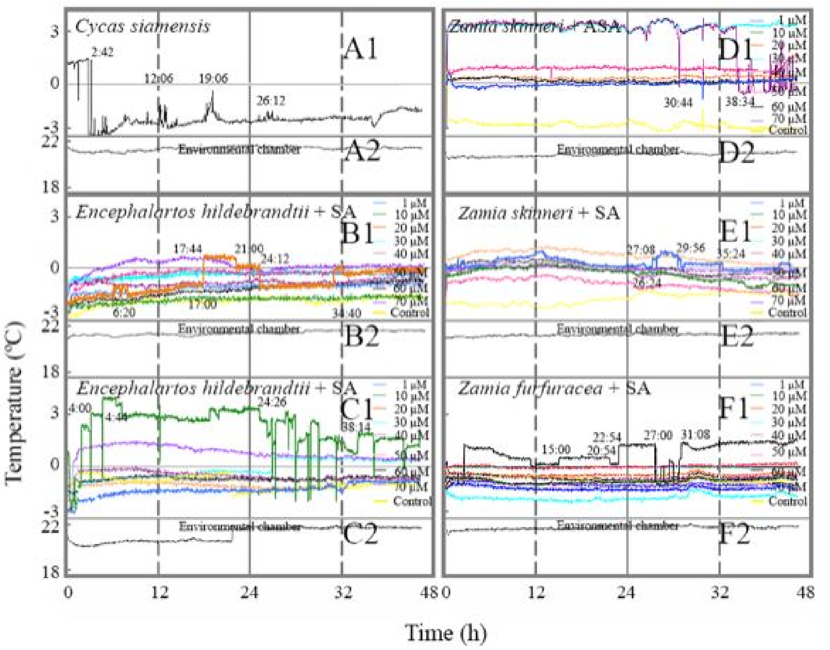

Fig.1 :

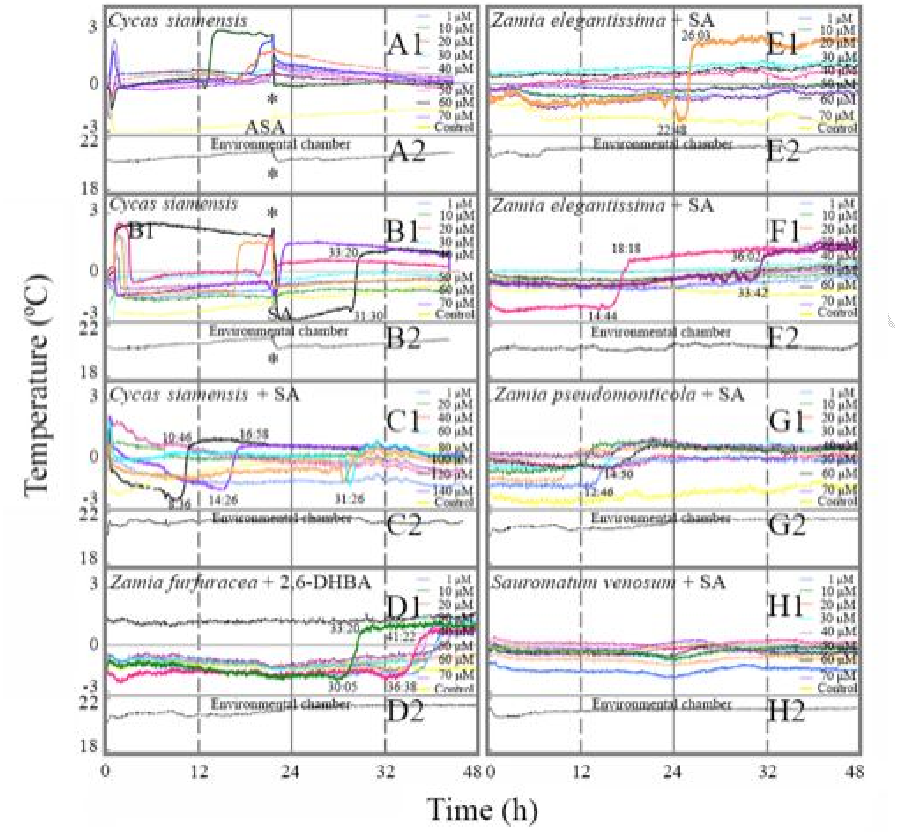
Cycas siamensis
A1
ASA
A2
Environmental chamber
Cycas siamensis
B1
33:20
31:30
SA
B2
Environmental chamber
Cycas siamensis + SA
C1
10:46
16:58
8:36
14:26
31:26
C2
Environmental chamber
Zamia furfuracea + 2,6-DHBA
D1
33:20
41:22
30:05
36:38
D2
Environmental chamber
Zamia elegantissima + SA
E1
26:03
22:48
E2
Environmental chamber
Zamia elegantissima + SA
F1
18:18
36:02
33:42
14:44
F2
Environmental chamber
Zamia pseudomonticola + SA
G1
14:30
12:40
G2
Environmental chamber
Sauromatum venosum + SA
H1
H2
Environmental chamber
1 µM
10 µM
20 µM
30 µM
40 µM
50 µM
60 µM
70 µM
80 µM
100 µM
120 µM
140 µM
Control
Temperature (°C)
Time (h)
3
0
-3
22
18
0
12
24
32
48

Fig. 2 :