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Induction of indole synthesis in the appendix of *Sauromatum guttatum* Schott

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Induction of indole synthesis in young appendix sections of *Sauromatum guttatum* Schott was studied. The inducer, known as calorigen, a compound synthesized in the mature staminate flower buds, is capable of triggering a rapid metabolic spurt in the appendix of the inflorescence. The induced indole-formation lasts for only a short period of time, and is similar to the normal pattern of indole synthesis. Under the experimental conditions, indole was detectable after 7 to 8 hr of calorigen treatment but had disappeared about 35 hr later. The moment when indole is present in maximal amount coincides with that of maximal heat production, i.e., about 27 hr after the application of calorigen. A simple and sensitive bioassay for detection of calorigen, based on the formation of indole, could be devised.

Calorigen also causes, to a lesser extent, a temperature-rise of those young floral parts (male, female and sterile tissues) where indole is not detectable at any time. The time sequence of the induced production of heat for the various floral parts mimics that of a natural metabolic flare-up.

Extracts prepared from the mature staminate flowers of *Arum maculatum* L., *Arum dioscoridis* Sibth and Sm., and *Dracunculus vulgaris* Schott were all able to induce the formation of indole in the appendix of *Sauromatum* in a fashion similar to that caused by the staminate flower extract of this plant.

In a previous publication we reported the presence of free indole in certain floral parts of some arum lily species (*1*). In *Sauromatum guttatum* Schott, production of free indole occurs in the appendix, and only during the first day of blooming when it forms part of the syndrome of dramatic metabolic changes (including the development of a respiratory climacteric, with generation of much heat and an unpleasant odor) which characterizes the appendix at that time.

The triggering of the metabolic outburst has been the subject of several studies. In 1937, van Herk (*2*) reported that excision of the staminate flowers or removal of the appendix from the inflorescence would prevent the appendix from warming up if carried out 24 hr or more before the expected time of blooming. If such experiments were done at a later time, e.g., 20–22 hr before the unfolding of the spathe, the appendix would still develop the characteristic metabolic flare-up. Excision of other organs did not affect the heating-phenomenon. From these facts, he concluded that the thermogenesis in the appendix is triggered by a substance which is synthesized in the staminate flowers and is released into the appendix

some 20 hr before the opening of the spathe. Later (*3*) he was able to induce the generation of heat in immature appendix halves by injection of an extract from the staminate flowers, assumed to contain the postulated triggering principle, "calorigen".

Further studies on calorigen were carried out in our Seattle laboratory. Modifying van Herk's bioassay for calorigen activity, Buggeln and Meeuse (*4*) were able to demonstrate the presence of a calorigen-like substance in the staminate flowers of several more arum lily species. However, their procedure, based on the monitoring of heat production and CO_2 evolution, is rather time-consuming, lacking in sensitivity, and expensive in terms of appendix material. In this paper we wish to report the development of a simple, sensitive and rapid bioassay, based on the hormone's ability to induce synthesis of indole in young appendix tissue.

Materials and methods

Plant materials

Methods for growing plants and for determining and indicating their flowering stages were as described previously (*1*). The day the spathe unfolds is referred to as D-day. Stages corresponding with a given number of days before and after this day are designated as D−1, D−2, etc., and D+1, D+2, etc., respectively.

Mature (D-day) staminate flower zones were used as the source of calorigen. The crude calorigen extracts were prepared according to the method of Buggeln and Meeuse (*4*). The final volume of the extract was adjusted in such a way that 1.0 ml extract corresponded with one excised staminate floral zone.

Bioassay of calorigen activity

a) *Induction of indole synthesis*: The appendix was cut off about 5 cm above the zone of the staminate flowers leaving the stump, still enclosed by the basal part of the spathe, for later (retroactive) determination of the flowering-stage. The appendix was used immediately, or if so desired, was kept in the laboratory with the cut end immersed in distilled water. In the latter case the upper part of the spathe was allowed to remain around the appendix to prevent it from drying out. Although, under these conditions, the spathe will open normally at a later date after the expected time of blooming, the appendix fails to develop the characteristic metabolic outburst.

The appendix was cut into 1.5 cm sections which were plunged directly into distilled water to wash off the starch grains released from the cut surfaces. Unless otherwise indicated, each of the sections was then allowed to stand vertically in a 10-ml beaker containing 0.5 ml of the solution to be tested and provided with a piece of pre-washed filter paper covering the bottom. The solutions also contained 1.0 mg/ml of streptomycin or, in some cases, 2.5×10^{-5} M penicillin. These additives showed no significant adverse effects on the phenomena studied. One drop of the test solution was placed on the upper cut surface of the section. The beaker was covered with a watch glass, still permitting proper aeration through the drain tip. The sections were incubated at room temperature (26±1°C) under constant

laboratory illumination for the period of time desired. Distilled water and phosphate buffer (0.1 M, pH 6.5) were used for controls in each experiment. The control series does not produce indole.

At the end of the incubation period, the appendix sections were rinsed with water, immediately ground into a paste, and 2.0 ml of toluene was added. The toluene layer was carefully poured into a test tube containing 0.5 ml of Ehrlich's reagent. A pink-red reaction was taken as indicative of the presence of indole and therefore of calorigen activity. For quantitative studies, 5.0 ml of toluene was used, and a 1.0 ml aliquot was taken for colorimetric determination of indole (*5*).

b) *Induction of heat production*: Heat production was used as an indicator of calorigen activity. Appendix sections 4 cm long were allowed to stand erect in a beaker containing the test solution described above. The upper surface of the section was enclosed in a layer of parafilm. The temperature-rise of the appendix section was measured according to the method of Buggeln and Meeuse (*4*).

Results

Thermogenesis

The temperature-rise displayed by the various floral parts of *Sauromatum* during the normal process of flowering has previously been recorded (*2*). In our laboratory, we have re-examined this phenomenon in order to compare it with the events of induced heat generation. The spathe of an inflorescence believed to be at the D—1 stage was cut off at the base of the floral chamber and the cut surface was covered with a thin layer of petroleum jelly. The thermometer probes were then attached to the surface of various parts of the central spadix; those attached to the stalk of the inflorescence or exposed to open air were used as references. The inflorescence was kept under continuous laboratory illumination.

Fig. 1 shows the typical pattern of temperature changes in various parts of the inflorescence (for a drawing of a *Sauromatum* inflorescence see Ref. *6*). The results confirmed those reported earlier (*2*) in that the temporal starting point, time span, and the magnitude of the temperature difference vary with the floral part tested. In most cases the temperature-rise occurred first in the zone of staminate flowers. According to van Herk (*2*), heat production in the staminate flowers starts around noon on D—1 and ends near noon on D+1, giving a total warm period of almost

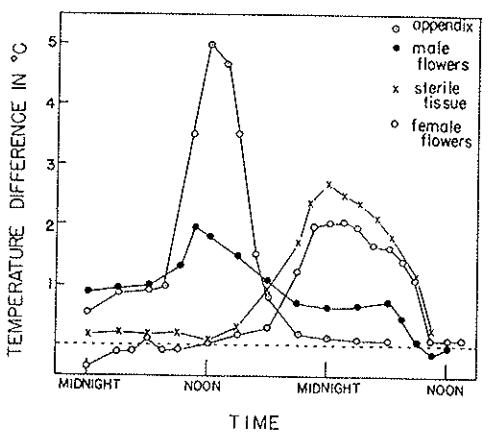

Fig. 1. *Temperature changes in various parts of the* Sauromatum *inflorescence during the time of blooming.* The thermoprobe attached to the stalk was used as tissue control and the data were calculated against room temperature (---). The temperature changes displayed by the appendix were measured at a level 10 cm above the male floral zone.

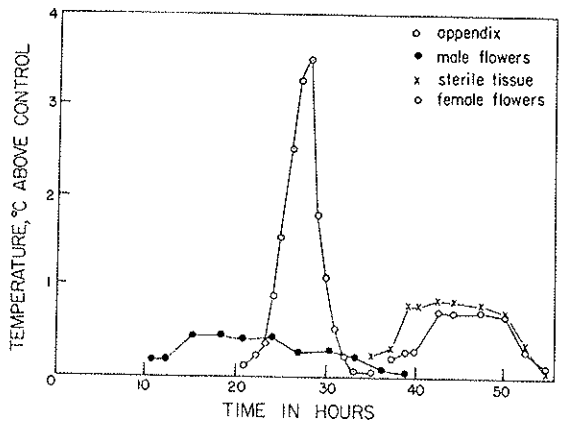

Fig. 2. *Calorigen-induced temperature changes in young floral parts of* Sauromatum. Two inflorescences at the stage of D—3 and D—4 were used for the test. The control for each part of the tissue tested was incubated in distilled water.

two days. The temperature peak, about 2°C over room temperature, occurs a little bit earlier than that of the appendix. In the latter organ the highest temperature is often 4° to 10°C over the ambient temperature. The warming period is relatively short and displays a sharp peak, usually reached around noon (with, sometimes, 2 to 3 hr variation). This peak is always associated with the most rapid respiration (*6*) and the strongest release of stench (*7*) and indole (*1*). Heating in both the female and the sterile region of the inflorescence occurs later, the peak being reached about 12 hr after the maximal temperature-rise in the appendix. The temperature of the stalk never rises. It is usually 0.2° to 0.5°C lower than that of the environment.

Induced metabolic changes

The calorigen-induced temperature changes in various isolated young floral parts are illustrated in Fig. 2. It shows that all of the floral parts tested are inducible, in terms of heat production. The pattern of the induced temperature-rise in these tissues resembles that of a natural cycle (Fig. 1). However, the induced temperature changes are relatively smaller. For example, the maximal temperature-rise in the female and the sterile regions is always less than 1°C, whereas under natural conditions they warm up to 2–3°C above room temperature. In appendix sections, the

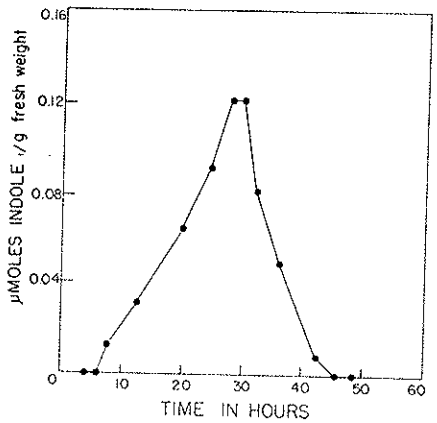

Fig. 3. *Calorigen-induced indole formation in young appendix sections of* Sauromatum. The tissue sections used were pooled from two D—2 appendices. Each value represents the average of two sections.

magnitude of temperature change varies considerably with the experiment, but usually ranges from 3°C to 6.5°C over the control. The temperature-rise of the materials is always accompanied by a distinct condensation of vapor on the inner wall of the beaker which contains the materials, apparently due to a rapid respiration. In the case of the appendix, the typical bad *Sauromatum* smell was particularly noticeable.

Our earlier report (*1*) has shown that the appendix is the only part of the inflorescence to produce indole at the time of the metabolic flare-up, suggesting that the process of indole production as it manifests itself in appendix sections can be used to assay for calorigen activity. Fig. 3 shows that indole was indeed produced during the induced period of metabolic changes. Furthermore, the induced indole cycle mimics the natural one, in that indole appears only for a short period of time (*1*). The peak time of indole formation coincides with the peak in heat production. All other parts of the inflorescence tested showed no indole production, although they can demonstrably be induced to develop heat, as already mentioned above.

The results also show that, in the induced process, the production of indole and of heat both last slightly longer than in the natural process, and that the time spans between the peaks in heat production displayed by various parts of the inflorescence are somewhat longer. For instance, in the normal flowering process the peak of heat production by the appendix is about 16 hr after the appearance of detectable indole in the tissue (*1*). In the induced cycle the time span is found to be about 20 hr, a delay of approximately 4 hr. In the case of the male flowers, the delay is not significant. The peak of heat production in female flowers and sterile tissue occurs about 45 hr after the treatment, i.e., with a delay of approximately 17 hr as compared with the natural cycle.

From the results it is clear that the process of indole production starts more than 10 hr before the heating process is observed in the appendix sections. Thus, by using indole formation as a criterion for calorigen activity one can shorten the time required for the experiment to as little as 8 hr.

Inducibility of the appendix

Fig. 4, illustrating the induction of indole synthesis in appendix sections of various stages, shows that all the immature appendices tested are susceptible to the action of calorigen. In all cases the induced indole cycle mimics the natural one as stated above. The sections of D−1 appendix are not suitable for the test because they already possess the ability to produce indole. Appendices harvested after blooming, i.e., in the stage of D+1 or older, cannot be induced to produce a second indole cycle. During this study it was noted that on the day of blooming, D-day, the basal part of an appendix left attached to the rest of the inflorescence always produces a considerable amount of indole; however, the terminal part of the same appendix, excised much earlier and kept with the cut end in water, fails to do so. This confirms the early observation of van Herk (*2*), that a prerequisite for the development of a metabolic flare-up is to leave the appendix attached to the inflorescence until one day before the day of blooming, or longer.

The response of appendix sections to the treatment with calorigen is further illustrated in the following experiment. Calorigen-induced appendix sections which had completed an indole cycle, were thoroughly rinsed with water and again

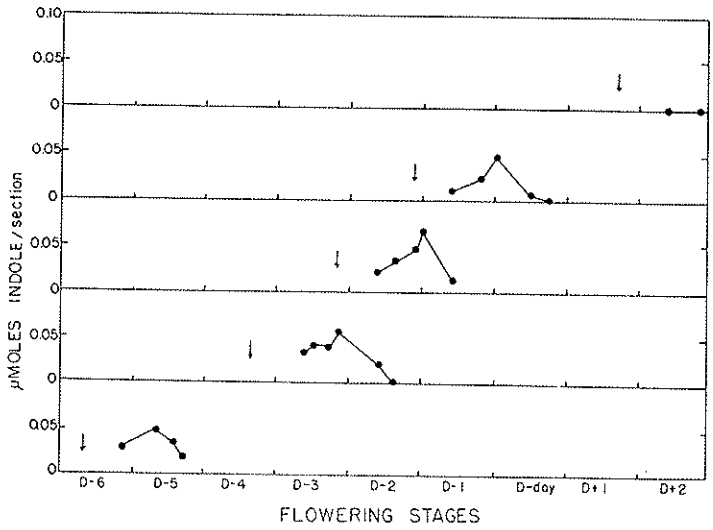

Fig. 4. *Induction of indole synthesis in the appendix by calorigen, for various flowering stages.* Each of the experiments was done separately but the calorigen extract was prepared from the same batch of male floral zones. The arrows indicate the time of calorigen application.

treated with calorigen. No indole, heat or stench was ever produced by these sections. On the other hand sections in the control series, which were incubated with water or buffer for 42 hr and were then treated with calorigen, produced an indole cycle similar to that of a normal induction. In fact, an appendix excised at the D—3 stage can be kept with the cut end in water, thus avoiding the development of a metabolic flare-up, for 9 days so that it is now equivalent to D+6; yet, it is still inducible! It appears that as long as the appendix remains in "pre-burst" metabolic condition it is suitable for indole induction. Therefore, one can keep the excised immature appendix in reserve for several days until the time desired. This is one of the advantages of our method. Buggeln et al. (*8*) delayed the development of a respiratory climacteric by continuous illumination of the inflorescence.

All parts of the appendix are suitable for the assay; sections taken at random all show indole production upon the treatment with calorigen. The actual amount of indole present at a given time varies somewhat as would be the case with different part of a normal appendix; e.i., those sections 10 to 25 cm above the male floral zone are the most active. In certain individuals, the variation among sections may be of the order of 20%. Thus for quantitative study the parts of the appendix used must be chosen so that they represent sections next to each other, and determinations should be run in multiplicate.

Time factors and the mobility of calorigen

It has been noted earlier (*3*, *4*) that there is always a lag period of approximately 24–27 hr between the moment of calorigen treatment and that of maximal heat production. The length of the lag period is not significantly affected by the amount of calorigen used (*3*). This is also true for the lag period in indole production. In

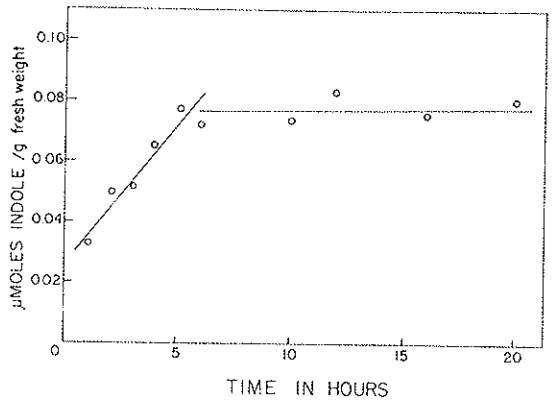

Fig. 5. *Effects of length of direct contact with calorigen on the amount of indole formed during a 20-hr incubation.* The details are described in the text.

one experiment we treated three batches of appendix sections with calorigen for various time periods. One batch was incubated continuously with calorigen during the experiment while the other two were treated for either 1 or 4 hr before they were transferred to water. One section from each series was taken for indole determination at various time intervals. It was found that all series started to produce indole after 7–8 hr and reached a maximal level at 26–27 hr. However, the amount of indole present at a given time is always highest in the continuously treated sections (which presumably took up the most calorigen) and lowest in the one-hour treated ones. In another series of experiments, the appendix sections were incubated continuously with either the calorigen preparation used in the above experiment, or with a ten-fold dilution of the preparation. The time-courses of indole formation were compared. It was found that in both cases the pattern of indole formation resembles that shown in Fig. 3, but the amount of indole formed at a given time is always higher in the series with the non-diluted preparation. Thus the amount of calorigen is a factor which does not control the *moment* of indole production, but the *amount* of indole produced. The sensitivity of our test is such that the extract from one male flower zone demonstrably induces indole production in sections from three complete appendices (i.e., in about 100 sections), all tried simultaneously.

Fig. 5 illustrates how long a period of exposure to calorigen is required to make the appendix sections fully responsive in terms of indole production. Appendix sections from two D—3 and two D—4 stages were incubated in a 250-ml beaker containing 10 ml of calorigen extract. At the time indicated, two sections were taken out and transferred to water. At 20 hr after the start of the experiment the sections from each series were weighed and the amount of indole determined. It was found that a direct contact with calorigen for a period as short as one hr was long enough to trigger the formation of indole. Within the first few hours of calorigen treatment, the amount of indole found is directly proportional to the length of the contact-period. This indicates that at least 4–5 hr of exposure to the calorigen is needed in order to activate the section fully.

The movement of calorigen in the appendix seems to be bidirectional. This is concluded from an experiment in which two 5-cm sections of the appendix (D—3) were treated with calorigen at one of the cut ends, either the basal or the tip end; it

was found that both sections were induced to produce heat in the same fashion. No difference in lag time was observed.

When two D—3 appendices measuring 24 and 43 cm were treated with calorigen at the cut end for 11 hr, we found that in both cases only the first 8 cm long sections showed positive indole test and the amount of indole decreased as the distance from the site of calorigen application increased. Since the lag time for indole production is about 8 hr, the rate of movement of calorigen in the system should be about 2–3 cm per hour.

Stability of calorigen

Our studies on the thermostability were prompted by van Herk's report (*3*) that crude calorigen loses its activity after one day's storage in the refrigerator. We were able to store crude calorigen (pH 6.5) in the presence of streptomycin at room temperature for as long as 7 days without loss of the hormone's ability to cause production of heat and indole. In fact, calorigen in dried form, after a sojourn at room temperature for up to 4 months, is still able to induce indole formation accompanied by production of stench and heat. Autoclaving for 15 min hardly affects calorigen. Staminate flowers, after having been stored in a deep-freeze for two years, still contain active calorigen. Thus the principle under study is very stable.

Calorigen acts over a range of pH values between 5.0 and 8.0. However, the highest activity occurs at relatively low pH (5.5–6.0), which is close to the pH of the tissue. Adjustment of the pH to extreme acidity or alkalinity, followed by neutralization, does not destroy the activity.

Calorigen-like substance(s) in other aroids

The presence of calorigen-like substances in the staminate flowers of other aroids has been reported (*4*). These substances, when assayed with *Sauromatum* appendix sections cause the same pattern of heat production and release of stench. Since indole production is associated with the metabolic changes it would be of interest to see whether extracts from other aroids also trigger indole production in the appendix of *Sauromatum*. In this study, mature staminate flowers of *Arum maculatum* L., *Arum dioscoridis* Sibth. and Sm., *Dracunculus vulgaris* Schott and the spadix of *Lysichitum americanum* Hult. and St. John were used to prepare calorigen extract. It was found that all extracts except that of *Lysichitum* are able to induce production of indole, in the same fashion as *Sauromatum* extracts.

Discussion

The system reported here offers considerable advantages in the study of the calorigen-induced metabolic events in excised *Sauromatum* appendix sections. It also serves as a rather convenient bioassay for calorigen activity. With this system, an average appendix is able to provide material for 10 to 20 tests. The presence of calorigen, as monitored by the formation of indole, can be detected after a period as short as 8 hr (although, in our experience, an overnight incubation is better). Furthermore, the material used, the young appendix, can be preserved for several days without losing its susceptibility to the action of calorigen which itself is very stable. We have taken advantage of these findings in an attempt to purify the

(*10*) Hess, C. M.: Glycolysis with its associated enzymes and intermediates in the appendix of *Sauromatum guttatum* Schott and other aroids. Ph. D. Thesis. University of Washington, Seattle, Washington. 1964.

(*11*) Wilson, R. H. and B. N. Smith: Uncoupling of *Sauromatum* spadix mitochondria as a mechanism of thermogenesis. *Z. Pflanzenphysiol.* 65: 124–129 (1971).