Chemical Nature of an Insect Gall Growth-Factor [1, 2]

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Introduction

Although insect galls on higher plants have been the subject of a large number of investigations and a vast descriptive literature on the subject has accumulated (3), our knowledge of the physiology of gall formation and development remains meager. It is, however, now generally agreed that gall formation is caused by chemical compounds of insect origin (see review in 14). Several workers have obtained abnormal proliferation of plant tissue by applying extracts of gall-forming insects or of organs of such insects (1, 7, 8, 9, 10, 12). The difficulties inherent in assaying such preparations for gall-forming activity are illustrated by the work of Leatherdale (7), who injected extracts of *Dasyneura urticae* into immature leaves of *Urtica dioica* by means of an ultrafine hypodermic needle. This treatment produced abnormal growth in only 12 out of 150 injections of whole insect extracts and in 9 out of 50 injections of extract of larval heads. No abnormal growth was observed in water-treated controls. The fact that such injections do not, in general, produce typical galls should not be considered surprising since gall formation probably depends upon the precise placing of the right amount of the appropriate chemicals in a way that is difficult if not impossible to duplicate experimentally (2).

Sawflies of the genus Pontania cause the formation of simple galls on the leaves and stems of various species of Salix (willow) (15). In the classic work of Beyerinck it was demonstrated that the initial stimulus for the formation of Pontania galls comes from fluid which is formed in an accessory gland and which is injected by the adult female into the plant together with the egg [quoted by Küster (6)]. This is an exceptional situation, for with most gall-forming Hymenopterous insects there is no proliferation of plant tissue until after emergence of the larva (16). Pontania galls do, however, require stimulation from the larva for complete development. Indeed, if the young larva is removed from a developing Pontania gall, the growth of that gall almost ceases. Hovanitz (4 & unpublished) has recently demonstrated that injection of extracts of Pontania-accessory glands into such larva-free galls suffices to maintain growth at a rate which is well above that characteristic of untreated larva-free galls and which, in some cases, approaches that of galls containing larvae. This response can be made to serve as the basis of a bioassay for gall-growth promoting substances, which avoids much of the uncertainty and labor involved in testing materials by simply injecting them into normal plant tissue.

In this paper we are concerned with the chemical nature of gall-growth promoting substances present in the accessory glands of Pontania.

Materials & Methods

► Plants & Insects. We collected willow leaves (*Salix alba*) bearing developing *Pontania pacifica* galls locally and placed them in covered plastic boxes containing several layers of paper towel. These boxes we stored at 7 C in the dark for several months. During this time the larvae complete their development, and leave the galls to pupate among the leaves and paper at the bottom of the box. The adults begin to emerge after about four to six months of pupation. Since the life span of adult Pontania is brief, we made daily collections of insects. The males we discarded and we either dissected or froze the females.

► Preparation of Material. The accessory glands and accompanying sacs, referred to jointly as glands, (fig 1) were carefully excised from female sawflies under a binocular microscope and immediately frozen. They were stored at −20 C until used.

In the initial stages of this work various extractive media were used. These included dilute sodium chloride solution, distilled water, aqueous phenol, and 80 % ethanol. Physiologically active material was recovered with each of these solvents. Because of its simplicity, the following method was used in subsequent experiments.

A small amount of 80 % ethanol was added to vials containing 50 or 100 frozen glands. The glands were then macerated in ethanol, ground in a small, all-glass homogenizer and the grindate centrifuged at 2,500 rpm in a clinical centrifuge. The supernatant was decanted, the residue washed with 80 % ethanol and

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again centrifuged. The combined supernatants were evaporated.

In experiments in which whole extract was used, a small volume of water was added to the dried supernatant, the mixture thoroughly stirred, centrifuged, and the pellet discarded.

In some experiments paper chromatography was used to fractionate the material. The dried residue was taken up in water and applied as a narrow band to washed Whatman 3MM filter paper (a band 5 inches long gave good results with an extract of 400 glands). The initial separation was carried out in *n*-butanol-acetic acid-water (4:1:1.8 v/v) solvent (BAW).

► Bioassay of Gall Growth Promoters. A bioassay devised by the third author was used. Cuttings of the willow trees (*Salix alba*) from which the insects had been collected were rooted and planted in a quartz-vermiculite mixture in plastic containers. They were watered daily with nutrient solution. Temperature was controlled at 13 C night and 20 C day. The plants were exposed to natural daylight supplemented with artificial light for a photoperiod of 16 hours. When the cuttings were well established, they were transferred to an artificial light room (light intensity about 500 ft-c, photoperiod 16 hr, temperature 23 C) where they remained until completion of the bioassay.

The plant was covered with a wire mesh cage into which a newly emerged female Pontania was introduced and allowed to remain for several hours. After a few days small galls could be found on some of the young leaves. After these had developed for 10 to 12 days (counting from the time of egg deposition) most of the galls were opened and the larva from each removed. The openings of these galls were carefully covered with small pieces of cover glass held in place with casein glue. The side of the gall opposite the cover glass was pierced with a fine glass needle and a solution of the material to be tested was injected into the hollow center of the gall (about 2 μl per injection). Injections were repeated every two or three days, usually until a total of seven injections had been made. At the same time, the length, width, and height of each gall were measured with a pair of fine calipers which were read to 0.1 mm. The three values thus obtained were multiplied together to give a volume parameter. Since the galls were not all of the same size at the start of the experiment the volume was converted to relative volume (i.e., volume of gall ÷ volume at first injection) for comparative purposes. Measurement was discontinued after about four weeks from the first injection since the larvae in the control galls complete their development and begin to emerge after about this time.

Galls injected with glass-distilled water served as controls while other galls from which the larva had not been removed were used as an indication of maximum growth expected under the conditions of a particular experiment.

Experience indicated that best results were obtained when only one gall per leaf was used. When more than one gall occurred on the same leaf all but one were left uncovered after removal of the larva.

While no special precautions were taken to prevent microbial contamination (indeed, sterile conditions would be exceedingly difficult to achieve) growth of such organisms was observed only when plants with very high water content were used. In the few cases where growth of mold was observed, the experiment was discarded.

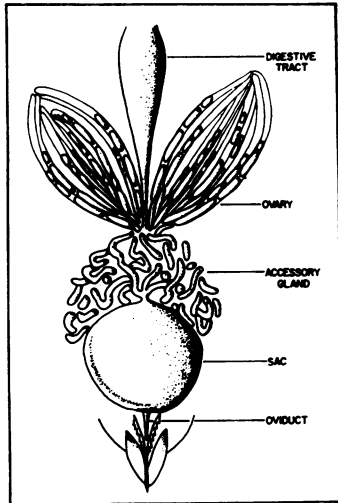
Results

► Experience With Bioassay. It must be emphasized at the outset that the bioassay is far from standardized. Different amounts of growth are obtained in successive experiments with the same material in galls on plants originating from cuttings taken from the same tree. Therefore, the results of different experiments can be compared from a qualitative point of view only.

Cuttings from some trees (especially those with soft tissue and high water content) are unsuitable for assay purposes since substantial growth occurred in most distilled water controls. Even when the most suitable plants were used, occasional distilled water controls grew far more than expected. For this reason, several distilled water treatments were included in later experiments.

► Preliminary Experiments on Nature of Growth-Promoting Factor. Figure 2 shows the growth-promoting activity of a 0.9 % sodium chloride extract of glands. The first experiments were directed toward determining whether the active material in this extract is a small molecule or macromolecule. Glands were macerated in 0.9 % sodium chloride solution and the mixture clarified by centrifugation. The extract was then dialyzed against salt solution of the same strength for 20 hours. The dialyzable fraction of extract possessed considerable activity although some activity also remained in the nondialyzable fraction. In a second experiment the protein of a similar extract was removed by shaking the saline extract with 95 % phenol. The phenol was then removed from the aqueous layer with diethylether. Material prepared in this way contained dialyzable growth-promoting material. From these results it appears that low molecular weight substances capable of promoting gall growth are present in the extracts. The ultraviolet-absorption spectrum of a crude extract of Pontania accessory glands (fig 3) shows rapidly increasing absorption from about 300 mμ down, with a peak at 260 mμ. As can be seen from figures 4 and 5, all the material giving rise to the 260 mμ peak was recovered in the dialyzable fraction and must, therefore, possess a low molecular weight.

Figure 6 shows that increasing concentrations of the dialyzable portion of the gland extract cause greater and greater gall growth up to the highest concentrations tested. Concentration is expressed in glands per injection, that is, the number of glands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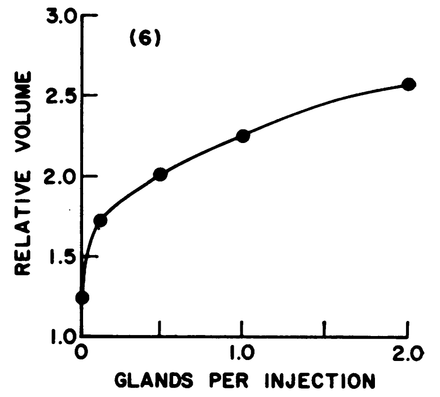
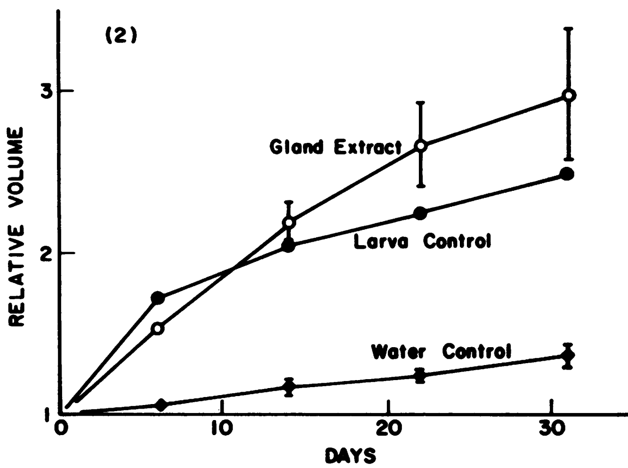
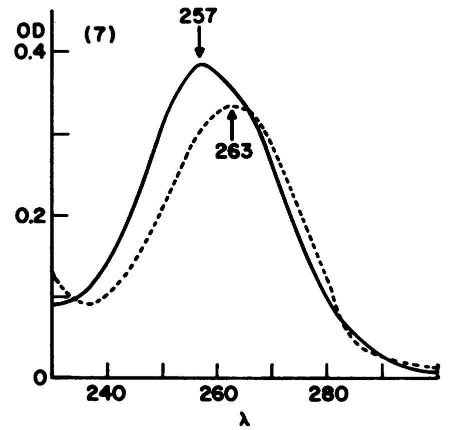

(6)
RELATIVE VOLUME
GLANDS PER INJECTION


(2)
RELATIVE VOLUME
Gland Extract
Larva Control
Water Control
DAYS


(7)
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263
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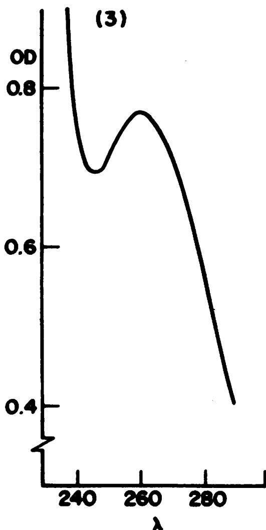
(3)
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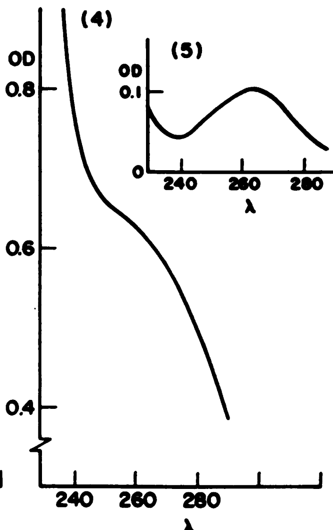
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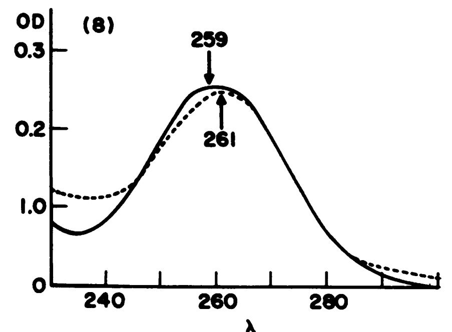
(8)
OD
259
261
λ

used to obtain the dialyzed material injected in a single injection (2μl).

The dialyzable fraction of the extract was subjected to a number of treatments and then assayed, with the following results: Physiologically active material was stable to heating at 100 C for 15 minutes in solution, stable to pH 13 or pH 1 for 15 minutes at room temperature, and to be adsorbed by charcoal from aqueous solutions. Attempts to purify the active factor by selective elution from charcoal failed, although some active material was eluted with 20 % ethanol, more with 80 % ethanol, and still more with 5 % aqueous phenol. Similarly, attempts to purify the active factor by means of ion exchange resins were unsuccessful. Active material was found in the acidic basic, and neutral eluates from the resin.

► Chromatographic Analysis of Gland Extract. Paper chromatography using *n*-butanol-acetic acid-water (4:1:1.8; v/v) and isopropanol-conc HCl-water (170:41:39; v/v) showed that the dialyzable fraction of the gland extract consists largely of six main components. One of these has been identified as glutamic acid on the basis of its paper chromatographic and electrophoretic properties. Each of the other major components absorbs ultraviolet light. In addition, several minor compounds are present. These include three ninhydrin positive compounds, four compounds which give a faint yellow color with *p*-dimethylaminobenzaldehyde (ureides or indoles) and some trace ultraviolet-absorbing compounds. Carbohydrates and phenolic compounds are not detectable.

The major ultraviolet-absorbing compounds have been examined in some detail. On the basis of their chromatographic (table I) and electrophoretic properties as well as their spectra in both acid and base, two of these compounds have been identified as uridine and uric acid. The other three ultraviolet-absorbing compounds (designated A, B, & C) have been partially characterized. The chromatographic properties of these compounds are given in table I and their spectra are shown in figures 7 to 9.

Treatment of compound A with 6 N HCl for 1 hour at 105 C produced free adenine. Tests for the presence of phosphate and for the ability to reduce periodate were negative so that compound A cannot be a ribotide or nucleoside. Its chromatographic properties are such as to exclude the possibility that it is deoxyadenosine. Thus, compound A appears to be an unusual adenine derivative.

Compound B likewise appears to be an unusual adenine derivative which does not reduce periodate or contain phosphate.

The remaining ultraviolet-absorbing compound has an absorption spectrum in acid which is suggestive of that of nicotinic acid. However, in contrast to the marked difference between the spectra of nicotinic acid in acid and in base, the spectrum of compound C in base is similar to that in acid. Treatment with 6 N HCl for 2-½ hours at 105 C produces two new compounds. One of these (Rf in isopropanol-HCl = 0.48) gives a bright blue fluorescence under ultraviolet light, the other (Rf = 0.70) absorbs ultraviolet light.

We attempted to isolate these unknown compounds from extracts of whole female Pontania, and thus to avoid the time-consuming dissection. Chromatographic techniques permitted the isolation of compound A only from such extracts. The other compounds were all obscured by a large quantity of material which is not present in the gland extracts. The preparation of the unknown compounds from extracts of excised abdomens was also attempted. This gave somewhat better results but the losses involved in purifying the unknowns are considerable so that, considering the labor involved in excising the abdo-

Table I

Chromatographic Properties of the Major Constituents of Pontania Gland Extract

Compound	Rf × 100 in: η-Butanol-acetic acid-water (4:1:1.8)	isopropanol-conc HCl-water (170:41:39)	η-Butanol: conc NH_4OH: water (86:41:13)
From extract:			
Uric acid	23*	20	...
Uridine	42	55	...
Glutamic acid	28	...	...
A	10	06	00
B	15	34	...
C	33	50	...
Pure compounds:			
Uric acid	20*	18	...
Uridine	38	58	...
Glutamic acid	29	...	...

* Elongated spot

Fig. 1 (*top left*). Accessory gland and sac of *Pontania pacifica*.
Fig. 2. Growth promoting activity of Pontania accessory gland extract.
Fig. 3. Spectrum of the crude extract of Pontania glands.
Fig. 4. Spectrum of the dialyzed gland extract.
Fig. 5. Spectrum of the dialyzable fraction of gland extract.
Fig. 6. Dose-response curve for the stimulation of gall growth by the dialyzable fraction of gland extract.
Figs. 7-8. Absorption spectra of compounds obtained from Pontania glands. *Solid line*, pH 1 to 2. *Dotted line*, pH 12. Figure 7, compound A. Figure 8, compound B.

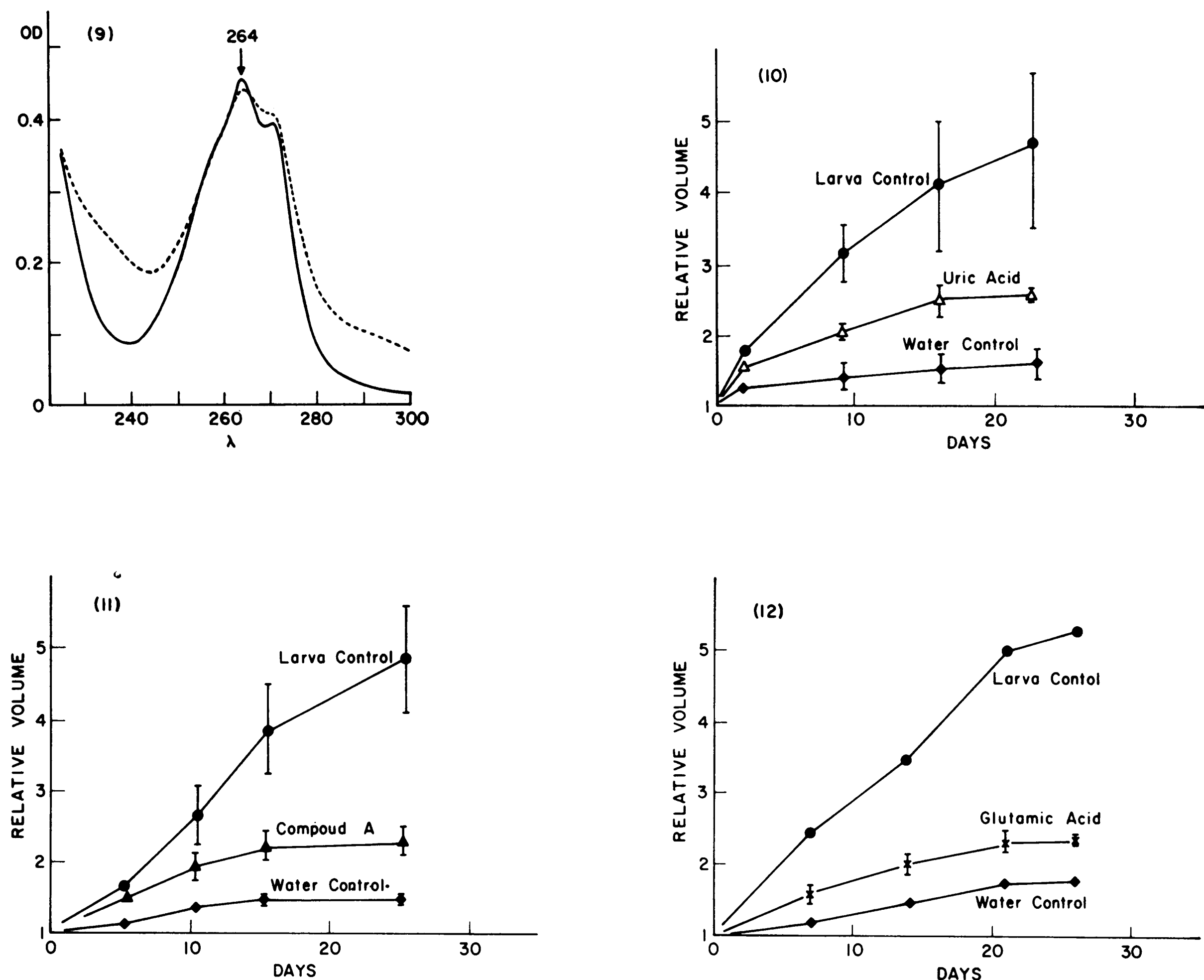

Fig. 9. Absorption spectra of compound C obtained from Pontania glands. *Solid line,* pH 1 to 2. *Dotted line,* pH 12.

Figs. 10-12. Growth rates of galls injected with various compounds. The time at which the first injection was made is given as day zero. Figure 10, growth with uric acid. Figure 11, growth with compound A. Figure 12, growth with glutamic acid.

mens, this procedure really offers little advantage over dissection and extraction of accessory glands.

From measurements of the optical density at 260 mμ, it is estimated that the main ultraviolet-absorbing compounds are present in the amounts of the order of 0.3 to 1 μg per gland. The amount of glutamic acid present (estimated by comparing the spot on paper chromatograms after ninhydrin treatment with those given by a glutamic acid concentration series applied to the same chromatogram) is approximately five μg per gland.

▶ Biological Activity of Compounds from Pontania Accessory Glands. The main constituents of Pontania accessory glands were tested individually for gall-growth promoting activity. At a concentration of about 50 mg/l (0.1 μg per injection), uric acid (fig 10) and the two adenine-containing compounds (A & B) are active in stimulating growth (data for A are shown in figure 11 and similar results were obtained with B), while uridine showed slight activity. Glutamic acid when supplied at the relatively high concentration of 400 mg per l (0.8 μg/injection) showed some growth-promoting activity (fig 12). Compound C possessed no detectable growth-promoting activity.

▶ Extract of Pontania Larvae. Young Pontania larvae were removed from developing galls and extracted with 80 % ethanol as described above. The extract showed considerable growth-promoting activity and yielded several ultraviolet-absorbing zones when chromatographed in BAW. These zones were not further examined.

▶ Biological Activity of Other Compounds. A number of other compounds have been tested for the ability to promote gall growth. The first of these to be tested, kinetin, is active, but no more so than adenine, adenosine, or deoxyadenosine supplied at the same

concentration. The pyrimidines, cytosine and uracil have slight growth-promoting activity as does indole-3-acetic acid.

Data obtained by application of the Xanthium leaf disc assay (11) for kinins to the Pontania gland extract indicate that such substances, if they are present at all, cannot exceed the equivalent of 0.004 μg kinetin per gland. Since the minimum amount of kinetin which stimulates gall growth is of the order of 0.1 μg per injection, it is apparent that kinins are not an important part of the Pontania gall-growth stimulating fluid.

It should be noted that no single compound consistently produced as great a response as did the mixture of materials in the dialyzable fraction of Pontania accessory gland extracts. However, rapid and sustained growth was obtained when a mixture of indole-3-acetic acid (10 mg/l), adenine (50 mg/l), and kinetin (10 mg/l) was injected (fig 13).

Fig. 13. Left, a mature gall which contains a live larva. Right, a gall of similar age from which the larva had been removed 12 days after the egg was deposited and which was injected at 2 to 3-day intervals with 2 μl of a solution containing IAA (10 mg/l), adenine (50 mg/l), and kinetin (10 mg/l).

Discussion & Summary

The results presented in the previous section demonstate that low molecular weight materials present in the accessory glands of Pontania possess gall growth promoting activity. It is also apparent from chromatographic analysis that six compounds make up the bulk of the dialyzable fraction of such extracts. The results of biological assays on these compounds show that four of them, uric acid, the two unidentified adenine derivatives, glutamic acid, and possibly uridine as well, are present in the gland in large enough amounts and have sufficient growth-promoting activity to be of physiological importance. Whether the compounds which stimulate the growth of preformed galls are also responsible for gall initiation is, at present, an open question.

Pelet et al. (13) have found that both auxin and kinetin are required for the growth of insect gall tissue in vitro and that addition of adenine and casein hydrolysate to the medium further stimulates growth.

It seems reasonable to assume that the growth substances which are supplied by the insect interact with those of the plant. In this connection it is worth noting that, while the Pontania gland extracts do not appear to contain material with kinin activity, these insects initiate their galls adjacent to vascular tissue which has been reported to be a source of kinin (5).

While detailed chemical knowledge is for the most part lacking, it is nonetheless apparent from a survey of the literature that different species of insects use different substances to stimulate gall growth on plants (14). Since normal growth can undoubtedly be disturbed by a number of chemicals, it is not surprising that in the course of evolution various species have developed different techniques by which they mold plant tissue to their own uses.

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