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Regulation of heat production in the inflorescences of an *Arum* lily by endogenous salicylic acid

(*Sauromatum guttatum*/thermogenic plants/flowering/plant growth regulation/cyanide-insensitive respiration)

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ABSTRACT **We have recently purified calorigen, the natural trigger for heat production in the inflorescences of *Sauromatum guttatum* Schott (voodoo lily), a thermogenic plant, and identified it as salicylic acid. Since then an analytical assay was developed that allows the quantitation of salicylic acid in plant tissues. This assay was used to demonstrate that on the day preceding the day of blooming the levels of salicylic acid in the thermogenic organs (appendix and lower spadix) of the voodoo lily increased almost 100-fold, reaching a level of 1 μg/g of fresh weight. The level of salicylic acid in the appendix started to rise in the afternoon and reached its maximum in the late evening, whereas the maximum accumulation of salicylic acid in the lower spadix occurred late at night. The increase in salicylic acid level in the appendix was followed the next morning by a spectacular metabolic burst that lasted for about 7 hr and at its peak increased the appendix temperature by over 12°C. The second, 14-hr-long, thermogenic episode in the lower spadix started late at night and ended on the following morning, after maximum temperature increases of more than 10°C. The concentration of salicylic acid in both thermogenic tissues promptly returned to basal, preblooming levels at the end of the thermogenic periods. The thermogenic response was under strong photoperiodic and developmental control, with salicylic acid eliciting much stronger thermogenic responses in light than in darkness. Similar surges in salicylic acid occurred in nonthermogenic male and female flowers, while the concentration of salicylic acid in the spathe remained consistently below 20 ng/g of fresh weight. Of 33 analogs of salicylic acid tested, only 2,6-dihydroxybenzoic acid and acetylsalicylic acid (aspirin) were thermogenic. The activity of 2,6-dihydroxybenzoic acid exceeded that of salicylic acid.**

The ability of certain *Arum* lilies to generate heat in the inflorescences during blooming has intrigued scientists from the times of Lamarck (1) and was recently reviewed by Meeuse and Raskin (2). Such heat production has been observed in the flowers and inflorescences of plants belonging to at least six families of angiosperms. In all studied cases, the plant thermogenesis was associated with an increase in the alternative or cyanide-insensitive respiratory electron transport system, which is unique to mitochondria of plants, fungi, and some protists (3–5). Recently, calorigen, the natural trigger of heat production in thermogenic plants, first demonstrated in physiological experiments by van Herk in 1937 (6, 7), has been purified from *Sauromatum guttatum* Schott (voodoo lily), a member of the Araceae family, and identified as salicylic acid (8). This work demonstrated an important regulatory role played by endogenous salicylic acid in plant development. (In this report, the terms calorigen and salicylic acid are used interchangeably.) Earlier studies showed that exogenously applied salicylic acid stimulated flowering in *Lemnaceae* (9, 10) and *Impatiens* (11), induced disease resistance in plants (12), exhibited allelopathic effects (13), and inhibited the biosynthesis of the plant hormone ethylene (14), stomatal closure (15), and root ion uptake (16).

The inflorescence of the voodoo lily develops from a large corm and can reach 80 cm in height (Fig. 1). The central column of the developing inflorescence (spadix) is tightly wrapped by a spathe (modified bract). The base of the spathe forms the floral chamber, which surrounds the lower spadix encircled at the bottom by a ring of female flowers and club-shaped organs. A dense yellow ring of highly reduced male flowers is located between the lower spadix and the appendix at the only entrance to the floral chamber.

The blooming sequence commences immediately after the beginning of the light period with the unfolding of the spathe, which exposes the appendix. After 2 hr of light, the appendix undergoes the salicylic acid-triggered metabolic explosion, which is characterized by a dramatic increase in respiration and heat production. At the peak of the heat production, the temperature of the appendix may increase by 14°C, and the rate of oxygen consumption approximates that of flying hummingbirds (17). The temperature of the appendix returns to ambient in the evening. The main function of heat is thought to be the volatilization of amines and indoles (18, 19), production of which is synchronized with thermogenesis and is also triggered by salicylic acid (8). The volatilization of these compounds produces the strong putrescent odor that attracts insect pollinators. The second heating episode, which occurs in the lower spadix late at night after blooming, has been reported but not studied (2, 8, 20). This report further elucidates the regulatory role played by salicylic acid in the flowering of voodoo lily by studying the changes in the levels of endogenous salicylic acid in relation to the thermogenesis. The comparative thermogenic activity of salicylic acid analogs is also investigated.

MATERIALS AND METHODS

Plant Material. Reproductive corms of *S. guttatum* (voodoo lily) were obtained from B. J. D. Meeuse (Botany Department, University of Washington, Seattle). Developing inflorescences were moved to a growth chamber programmed for the following conditions: day and night temperature of 20°C, a 15-hr photoperiod with a photon fluence rate of 200 μmol of photons·m^{-2}·s^{-1} of photosynthetically active radiation at the corm level, and 70% relative humidity.

Salicylic Acid Determination. Inflorescence tissue was either used directly or stored at −80°C. One gram of tissue samples was ground in 2.5 ml of 90% methanol in a Tissumizer (Tekmar, Cincinnati). The extract was centrifuged at 12,000 × *g* for 15 min. The pellet was resuspended in 100% methanol and reextracted at 12,000 × *g* for another 15 min. Supernatants from both extractions were combined and dried

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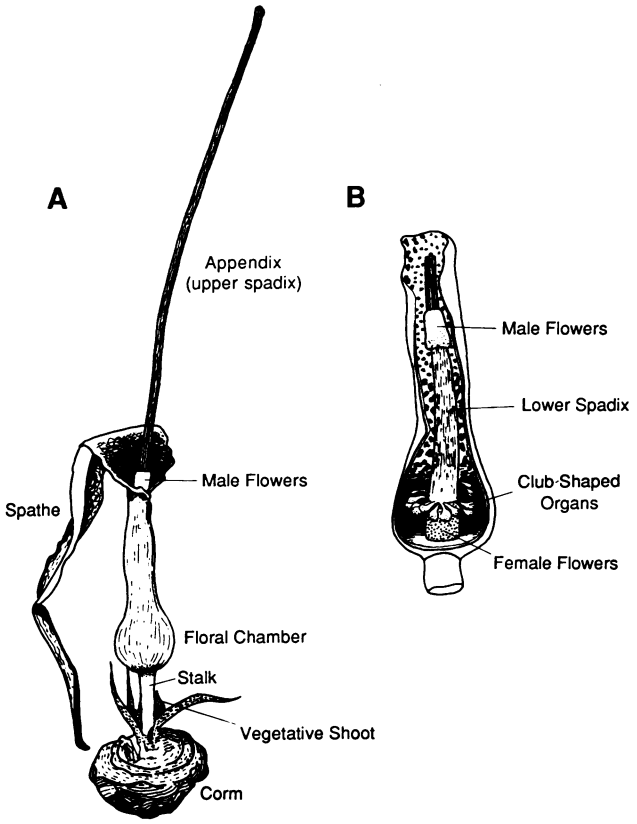

FIG. 1. The inflorescence of *S. guttatum* on the day of blooming and heat production. (*A*) Entire inflorescence. (*B*) Longitudinal cross section of the floral chamber. The drawing is not entirely to scale.

under N_2 at 40°C. The residue was resuspended in 2.5 ml of 5% (wt/vol) trichloroacetic acid and centrifuged at 1200 × *g* for 10 min. The supernatant was partitioned with 5 ml of a 1:1 (vol/vol) mixture of ethyl acetate/cyclopentane containing 1% (vol/vol) isopropanol. The top organic phase was passed through a BondElute 3 cc 2OH (Diol) solid-phase extraction column (Analytichem International, Harbor City, CA) preconditioned with 2 ml of a 1:1 (vol/vol) mixture of ethyl acetate/cyclopentane containing 1% (vol/vol) isopropanol. The column was washed with an additional 0.5 ml of extraction solution, and the wash eluate was combined with the sample eluate. The resulting 5.5 ml were dried under N_2 and resuspended in 0.5 ml of the HPLC mobile phase [45% water/55% methanol with 0.025% (wt/vol) H_3PO_4]. HPLC separation of salicylic acid was performed on a Varian liquid chromatograph (model 5060) equipped with a Zorbax phenyl guard column (4 mm × 1.25 cm, DuPont), a Zorbax phenyl cartridge column (4 mm × 8 cm), and a Zorbax ODS cartridge column (4 mm × 8 cm); all columns were maintained at 40°C with a mobile-phase flow rate of 1.2 ml/min. Salicylic acid concentration in 0.1-ml samples was determined with a HPLC spectrofluorescence detector (model FL750, McPherson, Acton, MA) equipped with a 200-watt xenon-mercury continuous arc lamp and a 360-nm cut-off filter. The best quantitation of salicylic acid was obtained with an excitation wavelength of 313 nm and an emission wavelength of 405 nm. This procedure had a 55% recovery rate for extractable salicylic acid as determined by spiking tissue samples with salicylic acid. The lowest concentration of salicylic acid reliably quantifiable by this procedure was 10 ng/g of fresh weight. Before tissue sampling, each inflorescence was carefully monitored to determine its developmental stage from a variety of morphological characteristics (2, 8). After the sampling of the appendix tissue, the inflorescence with the remainder of the appendix was kept in the growth chamber so that the estimated day of blooming could be verified retroactively.

Temperature Measurements. The temperatures on the surfaces of the inflorescence were measured with a thermal video system (model TVS-4300, Hughes Aircraft, Carlsbad, CA), which is capable of depicting and quantifying temperatures with 0.1°C resolution. Only the highest temperature observed on the tissue surface of the thermogenic organs was recorded (8). The temperature of the lower part of the spadix located below the male flowers was observed through a window cut in the wall of the pollination chamber made with a sharp scalpel just before the beginning of the temperature recording.

Screening of the Analogs. All tested analogs of salicylic acid were purchased from chemical suppliers and were of the highest purity available. All compounds were applied in freshly prepared aqueous solutions at a concentration of 15 μM. The appendix section bioassay was adapted from ref. 8. Two days before inflorescence unfolding and 4 hr after the beginning of the light period, the central portion of the appendix was sliced transversely into 3-cm-long cylindrical sections, each weighing ≈3 g. The inflorescence, with the remainder of the appendix, was kept in the growth chamber so that the estimated day of blooming could be verified retroactively. Two hundred microliters of the solution of the test compound were pipetted into the circular cavity carved into the top of each section. Water and 15 μM salicylic acid were used for the control sections. The sections were placed upright on moist filter paper and incubated overnight in the growth chamber. The next morning the highest surface temperature of each of the sections was monitored with the thermal video system. All thermogenically active compounds were retested along with salicylic acid at a concentration of 1.5 μM. At this concentration, salicylic acid produces about 50% of the maximum temperature increase. The relative activity was calculated by dividing the average temperature increase in degrees Celsius produced by 200 μl of a 1.5 μM solution of the active compound by the average increase produced by 200 μl of 1.5 μM salicylic acid.

RESULTS

Salicylic Acid Levels in the Inflorescence. Heating of the appendix, which directly follows the spathe unfolding, is the first and most spectacular thermogenic event in the blooming sequence of the voodoo lily inflorescence. The heat production is preceded by a dramatic rise in salicylic acid levels in all parts of the appendix (bottom, middle, and top) (Fig. 2 *A–D*). Endogenous salicylic acid started to increase sharply in the late afternoon of the day before blooming and reached its maximum during the evening when its level surged 100-fold above the level observed in the appendix 3 days before blooming. This increase was followed by an intense heat and odor production in the appendix the next morning (Fig. 2*A*). The heat production reached its peak at the period between the third and fifth hour of light exposure, when the average surface temperature of tested appendices increased by over 12°C. The temperature of the appendix returned to ambient in the late afternoon in parallel with the precipitous decrease in the amounts of salicylic acid in this tissue to the preblooming levels. On a microgram per gram of fresh weight basis, the increase of salicylic acid was largest in the top portion of the appendix. The period of heat production in the appendix was followed by a quiescent period, which lasted until the middle of the 9-hr dark period to which the plants were exposed. Thereafter, the second heating episode commenced in the lower portion of the spadix, located between male and female flowers in the center of the floral chamber (Fig. 3*A*). The maximum heat production in the lower spadix occurred during the hours immediately preceding and fol-

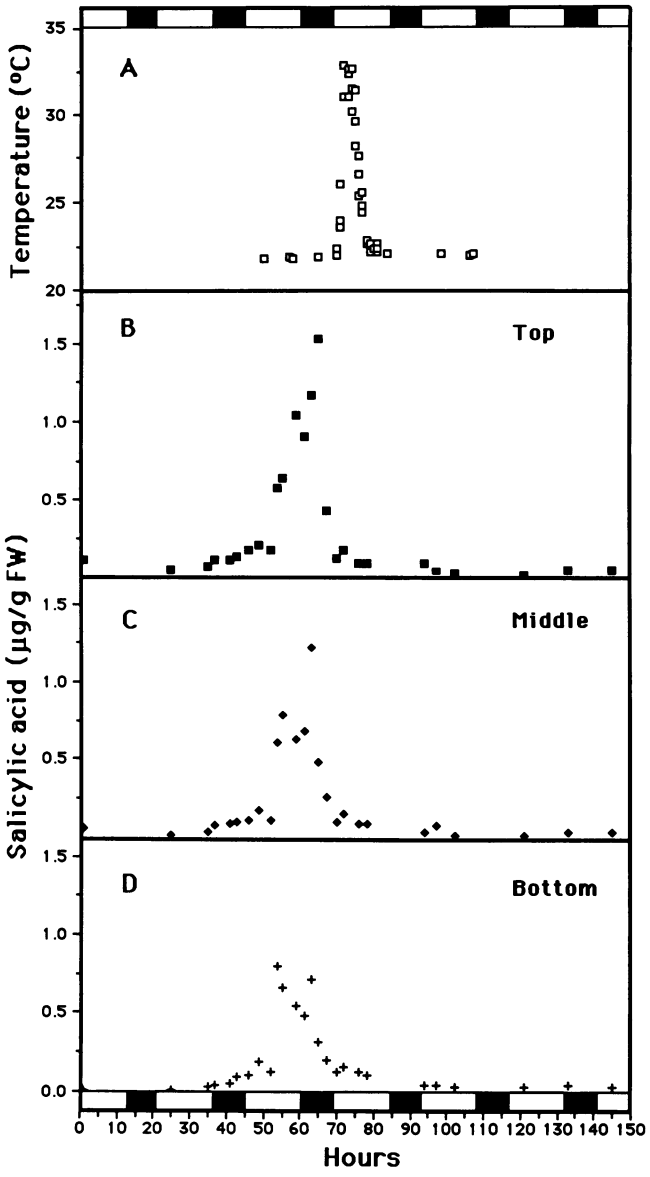

FIG. 2. Time course of heat production (*A*) and salicylic acid level in the top (*B*), middle (*C*), and bottom (*D*) parts of the appendix of *S. guttatum* inflorescence. (*A*) The temperature readings were obtained from three different inflorescences. Only one symbol is shown when data points overlap. This experiment was repeated three times with similar results. (*B–D*) One-gram sections from top, middle, and bottom regions of the appendices were excised from 25 inflorescences harvested over a period of 3 months. Each data point represents the salicylic acid concentration of an individual section. Zero hour on the horizontal axis indicates the start of the observations, and the black and white rectangles represent the periods of darkness and light, respectively. FW, fresh weight.

lowing the beginning of the light period and coincided with the release of pollen from the male flowers into the floral chamber below. Although the maximum temperatures of the lower portion of the spadix at the peak of heat production stayed slightly below those of the appendix (no more than 10°C above ambient), the thermogenesis in the lower spadix lasted for 14 hr, which was ≈2 times longer than the thermogenesis in the appendix. Analogous to that in the appendix, the thermogenesis in the lower spadix was preceded by a major rise in endogenous salicylic acid (Fig. 3*B*). The level of salicylic acid in the lower spadix reached its maximum during the night and early morning just before the inflorescence unfolding. In contrast to the appendix tissue, this nearly 100-

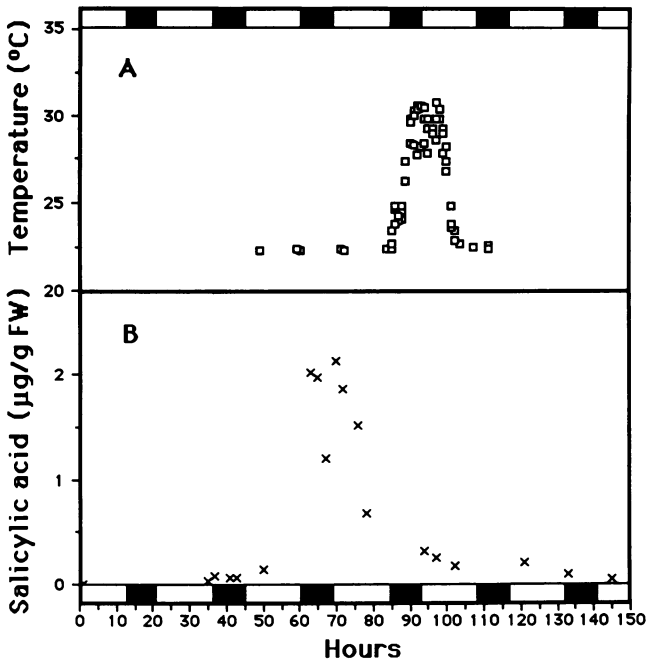

FIG. 3. Time course of heat production (*A*) and salicylic acid level (*B*) in the lower spadix of *S. guttatum* inflorescence. (*A*) Temperature readings were obtained from three different inflorescences. Only one symbol is shown when data points overlap. This experiment was repeated three times with similar results. (*B*) One-gram sections of lower spadix were excised from 18 inflorescences harvested over a period of 2 months. Zero hour on the horizontal axis indicates the start of the observations, and the black and white rectangles represent the periods of darkness and light, respectively. FW, fresh weight.

fold increase occurred significantly later, and the elevated levels of salicylic acid persisted over a longer period of time. Although no significant temperature increases in the male and female flowers were detected during flowering, these organs exhibited the largest increases in salicylic acid level (Fig. 4 *A* and *B*). The salicylic acid level in male and female flowers began to rise in the afternoon of the day preceding the day of inflorescence unfolding, eventually reaching 3 μg/g of fresh weight, which is about 100 times above the levels observed 3 days before and after blooming. The level of salicylic acid in these tissues returned to basal levels the morning after the morning of blooming. No changes were detected in the amounts of salicylic acid present in the spathe. Before, during, and after blooming, the salicylic acid in the spathe was barely detectable and never exceeded a level of 30 ng/g of fresh weight.

We have investigated the possibility that some portion of the salicylic acid in the spadix tissue may be present in the form of a storage or transport conjugate. Therefore extracts of the appendix and the male flowers were subjected to a variety of hydrolytic treatments in a series of replicated experiments. These treatments included hydrolysis with heated acid or base and enzymatic hydrolysis with α-glucosidase, sulfatase, α-mannosidase, β-galactosidase, β-glucosidase, fucosidase, and esterase. None of those treatments resulted in a significant increase in the amount of salicylic acid in the tissue extract, suggesting that most of the salicylic acid in the inflorescence tissue is present in the free form.

The concentration of salicylic acid measured in the appendix tissue 2 days before blooming was below 0.1 μg/g of fresh weight. However, the application of exogenous salicylic acid at only a slightly larger concentration (0.13 μg/g of fresh weight) to 3-cm-long sections excised from the appendix at this age and kept under the normal photoperiod was previously shown to induce production of considerable heat and

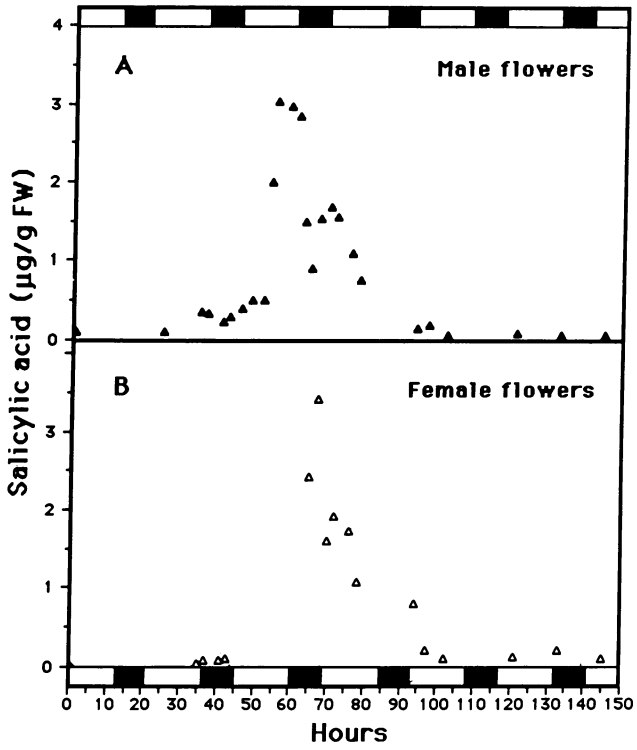

FIG. 4. Time course of changes in salicylic acid level in the male (*A*) and female (*B*) flowers of *S. guttatum*. (*A*) Data were obtained from one-gram sections of male flowers excised from 25 inflorescences harvested over a period of 3 months. (*B*) Data were obtained from one-gram sections of female flowers excised from 18 inflorescences harvested over a period of 2 months. Zero hour on the horizontal axis indicates the start of the observations, and the black and white rectangles represent the periods of darkness and light, respectively. FW, fresh weight.

odor (8). To explain this inconsistency, the appendix sections excised 2 days before blooming and treated with 200 μl of 15 μM salicylic acid (8) were put in constant darkness to mimic the conditions in the closed inflorescence. Although heat production in these sections was barely detectable (Fig. 5), sections excised from the same plants and kept in darkness

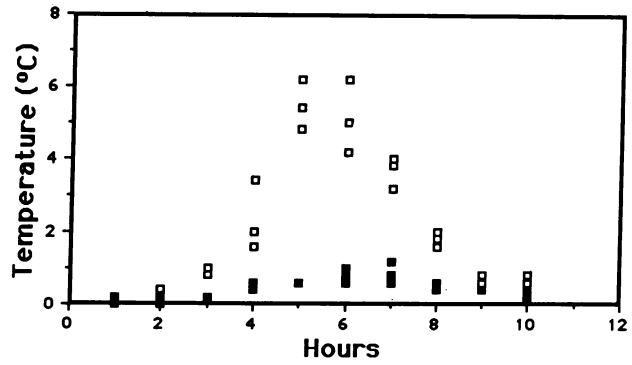

FIG. 5. Time course of temperature changes in 3-cm-long appendix sections excised from *S. guttatum* inflorescence 2 days before blooming, treated with 200 μl of 15 μM salicylic acid, and placed in darkness permanently (■) or until the beginning of the next light period (□), which is represented by zero hour on the horizontal scale. The numbers on the vertical axis are the differences between the surface temperature of the salicylic acid-treated and water (control) sections. The temperature measurements of the sections kept in darkness were performed in darkness. Each treatment was replicated on three sections (shown as separate data points) excised from three inflorescences. Only one symbol is shown when data points overlap. The experiment was repeated three times with similar results.

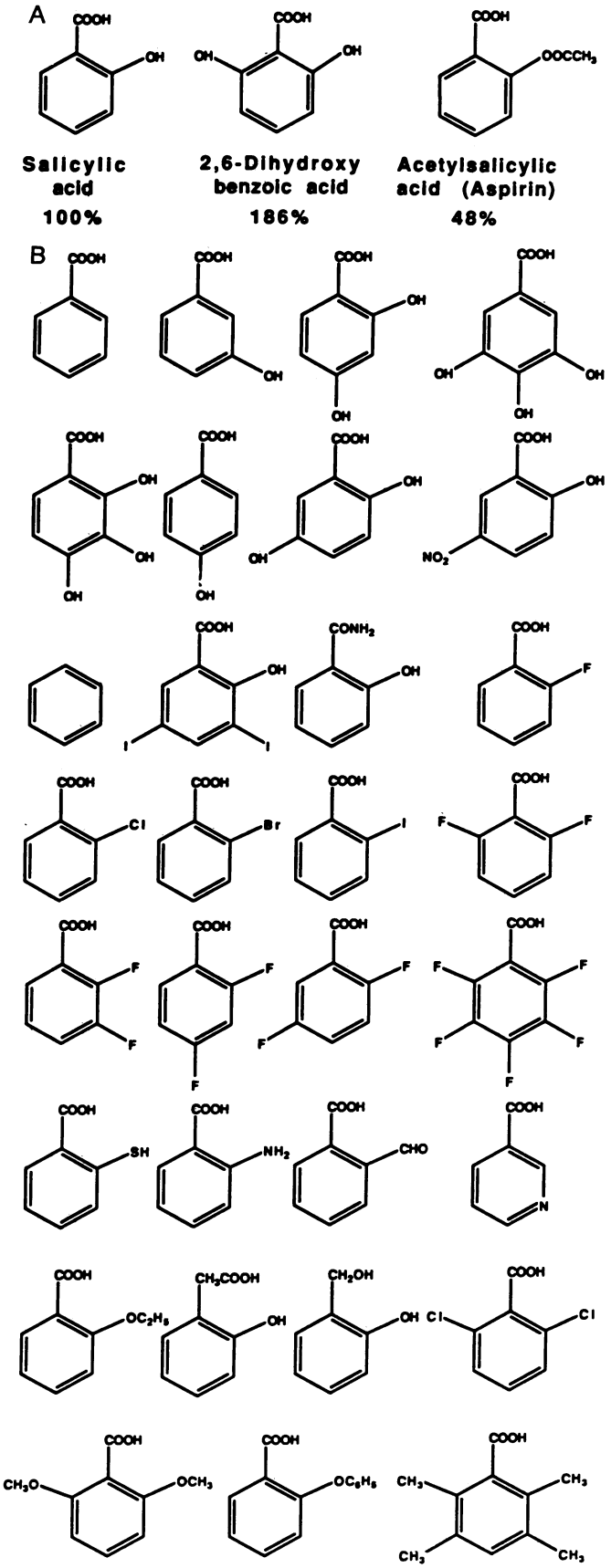

FIG. 6. Thermogenically active (*A*) and inactive (*B*) analogs of salicylic acid assayed in 3-cm-long sections excised from the appendix of *Sauromatum guttatum* 2 days before blooming. The percentages in *A* indicate the ability to induce heat production relative to salicylic acid (calorigen).

only until the beginning of the next photoperiod showed temperature increases of over 6°C 5 hr after the exposure to light, indicating that the unfolding of the spathe, which exposes the appendix to light, is essential for thermogenicity.

Relative Activity of Salicylic Acid Analogs. Thirty-three analogs of salicylic acid were compared with salicylic acid for their ability to induce heat production in 3-cm-long sections of the voodoo lily appendix (Fig. 6). Only two compounds showed thermogenic activity at the concentrations tested (Fig. 6*A*). 2,6-Dihydroxybenzoic acid was significantly more active than salicylic acid; at the same concentration it produced temperature increases that were larger than those produced by salicylic acid. Aspirin (acetylsalicylic acid) was about half as active as salicylic acid. Removing or changing the position of the hydroxyl group on the benzene ring, substituting it with halogens or other groups, or adding groups to any other positions on the benzene ring of the salicylic acid destroyed the thermogenic activity (Fig. 6*B*). A similar lack of activity was observed when the carboxyl group of salicylic acid was derivatized or substituted.

DISCUSSION

The temperature increases observed in the appendix and lower spadix of *S. guttatum* Schott (voodoo lily) were preceded by a dramatic rise in the level of endogenous salicylic acid (Figs. 1 and 2). We reported earlier that salicylic acid is a natural trigger for the thermogenic response in voodoo lily (8). Therefore, it can be concluded that the nearly 100-fold increase in the levels of salicylic acid, which starts on the day preceding the day of blooming, is directly responsible for triggering the sequential episodes of heat production in the appendix and lower spadix. The thermogenic effect of salicylic acid is further enhanced by the maximization of tissue sensitivity to this regulator on the day before blooming (8). Exposure of appendix to light, caused by the unfolding of the spathe, increases the tissue sensitivity to salicylic acid even further (Fig. 5). This combination of increased concentration of calorigen and the developmentally and photoperiodically regulated rise in tissue sensitivity to it results in the metabolic explosion, which has no parallel in the nonthermogenic species of the plant kingdom.

The transient rise in salicylic acid concentration in the lower spadix occurred later and lasted longer than that in the appendix. This observation may explain why the heating of the lower spadix is longer and happens after the heating of the appendix. The highest concentrations of salicylic acid were observed in the male and female flowers, although these tissues did not produce any heat during blooming. This observation agrees with the 50-year-old hypothesis of Van Herk (6, 7) that calorigen, identified today as salicylic acid, is originally produced in the male flowers and subsequently transported to the thermogenic organs. However, at this time, no evidence has been obtained on the export of salicylic acid from the floral structures to the other parts of the spadix. Also no evidence was found indicating the presence of the conjugated derivatives of salicylic acid, which might be used for transport.

The thermogenic effects of salicylic acid were very specific. Any modification in the structure of the molecule destroyed the activity of calorigen. 2,6-Dihydroxybenzoic acid, with hydroxyl groups at both ortho positions, was the only exception. This molecule was about 2 times more active than salicylic acid when applied to sections of the voodoo lily spadix. Aspirin (acetylsalicylic acid) also had some thermogenic activity. This probably can be explained by the rapid hydrolysis of aspirin to salicylic acid that occurs in biological tissues (21).

The precision, elegance, and complexity of the blooming cycle of the voodoo lily is most fascinating. While the first heating episode in the appendix lures the potential pollinators into the floral chamber, its perfect trap-like design with slippery concave walls and the hedge of club-shaped organs prevents the insects from escaping, while keeping them active. After enough insects are trapped in the floral chamber, the lower spadix starts to heat up, warming the pollination chamber and stimulating the activity of the trapped insects. The sweet odor emanating from the club-shaped organs is also thought to stimulate mating activity of the trapped insects (2). At the peak of heating, the sticky pollen is showered from the rapidly dehiscing male flowers down to the bottom of the pollination chamber to be picked up by the pollinators and deposited on the female flowers conveniently located at the entrance to the most inviting, but usually futile, escape route along the spadix. After the end of the second heating episode, the whole inflorescence starts to senesce and shrivels rapidly, allowing the insects to escape and cross-pollinate other inflorescences with the pollen adhered to their bodies. The discovery of the regulatory role of salicylic acid in the flowering sequence of *Arum* lilies provides the connection between the easily observable developmental phenomenon and the complex biochemical control behind it. While thermogenesis and production of chemical insect attractants are the most apparent events in the voodoo lily inflorescence controlled by calorigen, it is possible that endogenous salicylic acid plays a much broader hormonal role in regulating development in *Arum* lilies as well as in other plants.

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