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Isolation of Flower-inducing and Flower-inhibitory Factors from Aphid Honeydew[1]

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CHARLES F. CLELAND[2]
Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT

The aphid *Dactynotus ambrosiae* Thomas has been allowed to feed on vegetative or flowering plants of the short-day plant *Xanthium strumarium* L., and the honeydew which they produce is extracted and tested for an effect on flowering using the long-day plant *Lemna gibba L.*, strain G3 for the bioassay. One zone of flower-inducing activity and at least two zones of flower-inhibitory activity are consistently obtained from the honeydew extracts. The levels of flower-inducing and flower-inhibitory activity are not demonstrably different in vegetative and flowering honeydew. The honeydew extracts are inactive on *Xanthium* but do give some flower induction with the short-day plant *Lemna perpusilla* Torr., strain 6746. The flower-inducing activity is clearly of plant origin and is present in the phloem since the same active material can be obtained from vegetative or flowering *Xanthium* by methanol extraction, and honeydew produced by aphids feeding on a chemically defined synthetic diet is completely without flower-inducing activity. This is the first report of successful flower induction in the long-day plant *L. gibba* G3 by some means other than long-day treatment.

The chemical basis for the hormonal control of flowering has been the subject of numerous investigations (1, 11, 15, 20). The physiological evidence for the existence of flower-inducing substances is quite conclusive (19). There is a growing body of evidence that, at least in certain plants, flower-inhibitory substances may also play an important role in the control of flowering (8, 9, 28, 31, 32). Flowering may well be controlled not simply by the presence or absence of a single inductive substance but rather by the interaction of one or more flower-inducing and flower-inhibitory substances.

Most efforts to isolate the hormonal factors responsible for the control of flowering have utilized a variety of procedures for direct extraction of plant material (1, 15, 20). These studies have generally yielded only limited success and thus a different approach has been employed in the present study.

The hormonal factors responsible for the control of flowering move from the photoinduced leaf to the receiving buds in the phloem (19). Aphids feed on phloem sap and in the process excrete fairly large quantities of honeydew which is qualitatively quite similar to phloem sap but does show certain quantitative differences such as a reduced amino acid concentration (23, 33). Recently, it has been shown that cytokinins, gibberellins, indoleacetic acid, and abscisic acid can all be isolated from aphid honeydew with no apparent chemical change or loss in biological activity (2, 14, 22, 26). By analogy, it was hoped that the hormonal factors responsible for the control of flowering would behave in a similar manner and thus could be isolated from aphid honeydew. A preliminary report on this work has appeared elsewhere (3).

MATERIALS AND METHODS

Honeydew Production. The aphid *Dactynotus ambrosiae* Thomas was originally collected near Bloomington, Indiana in October 1966 feeding on *Xanthium* sp. It has been maintained since that time in the laboratory on the Chicago strain of the short day plant *Xanthium strumarium* L. The aphids are allowed to feed either on vegetative *Xanthium* maintained on continuous light for the production of vegetative honeydew or on flowering *Xanthium* (plants receive 5 short days before aphids are added) maintained on a 13L:11D regime for the production of flowering honeydew. The honeydew is collected on glass plates placed around the base of the plants, and every 1 to 2 weeks the honeydew is removed from the plates, dried in a desiccator, and stored at −10 C.

Aphids have also been grown on a chemically defined synthetic diet which is similar to diet A of Dadd *et al.* (7), except that the concentration of arginine and sucrose have been increased to 30 mg/100 ml and 20 g/100 ml, respectively. Individual diet sachets are prepared as previously described (24) by using sterile filtration to dispense 0.3 ml of the diet between two layers of surface-sterilized parafilm. Each sachet is placed over a 60 × 15 mm plastic Petri dish containing 20 to 30 young aphids. The diet honeydew that is produced is eluted with distilled water, freeze-dried, and stored at −10 C.

Extraction of Honeydew. For extraction, the dried honeydew was dissolved in 0.5 M phosphate buffer, pH 8.2. Preliminary experiments indicated that it was not necessary to partition against ethyl acetate at pH 8.2 since all of the flower-inducing activity could be obtained if the pH was lowered directly to 2.5 and partitioned 4 times against ethyl acetate (3). Although this fraction would be expected to contain both acidic and neutral substances, for simplicity it will be referred to as the acidic ethyl acetate fraction A. This fraction was further 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, starting at the origin, five 3-cm zones (A-1 to A-5) were scraped off the plate, eluted 3 times with 3 ml of water-saturated ethyl acetate and evaporated to dryness. In some experiments, one

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[2] Present address: Department of Botany, University of North Carolina, Chapel Hill, N.C. 27514.

or more of the zones was subdivided into two equal zones such as A-4a and A-4b.

In some experiments after taking the acidic ethyl acetate fraction, the aqueous phase was subjected to a 2-hr acid hydrolysis at pH 1 and 60 C. The pH was then readjusted to 2.5, and a second acidic ethyl acetate fraction was obtained which was purified by TLC in the same manner as for the first acidic ethyl acetate fraction.

Extraction of Xanthium. The young leaves and apices of vegetative or flowering *Xanthium* were harvested in late afternoon, quickly frozen in liquid nitrogen, and then lyophilized. The freeze-dried plant material was extracted by cold methanol, and an acidic ethyl acetate fraction was obtained according to the method of Cleland and Zeevaart (6). The buffer phase left after taking the acidic ethyl acetate fraction was subjected to acid hydrolysis (2 hr, 60 C, pH 1.0), the pH was readjusted to 2.5, and a second acidic ethyl acetate fraction was obtained. Both acidic ethyl acetate fractions were further purified by TLC in the same manner as for the honeydew extracts.

Bioassay. The primary bioassay plant was the long-day plant *Lemna gibba* L., strain G3, but in one experiment the short-day plant *Lemna perpusilla* Torr., strain 6746 was also used. For *L. gibba* G3 the plants were grown on 20 ml of E medium in 125-ml Erlenmeyer flasks (4), whereas for *L. perpusilla* 6746 the plants were grown on 20 ml of 0.5 Hutner's medium in 25 × 150 mm test tubes (27). The fractions from the honeydew or *Xanthium* extracts were dissolved in 3.3 ml of distilled water, gently shaken for 30 min, and the pH was adjusted to between 5 and 7. For each fraction, 1 ml plus 0.5 ml of distilled water was added by sterile filtration to each of three flasks or test tubes of autoclaved media. Control cultures received 1.5 ml of distilled water.

For *L. gibba* G3 each flask was inoculated with a single 4-frond colony and grown under previously defined conditions (4) for 7 or 8 days on the experimental photoperiod followed by 4 or 3 days of continuous light for a total of 11 days. The terminal 3 or 4 days of continuous light by itself causes no flowering, since the 7 short day-4 long day and 8 short day-3 long day controls were always completely vegetative. For *L. perpusilla* 6746, each test tube was inoculated with a single 3-frond colony and grown under the same conditions used for *L. gibba* G3 for 5 days on the experimental photoperiod followed by 2 short days. Control cultures given 5 long day-2 short day treatment were completely vegetative. Flowering number of vegetative fronds in a culture (4). The No. VF[3] is a measure of both growth and flowering, but significant flower induction will always be indicated by the simultaneous increase in the FL% and decrease in the No. VF. Three cultures were used for each treatment with the variation between cultures usually being quite small. Each experiment was repeated several to many times with good agreement between replicate experiments.

RESULTS

Preliminary attempts to induce flowering in *Xanthium* with honeydew extracts proved unsuccessful (3). However, when extracts of 1.5 to 5 g dry weight of vegetative or flowering honeydew were applied to *L. gibba* G3, a striking promotion of flowering, as indicated by the increase in the FL% and decrease in the No. VF, was obtained in nearly every experiment by fraction A-4 and in some experiments also by fraction A-1 (Table I). The flower-inducing activity in fraction A-4 rechromatographs to the same R_F region and corresponds to a narrow band of bright blue fluorescence under UV light of 254 nm. In addition to the flower-inducing activity, there is usually also flower-inhibitory activity in fractions A-2 and A-3.

Table I. *Influence on Flowering in Lemna gibba G3 of Acidic Ethyl Acetate Fraction Obtained from Extracts of Flowering or Vegetative Honeydew*

Photoperiod	Starting Material		Treatment					
			Control	A-1	A-2	A-3	A-4	A-5
	g dry wt							
Flowering honeydew								
9L:15D	5.0	FL%	0		0	0	18	3
		No. VF	90		74	83	44	76
10L:14D	2.0	FL%	0	14	0	0	53	0
		No. VF	145	83	118	114	25	129
11L:13D	2.0	FL%	8	52	0	1	55	0
		No. VF	130	46	141	150	48	166
12L:12D	5.0	FL%	52		0	0	68	1
		No. VF	111		90	98	29	170
Vegetative honeydew								
9L:15D	1.5	FL%	0	0	0	0	3	0
		No. VF	101	86	146	120	74	118
10L:14D	2.0	FL%	8	14	0	0	40	4
		No. VF	78	97	89	118	45	87
11L:13D	1.5	FL%	16	11	0	0	55	3
		No. VF	144	145	119	163	32	151

Preliminary experiments indicated that the plants are more sensitive on daylengths slightly longer than the critical daylength for *L. gibba* G3 (about 9.5 hr) where the controls usually show a low FL%. Therefore, most experiments have used 10L:14D or 11L:13D regimes. However, flowering has also been obtained on a strict short day regime of 9L:15D, so the effect is clearly on flower induction.

As reported earlier (3), flower promotion has also been obtained with *L. perpusilla* 6746. However, while fraction A-4 is the most active on *L. gibba* G3, for *L. perpusilla* 6746, the best activity appears to be in fraction A-1. This difference is illustrated in Table II in an experiment where each zone on the TLC plate was divided in half so that 10 fractions were taken instead of 5. Each fraction was divided with half being tested on *L. gibba* G3 and the other half being tested on *L. perpusilla* 6746. The significance of the differences in the response of *L. gibba* G3 and *L. perpusilla* 6746 to the honeydew extracts remains to be determined.

Hendricks (12) has suggested that materials moving in the phloem, such as the flowering stimulus, might be solubilized for transport by the formation of glycosides. To test this possibility the aqueous phase left after taking the first acidic ethyl acetate fraction was subjected to acid hydrolysis and then a second acidic ethyl acetate fraction was obtained. Table III shows that acid hydrolysis does yield additional flower-inducing and flower-inhibitory activity. Preliminary experiments with a crude glucosidase (Sigma) preparation suggest that at least part of this additional flower-inducing activity in fraction A-4 was present in the honeydew as a glycoside.

Although flowering and vegetative honeydew do not exhibit any apparent qualitative differences in their effect on flowering

[3] Abbreviations: No. VF: number of vegetative fronds; FL%: flowering per cent.

Table II. *Influence of Acidic Ethyl Acetate Fraction Obtained from Extract of Flowering Honeydew on Flowering in Lemna gibba G3 and Lemna perpusilla 6746*

The experiment was started with 2.4 g dry weight of flowering honeydew.

Photoperiod		Treatment										
		Control	A-1a	A-1b	A-2a	A-2b	A-3a	A-3b	A-4a	A-4b	A-5a	A-5b
L. gibba G3												
11L:13D	FL%	0	37						49	0	0	0
	No. VF	148	41						33	129	140	144
13L:11D	FL%	49			5	1	28	0				
	No. VF	85			153	147	122	169				
L. perpusilla 6746												
13L:11D	FL%	1	35	8	12	0	2	0	4	2	0	0
	No. VF	133	64	99	106	85	131	128	128	136	132	138

Table III. *Comparison of Flower-inducing and Flower-inhibitory Activities in Lemna gibba G3 by Acidic Ethyl Acetate Fractions*

Fractions were obtained from an extract of flowering honeydew either before or after acid hydrolysis. The experiment was started with 1.2 g dry weight flowering honeydew.

Treatment	FL%	No. VF
Before acid hydrolysis		
9L:15D control	0	134
A-1	0	142
A-4a	0	126
A-4b	13	74
13L:11D control	62	78
A-2	1	144
A-3	38	109
A-5	10	225
After acid hydrolysis		
9L:15D control	0	134
A-1	0	115
A-4a	0	94
A-4b	10	62
13L:11D control	62	78
A-2	4	323
A-3	0	141
A-5	5	252

(Table I), the possibility that there may exist consistent quantitative differences in the level of flower-inducing and flower-inhibitory activities between flowering and vegetative honeydew has been examined. Although a few experiments seemed encouraging, more often the results were inconclusive (3), and thus if such differences do exist they must be small and hard to resolve.

In attempting to judge the significance of these results it is important to know if the flower-inducing and flower-inhibitory activities obtained from the honeydew are really of plant origin or are instead aphid excretory products. Methanol extracts of flowering or vegetative *Xanthium* were prepared. As was the case with the honeydew extracts, the flower-inducing activity could be obtained from either flowering or vegetative *Xanthium* and there was little or no difference in the level of flower-inducing activity in the extracts of flowering and vegetative plants (Table IV).

The flower-inducing activity in the *Xanthium* extracts appears to be the same as that obtained from the honeydew since it exhibits the same blue fluorescence under 254 nm UV light and shows similar chromatographic behavior on both TLC and GLC (Cleland and Ajami, unpublished). The flower-inducing substance in the honeydew appears to be present in *Xanthium*, but this does not necessarily mean that it is present in the phloem.

To try and answer this question, aphids were allowed to feed on a completely defined synthetic diet, and the diet honeydew which they produced was then compared to honeydew from aphids feeding on flowering *Xanthium*. The results of one experiment are given in Table V and as can be seen only the flowering honeydew had any flower-inducing activity. Additional experiments have shown that the diet honeydew produced during the first 2 days after the aphids are placed on the diet does occasionally show some slight flower-inducing activity. However, this activity is almost certainly of plant origin, since diet honeydew collected starting with day 3 is completely without activity. Thus it seems clear that all of the flower-inducing activity in fraction A-4 in vegetative or flowering honeydew is of plant origin.

In fraction A-2, the diet honeydew had no apparent flower-inhibitory activity and thus this activity is also of plant origin. However, fraction A-3 of the diet honeydew did have good flower-inhibitory activity. It appears that at least part of the inhibitory activity in this zone obtained from flowering or vegetative honeydew is produced by the aphids and does not come from the plants.

Table IV. *Influence on Flowering in Lemna gibba G3 of Acidic Ethyl Acetate Fraction*

Fraction was obtained from methanol extracts of flowering or vegetative *Xanthium* following acid hydrolysis. The experiment was started with 25 g dry weight vegetative or flowering *Xanthium* apices and young leaves.

Treatment	Control		Vegetative Extract		Flowering Extract	
	FL%	No. VF	FL %	No. VF	FL%	No. VF
11L:13D control	29	61				
A-1			3	89	2	109
A-2			2	97	9	98
A-3			0	89	0	101
A-4a			0	110	0	119
A-4b			58	27	51	32
A-5			1	114	1	120

Table V. *Comparison of Flower-inducing and Flower-inhibitory Activities in Lemna gibba G3 by Acidic Ethyl Acetate Fractions*

Fractions were obtained from extracts of flowering or diet honeydew. The experiment was started with 1.5 g dry weight flowering or diet honeydew.

Treatment	Control		Flowering Honeydew		Diet Honeydew	
	FL%	No. VF	FL%	No. VF	FL%	No. VF
Before acid hydrolysis						
9L:15D control	0	78				
A-1			0	56	0	67
A-4			11	39	0	61
13L:11D control	58	46				
A-2			1	75	59	40
A-3			0	86	1	81
A-5			10	90	3	95
After acid hydrolysis						
9L:15D control	9	88				
A-1			0	61	0	74
A-4			10	43	0	76
13L:11D control	56	58				
A-2			15	140	49	76
A-3			0	84	26	90
A-5			41	84	54	58

DISCUSSION

The present study provides evidence for the existence of a substance in the phloem of vegetative and flowering *Xanthium* that can be recovered from aphid honeydew and is capable of inducing flowering in *L. gibba* G3. None of the known plant hormones are able to induce flowering in this plant. Gibberellic acid, which can induce flowering in some long-day plants, is inhibitory for flowering in *L. gibba* G3 (5, 29, 30). Flower induction by cytokinins has been reported for *Wolffia microscopica* (21) and *Lemna paucicostata* (10). In *L. gibba* G3, treatment with kinetin or zeatin may produce a slight increase in flowering on marginal daylengths such as 11L:13D but has never caused any flower induction on strict short days (Cleland, unpublished). Both indoleacetic acid (25) and abscisic acid (Cleland, unpublished) are inhibitory for flowering. The results of the present study provide the first evidence for the induction of flowering in *L. gibba* G3 by some means other than long-day treatment.

The response of the short-day plant *L. perpusilla* 6746 to the honeydew extracts is somewhat different from that of *L. gibba* G3. Whereas fraction A-4 is consistently the most active on *L. gibba* G3, fraction A-1 has been the most active in most experiments with *L. perpusilla* 6746. The significance of this difference is not understood. However, since a variety of chemical substances are known that are capable of promoting flowering in *L. perpusilla* 6746 such as copper (13), lithium (16), ascorbic acid (17), and acetylcholine (18), promotion of flowering in this plant is perhaps not as significant as in *L. gibba* G3.

The lack of consistent differences in the flower-inducing and flower-inhibitory activities in flowering and vegetative honeydew can be explained in at least two ways. First of all it may be that the active factors isolated from aphid honeydew do not have any controlling influence on flowering in *Xanthium*. On the other hand, flowering may be controlled by several interacting substances which show only a quantitative change in response to a change in daylength. It is possible that the concentration of these active factors does change with photoinduction but that the size of the changes is so small that it has not yet been possible to resolve them with the approach used in this study.

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