

Evidence for the role of salicylic acid in thermoregulation in sporophylls of male cones of six cycad species

Abstract

The present study describes preliminary experiments that provide a framework for the study of induction of thermogenesis by aspirin, salicylic acid, and 2,6-dihydroxybenzoic acid in cycads. Temperature of single sporophylls of six cycad species was 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, and *Cycas siamensis* Miq. Temperature pattern was used to distinguish between different waves. Male sporophyll tissue of six species treated with salicylic acid generated waves, asymmetric square and sinusoidal waves. The sporophylls also generated a jump in temperature at a rate of ~ 1 °C/h when 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.

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 *Sauromatum venosum* (Aiton) Kunth and *Arum italicum* Mill inflorescences (Araceae; Skubatz 2014). Three mitochondrial sources of heat were identified in the *Sauromatum* appendix: F₀F₁-ATP synthase (CV), adenine nucleotide translocator (ANT), and alternative oxidase (AOX). It has been shown that SA is present in 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 mitochondrial heat-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) and UCP is also likely to be present in cycads (Zhu *et al.* 2011).

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 (Tang 1987, Roemer *et al.* 2008, Suinyu *et al.* 2013). In a previous paper a rhythmic thermogenic activity in male sporophylls attached to the cones

was detected in four species using thermocouples (Skubatz *et al.* 2019). A microcalorimetric study provided more information on thermal energy in single sporophylls detached from their cones from three cycad species (Skubatz *et al.* 1993). 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 the three inducers induced square waves and temperature 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 ~ 21 °C 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.Bouché are 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 stages at 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 cones were kept at ~ 21 °C under 12 h light / 12 h dark for ~ 7 d (Skubatz 2019).

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 darkness for 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 was immersed 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

HEPES buffer, pH 7.0 with or without one of the three inducers at 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 ~ 21 °C. 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 sizes 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*.

TEMPERATURE MEASUREMENT.

Temperature was recorded using T-type thermocouples with an accuracy of ± 0.5 °C (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 $\Delta^{\circ}\text{C}/\text{h}$. 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 temperature in sporophylls of male cycads.

Clusters of square wave temperature (a cycle with a steep increase in amplitude and a steep decrease 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 μ M SA the temperature pattern also followed a square wave (Fig. 1B1). The temperature jumped ~ 0.5 $^{\circ}$ C 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 (thermogenic state). The temperature dropped the second time to the basal temperature after 24:12 h. The two flat maxima may represent one complete cycle with 2 stair-steps down and its direction was from a high to low maximum. Two additional small square-like patterns with complete cycles were observed at 6:20 h and 34:40 h with a 0 stair-step. Each square wave may arise from a sum of spikes with similar amplitude in the presence of SA. The 2 stair-steps down maybe a result of drops in temperature amplitude of one cycle.

Male sporophylls of *Z. skinneri* treated with ASA generated an asymmetric square-like pattern (the first half cycle is wider than the second half) with a 2 stair-steps temperature drop (Fig. 1D1). The drops were generated at 30:44 h and 38:34 h. Subsequently, after the second drop spikes in temperature appeared. These steep drops of short duration generated an asymmetric square wave suggesting two complete cycles of heat. Salicylic

acid treated *Z. skinneri* sporophylls also generated a square-like pattern (Fig. 1E1). The temperature jumped ~ 0.5 °C in ~ 0.5 h and stayed at this level for ~ 3 h. 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.

A male sporophyll of *Z. furfuracea* (Fig. 1F1) generated a square wave with some modifications. The first jump of temperature was at 15:00 h. Subsequently, the temperature dropped to the basal temperature. Second temperature jump was to a higher temperature level was 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 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 patterns were detected between 27:00 h and 31:00 h. Another temperature square-like pattern with 3 stair-steps up and multiple stair-steps down (more than 5) were observed in another cone of *E. hildebrandtii* (Fig. 1C1). These asymmetric cycles have large upper plateau length relative to the lower small plateau length and thus, the downward spikes can generate a cooling effect in the thermogenic mitochondria. 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).

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

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 the right stage of development, were immersed in a buffer solution without any inducer the (Fig. 2A1 and B1). The temperature jumped $\sim 3\text{ }^{\circ}\text{C}$ in 3 h (green line in Fig. 2A1) and $\sim 4\text{ }^{\circ}\text{C}$ 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 $\sim 21:00$ 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 at $20\text{ }\mu\text{M}$ and $60\text{ }\mu\text{M}$, respectively (Fig. 2B1). A drop in temperature preceded the jump in temperature in (Fig. 2A1) and (Fig. 2B1). A $\sim 0.5\text{ }^{\circ}\text{C}$ decrease in temperature was observed in sporophyll tissue treated with $20\text{ }\mu\text{M}$ and $40\text{ }\mu\text{M}$ SA at 31:36 h (Fig. 2C1) and another decrease with $120\text{ }\mu\text{M}$ and $140\text{ }\mu\text{M}$ SA at 8:36 h and 14:26 h. The temperature of sporophyll tissue jumped at 8:36 h $\sim 3\text{ }^{\circ}\text{C}$ in 2 h and at 14:26 $\sim 2\text{ }^{\circ}\text{C}$ in 2 h. This behavior can be attributed to a new form of an asymmetric sinusoidal wave followed by an increase and prolonged flat temperature level.

In male sporophylls of *Z. elegantissima* the temperature jumped $2\text{ }^{\circ}\text{C}$ in 4 h (Fig. 2E1 and 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 $20\text{ }\mu\text{M}$ (Fig. 2E1) and $50\text{ }\mu\text{M}$ SA (Fig. 2F1). A moderate jump to a new steady state temperature was observed in male sporophyll tissue of *Z. pseudomonticola* treated with $1\text{ }\mu\text{M}$, $10\text{ }\mu\text{M}$, $30\text{ }\mu\text{M}$, and $60\text{ }\mu\text{M}$ SA, $\sim 0.5\text{ }^{\circ}\text{C}$ in ~ 2 h (Fig. 2G1) and in male sporophyll tissue of *Z. furfuracea* treated with $10\text{ }\mu\text{M}$ and $50\text{ }\mu\text{M}$

2,6-DHBA, ~ 1.5 °C 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 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 (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 *Ceratozamia miqueliana* 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). Waves with such frequency are not

resolvable using thermocouples and can appear as spikes. Spike clusters can form asymmetric square and sinusoidal waves. It has been shown that mitochondrial membrane potential and different mitochondrial 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 °C/h. In *C. miqueliana* sporophylls attached to central cone axis the higher rate was ~ 0.09 and the lowest ~ 0.01 °C/h (Skubatz *et al.* 2019). In the *S. venosum* appendix temperature rate was ~ 0.04 °C/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 of thermogenesis.

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* showed an 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 may be 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). A cyclooxygenase (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 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* (L.) Heynh is affected by temperature and is dependent transcription factors by an unknown mechanism (Thines *et al.* 2014). In *Neurospora crassa* 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 waves seen 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 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 (Sokolov 2013).

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Figures

Fig. 1. Temperature pattern 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).

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).

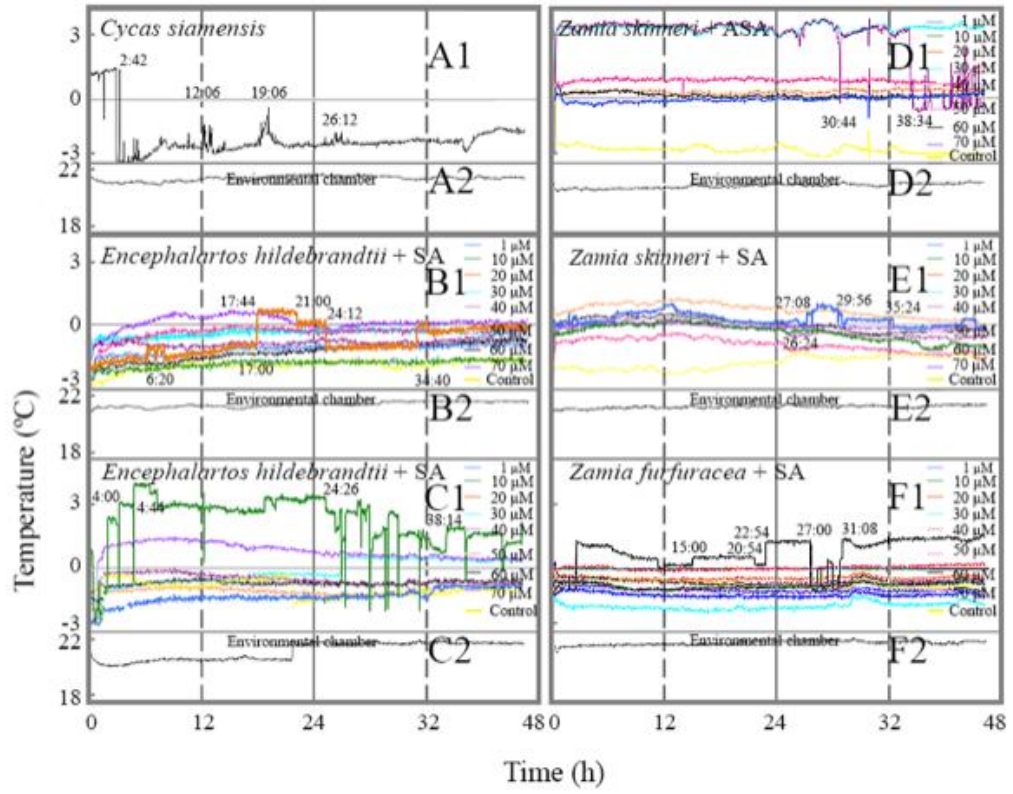


Fig.1 :

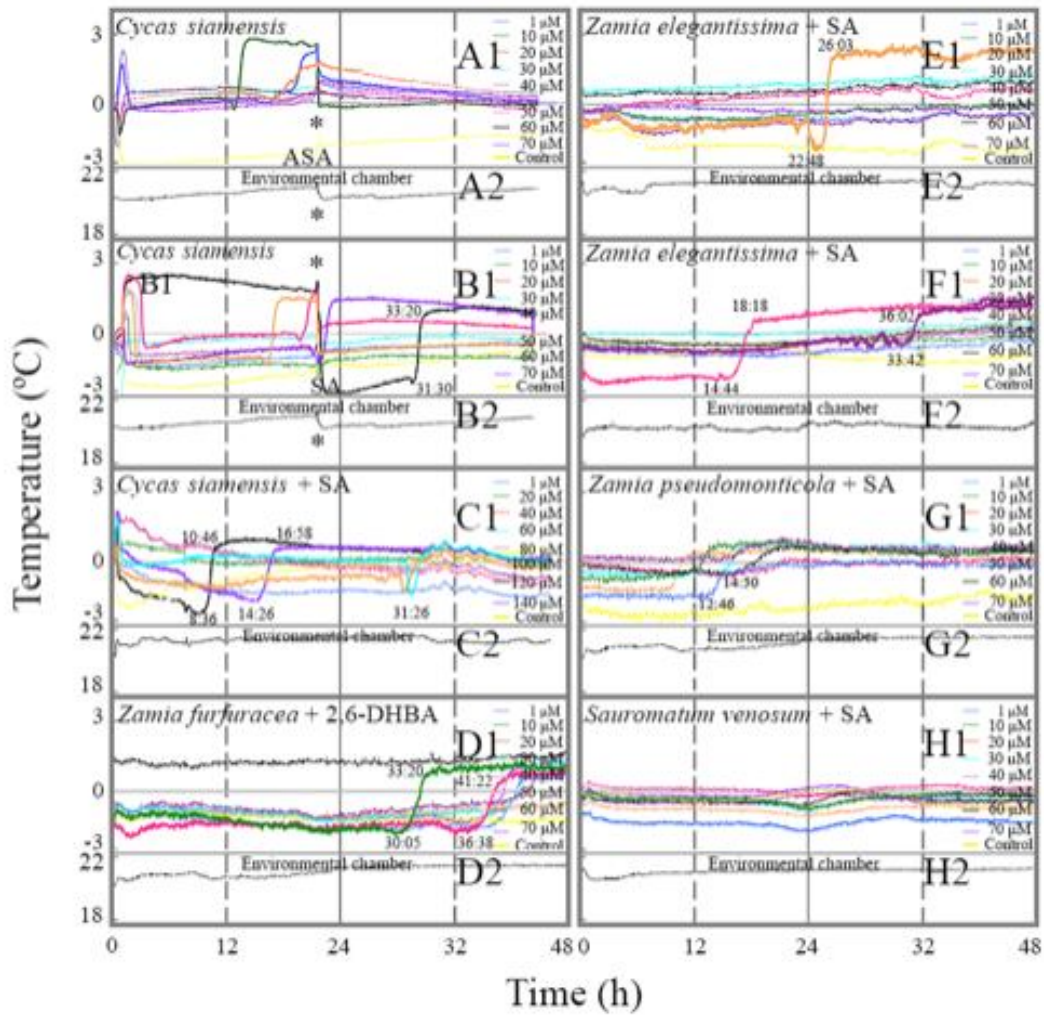


Fig. 2 :

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