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Identification of the Flower-inducing Factor Isolated from Aphid Honeydew as being Salicylic Acid[1]

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ABSTRACT

Honeydew produced by the aphid *Dactynotus ambrosiae* when feeding on flowering or vegetative plants of the short day plant *Xanthium strumarium* contains an active substance capable of inducing flowering in the long day plant *Lemna gibba* G3. In the present study, this active material has been identified as salicylic acid through the use of gas-liquid chromatography and mass, infrared, and ultraviolet spectrometry. Authentic salicylic acid induces flowering in *L. gibba* G3 under strict short day conditions with an optimal response at about 5.6 μM. The possible significance of salicylic acid for the control of flowering in *Xanthium* or *L. gibba* G3 is discussed.

Previous work has shown that the phloem of vegetative or flowering *Xanthium strumarium* contains an active substance capable of inducing flowering in the long day plant *Lemna gibba* G3 (5). This material can be obtained either from methanol extracts of young leaves and apices of vegetative or flowering *Xanthium* or by extraction of honeydew produced by the aphid *Dactynotus ambrosiae* when feeding on vegetative or flowering *Xanthium*. In the present study, the active substance obtained from the aphid honeydew has been purified by TLC and identified through the use of GLC and mass, infrared, and UV spectrometry.

MATERIALS AND METHODS

Extraction of Honeydew. The honeydew was collected and extracted as previously described (5). The acidic ethyl acetate fraction obtained either before or after acid hydrolysis of the aqueous extract (2 hr, 60 C, pH 1) was purified by TLC on Silica Gel H in the solvent system of chloroform-ethyl acetate-acetic acid (60:40:5, v/v). The TLC plates were allowed to develop for 15 cm and then examined under 254 nm UV light. Previous work (3, 5) has shown that the flower-inducing activity obtained in fraction A-4 either before or after acid hydrolysis corresponds to a thin, bright blue fluorescent band as seen with 254 nm UV light at about R_F 0.70. Thus this band was scraped off the plate, eluted 3 times with 3 ml of water-saturated ethyl acetate and evaporated to dryness. The crystalline material contained in this fraction was recrystallized once from 95% ethanol. The crystals were dried under N_2 prior to subsequent analysis.

Analytical Procedures and Instrumentation. Melting points were determined in a Buchi apparatus fitted with an oil bath. Ultraviolet and visible absorption spectra were taken in a Cary 14 spectrophotometer fitted with 0.1-cm cells and a microbeam attachment. Infrared absorbances were measured in solutions of CCl_4 on a Perkin-Elmer 727 spectrometer. Mass spectra were determined by direct insertion of samples on an AEI Model MS-9 double focusing spectrometer with 70 ev beam intensity.

Analytical GLC was performed on a Barber-Coleman flame ionization detector, Model 5000, fitted with 183-cm glass columns, 0.318 cm in diameter and with N_2 (40 ml/min) as carrier gas. The injection port temperature was 200 C, and the detector was 240 C. Column temperatures were isothermal at 140 C. The packing consisted of 3% OV 17 on gas-chrom Q.

Prior to GLC, all samples, unknowns and standards, were derivatized with Trisil silylating reagent (Pierce Chemical) by the procedure of Coward and Smith (7) so as to yield a 10 to 50 ng/μl solution of derivatized compound in pyridine. The latter was injected in 1-μl aliquots.

***Lemna* Bioassay.** The long-day plant *Lemna gibba* L., strain G3, was used as the bioassay plant. The growth medium and culture conditions were the same as previously described (5, 6). Salicylic acid was added by sterile filtration to previously autoclaved medium. The appropriate concentration was prepared so that with the addition of 1 ml of the salicylic acid solution plus 0.5 ml of distilled water to 20 ml of culture medium the desired concentration of salicylic acid would be obtained in the resulting 21.5 ml of medium. Control flasks received 1.5 ml of distilled water. Flowering was evaluated as previously described (5, 6).

RESULTS

Preliminary studies on the active material indicated a λ_{max} in the UV at about 295 nm, a single fluorescence excitation peak with a λ_{max} at about 295 nm, and a single broad band of emission in the blue with a λ_{max} at about 410 nm.

The active crystalline material from the honeydew had a melting point of 159 ± 0.5 C. It was examined with GLC as the trimethylsilyl derivative and established to be at least 98% pure. Comparison with several different compounds suggested that the active material might be salicylic acid (*o*-hydroxybenzoic acid) since it co-chromatographed with trimethylsilyl-salicylic acid (Fig. 1).

To examine this possibility further, a sample was subjected to mass spectral analysis. A fragmentation spectrum was obtained that coincided exactly with the published mass spectra

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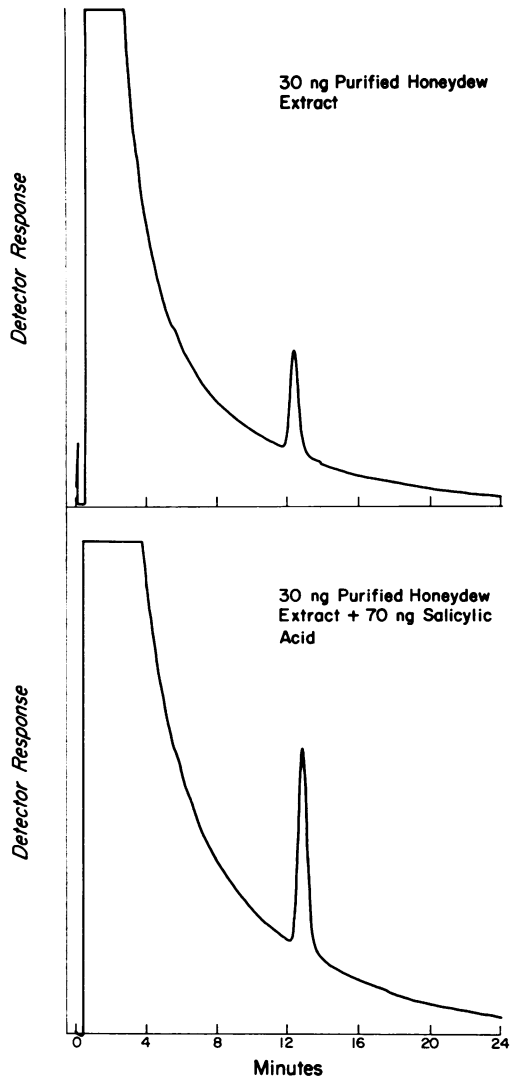

FIG. 1. Comparative GLC on the trimethylsilyl derivatives of the active flower-inducing substance from the honeydew extracts and authentic salicylic acid.

of salicylic acid (1, 12) (Fig. 2). It was characterized by a strong parent ion at m/e 138 (M^+) which underwent a multicentered elimination of water to yield a secondary fragment at m/e 120 ($M^+ - 18$). Loss of CO from this latter ion in turn accounts for the third prominent peak at m/e 92 ($M^+ - 18 - 28$).

The active material was also examined by infrared spectrometric analysis. A sample was dissolved in CCl_4 and it showed prominent absorption maxima typical for an *o*-hydroxybenzoic acid in solution (11). They were as follows: 3536 cm^{-1}, 3206 cm^{-1}, 1694 cm^{-1}, and 1662 cm^{-1}.

As a final confirmation of the structural identity of the active material the UV absorption spectrum at three pH values was obtained. The shifts in λ_{max} and E associated with each solvent were identical with those reported for salicylic acid (8).

On the basis of these analyses, the active natural material was identified as salicylic acid. As expected, authentic salicylic is able to induce flowering in *L. gibba* G3 both on a marginal daylength of 10L:14D and also on a strict short day of 9L:15D (Fig. 3). At the optimal concentration of about 5.6 μM, the flowering response is as good as that obtained in the best experiments with honeydew extracts (3, 5).

Treatment of *L. gibba* G3 with the active fraction from aphid honeydew results not only in flower induction but also in a dramatic decrease in total frond number and number of vegetative fronds and an increase in frond gibbosity (4). Despite this inhibition of growth rate, there is usually no significant decrease in average frond size. Treatment with salicylic acid at the optimal concentration results in the same changes in growth rate and frond morphology, and these observations further substantiate the conclusion that the active flower-inducing substance in the honeydew extracts is salicylic acid.

DISCUSSION

When *L. gibba* G3 is grown under long days on a simple Hoagland-type medium, flowering can often be promoted by the addition of various chelating agents to the medium (9, 10, 13). In each case, the plants were grown under photoinductive conditions but in media that did not allow good flowering. The effect of the chelators was exerted in the medium to overcome the inhibitory effect of the medium on flowering rather than in the plants to cause direct flower induction. Furthermore, the chelator effect can not be obtained on strict short days.

The medium used in the present study contains 30 μM EDTA and usually supports a level of flower induction on long days that is essentially 100%. The effect of salicylic acid on flowering is not due to any chelating properties it may have. It is

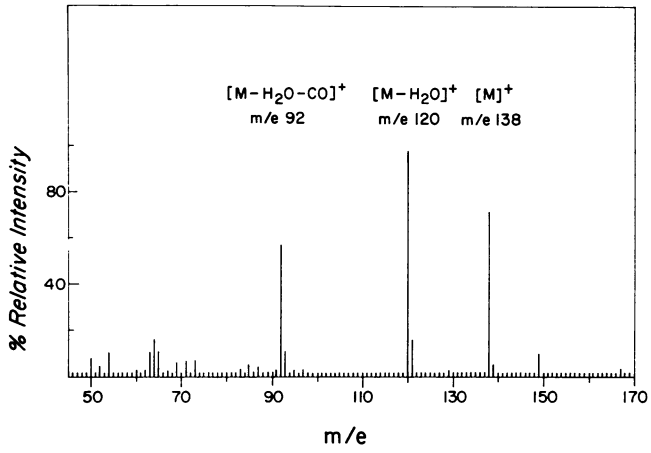

FIG. 2. Mass spectrum of the flower-inducing substance isolated from aphid honeydew.

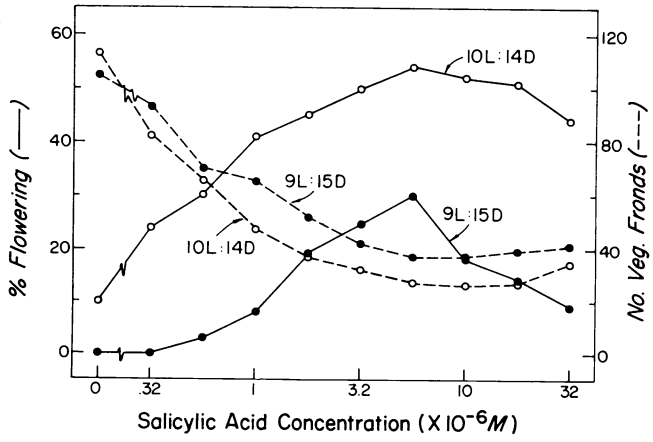

FIG. 3. Influence of salicylic acid on flowering and growth in *Lemna gibba* G3 on marginal daylength of 10L:14D and strict short day of 9L:15D.

clear that its effect is exerted on the plants directly, and for the first time a chemical substance has been identified that is capable of inducing flowering in *L. gibba* G3 on strict short days.

Salicylic acid has been tested on several other Lemnaceae for a possible effect on flowering. In preliminary experiments, it had little or no effect on flowering in two species of *Spirodela* and had only a small effect on *Lemna perpusilla* 6746. However, salicylic acid does cause quite substantial flower induction in at least one strain of *Lemna minor* and *Lemna obscura* (Cleland, unpublished).

The results of this study together with those of previous work (3, 5) clearly indicate that salicylic acid is present in the phloem of both flowering and vegetative *Xanthium* and can be obtained either by extraction of plant material or by extraction of aphid honeydew. It is interesting to note that while part of the salicylic acid in the aphid honeydew is present in the free form and thus will partition into ethyl acetate without acid hydrolysis, apparently most, if not all, of the salicylic acid in *Xanthium* is present in a bound form, presumably as a glycoside (5). This raises the question of whether the free salicylic acid in the honeydew was formed as the result of glycosidase activity in the aphid digestive tract or occurred as the honeydew was being collected on glass plates over a period of 1 to 2 weeks. The evidence strongly favors the former possibility, since free salicylic acid is found in the honeydew whether it is removed from the plates on a daily basis or allowed to accumulate on the plates for a period of up to 2 weeks. Also when all the free salicylic acid is removed from the honeydew solution by partitioning against ethyl acetate at pH 2.5, acid hydrolysis is required to obtain any additional free salicylic acid. Allowing the honeydew solution to sit at room temperature for several days does not result in the formation of additional free salicylic acid. Aphids are well known to possess glycosidase activity in their digestive tracts (2), and thus this would appear to be the main mechanism by which the free salicylic acid in the honeydew is produced.

One important question raised by this work is whether salicylic acid plays any role in the control of flowering in *Xanthium*. As already noted salicylic acid is present in both flowering and vegetative *Xanthium* and does not appear to change in concentration upon photoinduction. Application of salicylic acid to vegetative *Xanthium* either alone or in combination with gibberellic acid and/or kinetin has no effect on flowering (Cleland, unpublished). Possibly the native salicylic acid glycoside would have a different effect on flowering from that of the free salicylic acid but such experiments must await the actual identification of the salicylic acid glycoside. It seems clear that although salicylic acid in combination with other, as yet unidentified substances, might have some influence on flowering in *Xanthium*, it is not the critical limiting factor for flowering on long days in *Xanthium*.

When flowering is induced in *L. gibba* G3 by a photoperiodic regime such as 6L:7D:3L:8D there is a considerable reduction in total frond number and number of vegetative fronds as compared to 9L:15D short-day controls. Induction of flowering by salicylic acid also leads to a considerable reduction in total frond number and number of vegetative fronds. Both long days and salicylic acid cause a striking increase in frond thickness or gibbosity (4). In addition to stimulating flower initiation, long days also stimulate flower development in *L. gibba* G3 (6), but preliminary experiments suggest that salicylic acid has only a small effect on flower development. It is clear that salicylic acid is able to mimic the effect of long days on flowering and growth to a remarkable degree. This fact raises the possibility that salicylic acid or some related substance is involved in some way in the control of flowering in *L. gibba* GS. This question is currently under investigation in this laboratory.

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