THESIS

# PLANT GROWTH HORMONES IN PINYON INSECT GALLS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JOHN ALLEN BYERS ENTITLED PLANT GROWTH HORMONES IN PINYON INSECT GALLS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

PLANT GROWTH HORMONES IN PINYON INSECT GALLS

The larvae of the midge, Janetiella sp. near coloradensis Felt. cause galls at the base of young pinyon needles. Some chemical substance induced in the needle tissue or secreted by the larvae is responsible for initiating and sustaining gall formation. Plant growth hormones in normal pinyon needles and round galls were investigated to determine their role in gall growth. Auxin and gibberellic acid were assayed in pinyon tissue. Round gall tissue, on a weight basis, had from 2.5 to 6.5 times more auxin than normal needle tissue; gibberellic acid was found to increase from 2 to more than 7-fold in gall tissue. On a per needle basis, round galls had from 11 to 27 times more auxin and 6 to 31 times more gibberellin than normal needles. The insect larvae did not contain indoleacetic acid in detectable quantities while it was not known whether traces of gibberellin-like activity found in larvae showed that they secrete gibberellins.

Peroxidase isozyme patterns in polyacrylamide gels were found to change as the gall formed. One peroxidase isozyme appeared to increase in gall tissue while a second peroxidase normally present was missing. Peroxidase can act as an indoleacetic acid oxidase to destroy auxin (IAA) and its growth promoting activity. The possible

role of orthophenols and peroxidases in auxin metabolism are discussed as a mechanism for gall formation.

Round galls, stubby galls, and normal needles were thin sectioned and stained to show the morphogenetic changes in gall tissues. Both cell enlargement and cell division resulted.

The high concentrations of auxin and gibberellin in round galls strongly implicate these hormones in gall formation. However, the levels of the hormone found in the gall tissue may not be high enough to be the direct cause of gall formation and may just be present as a secondary response.

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# INTRODUCTION

Trees and other plants may be attacked by insects, bacteria, or fungi that induce or promote abnormal growth of the plant tissue. The abnormal growth, often called a tumor or gall, is detrimental to the plant or tree because of the associated slower growth and deformity.

Pinyon trees, Pinus edulis Engelmann have several gall midge species which inhabit needles and induce the formation of galls. The larval midge brings about abnormal needle growth, presumably through some chemical interaction, and the chemical differences between normal and galled needles should provide much needed information on the physiology of gall formation.

In this study, the nature of the chemical interaction is presumed to be hormonal. The gall midge may either induce the plant to produce extra growth hormones or the midge may secrete these specific hormones directly. A change in hormone type or content in gall tissue would strongly suggest their prominent role in gall formation. Therefore, the types and quantities of the plant growth hormone classes, auxins and gibberellins, were determined in extracts of the gall midge larva and its respective gall (the round needle gall) and compared to the growth hormones in normal pinyon needles. The peroxidase isozymes were investigated in normal and galled

tissue to see if substantial changes in this family of enzymes could account for an alteration in auxin metabolism. Additional evidence for the hormonal theory of gall formation is presented in the form of histological and chemical differences between normal and galled tissue.

# REVIEW OF LITERATURE

## Pinyon

Pinyon pine, Pinus edulis Engelmann 1948, is a xerophilic tree growing at elevations from 5,000 to 9,000 feet throughout southern Colorado and southwestern United States (Sudworth 1917, Phillips 1909). It has two and occasionally three needles per fasicle. The needles are sharp pointed, curved, and range in length from 7/8 to 1 3/4 inches with smooth margins and two resin ducts in cross section (Sudworth 1917). Rehder (1940) reported that needles do not have stomata on their back. However, sectioned needles examined under a microscope showed stomata on all sides of the needle (Fig. 17 B page 87). Most needles remain on the tree for at least four years while many remain up to nine years (Sudworth 1917). However, if the needles are galled they fall from the tree in a year or less (Brewer 1972).

## Gall Midges in Pinyon

The family Cecidomyiidae (also know as Itonididae) is a group of nematocerous Diptera containing more than 5,000 species. While various species are fungus feeders or predators, the majority are gall makers. The legless larvae have a small dorsal sclerotic

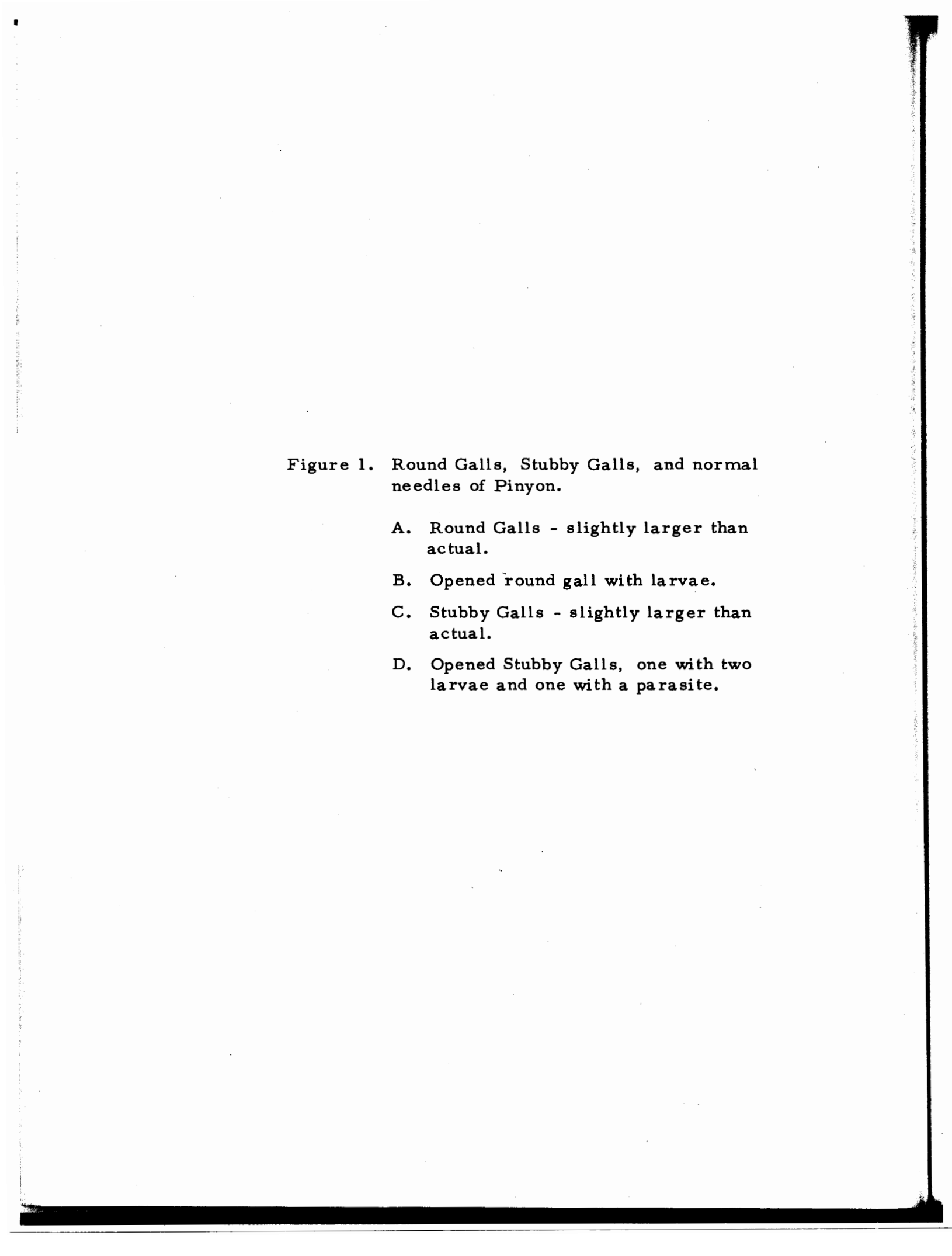


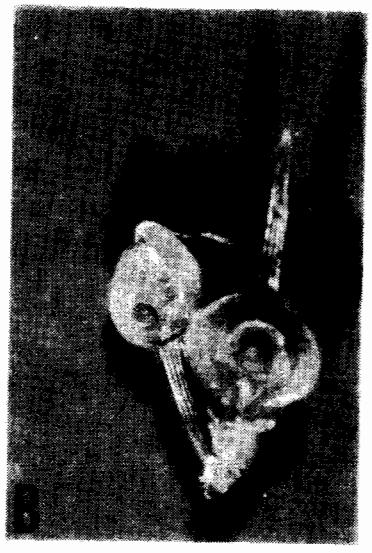
Figure 1. Round Galls, Stubby Galls, and normal needles of Pinyon.

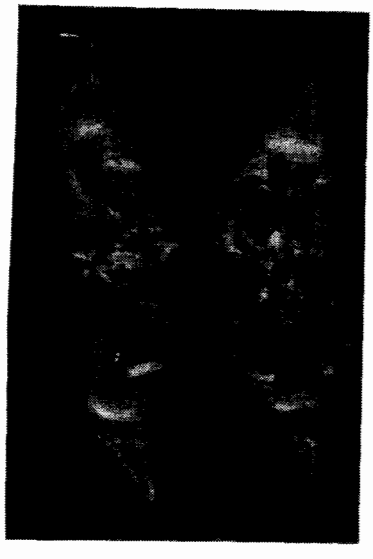
A. Round Galls - slightly larger than actual.

B. Opened round gall with larvae.

C. Stubby Galls - slightly larger than actual.

D. Opened Stubby Galls, one with two larvae and one with a parasite.





spatula behind the head and very well-developed salivary glands (Mani 1964). The glands are used for the secretion of digestive enzymes and perhaps the chemical factor(s) responsible for gall formation.

Over 20 species of gall midges are associated with the genus Pinus (Barnes 1951). There are five species of gall midges (Diptera:Cecidomyiidae) known to produce characteristic galls in pinyon (Brewer 1971, Brewer and Houseweart 1972, Little 1943, Doane et al. 1936, Houseweart and Brewer 1972).

Pinyon Round Needle Gall and Midge

The pinyon round needle gall is caused by one or more cecidomyiid larva (ae) reported tentatively as Janetiella coloradensis (Little 1943), because the adults have never been reared (Brewer 1972). Little (1943) reported that this midge produces a "spherical, hard, almost solid brown swelling about 3/16 inch in diameter" at the base of two needles. Little's description is correct for round galls that have matured and senesced. As the galls are forming, however, they are either green or red and medium soft with watery tissue (Fig. 1 A, B page 5).

The midges pupate in the soil and adults emerge usually in May or June. The midges mate and the female lays eggs in the candle (forming bud with immature needles) about the middle of June. The eggs hatch in about one week followed by gall formation. The

round galls grow rapidly until mid-September and senesce during September and October. The midge larvae leave the gall and overwinter in the soil as mature larvae (Brewer 1972).

Pinyon Stubby Needle Gall and Midge

The gall is produced by Janetiella sp. near coloradensis Felt (Brewer 1972). The closest description of the stubby gall is given by Doane et al. (1936) who reports that, "kidney-shaped enlargements 7 mm long and 4 mm in diameter at the base of the needles of Pinus edulis are caused by Thecodiplosis cockerelli Felt." The taxonomy of this midge is contradictory since two different names have been given to the stubby gall midge. The life history of the stubby gall midge is similar to the round gall midge but unlike the latter it overwinters in the gall (Brewer 1972, Fig. 1 C, D page 5).

## Plant Galls

Mani (1964) stated that "galls are pathologically developed cells, tissues or organs of plants that have risen mostly by hypertrophy (cell enlargement) and hyperplasia (cell proliferation) under the influence of parasitic organisms like bacteria, fungi, nematodes, mites, or insects."

Plant galls are for the benefit of the parasitic gall-maker and not the host plant. The gall-maker derives nourishment and obtains shelter from the plant gall. The gall protects the larvae from

predators and parasites and prevents desiccation (Frost 1942). On the other hand, the plant diverts its resources to the gall, loses substance, has impaired sap flow, and incures other injuries (Mani 1964). Only in the case of leguminous plants does galling provide some benefits since Rhizobium fixes $N_2$ for the plant (Mani 1964). However, Zweigelt (1941) proposed that the plant benefits from galls which isolate the gall-former.

Galls occur on all plants from algae and fungi to higher plants. Insects, however, predominately incite galls on gymnosperms and angiosperms. Dicotytedons have over 92% of all types of plant galls while monocotyledons account for about 6% and gymnosperms 2%. (Mani 1964). At least one-half of the higher plant families are attacked by gall insects. Frost (1942) stated that, "The Cecidomyiidae alone attack 69 families and 202 genera" of plants.

Although all parts of the plant may be galled, the abundance of gall types varies with the plant part. For instance, cynipid wasps on Quercus, which represent 5% of the world's galls, have 5% of the species on roots, 5% on buds, 22% on branches, 2% on flowers, 4% on acorns and 63% on leaves (Mani 1964).

Six orders of insects cause plant galls: Coleoptera, Lepidoptera, Homoptera, Thysanoptera, Diptera, and Hymenoptera (Frost 1942). Felt (1917) lists 1,278 insect gall species in North America of which there are 12 Coleoptera, 17 Lepidoptera, 60

Homoptera, 701 Diptera, and 488 Hymenoptera. Six hundred eighty-two of the Diptera are in the family Cecidomyiidae.

Mani (1964) divides galls into two basic groups: organoid and histioid. Organoid galls are histologically identical to normal tissue but are shaped into abnormal looking structures. Examples of organoid galls are abnormally shaped leaves, elongated or stunted internodes, greening of petals, and witches' brooms. Parasitic fungi (Uredinaceae, Ustilaginacea, Exoascaceae, etc.), mites, and aphids are the incitants in organoid galls. Histioid galls are composed of abnormal cells which have undergone hypertrophy and hyperplasia (not always both). These galls are produced mainly by Diptera (Cecidomyiidae) and Hymenoptera (Cynipidae) (Mani 1964).

Histioid galls show practically all gradations of tissue differentiation from the normal state. The epidermal cells of galled tissue usually are larger and sometimes several cell layers thick (Kuster 1925). The number of stomata usually is decreased in gall tissue as well as the presence of chlorophyll (Mani 1964). Cells from various galls often are reported to contain larger nuclei and sometimes several nuclei, a change in chromosome number, and disintegration of plastids and mitochondria (Mani 1964).

Using the classification of Frost (1942), histioid galls can be subdivided into open and closed type galls. Open galls are formed by insects, etc., initially feeding on the tissue surface but as feeding

continues, the tissue envelopes the gall-formers. Examples are haustellate forms such as aphids, psyllids, coccids, and mites. Young are often produced within the galls and several individuals inhabit each gall. Closed galls are formed by larval insects that bore into the tissue which subsequently galls. Coleoptera, Lepidoptera, Diptera, and Hymenoptera are the orders of insects whose larvae cause closed galls. Typically, only one (monothalamous) or a few (polythalamous) larvae inhabit the closed gall. No mating of adults occurs in these galls, only the larval development (Frost 1942).

Galls arise almost exlusively in meristematic tissue (Frost 1942). Anaplasia refers to tissue which is in the juvenile or meristematic state. The tissue is galled when it is young and often the tissue remains in the anaplastic state (Stonier 1969, Hovanitz 1959).

## Gall Formation

The formation and development of plant galls involves an intricate relationship between the organism inducing the gall and the plant forming the gall. The organism must impart something that interacts with the plant to evoke the gall.

The stimulus for gall formation was suggested first by Malpighi (1687) to be of animal origin. Carter (1962) stated the four main hypotheses for the stimulation of galls: (1) "the physical irritation

set up by the presence of a foreign body", (2) "the injection of a stimulating fluid at the time of oviposition", (3) "the excretion of metabolic products", and, (4) "the secretion of an active saliva." The idea that a physical irritation by a foreign body or insect could cause gall formation has been discredited (Hovanitz 1959, Carter 1962). Physical callus galls occur due to mechanical injury but these are non-biological and only serve in wound healing. Furthermore, wounding causes the release of cell contents which can then affect other normal cells (Mani 1964). Other investigators have tried to induce galls by poking and pricking, but to no avail (Horanitz 1959, Carter 1962). Finally, it commonly is observed that not all insects of the same family induce galls even though they feed in the same manner (Mani 1964).

Wasps of the genus Pontania have growth promoting substances which are injected with their ovipositor. The eggs are laid during the injection and when they hatch, a partial gall has already formed. If the eggs are removed, however, the gall stops development indicating a continued need for a gall-inducing chemical from the larvae (Horanitz 1959, McCalla et al. 1962, Carter 1962). Gall formation caused by egg injection is not common and in any case does not form the entire gall but only initiates it. In the Cecidomyiidae the eggs are laid on the plant tissue (no injection) (Snodgrass 1935) so the gall is induced entirely by the larvae (ae).

There are few reports concerning gall forming chemicals in excretory waste. Kuster (1911) reported that the excrement of Pontania salicis can produce hyperplasia. Leaf-mining larvae also cause outgrowths in poplar leaves which are in contact with fecal pellets (LaRue 1935).

Most galls are probably induced by chemicals injected or secreted from the salivary glands. Parr (1939) injected pine needles with extracts of salivary glands of Matsucoccus gallicolus and produced galls somewhat typical of the insect. Cosens (1912) showed that cynipid saliva will induce gall-like formations on oak leaves. Mani (1964) reported several workers have induced artificial galls from salivary extracts of gall insects. It is generally accepted that the chemical which causes galls is usually found in the saliva but the nature of the chemical is not well understood.

## Cecidotoxins

A cecidotoxin or cecidogen is a chemical substance which upsets the normal physiology of the plant tissue and brings about gall formation.

### Organic and Inorganic Chemicals

Several investigators have tried to determine the natural cecidogen by applying various amounts of practically any type of chemical to plants. Silbenberg (1909) found that solutions of zinc

sulphate cause tubers of Solanum tuberosum to form callus tissue. Perti (1923), using a 0.2% solution of sodium glycolate, induced swellings on Vitis vinifera. A 1% lactic acid solution applied to tubers of Daucus carota formed callus tissue similar to crown gall (Blumenthal and Meyer 1924). Komuro (1931) immersed seedlings of Vicia faba and Pisum sativum in coal-tar and produced giant galls. Painting plants with Scharlach-red, coal-tar and 1,2,5,6-benzopyrene increased division of cells and produced galls (Gavaudan and Gavaudan 1939). Application of 2% L-tryptophan in lanolin induced tumors in Phaseolus (Murray and Whiting 1946). Meristem tissue produced crown gall like tissue when 1,2,5,6-dibenzanthracene, 3,4-benzopyrene, and 20-methylcholanthrene were applied (Levine 1950). The diversity of chemicals that will induce gall-like tissue probably indicates that these cecidogens work by partially destroying the tissue which reacts by forming callus, as if wounded. These chemicals tell us nothing about the mechanism of gall formation or that they are really involved in the natural system.

Another group of investigators looked for substances in gall tissue which were not found in normal plants. Most of the work has been done with the saliva of gall aphids. This saliva has a high enzyme and amino acid content. However, the presence of invertase, protease, and amylase enzymes in aphid saliva has been disregarded as an active cecidogen since other aphids which are not associated

with gall formation have these enzymes also (Mani 1964). Anders (1961), however, thinks that the amino acids lysine, histidine and tryptophan are cecidogenic. Anders found that these amino acids are not present in gall aphid saliva but are breakdown products of the cell due to the aphid's proteolytic enzymes. The gall aphid must regurgitate the amino acids or be inefficient in sucking them out of the plant in order for gall formation to result (Mani 1964). Therefore, it seems unlikely that amino acids are the cecidogen in most galls since amino acids have no known hormonal activity (except tryptophan if it is converted to indoleacetic acid, Leopold 1960) and would be metabolized rather quickly.

The most promising cecidogen candidates are the phytohormones. Auxins (derivatives of indole-3-acetic acid) and cytokinins have been accused of causing galls while the gibberellins and other known growth regulators have been largely ignored.

## Phytohormones

Phytohormones regulate development and growth in plants. If the growth hormones in plant tissue were changed in amount or type, the tissue would probably change to new morphological forms, expecially if it was young (Salisbury and Ross 1969). Thus, a gall forming insect could secrete or induce plant synthesis of growth hormones which would cause the young needle to form a gall. However, while plant growth hormones may be a causal factor in gall

formation, it is also possible that growth hormones change as a result.

Plant growth hormones may affect tissues and cells in several ways: (1) direct effect on gene expression, (2) interaction with various enzymes, and (3) change membrane permeability. The normal repression of DNA could be altered so that new enzyme systems are synthesized or shut down and thereby change the physiology and morphology of the tissue (Villee 1961, Lehninger 1970). Auxins and gibberellins, two classes of plant growth hormones, are thought to interact with protein repressors which alter the transcription of DNA so that new m-RNA and consequently new protein synthesis can occur (Salisbury and Ross 1969). In any case, application of phytohormones to many plant systems has induced m-RNA and protein synthesis (Key 1964, Varner 1964, Galston and Davies 1969). It also is possible that hormones could cause sites on the DNA strand to initiate the protein synthesis process and make new repressor proteins which would stop certain enzyme syntheses (Lehninger 1970, Hartman and Suskind 1969). Thus, a promotion or inhibition of m-RNA synthesis, and subsequently enzyme synthesis, could radically change the physiology of the cell and tissue.

Growth hormones could combine allosterically with certain enzymes decreasing or increasing their activity (Villee 1961). Other hormones, at least in animal systems, release c-AMP which causes

kinase enzymes to become more active in phosphorylating other enzymes. These enzymes when converted to the phosphorylated form become more or less active than before (Lehninger 1970). Still other growth hormones may act in some unknown way with enzymes and proteins in influencing the cell membrane permeability (Villee 1961) to H+ ions (Cleland 1971). According to Cleland (1971), H+ ions cause the cellulose and hemicellulose microfibrils to loosen permitting rapid cellular growth. The mode of action of plant growth hormones is not well understood with much contradictory evidence probably due to the complicated plant systems (Galston and Davies 1969).

The gall midge, as stated earlier, may secrete active growth hormones directly into the surrounding cells. Alternatively, hormone precusors may be secreted. The midge may secrete chemicals which inhibit feedback mechanisms at branch points in hormone synthesis thus diverting all the precusors to one hormone (Villee 1961). Also, the midge could inhibit degradative enzymes of these hormones thus obstructing the breakdown of hormones and increasing their abundance (Galston and Davies 1969). In any case, when gall formation occurs, an increase in certain growth hormones should result in galls regardless of the insect's mechanism. Therefore, it is important to first know that hormonal or chemical changes occur in the gall before one can understand what mechanisms the gall midge employs in gall formation.

The known morphogenic effects of various hormones on plants can be compared to the tissue of pinyon galls to predict which types are involved in gall formation. Auxins and gibberellins are the most promising suspects in cecidogenesis while cytokinins and other growth regulators may also play a role. The possible function of plant growth hormones in gall formation will be discussed below.

Auxins

A compound is termed an auxin if it is able to cause an oat coleoptile to bend away from the site of application on the stem (Wilkins 1969). Using the oat coleoptile bending test (bioassay), Went and Thimann (1937) found auxins in all plants with meristematic tissue. The natural compound believed to be auxin is indole-3-acetic acid (IAA). IAA has been extracted from numerous plant systems and it has auxin activity in the oat bending and elongation tests (Bentley 1958, Burnett and Audus 1964, Fawcett 1961). Other indole derived auxins are found in plants, but their growth promoting activity is generally less than IAA (Leopold 1960).

Other chemicals such as naphthaleneacetic acid (NAA) or 2, 4-dichlorophenoxyacetic acid (2, 4-D) mimic auxin effects on plant growth but never are found naturally in plants (Salisbury and Ross 1969). NAA and 2, 4-D, as synthetic auxins, are often used in studies concerning the effects of exogenous application of auxin on plants because they are more stable chemically and biologically than the

natural indole-3-acetic acid (IAA) (Straus and Gerding 1963). See Figure 2 A page 29 for structure of IAA.

IAA has been found in minute quantities primarily in meristematic regions of plants (Went and Thimann 1937). For instance, one of the smallest concentrations of IAA has been found in oat coleoptiles which contain only .06 ug IAA/kg of fresh tissue (Salisbury and Ross 1969). A higher value was reported in corn where there were 1000 ug IAA/kg material (Stowe et al. 1967). An average value which corresponds to concentrations in the majority of plants was found in pineapple which had 6 ug IAA/kg (Overbeek et al. 1947). J. P. Nitsch stated that the IAA in pineapple was proportional to the weight of a needle in a 22 ton haystack (Overbeek 1966).

Auxins and IAA have been reported to affect plants in a wide variety of ways (Galston and Davies 1969). However, their primary effect is on cell enlargement (hypertrophy) and cell elongation as well as cellular division (hyperplasia) (Sachs 1961, Adamson et al. 1967, Salisbury and Ross 1969, Overbeek 1966, Yanagishima and Shimoda 1968, Nitsch 1968, Jablonski and Skoog 1954). Auxins also help produce abscission and senescense in plants presumably through an affect on ethylene production (Overbeek 1966, Burg and Burg 1966, Salisbury and Ross 1969, Abeles and Holm 1966).

Auxin has been implicated in causing plant galls. Several workers have induced plant galls by applying IAA and other auxins

(Salisbury and Ross 1969). For example, Arrillage (1949) obtained stem galls on sugarcane by injecting 2,4-D, IAA, and NAA (all auxins). Furthermore, cell hypertrophy and hyperplasia commonly observed in plant galls (Mani 1964) can also be produced by application of IAA (Nitsch 1968). Finally, the internal anatomy of naturally occurring plant gall cells (Mani 1964) is similar to auxin induced gall cells (Nitsch 1968).

Very little work has been done on determining the cecidogen in insect plant galls. Nysterakis (1946) suggested that indole-3-acetic acid was the cecidogen in gall aphid saliva. He based his theory on previous work which showed auxins in mixtures of saliva and honeydew extracts. Weidner (1950) suggested that the stimulating chemical was an anti-auxin that promoted auxin activity. The anti-auxin (used incorrectly in this context since anti-auxins inhibit auxin activity) would be analogous to an indoleacetic acid oxidase inhibitor.

Mani (1964) in opposition to the idea that an auxin is the active cecidogen, believes that the tumors induced by auxin and those by insects are different. In gall tissue cells, the nucleus is central and rounded while IAA-produced galls have peripheral and somewhat rectangular nuclei. Also, the presence of IAA in saliva of gall aphids has never been proved (Mani 1964). However, not much sensitive work has been done to test aphid salivas for IAA and the presumed absence of IAA in gall aphids certainly does not rule out IAA in connection with other gall-formers.

Auxins have not been identified in insect galls. Most of the work on growth hormones in galls has been done with bacterial, fungal, or nematode galls. Viglierchio and Yu (1968) concluded that three species of nematodes infecting Brussel sprouts, sugar beet, and tomato produced the indole auxins: IAA, indoleacetonitrile, indoleacetic acid ethyl ester, and indolebutyric acid. The type of auxin depended on the nematode species and was not produced by the infected plant. Wong and Willetts (1969) reported hypertrophy and hyperplasia of the cells surrounding these nematodes. Galls induced by fungi in corn have 16 times as much IAA per unit weight of tissue as normal tissue (Wood 1967). There are several reports of higher concentrations of IAA in various crown galls caused by Agrobacterium tumefaciens or Impatiens balsamina (Bouillenne and Gaspar 1970, Goodman et al. 1967). Furthermore, maleic hydrazide and 2,4,6-trichlorophenoxyacetic acid both of which inhibit IAA action also stopped crown gall tissue formation (Waggoner and Dimond 1953, Wood 1967). Plants inoculated with attenuated strains of A. tumefaciens would not develop galls until IAA was applied exogenously (Klein and Link 1952).

On the other hand, IAA not always is found to increase in galled tissue since Rowan (1967) found no auxin difference in rust galls of loblolly pine. Ignoffo (1957) also found no growth hormones in aphid galls, however, his pea epicotyl sections were inhibited in growth

probably indicating plant inhibitors which counteracted any potential growth hormone effects.

IAA and its derivatives seem to be found in many plant galls at higher concentrations than normal. The fact that IAA inhibitors stop gall formation, and that exogenous application of IAA produce galls under certain circumstances indicate IAA must either allow gall formation or actively promote it. Therefore, IAA is a prime cecidogen suspect, however, very little work has been done on insect gall formation and virtually none has been done on cecidomyiid galls so other growth hormones or substances may be involved.

Many investigators have reported auxin differences between galls and normal tissue but the mechanism of auxin induction usually is speculative or ignored. As mentioned before, certain species of nematodes, fungi and bacteria may produce auxin directly (Viglierchio and Yu 1968, Yu and Viglierchio 1964, Turian and Hamilton 1960, Wilson 1965) or induce plant tissues to produce auxin (Lipetz 1959). The majority of reports do not say whether the causative agent secretes the cecidogen or induces the surrounding tissue to produce the cecidogen (growth hormone). If the gall maker does not directly secrete an auxin, then another secreted chemical must ultimately interact with the regulatory mechanisms of IAA levels in plant tissue and increase IAA concentration to form the gall.

Regulation of IAA in Plants

The effective concentration of IAA may be increased by (1) synthesizing more IAA, (2) releasing competition at the IAA active site, or (3) inhibiting breakdown of IAA by inhibiting degradative enzymes (Villee 1961, Galston and Davies 1969). The cecidomyiid larva could secrete chemicals which would inhibit negative feedback mechanisms of IAA synthesis or these secreted chemicals could induce synthetic enzyme systems for IAA. The insect larva could also bind chemical inhibitors which normally compete with IAA at its active site (Villee 1961). Thus, IAA would have more sites of action available and cell hypertrophy might result.

Most of the speculation in the literature, however, concentrates on the theory that gall organisms inhibit enzymes which normally break down IAA and regulate its concentration (Bouillenne and Gaspar 1970, Stonier 1969). The degradative enzyme is called IAA oxidase which is generally believed to be a peroxidase (Galston and Davies, 1969). In fact, most if not all peroxidase isozymes have some degree of IAA-oxidase activity converting IAA into another indole chemical probably 3-methylene oxindole which does not have hormonal growth properties (Wilkins 1969).

IAA presumably will build up (no feedback mechanism) if peroxidase is inhibited by certain polyphenols. Caffeic acid, quercetin, chlorogenic acid, scopoletin, and catechol all have a free hydroxyl group in the ortho position on the aromatic ring and they can

competitively inhibit IAA oxidase (peroxidase) (Andreae and Collet 1967, Salisbury and Ross 1969, Furuya et al. 1962, Thimann et al. 1962). See Figure 2 D page 29 for example of an orthophenol. On the other hand, monophenols such as kaempferol derivatives act as co-factors in the IAA oxidase system and accelerate IAA breakdown (Furuya et al. 1962). Therefore, peroxidase acts as an IAA-oxidase to oxidize or breakdown IAA into an inactive form, but peroxidase can be inhibited by orthophenols thus allowing IAA concentrations to increase.

Detection of IAA in Plants

IAA occurs naturally in plant tissue in such minute quantities that very sensitive methods or large quantities of plant material must be employed to detect the hormone. Most of the findings discussed previously were of a purely qualitative nature. The investigators performed bioassays using various plant parts found to be highly sensitive to the growth hormone, but only semi-specific in differentiating various growth hormones. To alleviate this problem, qualitative analysis of the test solution was performed, first by paper chromatography and later by thin-layer chromatography in conjunction with solvent partitioning purification (Burnett and Audus 1964). The latest methods use a more rigorous purification procedure with gas chromatography and spectrophotofluorimetry to quantitate the hormone. However, spectrophotofluorimetry is still not as sensitive as

certain bioassays and contaminating fluorescent compounds may confuse the results (Burnett and Audus 1964, Powell 1964).

Most bioassays for IAA use oat coleoptiles which are extremely sensitive to IAA (5 to 10 ng/ml). An oat coleoptile is the young shoot which drives upward through the soil protecting the delicate leaves within. The tubular coleoptile section elongates in response to several nanograms of IAA or other synthetic auxins. Unfortunately, extracts must still be quite pure since non-specific inhibitors will mask auxin effects (Sirois 1966 and 1967).

Gibberellins

Today at least 36 naturally occurring homologues of gibberellic acid (GA) are known with various effects on growth rate in different plants and within the same plant. Gibberellins have been found in over 100 species of dicotyledons, over 30 species of monocotyledons, and several conifers (Goodwin 1971). Furthermore, all angiosperms and gymnosperms that were carefully examined possessed at least one gibberellin (Wilkins 1969). Plants vary as to the types and quantities of the various gibberellins (Goodwin 1971, Wilkins 1969). See Figure 2 B page 29 for diagram.

Gibberellins, like auxins, induce a wide variety of growth and developmental changes within plants. Gibberellic acid often acts synergistically or additively to auxin in promoting cell expansion and cell division (Sachs 1961). Conifer species, except loblolly pine

(Pinus taeda) show less response to exogenous gibberellins than angiosperms (Roberts et al. 1963).

To my knowledge gibberellins have never been looked for in insect, bacterial, nematode, or fungal galls. Fungal galls would be the most likely to contain higher concentrations of gibberellins since the hormones were first isolated from the fungus Gibberella fujikuroi in rice seedlings (Salisbury and Ross 1969). If gibberellic acid is not the active cecidogen it is possible that the cecidomyiid larva may secrete large quantities of ecdysone. Carlisle et al. (1963) showed that the insect moulting hormone had gibberellin-like activity in the dwarf pea bioassay. Consequently, ecdysone or ecdysterone might be expected to produce cell enlargement and subsequent gall formation by acting like gibberellic acid. See Figure 2 E page 29 for structure of ecdysone.

Regulation of Gibberellin in Plants

Less is known about the regulation of gibberellins than auxins. The biosynthesis of gibberellic acid is known and certain chemicals, AMO-1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine-carboxylate methyl chloride), CCC (trimethylammonium chloride), and phosphon D (2,4-dichlorobenzyl-tributylphosphonium chloride) inhibit various enzymes involved in its synthesis thus decreasing GA levels (Goodwin 1971). Other chemicals may exist which

could increase GA levels by interacting with synthetic or degradative enzymes.

Detection of Gibberellins in Plants

Brinks et al. (1969) have described techniques using gas chromatography and mass spectrometry to characterize gibberellins in plants. The test is definitive but relatively large amounts of comparatively pure gibberellin (0.05 to 1 ug.) are needed. However, a gibberellin can be identified using thin-layer chromatography with several solvent systems (Paleg 1965) and different bioassays (Crozier et al. 1970). Bioassays are more sensitive (0.001 ug/ml) but less specific and are at best a qualitative indicator of whether or not gibberellins are present (Jones and Varner 1967). An advantage of bioassays over purely analytical techniques is that the plant extract does not have to be rigorously purified and more results are obtained with less work and time. Also analytical techniques necessitate expensive instruments not available to many researchers. A combination of several bioassays in conjunction with thin-layer chromatography is quite effective in identifying gibberellins (Paleg 1965, Crozier et al. 1970).

Perhaps the most popular bioassay for gibberellins involves the use of barley seeds. The barley endosperm is surrounded by a thin layer of living cells called the aleurone layer which is extremely sensitive to small amounts of GA secreted by the embryo. Therefore

the embryo is excised from the seed so no endogenous gibberellin will contaminate the sample being bioassayed. If as little as 0.1 nanogram gibberellin per ml is applied to aleurone cells, they synthesize starch hydrolyzing enzymes, particularly α-amylase, and release them into the medium. The α-amylase activity is measured as a function of starch breakdown and a standard curve of α-amylase production versus gibberellin concentration can be made for a particular variety of barley seeds. Then extracts of experimental material can be added to barley endosperm half-seeds, the α-amylase production measured, and the amount of GA present in the extract calculated from the standard curve (Jones and Varner 1967, Chrispeels and Varner 1967, Varner 1964).

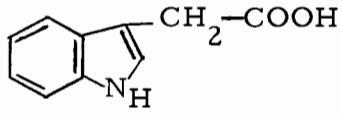
Cytokinins

The first natural cytokinin was isolated and identified by Letham et al. (1964) and called zeatin. See Figure 2 C page 29 for diagram of zeatin. Cytokinins usually must be present for plant cell division to occur but IAA is also necessary. For instance, Jablonski and Skoog (1954) working with tobacco pith tissue found that kinetin (a synthetic cytokinin) plus auxin induced rapid cell division while kinetin alone had no effect. Gibberellic acid could replace auxin with the same effect on cell division. Das et al. (1956) showed that auxin also caused nuclear kinesis but auxin and kinetin caused cytokinesis.

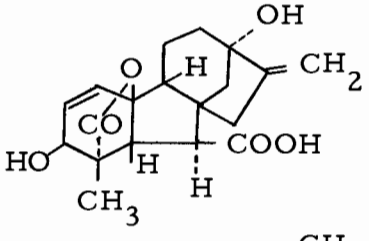
Cytokinins are also reported to cause cell enlargement (Arora et al. 1959).

Another important effect of cytokinins is their ability to delay senescence in plants. Richmond and Lang (1957) using kinetin delayed senescence in detached leaves of Xanthium for more than 20 days. The delay of senescence can be used as a bioassay for detecting cytokinins (Osborne et al. 1961).

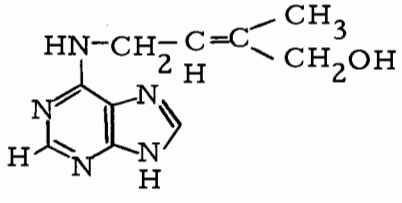
Rowan (1970b) found a 10 fold increase in cytokinin activity in fusiform rust galls (fungal) on loblolly pine. McCalla et al. (1962) tested for cytokinins in willow galls caused by sawflies of the genus Pontania and found none. However, a mixture of kinetin and auxin promoted growth on preformed galls, but to a lesser extent, so did uric and glutamic acid. Pinyon galls, especially the round variety, senesce and dry out in mid-September so if cytokinins were responsible for gall formation they would have to be destroyed to allow senescence. Since very little work has been done on cytokinins in galls, future cytokinin studies might be of value.

A. 

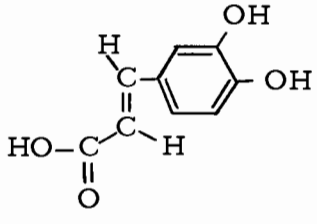
Indoleacetic acid (IAA)

B. 

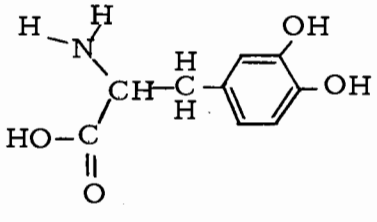
Gibberellic acid 3($GA_3$)

C. 

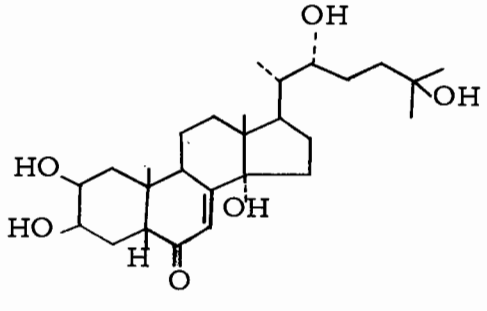
Zeatin

D. 

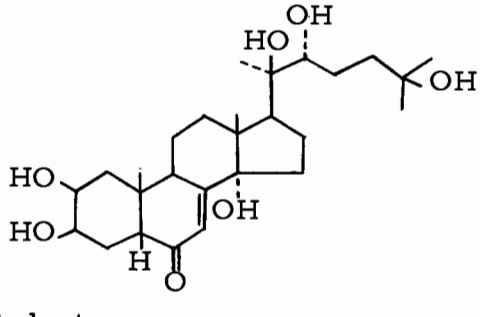
Caffeic acid



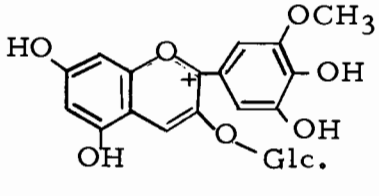
Dihydroxyphenylalanine

E. 

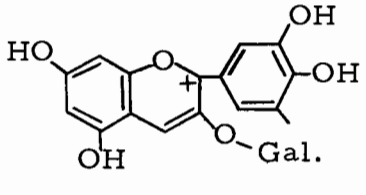
Ecdysone



Ecdysterone

F. 

Petunidin 3-glucoside



Cyanidin 3-galactoside

Figure 2. Various chemicals involved in plant growth (A, B, C, D) insect growth (E), or gall color (F).

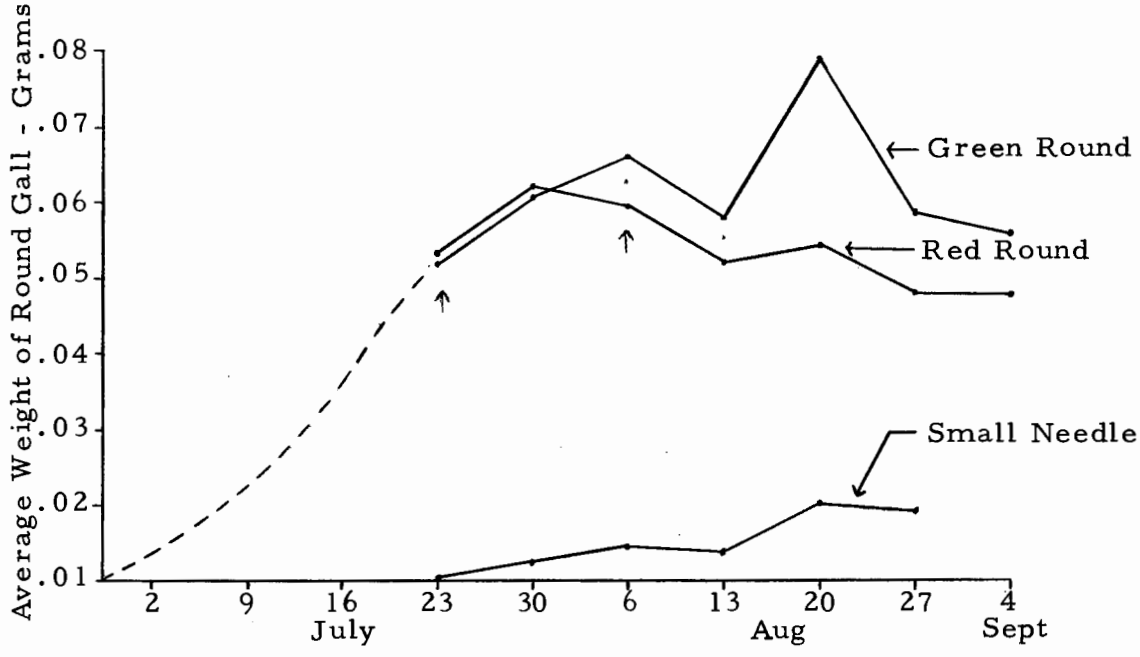
## METHODS AND MATERIALS

### Collection and Processing of Pinyon Insect Galls

Weekly trips were made to Salida, Colorado during the summer of 1972 for the collection of pinyon pine needles and insect needle galls. The pinyon forests were located approximately 15 km. NNW of Salida at an elevation of 2,350 m. At least 50 trees were sampled each week and no more than half of each tree's galls were taken. The ends of pinyon branches containing the needle galls were cut off at 15 cm lengths and placed in styrofoam coolers filled with crushed ice. The coolers kept the pine twigs fresh for 18 hours until the galls and normal needles were hand picked and separated into the different types and colors, weighed, and quick frozen in liquid nitrogen. The pine material was then crushed along with dry ice into a powder-like consistency in a large mortar with a pestle. The material was subsequently lyophilized and stored at -20° C until needed.

The total weight of normal needles and round galls (red and green) was recorded for each week (Fig. 4 A). The weight of an average needle or gall was obtained by dividing the weight of a sample by the number of needles or galls in the sample (Fig. 3 A). The total number of needles or galls was then found by dividing the weight of

A. Change in weight of round gall and small normal needle during Summer 1972.




B. Increase in round gall volume during Summer 1972.

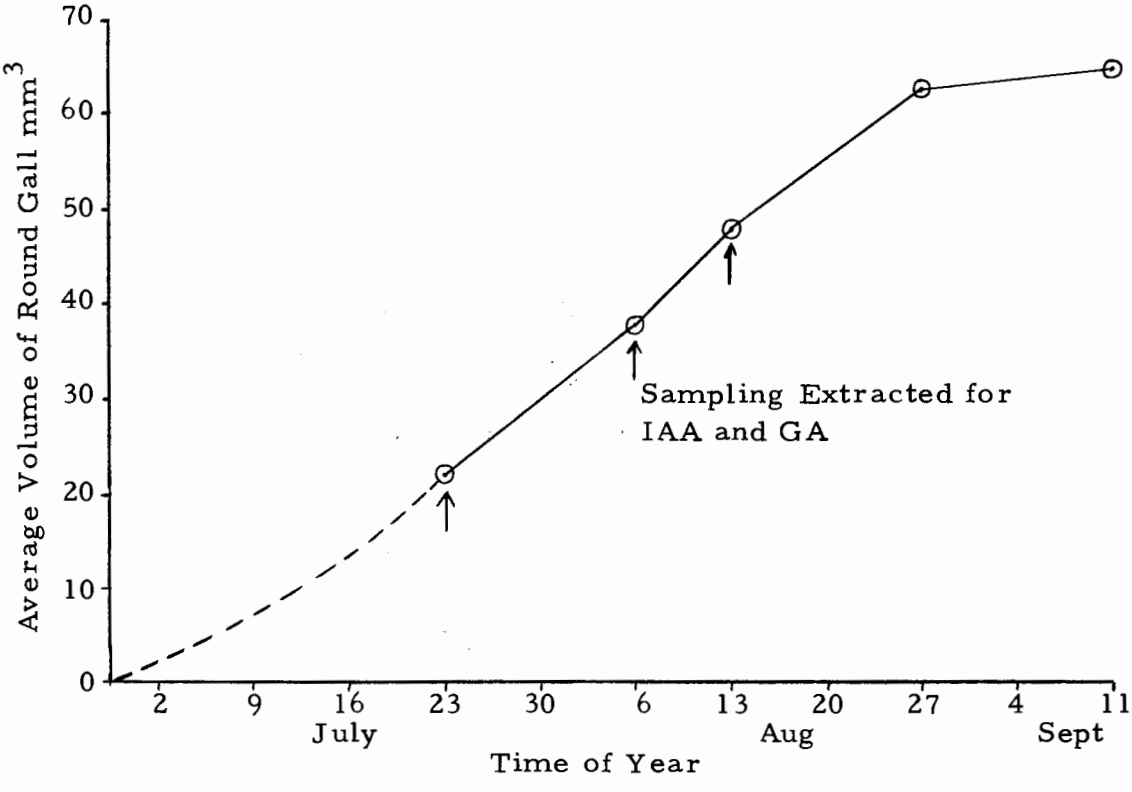



Figure 3. Weight and size of round galls during Summer 1972.

the entire collection by the weight of an average needle or gall. Therefore, on July 23, 1972, 1,305 round galls weighed 67.6 grams and 5,772 normal needles weighed 59.8 grams; on August 6, 1972, 894 round galls weighed 59.1 g and 4,625 needles weighed 67.9 g; on August 13, 1972, 1,369 galls weighed 79.4 g and 4,988 needles weighed 69.5 g. The entire sample of normal needles and galls from each collection date was extracted for auxins and gibberellins.

The increase in volume of round galls was calculated by measuring the diameter of about 50 galls from each week. The volume was calculated from the equation: $(4/3)\pi r^3$ (Fig. 3 B).

## Extraction, Purification, and Detection of IAA and GA

The methods for extraction and purification of gibberellins and auxins were modified from the technique described by R. L. Jones (1968). His techniques were designed primarily for extraction and purification of the gibberellins but acidic indole auxins were also found to separate along with the gibberellins. IAA (1000 μg) was added to pinyon needles and the amount of IAA left behind at various solvent partitioning steps was tested to determine purification losses. The only appreciable loss of IAA occurred when IAA in ethyl acetate was partitioned with an alkaline aqueous solution. A 2% loss resulted as tested with the Ehrlich colorimetric reaction (Powell 1964). Therefore it was concluded IAA and gibberellin can be simultaneously

extracted and purified and later separated with thin-layer chromatography.

Extraction and Purification

A known amount of lyophilized material (59.1 to 79.4 grams, see previous page) was homogenized in 125 ml of 80% aqueous methanol with a Waring blender for 3 min. at high speed. After an hour incubation period and an additional 3 min. of homogenization, the crude methanolic slurry was filtered through a wad of glass wool.

The crude fiber residue was washed with methanol to remove any remaining free indoles and gibberellins. The aqueous methanol was then evaporated on a Rinco flash evaporator until only the water remained. Fifty ml of water were added to the water extract which was then centrifuged at 12, 000 x g for 10 min.

Solvent Partitioning Purification

The supernatant from the previous centrifugation step was acidified with 2N HCl to pH 2,5 and partitioned three times against ethyl acetate. The ethyl acetate fractions were combined and partitioned two times against alkaline water (100 ml $H_2O$ plus 30 ml 2N NaOH first partition, and 100 ml $H_2O$ plus 10 ml 2N NaOH second partition). The aqueous solution must remain basic (pH 8.5) after the partitioning. The alkaline solution was then re-acidified to pH 2.5 with 2N HCl and partitioned two times against ethyl acetate (Fig. 5 page 36).

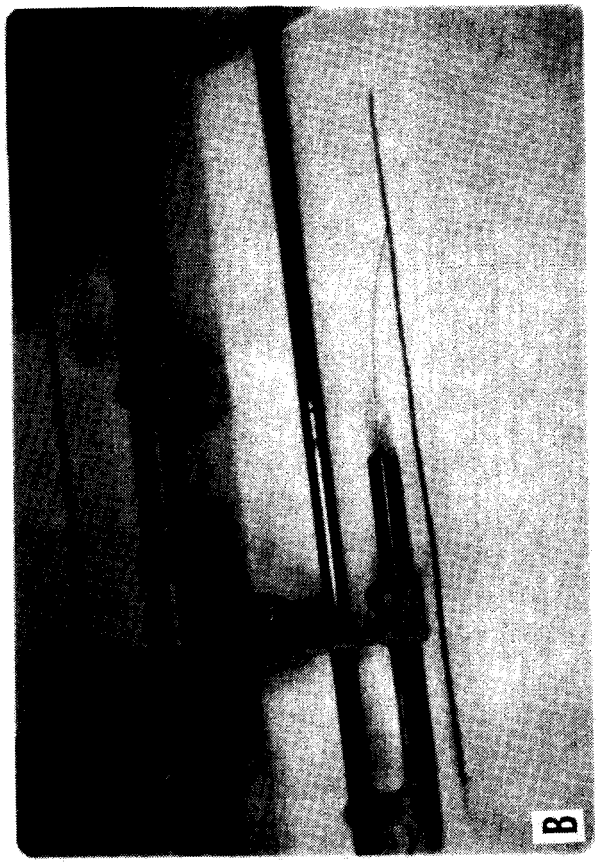
Figure 4. Purification of plant hormones.

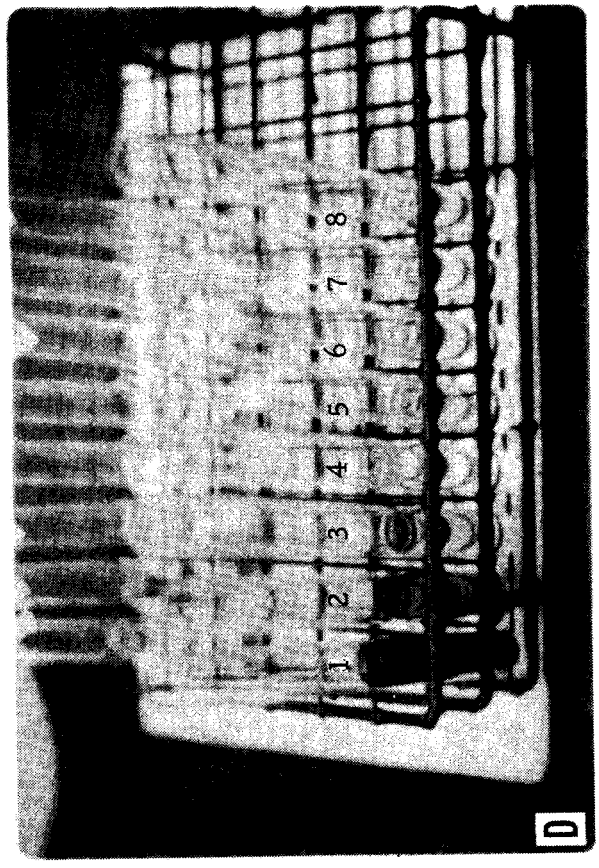
A. Weighing small normal needles and galls.

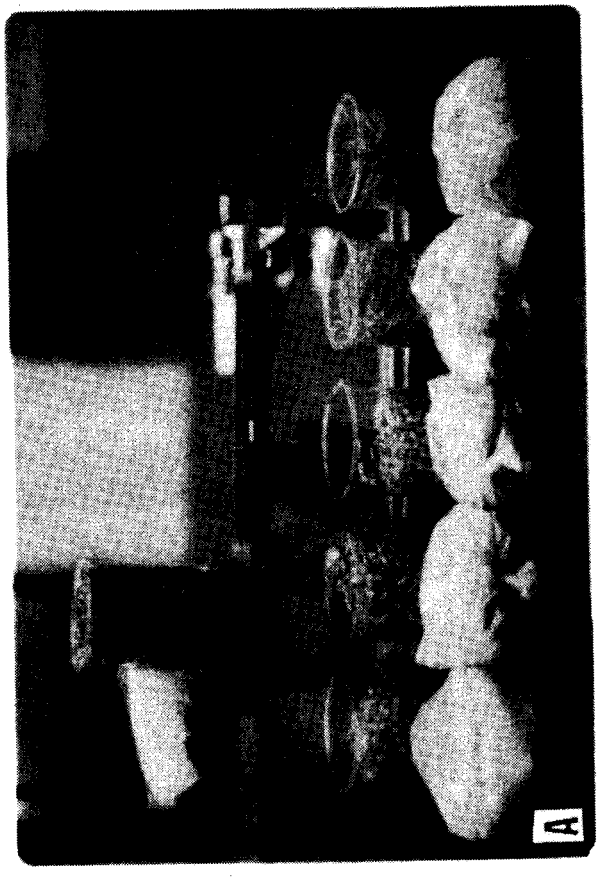
B. Streaking chromatography paper.

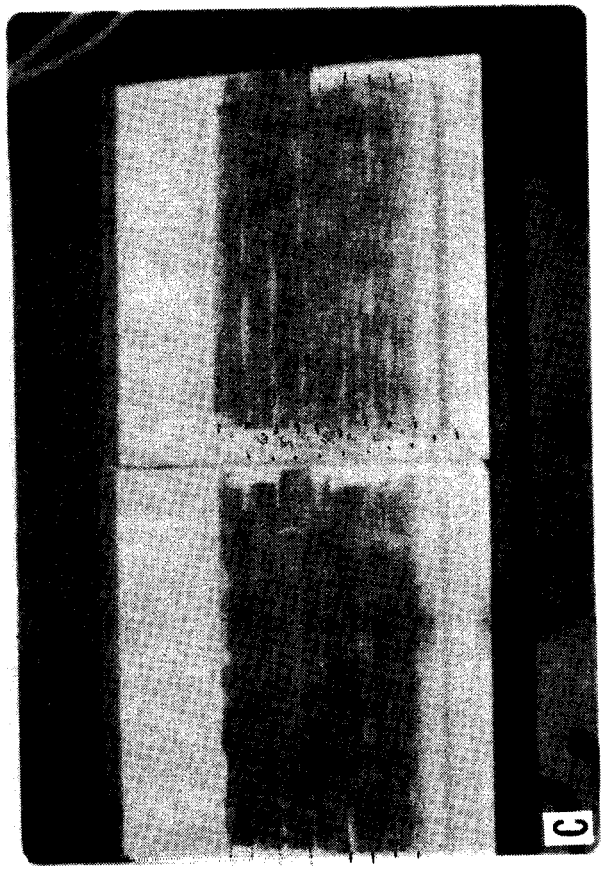
C. Thin-layer plates scraped of silica gel.

D. Pinyon extracts eluted from silica gel bands. Gall foreground, small needle background.


B


1 2 3 4 5 6 7 8
D


A


C

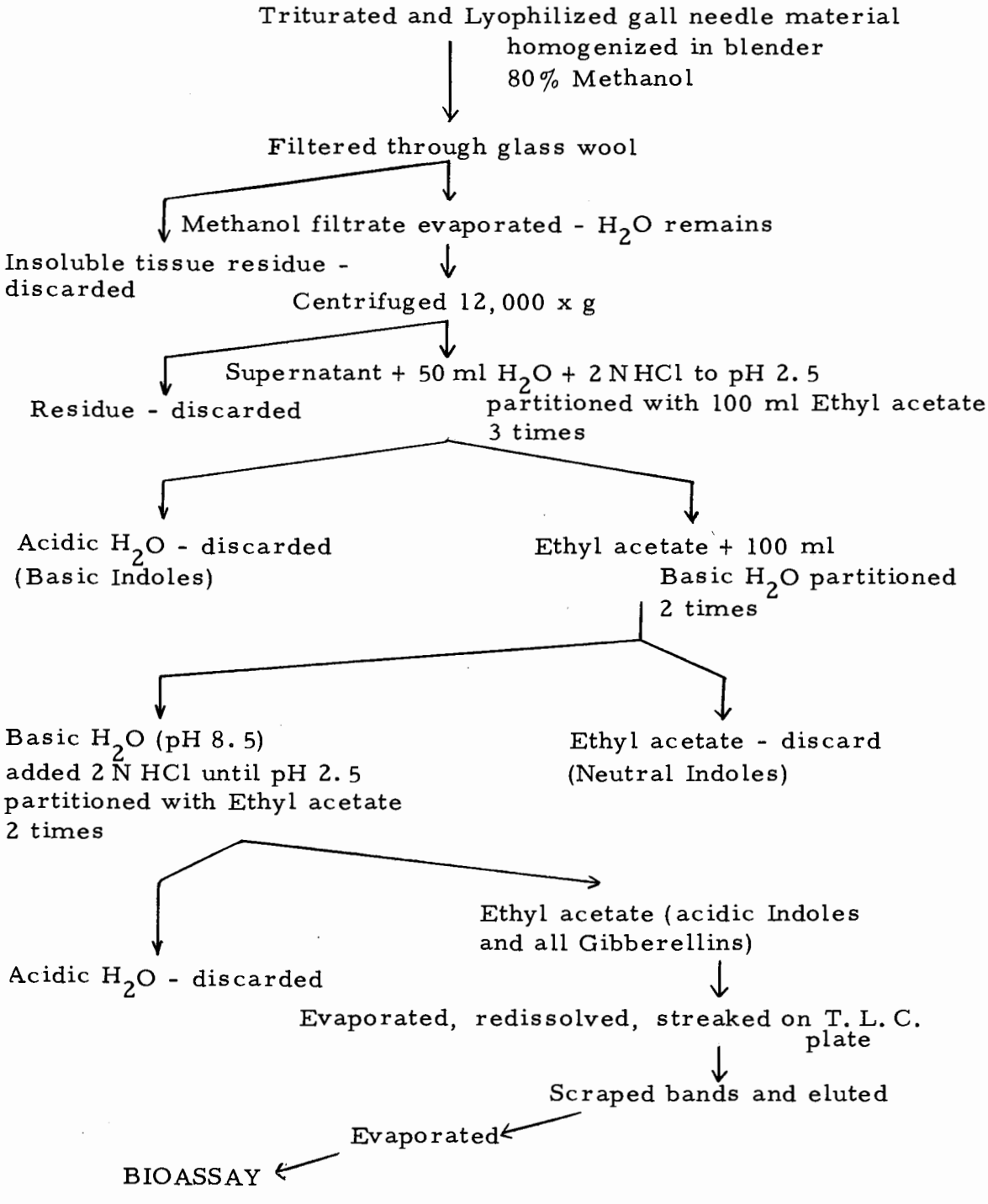



Figure 5. Gibberellin and acidic Indole extraction - Flow diagram.

Using the methods of Jones (1968), emulsions of water and ethyl acetate created difficulties in extraction and solvent purification steps. The emulsion kept the water and ethyl acetate fractions from separating and prevented solvent purification. However, by switching the steps so that the basic extraction followed the acidic extraction, permanent emulsions rarely occurred. Furthermore, emulsions which did occur in the modified procedure could be easily broken down by either vigorously shaking the emulsion, or by adding 2 - 3 times more ethyl acetate than water and re-partitioning.

The acidic ethyl acetate fractions were combined and evaporated to dryness and redissolved in 2 ml of methanol. The 2 ml of methanol containing IAA and GA then was filtered through a 1 cm dia Whatman #1 filter (retention 11 $\mu$) with a syringe equipped with a Swinny adapter. Additional methanol (1 ml) was used to wash the evaporating flask and strained through the filter.

Chromatography and Elution of Hormones

The filtered methanol extract was then streaked on 20 x 20 cm silica gel H plates (250 u thick) with a mechanical streaker. The streaker produced a line of uniform width (2 mm) at a constant distance parallel to the plate's edge (Fig. 4 B page 35). A spot of authentic $GA_3$ (one of the gibberellins) and IAA was applied on the origin at the right of the plate as a check. The streaked plates were developed in a chromatography chamber containing benzene, isopropanol,

and water (60:20:5). The plates were developed at 25° C to a distance of about 15 cm from the origin.

Solvent systems for developing thin-layer plates with gibberellins and auxins are numerous but few systems will simultaneously separate both (Stahl 1969, Paleg 1965). Solvent systems with acetic or formic acid are excellent in promoting good resolution of growth hormones on silica gel plates but these acids cause indoleacetic acid to partially decompose, turn red, and lose its biological activity (Stahl 1969). However, if microgram quantities of IAA are present and biological activity has been shown, then acetic or formic acid can be used to prevent tailing and chromogenic sprays may be used to localize the various indoles.

The problem with finding a suitable solvent system was further complicated by a phenomenon resembling displacement chromatography. In displacement chromatography, a column is continuously supplied with a displacer chemical which competes with the compound of interest allowing it to increase its $R_f$ above normal (Snyder 1968). In the pinyon extracts there appeared to be a displacer substance which completed for the adsorptive sites in the silica gel and caused IAA and $GA_3$ to move to a much higher $R_f$ than in a pure system of standards. Therefore, increasing the concentration of pinyon extract and IAA (by applying more sample per spot) caused the IAA to move higher on the plates (Fig. 6 B page 41). Consequently, none of the

published solvent systems for separating auxins or gibberellins could be compared to IAA and GA in pinyon extracts (Stahl 1969, Paleg 1965). A solvent system was finally obtained which permitted optimal purification and separation of IAA and $GA_3$ by running various chromatograms with IAA, $GA_3$, and pinyon extract in solvent systems with different ratios of benzene to isopropanol (Figure 6 A page 41).

Silica gel G was not used in thin-layer chromatography of the growth hormones because it has a phytotoxic effect on many bioassay plants (Stahl 1969). The "G" stands for gypsum or $CaSO_4$ which acts as a binder for the silica and is usually 13% by weight. Both the sensitivity and the amount of coleoptile growth were decreased when silica gel G was used as the chromatographic media. Using silica gel H (no gypsum) there was very little inhibition even when larger amounts of silica gel were eluted.

After development, the chromatographic plate was divided into 8 contiguous lateral bands each 1.5 cm wide with the first band directly above the origin (Fig. 4 C). Each band of silica gel H was scraped off with a spatula onto a sintered glass filter. IAA and gibberellins were eluted from the silica gel by adding methanol and vigorously swirling. The resulting slurry was vacuum filtered into appropriate bioassay tubes with 3 methanol washings totaling 10 ml. The bioassay tubes or flasks were chilled to prevent boiling and placed in a vacuum desiccator until the methanol was evaporated to

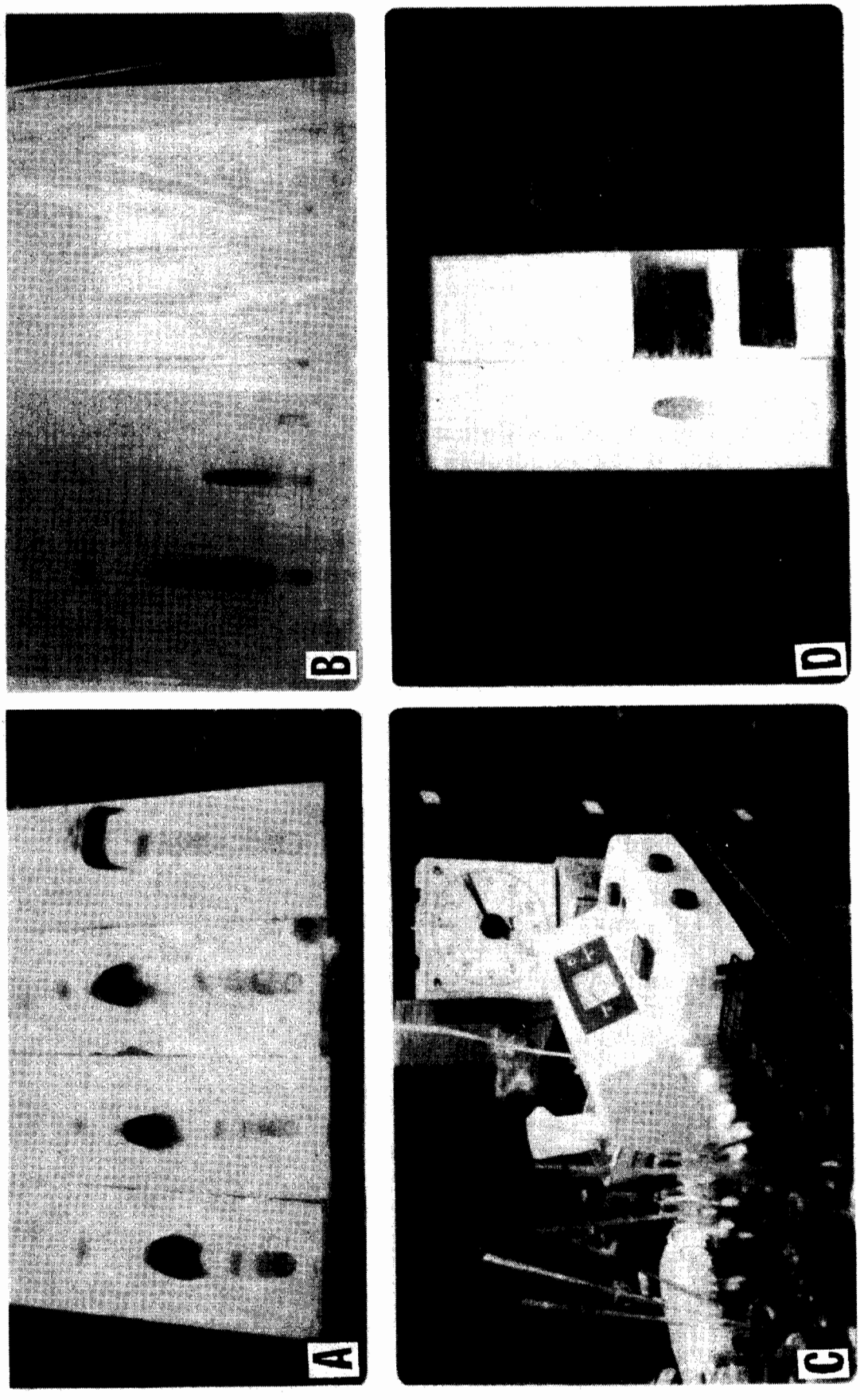
Figure 6. Miscellaneous (Thin-layer chromatography; measuring α-amylase).

A. Finding optimal ratio of benzene to isopropanol for separating IAA.

B. Increase in $R_F$ of IAA with increased amounts of pinyon extract.

C. Assaying α-amylase activity in solutions from barley endosperm incubated with $GA_3$.

D. IAA and $GA_3$ standards (left) compared to silica plate scraped for midge larval hormones.


B
D
A
C

dryness. The tubes containing the various chromatographic fractions then were bioassayed for plant growth hormones.

Visualization

Various chromogenic spray reagents were used to indicate where on the plate the indoles and gibberellins were migrating for both authentic hormones and pinyon extracted hormones.

Indoles: Ehrlich Test
1 ml aqueous sample + 2 ml Ehrlich reagent, mix, and read absorbance at 530 nm on Bausch and Lomb spectronic 20.

Ehrlich reagent: 10% p-dimethylamino-benzaldehyde in concentrated HCl (Powell 1964).

Ehrlich spray reagent: 2.5% p-dimethyl-aminobenzaldehyde in 3 N HCl - (1:4 dilution of Ehrlich reagent).

Gibberellins: $H_2SO_4$
75% $H_2SO_4$; spray plate and heat to 115° C for 1 min. The gibberellin product fluoresces in $UV_{366}$ light (Stahl 1969).

Extraction of IAA and GA From Round Gall Larvae

Round galls were partially freeze-dried and then opened. From 136 round galls 204 larvae were removed and placed in methanol. The larvae were crushed in the methanol to release possible plant growth hormones. The crude extract then was applied to a silica gel H plate and developed in benzene, isopropanol, and water (50:40:5). The bands corresponding to the Rf's of $GA_3$ and IAA were scraped, eluted, and bioassayed for these growth hormones (Fig. 6D page 41).

Seventeen sugar beet root maggots, Tetanops myopaeformis Röder, which weighed 0.34 grams also were extracted for growth hormones and bioassayed as a control.

## Bioassay of Auxins

The bioassay of indole growth hormones was modified slightly from that developed by Nitsch and Nitsch (1956) and Sirois (1966 and 1967). Oat seeds, Avena sativa, of the hulless cultivar 'Brighton' were used in all bioassays. The seeds were obtained from Mr. O. McNaughton, Box 3191 Postal Station C, Ottawa, Canada.

The oat coleoptile straight growth bioassay for detecting auxins is sensitive and reliable but variability in coleoptile lengths within tubes and between successive weeks can make analysis difficult. The variability of coleoptile lengths may result from several factors. The inherent ability for growth differs in coleoptiles so some uncontrolled variability should always occur. Often coleoptiles are stunted due to cracks or holes in the embryo and primordial radicle (root) of the oat seed causing unhealthy coleoptiles which may elongate poorly in response to IAA.

Oat seeds of uniform size and intact embryos were selected based on microscopic observation. The seeds were surface-sterilized for 5 minutes in 2.6% sodium hypochlorite (commercial bleach diluted 1:2). The seeds then were placed in cheesecloth and rinsed in tap

water for 1 hr and soaked in distilled water 4 hr more. Three hundred milliliters of distilled water were added to a germination pad* contained in a 32 cm x 9 cm x 24 cm clear plastic box insuring constant humidity (approximately 100%). The seeds were planted at a 45° angle with the embryo down and the grooved side facing downward in soggy tissue paper. The plastic boxes were then placed in a coleoptile growth chamber and exposed to red light for 22 hr at 29° C. The red light, which inhibits the first internode growth, was provided by one 25 watt red bulb for two boxes, each box approximately 40 cm from the light source. A timer automatically shut off the red light after 22 hr and the seedlings remained in darkness 54 hr at 24° C until cutting. The subsequent work was done in the same coleoptile room at 24° C under dim green light. The coleoptiles are insensitive to this wavelength (540 nm.).

Oat coleoptiles approximately 2 cm tall were harvested from the boxes and cut cross-sectionally into sections with razor blades mounted between plastic blocks. The apical 3 mm of each coleoptile was discarded and the two 6 mm sections below, designated A and B, were cut and placed in beakers of distilled water.

The most important factor in reducing the variability in coleoptile lengths was the use of short, uniform coleoptiles (2 cm).

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*From Seedburo, 618 W. Jackson Blvd., Chicago 6, Ill.

Longer coleoptiles have less potential for growth and are less sensitive to auxins (Nitsch and Nitsch 1956). At 100 ng IAA, sections from long coleoptiles elongated about half as much as sections from short coleoptiles. Therefore, it was very important to harvest the coleoptiles at the shortest possible height that would yield 2 sections. By varying the temperature or the time of harvesting, coleoptiles would double in length after cutting when placed in optimal concentrations of IAA (100 ng per ml).

The coleoptile sections were floated in beakers A and B for 3 hr and then 5 sections were placed in each 15 x 150 mm test tube containing 1 ml buffer and 1 ml of various amounts of IAA as standards. The buffer contained a mixture of 0.01 M $K_2HPO_4$ and 0.005 M citric acid. The standards of IAA were made from serial, 1:10 dilutions of freshly prepared IAA, 0.1 g/l (Eastman organic chemicals). In the case of gall or normal needle material, 1 ml of distilled water instead of the standards was added to the test tube.

The test tubes were slowly rotated at a $10^{o}$ angle from the vertical at 0.5 revolution per minute for 19 hr. The tubes were then removed from the dark coleoptile room ($24^{o}$ C) and measured for increased length with a microscope equipped with an ocular micrometer. The coleoptile lengths were expressed in units (1 unit = 0.12 mm).

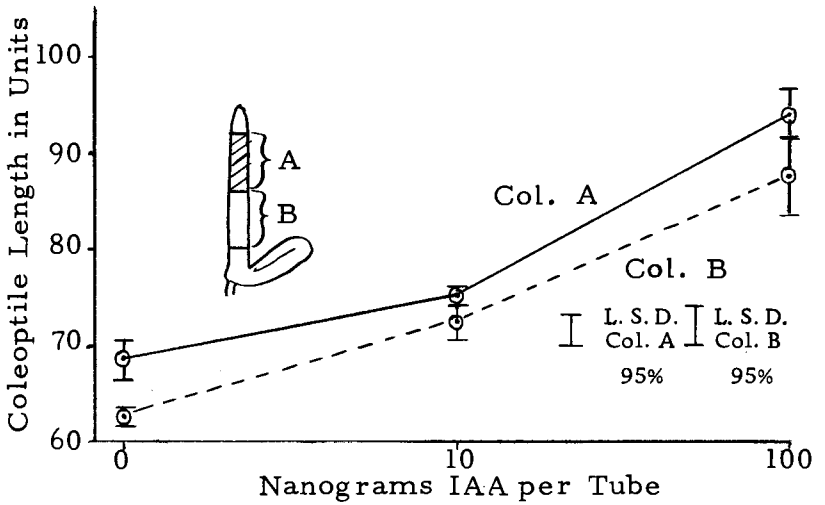
## Calculation of IAA Levels in Pinyon

A standard curve showing coleoptile growth (sections A and B) in response to added quantities of pure IAA was determined for each of the 3 experiments involving pinyon material (Table 6, page 108) and Figure 7 A, B, C page 47). The nanograms of IAA in pinyon extracts were interpolated from the standard curves by comparing the average coleoptile length from various pinyon extracts (Table 7 page 109) to the appropriate standard curve.

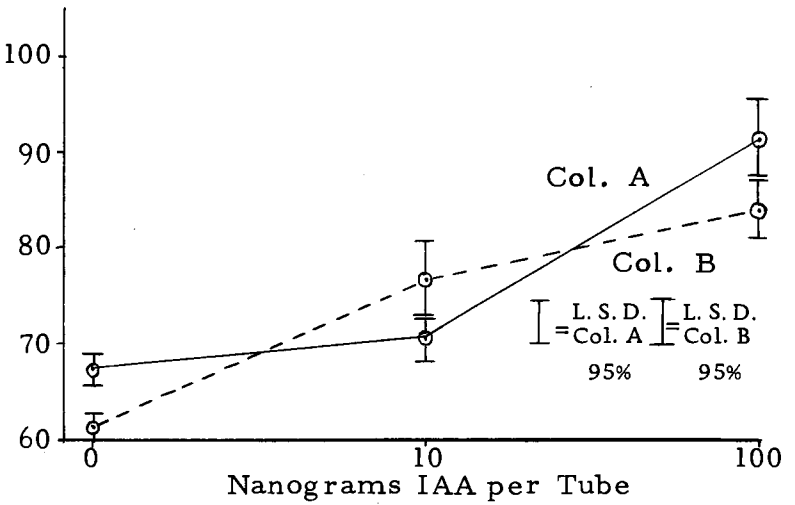
Two different methods were used to calculate the amount of auxin in pinyon extracts. The average coleptile lengths from the various pinyon extracts for each week were compared to the standard curve for that week and the nanograms of IAA were interpolated (Table 1 page 66). The bioassay conditions (temperature, etc.) for the coleoptiles in the standard curve tubes and the pinyon extract tubes were identical, therefore, comparisons should be valid. Secondly, the coleoptile lengths from pinyon extracts of all three experiments were compared to a second standard curve averaged from five separate standard curves. (The five separate standard curves were a compilation of the three experiment bioassays and two previous trial runs). Because 55 coleoptiles were used for each IAA concentration the standard curve more closely approximates the true curve (Figure 7 D). Each method of calculating the amount of IAA in pinyon extracts had advantages so both ways were used.

1 unit = 0.12 mm

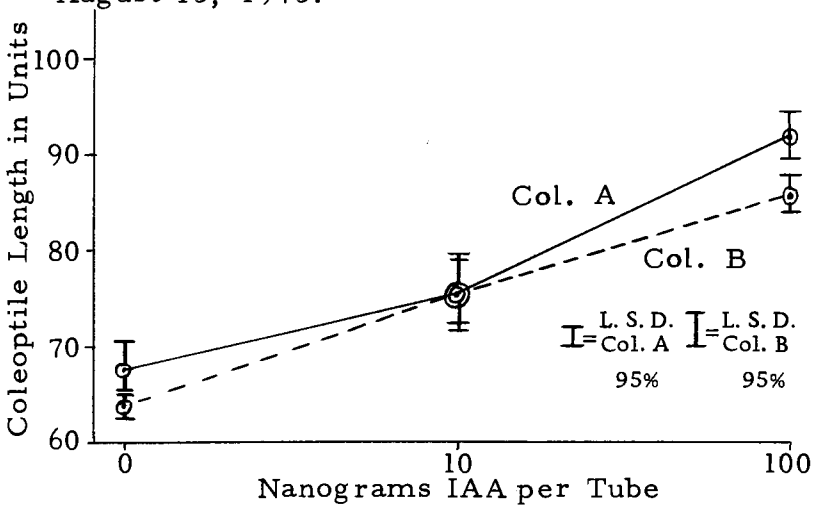
Auxin standard curve for Pinyon leaves collected July 23, 1972.




Auxin standard curve for Pinyon leaves collected August 6, 1972.




Auxin standard curve for Pinyon leaves collected August 13, 1973.




Auxin standard curve - Average of five separate standard curves.

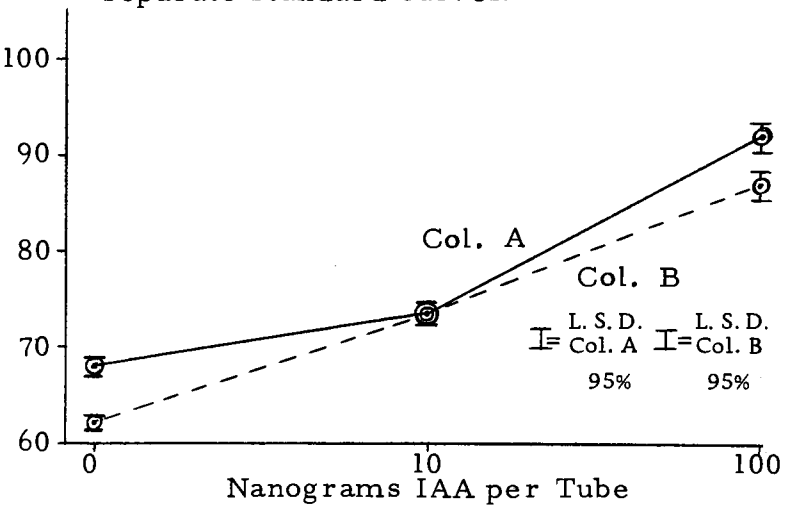
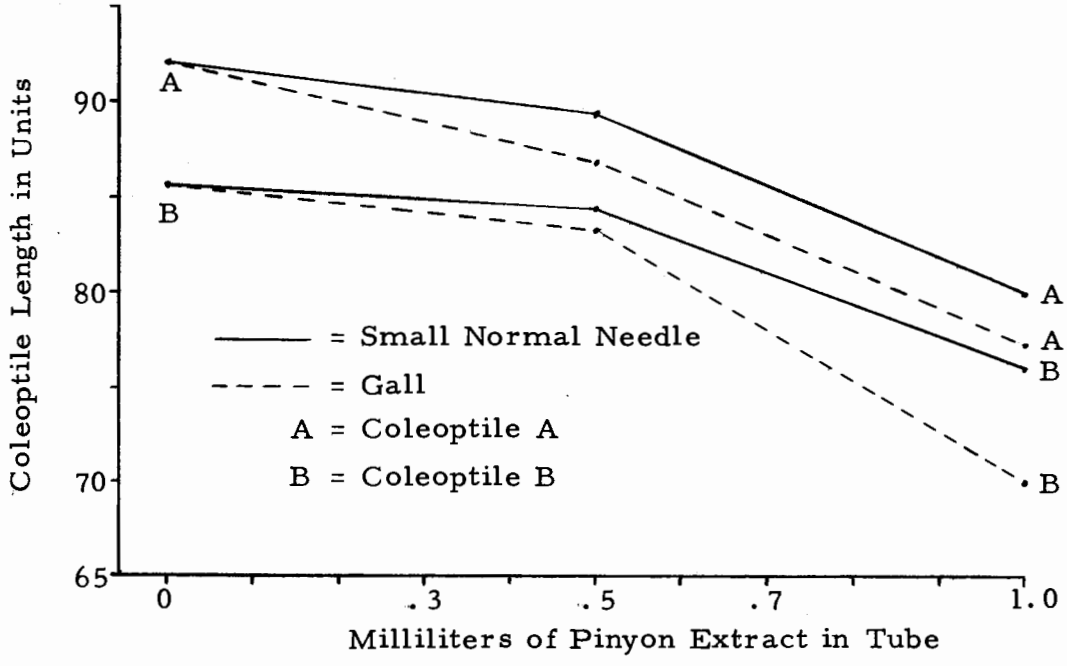



Figure 7. Oat Coleoptile growth in response to IAA - Standard curves used to calculate Auxin content of Pinyon leaves.

The amount of IAA per gall or normal needle was calculated by dividing the total amount of IAA in the galls or needles by the number of galls or needles. For instance, 1,305 galls from July 23, 1972 had 586 ng IAA or 0.265 ng per gall. A correction factor (due to an estimated 10% extraction and purification loss) of 1.1 was multiplied in all cases by the ng of IAA per gall to yield a final amount. Therefore, 0.265 ng per gall when multiplied by 1.1 equaled 0.295 ng of IAA per gall (Fig. 11 and Table 1). The amounts of IAA per gram of gall or normal tissue also was calculated (Table 2 page 67). The X-fold increase in IAA was calculated from the amounts of IAA per round gall or tissue weight divided by the amount per small normal needle (Fig. 11 page 68).

To determine the amount of inhibition in coleoptile growth, tubes containing various amounts and types of pinyon extract from August 13, 1972 plus 100 ng IAA were incubated with coleoptile sections. A graph of the inhibition for different amounts of pinyon extract is shown in Figure 8 A page 49. An inhibition correction factor was calculated for each type and amount of pinyon extract and each coleoptile type (A or B) by dividing the standard coleoptile length at 100 ng IAA with the coleoptile length for each pinyon extract type and amount (Table 8 page 49). The appropriate coleoptile lengths were multiplied by the corresponding inhibition correction factor so that the coleoptile length could be compared to the standard curve as

A. Coleoptile growth inhibited by pinyon needle extract with 100 ng IAA.




B. α-Amylose release from barley half-seeds inhibited by pinyon needle extract with 10 ng GA.

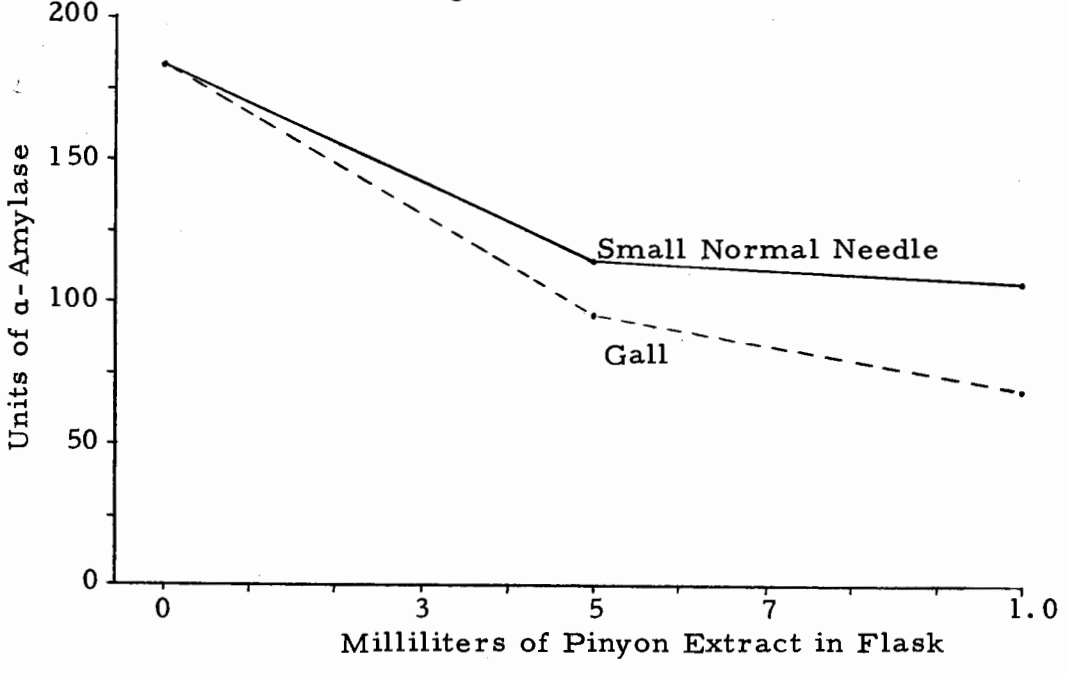



Figure 8. Inhibition curves of coleoptile growth or α-amylase release due to various amounts and types of pinyon extract.

if inhibition did not occur. A second correction factor must be considered which accounts for weight differences of extracted material. The other two experiments used less pinyon material than from August 13, 1972 so the inhibition should be proportionately smaller. Following is the equation describing the correction for inhibition:

$$\left[\left(\begin{array}{c}\text{inhibition}\\ \text{correction}\\ \text{factor}\end{array} \times \begin{array}{c}\text{av. col. lg.}\\ \text{for sample}\\ \text{being corrected}\end{array}\right) \begin{array}{c}\text{av. col. lg.}\\ \text{for sample}\\ \text{being corrected}\end{array}\right] \times \frac{\begin{array}{c}\text{wt.}\\ \text{sample}\\ \text{being}\\ \text{correc.}\end{array}}{\begin{array}{c}\text{wt.}\\ \text{of sample}\\ \text{Aug. 13th}\end{array}} +$$

$$\begin{array}{c}\text{av. col.}\\ \text{for sample}\\ \text{being corrected}\end{array}$$

The above equation reduces to:

$$\begin{array}{c}\text{av. col. lg.}\\ \text{of sample}\\ \text{being corr.}\end{array} \times \left[\left(\begin{array}{c}\text{inhibition}\\ \text{correction}\\ \text{factor}\end{array} - 1\right) \frac{\begin{array}{c}\text{weight of sample}\\ \text{being corrected}\end{array}}{\begin{array}{c}\text{weight of sample}\\ \text{Aug. 13, 1972}\end{array}} + 1\right] =$$

which equals:

coleoptile length corrected for inhibition.

By comparing the corrected coleoptile length to the standard curve, the ng of IAA in the bioassay tubes were obtained. The ng of IAA were multiplied by a volume factor [(1 ÷ mls of extract) x total mls used to elute IAA from silica] to get the total ng of IAA in pinyon galls or needles (Table 1 page 66).

## Bioassay of Gibberellins

The bioassay of gibberellin hormones was essentially that of Jones and Varner (1967) and other investigators (Chrispeels and Varner 1967, Coombe et al. 1967a, b). Naked seeds of Hordeum vulgare (cv 'Nepal') were obtained from the Agronomy Department of Colorado State University. The seeds were selected for uniform size and cut transversely in half and the embryo discarded. The half-seeds were weighed in groups of 10 and placed in test tubes. The half-seeds were surface sterilized with 1.3% sodium hypochlorite (1:4 dilution of commercial bleach) for 15 minutes and then washed with 10 ml of sterile water three times. The seeds were planted with the grooved side down in autoclaved 10 cm petri dishes (40 seeds to a dish) on moist tissue paper (30 ml of $H_2O$). The planting was performed in an isolation chamber to reduce fungal spore contamination of the petri dishes.

The half-seeds remained in darkness for a 72 hr imbibition period at 23° C. Ten half-seeds then were transferred to each of the 50 ml Erlenmyer flasks with 1 ml of buffer containing 2 uM sodium acetate and 20 uM $CaCl_2$, and 1 ml of various amounts of gibberellin in a final volume of 2 ml. Gibberellic acid standard solutions were made by 1:10 serial dilutions of the stock solution 0.1 g $GA_3$/liter (Eastman organic chemicals). In the case of flasks with dried gall or normal needle extracts, 1 ml of water replaced the 1 ml of $GA_3$

standard solution. Ten ul containing 20 ug chloramphenicol was also added to inhibit bacterial growth.

The flasks were stoppered and incubated for 24 hrs, in the dark, at 31° C on a Dubnoff shaker operating at 50 oscillations per minute. The solutions were then decanted and the flasks washed with 3 ml of water. A total of 5 ml of enzyme solution from each flask was centrifuged at 6,000 x g for 10 minutes and the supernatant decanted into a test tube. The test tubes, each containing the enzyme from 10 half-seeds, were kept in a refrigerator until measured for α-amylase activity (2-5 hr later).

The α-amylase activity was measured with the following procedure. One ml from each of the above extracts was diluted anywhere from 1:10 to 1:150 depending on what concentration of $GA_3$ was used in the incubation medium. One ml of the final dilution was then added to 1 ml of starch substrate and incubated at room temperature (25° C) for 6 min. until the reaction was stopped with the addition of 1 ml of iodine reagent. Five ml of water were added to the reaction mixture (final volume 8 ml) and after mixing, the optical density of the solution was measured on a Bausch and Lomb Spectronic 20 at 620 nm (Fig. 6 C page 41).

The starch substrate was prepared with 150 mg of Manischewitz potato starch, 4.41 mM $KH_2PO_4$, and 200 uM of $CaCl_2$ in a total volume of 100 ml water. The solution was then boiled

1 min., cooled, and centrifuged at 12,000 x g for 10 min. The supernatant was used as the starch substrate.

The iodine reagent was prepared by mixing 1 ml of iodine stock solution and 99 ml of 0.05 N HCl. The iodine stock solution contained 6 g of potassium iodide, 600 mg of iodine, and water to 100 ml.

Barley half-seeds produced more α-amylase at higher concentrations of gibberellin. The slope of this relationship was different each time the bioassay was performed (Figure 9 B page 55). Apparently, some factor was equally affecting all the flasks with different concentrations of GA. A basic factor might be the temperature during the incubation period or the time of sodium hypochlorite sterilization. The temperature was always $30^{\circ} \pm 2^{\circ}$ C and the tray of flasks was rotated after 12 hr to equalize any temperature differences. On the other hand, the length of time the half-seeds were immersed in sodium hypochorite (15 ± 3 min) probably accounts for the different rates of α-amylase release between bioassays. After sterilization, a ring of presumably dead aleurone cells occurred near the cut edge of barley seeds. A wider ring corresponding to longer sodium hypochlorite exposure and less living aleurone cells would release less α-amylase in response to gibberellin than a narrow ring. Other factors which affect α-amylase release are discussed by Jones and Varner (1967), Coombe et al. (1966a, 1966b), and Chrispeels and Varner (1967).

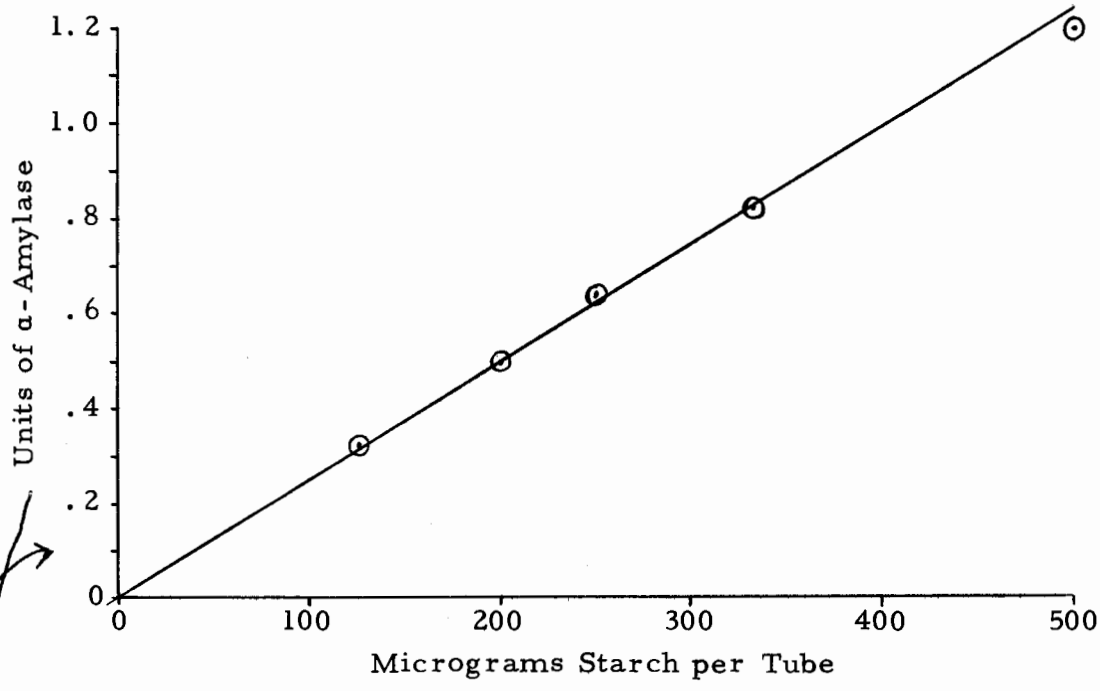
The release of α-amylase is usually reported in micrograms per barley half-seed by comparing the optical density values of the starch-iodine complex after incubation with the unknown with the O. D. values of the complex following incubation with known quantities of pure α-amylase. Problems arise with this method since pure α-amylase samples do not exist commercially and are often different at each laboratory. Furthermore, the barley seeds also secrete smaller amounts of β-amylase (Jones and Varner 1967). Finally, the number of micrograms of α-amylase calculated at one dilution (from optical density values and the standard α-amylase curve) of the barley enzyme medium were quite different from values obtained at another dilution.

Using starch breakdown or the amount of starch hydrolyzed per minute as the unit of α-amylase activity, different dilutions of the barley enzyme medium always showed the same amount of activity.

## Calculation of GA Levels in Pinyon

A standard curve of the amount of starch versus the optical density was made by varying the starch concentration and keeping the iodine constant (Fig. 9 A page 55). The optical density of the starch-iodine complex indicates how much starch is left after the 6 min incubation with α-amylase. Since the amount of starch added to the barley α-amylase dilutions was always constant, a decrease in starch (or O.D.) would indicate the amount of α-amylase. A unit of

A. Standard curve of starch - Iodine color (amount of iodine constant).




B. Variation in standard curves of α-amylase release by Barley endosperm in response to $GA_3$.

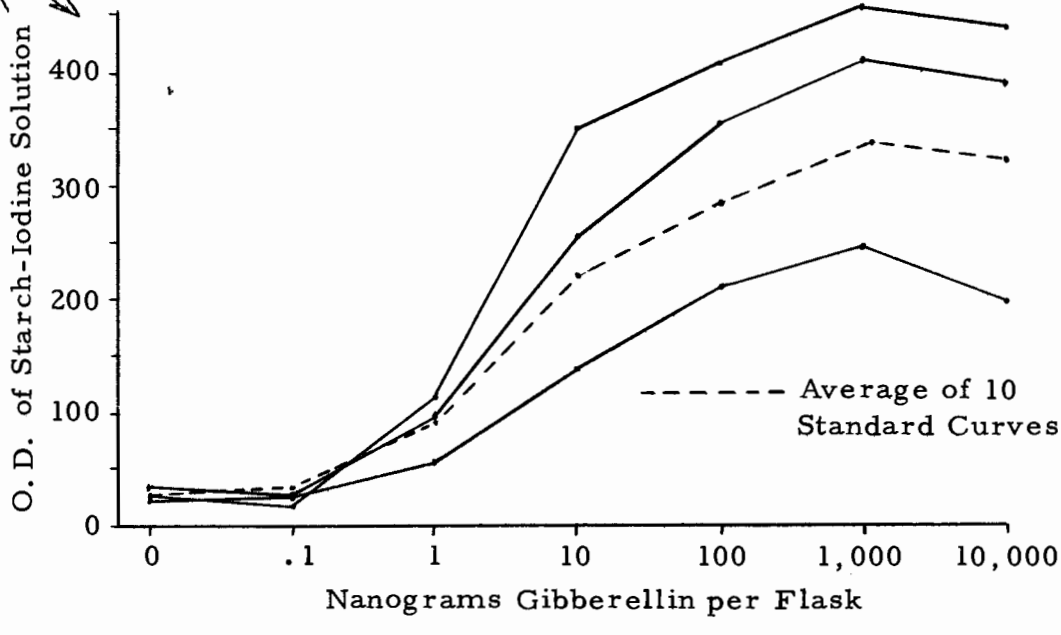



Figure 9. Standard curves used to determine GA content of pinyon leaves.

α-amylase was defined as the amount of enzyme able to convert 100 ug starch to glucose per minute (Nickells et al. 1971). In other words, the amount of starch hydrolyzed per minute was proportional to the amount of α-amylase present in the tube. The starch hydrolyzed was then found by subtracting the amount of starch present after the 6 min incubation period from the initial amount of starch (500 ug). This difference was then divided by 6 min to obtain the units of α-amylase present in the barley enzyme dilution. The total α-amylase units were obtained by multiplying the above units by a dilution factor (x 10 to x 150), a weight correction, and finally by the number of milliliters in the barley supernatant (5), (See Appendix Tables 9, 10, 11). The standard curve of the α-amylase release was performed for each week that pinyon material was extracted (Appendix Tables 10 and 11). The standard curves for each bioassay and the average of 10 separate bioassays are shown in Figure 10 page 57. The units of α-amylase activity calculated for each extract sample (Appendix Tables 9, 10, 11) were compared to the appropriate standard curve and the amount of gibberellin corresponding to the number of units was read from the standard curve. None of the results were compared to the average of 10 standard curves since each curve had a characteristic slope (Figure 9 A page 55). Therefore, the results from pinyon extracts for each week would closer approximate their particular standard curve.

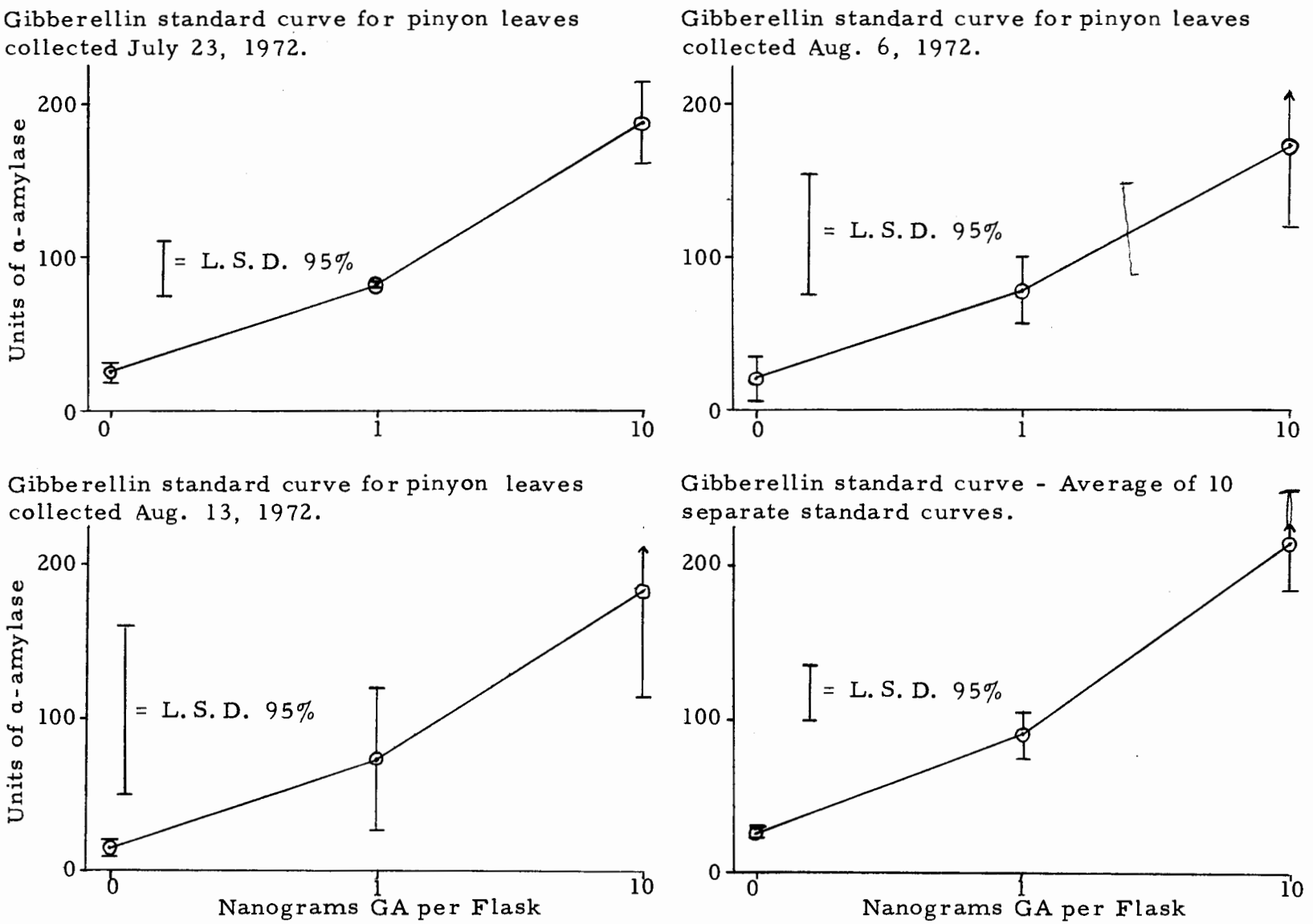



Figure 10. α-amylase release by barley endosperm in response to gibberellin$_3$ - Standard curves used to calculate GA content of pinyon leaves.

The nanograms of gibberellin in pinyon extracts were obtained from the standard curves and amounts per needle or gall were calculated and corrected for volume and purification loss (about 10%) Tables 3 and 4 pages 77, 78).

Inhibition corrections much like those described for auxins were calculated from inhibition curves (Figure 8 B page 49) constructed from results shown in Table 8 page 110. The nanograms of gibberellin per gram tissue as well as per round gall or small needle and the X-fold increase of GA in round galls compared to normal needles were corrected for inhibition (Tables 3 and 4 pages and Figure 14 page 79).

## Polyacrylamide Gel Electrophoresis of Peroxidases

Peroxidase isozymes in samples of normal needles and round and stubby galls were separated using polyacrylamide gel electrophoresis. Merry Alexander, Colorado State University, prepared, ran, and stained the polyacrylamide gels for peroxidases. The techniques and equipment* used are described by Denna and Alexander (1973). Cecidomyiid larvae were dissected from the galls so that any peroxidase differences would be due only to the plant tissue. Approximately equal weights of frozen pinyon needles and galls were macerated using a mortar and pestle with various amounts

*Ortec Inc., 100 Midland road, Oak Ridge, Tenn. 37830.

of water (1 to 10 ml). The pulp was squeezed through cheesecloth and 1 ml of this extract was mixed with 0.5 ml 80% sucrose. A sample of each mixture was applied to sample wells in the center of a polyacrylamide gel formed between quartz plates. The samples were then overlayered with another polyacrylamide gel. After polymerization, the gel slab was placed in an electrophoretic field produced by an Ortec 4100 pulsed constant power supply. Later, the gel was removed from the quartz casing and stained for peroxidases with o-tolidine and hydrogen peroxide. Peroxidases will oxidize o-tolidine into a yellow-brown product which can easily be seen (Fig. 13 page 71).

## Pinyon Thin-section Preparation and Observation

The following techniques are basically from Sass (1958).

Round galls, stubby galls, and young needles were fixed and stored in FAA solution (50% ethanol, 35% water, 10% formaldehyde 37% and 5% acetic acid). After several weeks, the galls or needles were passed through a series of dehydration steps of ethanol from 50% to 75%, 95%, and 100%. The needles remained at each concentration for at least 6 hr. Xylene was next substituted for ethanol by the following changes: 25% xylene and 75% ethanol, 50% xylene and 50% ethanol, 75% xylene and 25% ethanol, finally 100% xylene. The pinyon material in xylene was heated to 60° C and two volumes of

paraffin (m.p. 57° C) were added per volume of xylene. The mixture containing the tissues was kept at 60° C for 4 days until the xylene evaporated and the tissues were completely embedded with paraffin.

The paraffin was cooled and sectioned with an A.O. Spencer microtome at a thickness of 25 μ. The sections were placed over a drop of water on a slide with a film of "Tissue-Tac" adhesive. The sections were affixed to the slide by heating to 56° C for 4 hr.

The slides then were dipped in xylene for 5 min. to remove the paraffin. Next, the slides were passed through a series of decreasing concentrations of ethanol: 100%, 95%, 70%, 50%, 30%, for 3 minutes at each step. The slides then were dipped in distilled water for 2 min and stained in 1% safranin-O for 30 min. After staining, the slides were placed in distilled water for 1 min and then passed at 1 min intervals from 30% ethanol to 50%, 70%, 95%, and into 95% ethanol with 0.6% acid fast green. The slides were stained for 30 seconds and passed into two changes of 100% ethanol (1 min) and then into phenol : xylene (1:4) for 1 min to clear the tissue. Finally, the slides were dipped in two xylene changes (1 min). A cover slip was applied using canada balsam as the adhesive.

Observations of the slides were made with a microscope at 100x and 400x. Various types of cells were counted in a cross section of a round gall and stubby gall collected July 30, 1972. The average area of a cell outside the endodermis (mesophyll and

epidermal cells) was calculated by dividing the total area by the number of cells. The area of gall tissue was determined by using a microscope with a movable grid at 100x. Each square of the grid contained 0.0277 square mm. The average diameter of a cell was then found by multiplying 2 by the square root of the average cell area divided by $\pi$. The average volume of a cell outside the endodermis was obtained with the following equation: $(4/3)\pi\left(\frac{d}{2}\right)^3$ where d = average diameter. The X-fold increase in cell volume of gall mesophyll cells over normal needle cells was found by simply taking the ratio of the volumes. Photomicrographs were taken of some of the slides under normal and polarized light.

## Characterization of Anthocyanins

Red pigment was extracted with methanol from lyophylized red round galls and stubby galls. The methanolic extract from the filtered material was bright red. The extract was concentrated "in vacuo" to 3 ml and streaked on Whatman #1 chromatography paper (Fig. 4 B page 35). The red pigment was subjected to various solvent systems using descending chromatography (Harborne 1967). Red petunia flowers also were extracted as a control. After development of the chromatogram, the pigmented band was cut out and eluted with methanol. The methanol was concentrated "in vacuo" and the resulting orange-red solution was measured for light absorption with a

dual-beam Bausch and Lomb recording spectrophotometer. The pigment then was acid-hydrolyzed to remove the glycosides, concentrated "in vacuo", streaked on chromatography paper, and developed in various solvent systems (Harborne 1967). The addition of HCl or NaOH was used to determine the pH at which color transitions occurred in the pigment. Ultraviolet light was used to determine fluorescent colors for comparison to known anthocyanins (Harborne 1967).

## RESULTS AND DISCUSSION

### IAA in Round Galls and Small Normal Needles

The auxin, indoleacetic acid, was found in larger amounts in extracts from round galls than in small normal needles (Appendix Table 5). The dramatic increase of auxin in round galls compared to normal needles is shown in Figures 11 and 12 pages 68, 69). The general increase in levels of IAA in galls was not altered when a correction for inhibition was made. The higher auxin levels in galls must occur at the start of gall formation since high levels were found in all samples.

The $R_f$ values of bands showing auxin activity corresponded to where pure IAA migrated when chromatographed with pinyon material. Pure IAA standard normally would migrate to band 4 or 5 but due to pinyon displacer substances, the IAA went to band 6 with substantial tailing in band 5.

The large increases of IAA in galls may be somewhat exaggerated since the lower values of IAA found in small needles were at the lower limit of detection. Therefore, the results were more variable for the small normal needle making the X-fold increase of IAA in galls more variable and subject to error. On the other hand, by considering inhibition, the calculated amounts of IAA in small normal needles

may be inflated by the inhibition correction factor since non-significant values of coleoptile lengths became statistically significant when compared to standard inhibition curves.

Inhibition of coleoptile growth by non-specific inhibitors in pinyon extracts was tested by the addition of 100 ng IAA to these extracts and doing a bioassay. The relative purity of the small needle and gall extracts can be seen in Figure 4 D page 35 in tubes 5 through 8. When more than 1 ml of any of these tubes was used in the oat coleoptile bioassay, severe inhibition of growth or death of the coleoptile occurred. Consequently, 0.3 to 0.5 ml of extract had the least inhibition and yielded the best results in the bioassay (Table 6 page 108. Smaller volumes of extract were not tried but 0.1 to 0.2 ml volumes would be expected to contain less auxin and thus would allow for less coleoptile growth.

In correcting for inhibition several assumptions were made. First, it was assumed the inhibition at 100 ng of IAA was proportionate to extracts with other amounts of IAA. Second, the decrease in weight of pinyon material used was assumed to be proportional to a linear decrease in inhibition. Finally, it was assumed the inhibitory substances were about the same from week to week during the summer of 1972.

More stringent purification of the pinyon extracts with column chromatography or rechromatography with thin-layer plates would

improve the accuracy of the results. Also, standard curves of inhibition at several concentrations of added IAA would allow more accurate determination of the hormone.

Radioactive IAA could be used to determine purification losses since basic and neutral indole compounds react colorimetrically with Erhlich's reagent thus frustrating the detection of IAA in the first stages of solvent purification. Finally, several concentrations of IAA near those found in pinyon galls could be applied to the just forming needles during June to try and mimic gall midge effects. If gall formation and anthocyanin production occurred then IAA is more strongly implicated in gall formation, however, the mechanism of IAA induction still is unknown.

## IAA in Cecidomyiid Larvae

The entire larval extract was bioassayed with 5 coleoptiles. Inhibition should have been minimal since 204 larvae weighed only a few mg and no plant inhibitors were present. The coleoptiles were inhibited slightly (col. A = 63.8) showing that there was either no IAA or that some specific inhibitor blocked IAA action. However, it is more likely that no auxin was present since any inhibitors may not have chromatographed with IAA and the larval weight was quite small (about 2 mg).

Table 1. Amount of Indoleacetic Acid per Gall or Small Normal Needle and X-fold Increase of IAA in Round Gall Compared to Small Normal Needle (S. N. ).

| $R_f$ Band | Sample | Vol. | Col. | Total ng IAA | Total ng IAA inhibition | IAA/gall or needle (ng) | IAA/gall or needle inhibition (ng) | X-fold increase IAA in gall | X-fold increase IAA in gall inhibition |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | July 23, 1972 | | | | |
| 5 | S. N. | 0.5 | A | 00 | 68 | 0.014 | 0.040 | | |
| 6 | S. N. | 0.5 | A | 73 | 140 | | | | |
| 5 | Gall | 0.5 | A | 140 | 270 | 0.295 | 0.499 | 21.0 | 12.5 |
| 6 | Gall | 0.5 | A | 206 | 316 | | | | |
| | | | | | August 6, 1972 | | | | |
| 6 | S. N. | 1.0 | B | 25 | 101 | 0.006 | 0.024 | | |
| 5 | Gall | 1.0 | B | 19 | 132 | 0.183 | 0.674 | 30.4 | 27.8 |
| 6 | Gall | 1.0 | A | 128 | 410 | | | | |
| | | | | | August 13, 1972 | | | | |
| 6 | S. N. | 0.3 | A | 83 | 120 | | | | |
| 6 | S. N. | 0.5 | A | 68 | 130 | 0.014 | 0.019 | | |
| 6 | S. N. | 0.5 | B | 31 | 40 | | | | |
| 6 | Gall | 0.3 | A | 333 | 438 | | | 12.1 | 11.5 |
| 6 | Gall | 0.3 | B | 115 | 153 | 0.164 | 0.247 | | |
| 6 | Gall | 0.5 | A | 276 | 490 | | | | |
| 6 | Gall | 0.5 | B | 84 | 136 | | | | |

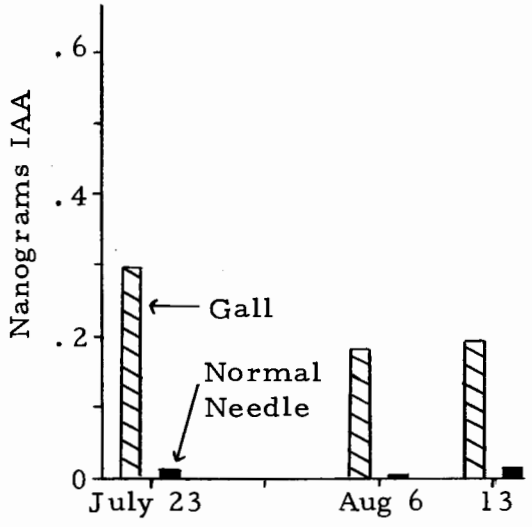
Vol. = mls. of extract in bioassay tube

Col. = type of coleoptile section used in bioassay tube

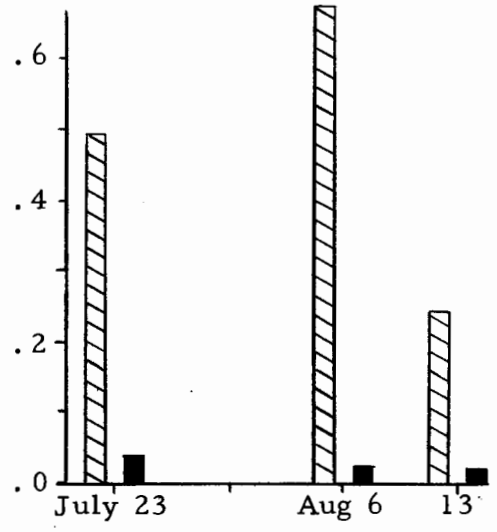
Table 2. Amount of Indoleacetic Acid per Gram of Round Gall or Small Needle Tissue (S. N. ) and X-fold Increase of IAA in Gall Compared to Small Normal Needle Tissue.

| Sample | ng IAA/gram tissue assumed no inhibition | ng IAA/gram tissue corrected for inhibition | X-fold increase IAA in gall tissue assumed no inhibition | X-fold increase IAA in gall tissue corrected for inhibition |
|---|---|---|---|---|
| | | July 23, 1972 | | |
| S. N. | 1.22 | 3.48 | | |
| | | | 4.2 | 2.5 |
| Gall | 5.12 | 8.67 | | |
| | | August 6, 1972 | | |
| S. N. | 0.37 | 1.49 | | |
| | | | 6.7 | 6.15 |
| Gall | 2.49 | 9.17 | | |
| | | August 13, 1972 | | |
| S. N. | 0.87 | 1.39 | | |
| | | | 2.9 | 2.8 |
| Gall | 2.54 | 3.83 | | |

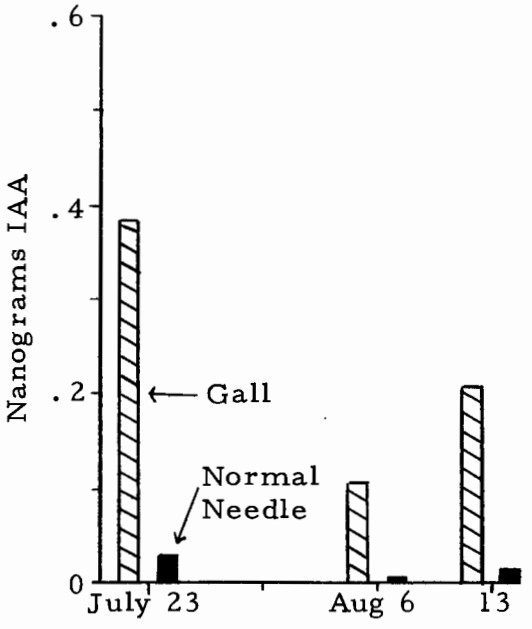
Amount of IAA per gall or normal needle - Results calculated from each week's standard curve - assumed no inhibition.




Amount of IAA per gall or normal needle - Results calculated from each week's standard curve, corrected for inhibition.




Amount of IAA per gall or normal needle - Results calculated from average of 5 Standard curves - assumed no inhibition.




Amount of IAA per gall or normal needle - Results calculated from the average at 5 Standard curves - corrected for inhibition.

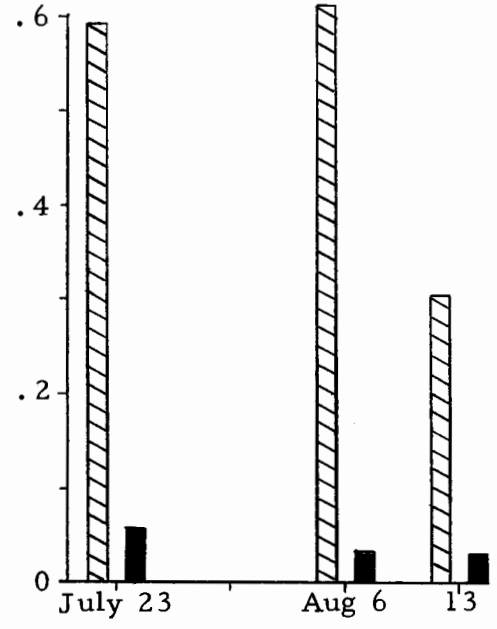
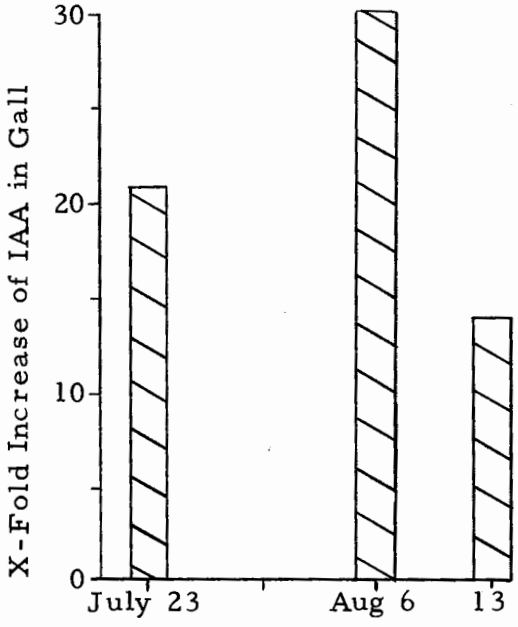
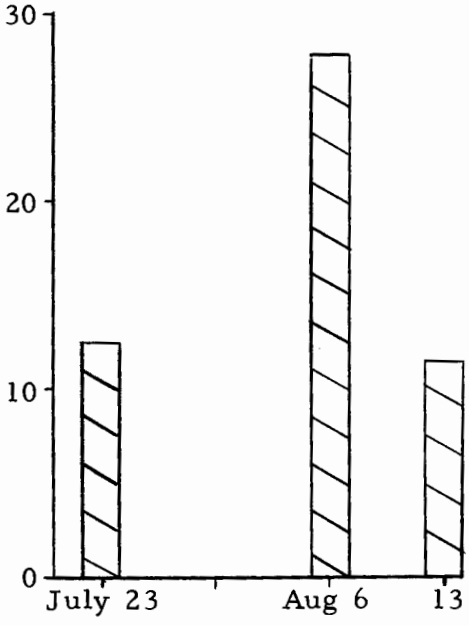



Figure 11. Amount of IAA per round gall or small normal needle.

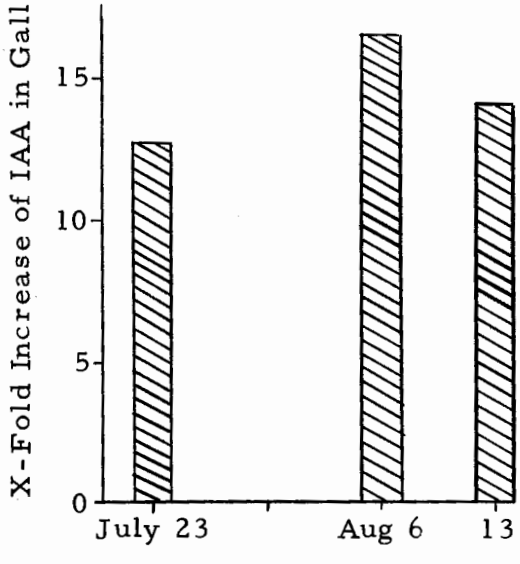
X-Fold increase of IAA in round gall - Results calculated from each week's standard curve - assumed no inhibition.




X-Fold increase of IAA in round gall - Results calculated from each week's standard curve - corrected for inhibition.




X-fold increase of IAA in round gall - Results calculated from each week's standard curve - assumed no inhibition.




X-fold increase of IAA in round gall - Results calculated from average of 5 standard curves - corrected for inhibition.

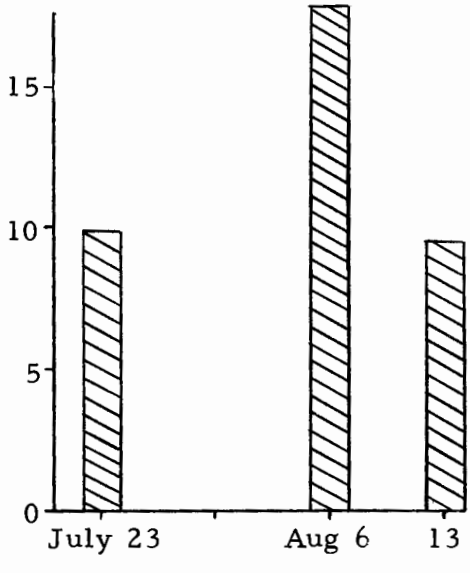
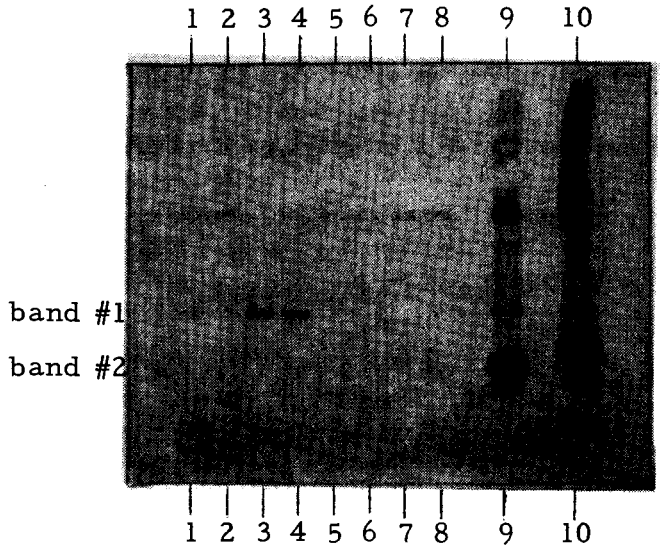



Figure 12. X-fold increase of IAA in round gall compared to small normal needle.

## Peroxidase in Galls and Small Normal Needles

Peroxidase isozymes are involved in several important morphogenetic actions in plant cells. Peroxidases oxidize several compounds such as reduced nicotinamide-adenine dinucleotide and convert coniferyl alcohol into lignin material (Galston and Davies 1969, Siegel 1955). As mentioned before, peroxidases act as IAA-oxidases and destroy the biological activity of IAA. Therefore, it is conceivable that the cecidomyiid larvae could inhibit peroxidase enzymes and cause IAA to build up to toxic or gall-forming quantities.

Round galls, stubby galls, and small normal needles were tested for peroxidase isozyme differences using polyacrylamide gel electrophoresis. Using this technique, galled tissue was found to contain higher concentrations of one peroxidase than normal tissue but was missing an anodic peroxidase isozyme. From the picture in Figure 13 page 71, one can see the darker staining (peroxidase in) band #1 from stubby and round galls. This same band also was present weakly in normal needles (too weak to see in photograph) indicating its induction or activation in gallous tissue. The stubby galls probably have more of this enzyme (#1) than round galls but the round galls were frozen for one year which could account for the loss of some of the peroxidase. The normal needles when concentrated had a peroxidase (#2) not found in either round or stubby galls.




1. Green Stubby Galls - dilute
2. Small Normal Needles - dilute
3. Green Stubby Galls - concentrated
4. Red Stubby Galls - concentrated
5. Small Normal Needles (Frozen 1 Year) - medium concentration
6. Red Round Galls (Frozen 1 year) - concentrated
7. Small Normal Needles - medium concentration
8. Small Normal Needles - concentrated
9.-10. Peroxidase Bands from Young Squash

Figure 13. Peroxidase isozymes from round galls, stubby galls, and small normal needles.

Peroxidase #1 from gall material could have been induced by high concentrations of IAA found in the round gall. Galston and Dalberg (1954) showed that IAA-oxidase production could be induced by exogenous IAA in young tissue. Ockerse et al. (1966) and Galston et al. (1967) have added further evidence that IAA can induce IAA-oxidase but they also stated that IAA can inhibit peroxidase isozymes in tobacco pith tissue. Therefore, the peroxidase isozyme differences could be due to high concentrations of IAA. On the other hand, peroxidase differences could result as a secondary response to changes in tissue physiology as gall formation occurs. An alternative idea is that the insect produces a chemical which inhibits peroxidase #2 in normal needles and IAA builds up and induces peroxidase #1. This idea is valid only if peroxidase #1 is ineffective in catabolyzing IAA.

Several diphenols are known to inhibit IAA-oxidase such as scopoletin, caffeic acid, chlorogenic acid, and catechol (Andreae and Collet 1967, Salisbury and Ross 1969, Furuya et al. 1962, Thimann et al, 1962). Insects are known to contain the orthophenols dihydroxyphenylalanine and dopamine which are precursors to a quinone which combines with proteins in the cuticle to form sclerotin (Wigglesworth 1970). These two orthophenols could be the chemicals responsible for the initiation of gall formation. Supporting the idea that orthophenols may cause gall formation, Anders (1960) found three

orthophenols in gall aphid saliva. Rowan (1970a) found different phenolics in fusiform rust gall tissue than in normal pine tissue. There is still very little evidence to support the hypothesis that orthophenols are the cause of IAA increase in gall tissue, but it is a reasonable idea since orthophenols increase IAA concentrations in other plant systems (Andreae and Collet 1967, Bouillenne and Gaspar 1970, Furuya et al. 1962).

## Senescence of Galls

Most pinyon needles remain on the tree for at least four years and often up to nine years (Sudworth 1917). However, the round gall which forms in the young needle, senesces early in September and dies by October or November while the stubby gall usually dries out by spring. Ethylene has been known since 1924 to hasten ripening in fruits and many investigators have reported ethylene causing senescence in fruit and flowers (Salisbury and Ross 1969, Hansen 1946). Applications of auxin to various plant tissues often results in the production of ethylene (Abeles and Rubinstein 1964, Burg and Burg 1966, Morgan and Hall 1962). Thus, the high levels of auxin in round galls may induce ethylene production by the tissues leading to senescence and abscission (Hansen 1946).

## GA in Round Galls and Small Normal Needles

Gibberellin was also found to increase in round galls compared to normal needles (Tables 3 and 4 pages 77, 78 and Figure 14 page 79). The results were similar whether inhibition was considered or not. No general trends in amounts of GA during the summer were observed. Therefore, the hormone levels must change drastically before July 23, probably at the initiation of the gall.

Band #2 had the highest gibberellin activity from pinyon extracts (Table 3 page 77). $GA_3$ was also found to migrate to band #2 when chromatographed with displacer compounds in the pinyon extract. Thus, gibberellin from pinyon behaved chromatographically like pure $GA_3$.

The nanogram quantities found in round galls and normal needles were in error at least 7% since the standards were only 93% $GA_3$. Furthermore, the amounts of GA calculated could be in even larger error since the gibberellin activity in pinyon needles may be due to one or more of the 35 other known gibberellins. One group of gibberellins (GA 1, 2, 4, 7, and 22) has activity similar to $GA_3$ in the barley endosperm bioassay while a second group (GA 5, 6, 8, 9, 10, 11, 13, 20, and 23) has less activity (Crozier et al. 1970). It was impossible with the methods used to determine whether the observed GA activity was due to the first group of gibberellins or to the second group. If the pinyon gibberellin activity was due to the second group

then the actual amount of GA would be much larger than calculated. However, the relative amounts of GA in normal needles and round galls are still valid and the excess amounts of GA in round galls may indicate some relation to gall formation. Other gibberellins (12, 14, 15, 17, 18, 19, 21, 24, 25, 26, and 27) are inactive in the barley endosperm bioassay and therefore are probably not responsible for the observed GA activity in pinyon, however, they still could be present (Crozier et al. 1970).

## GA in Cecidomyiid Larvae

Several workers have reported ecdysone and ecdysterone in higher concentrations in plants than in insects (Carlisle and Ellis 1968, Ohtaki et al. 1967, Takemoto et al. 1967). Since some plants contain ecdysone, it seems possible that insects may contain high concentrations of plant growth hormones (GA) used to form galls.

About 0.4 ng GA was found in 204 round gall larvae. If the larvae were responsible for the gibberellin found in the round gall extracts (which contained about 1,000 galls) then 0.4 ng/204 gall larvae calculates to about 2.9 ng from the larvae in 1,000 galls. However, the lowest amount of GA found in galls was 6 ng and after correcting for inhibition, the lowest value was 20 ng. The greatest amount of gibberellin found in galls (August 6, 1972) was at least 15 times more than in a comparable amount of larvae. Inhibition may

have reduced the gibberellin activity in crude larval extracts since solvent partitioning purification was not carried out. However, the 204 larvae weigh only a few milligrams and inhibition would not be expected at this level. Therefore, the increased levels of GA in round galls was probably not due solely to gibberellin from the cecidomyiid larvae.

It is possible that the relatively low amounts of gibberellin found in gall larvae is a residual amount contained in the salivary glands that is continually secreted to sustain gall formation. In other words, the rate of GA production and release by the cecidomyiid larvae may be quite rapid so that relatively small amounts of GA would be expected in the larvae but higher amounts would accumulate in the gall tissue. However, the gibberellin activity found in cecidomyiid larvae could conceivably have been due to ecdysone.

Carlisle et al. (1963) reported that ecdysone, the insect moulting hormone, had growth promoting activity about 10% of that exerted by $GA_3$ on the dwarf pea gibberellin bioassay. Conversely, gibberellins had ecdysone activity in certain grasshoppers. Therefore, the gibberellin-like activity from round gall larvae could be due to either normal levels of ecdysone or superoptimal levels which might have gall forming effects. On the other hand, the cecidomyiid larva could secrete homologues of ecdysone which had some gibberellin activity but even more pronounced effects in pinyon needles where galling

Table 3. Amount of Gibberellin per Round Gall or Small Needle (S.N.) and X-Fold Increase of Gibberellin in a Gall Compared to a Small Normal Needle.

| Sample | $R_f$ Band | ~~Total~~ α-amylase | ng GA | ng GA corrected for inhibition | ng GA/gall or needle | ng GA/gall or needle inhibition | X-fold increase GA in gall | X-fold increase GA in gall inhibition |
|---|---|---|---|---|---|---|---|---|
| | | per sub-sample 5-7 ml | per sample ~10 ml | July 23, 1972 | | | | |
| S.N. | 2 | 48.3 | 5.3 | 12.4 | 0.00051 | 0.00119 | | |
| S.N. | 2 | 19.9 | 0.0 | 0.0 | | | | |
| Gall | 2 | 38.9 | 5.8 | 20.8 | | | 10.0 | 14.4 |
| Gall | 2 | 55.2 | 12.2 | 28.1 | 0.00511 | 0.01716 | | |
| Gall | 2 | 31.1 | 0.0 | 11.5 | | | | |
| | | | | August 6, 1972 | | | | |
| S.N. | 2 | 65.6 | 4.6 | 15.4 | 0.00142 | 0.00401 | | |
| S.N. | 1 | 35.1 | 1.3 | 1.3 | | | | |
| Gall | 2 | 137.8 | 40.8 | 100.0+ | 0.05478 | 0.12430 | 38.6 | 31.0+ |
| Gall | 1 | 50.6 | 3.3 | 3.3 | | | | |
| Larvae | 1-3 | 44.8 | 0.4 | 0.4 | | | | |
| | | | | August 13, 1972 | | | | |
| S.N. | 1 | 45.2 | 3.2 | 3.2 | | | | |
| S.N. | 2 | 41.7 | 0.9 | 9.2 | 0.00092 | 0.00175 | | |
| S.N. | 2 | 23.4 | 0.0 | 4.8 | | | | |
| S.N. | 2 | 15.8 | 0.0 | 0.0 | | | 6.4 | 14.8 |
| Gall | 2 | 52.5 | 8.8 | 32.5 | | | | |
| Gall | 2 | 54.6 | 9.4 | 35.4 | 0.00584 | 0.02584 | | |
| Gall | 2 | 46.7 | 3.4 | 27.6 | | | | |
| Sugar Beet Larvae | 1-3 | 45.7<br>58.8 | 1.5 | 1.5 | | | | |

Table 4 Amount of Gibberellin per Gram of Round Gall or Small Needle Tissue (S. N. ) and X-fold Increase of GA in Gall Compared to Small Normal Needle Tissue.

| Sample | ng GA/gram tissue assumed no inhibition | ng GA/gram tissue corrected for inhibition | X-fold increase GA in gall tissue assumed no inhibition | X-fold increase GA in gall tissue corrected for inhibition |
|---|---|---|---|---|
| | | July 23, 1972 | | |
| S. N. | 0.044 | 0.140 | | |
| | | | 2 | 2.9 |
| Gall | 0.088 | 0.298 | | |
| | | August 6, 1972 | | |
| S. N. | 0.087 | 0.246 | | |
| | | | 8.6 | $7^{+}$ |
| Gall | 0.746 | $1.727^{+}$ | | |
| | | August 13, 1972 | | |
| S. N. | 0.050 | 0.113 | | |
| | | | 1.8 | 3.5 |
| Gall | 0.091 | 0.401 | | |

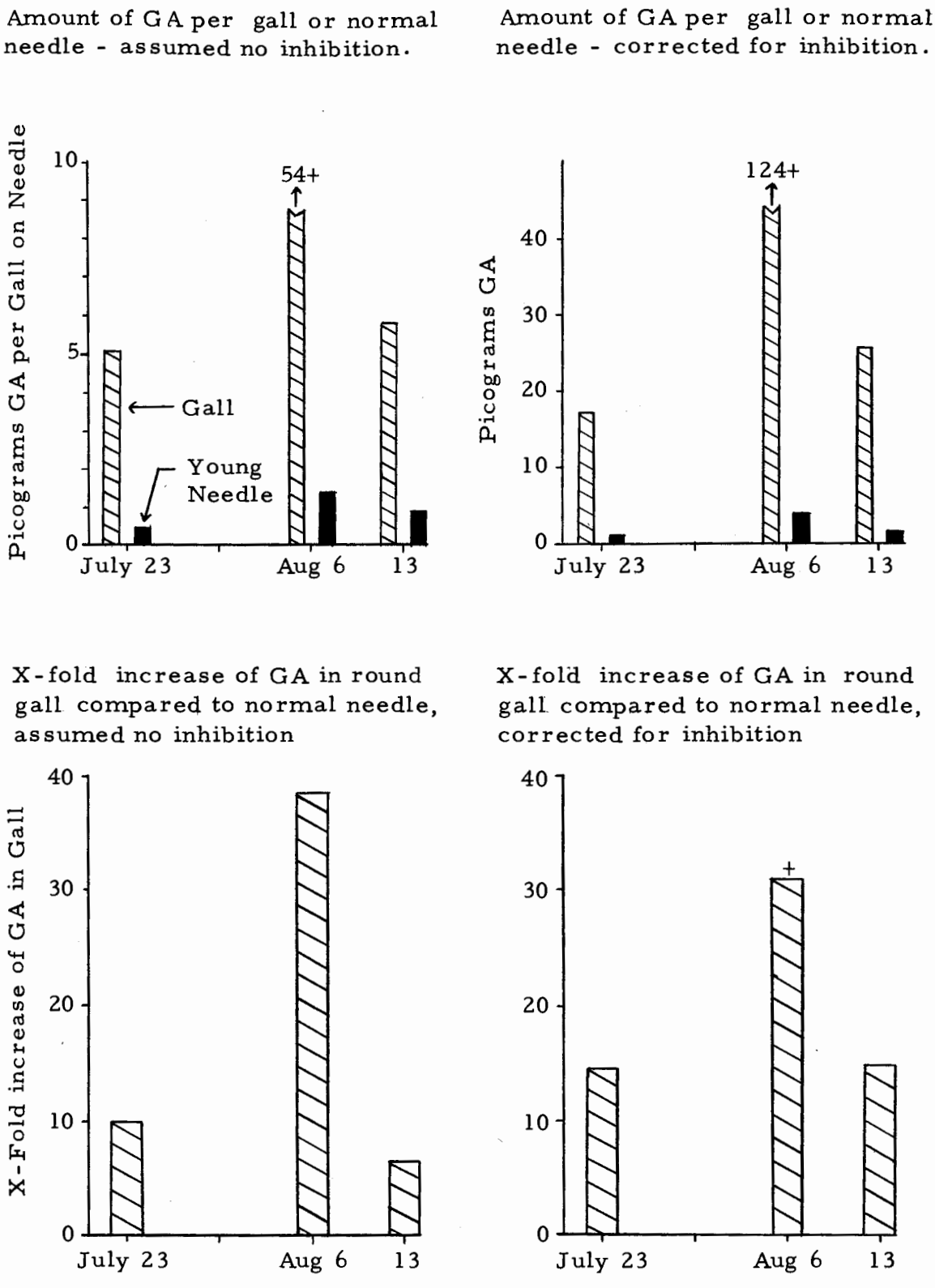



Figure 14. Amount of Gibberellin per round gall or small normal needle and X-fold increase of GA in round galls.

would result. To check the findings of gibberellin in cecidomyiid larvae, sugar beet root maggots (Diptera) were bioassayed for gibberellin.

Sugar beet root maggots were processed exactly like cecidomyiid larvae and 1.5 ng of gibberellin-like activity were detected. Only 17 larvae were used but they weighed 340 mg. Since these insects are not gall makers, the "gibberellin" is probably ecdysone. Ecdysone and ecdysterone may behave like gibberellin on silica gel plates due to several hydroxyl groups making it rather polar but the chromatographic properties were not tested. Ecdysone, however, would probably remain in the aqueous phase of the first solvent partitioning since ecdysone is soluble in water and not acidic (Wigglesworth 1970). Therefore, ecdysone and ecdysterone probably do not account for the gibberellin activity in round gall extracts but in the larval extraction (no solvent partitioning), ecdysone may act as the "gibberellin". Extraction of a larger number of cecidomyiid larvae, followed by solvent partitioning purification and GA bioassay might determine whether GA or ecdysone was responsible for the gibberellin-like activity.

The mechanism by which cecidomyiid larvae induce higher levels of gibberellins in round galls is unknown. However, high levels of gibberellin are known to increase auxin levels (Kuraishi and Muir 1964a, b, Sastry and Muir 1963) by increasing the diphenols

which inhibit peroxidase (Kogl and Elema 1960). Thus, the larvae could secrete or induce gibberellin which would then increase auxin levels so galling could result.

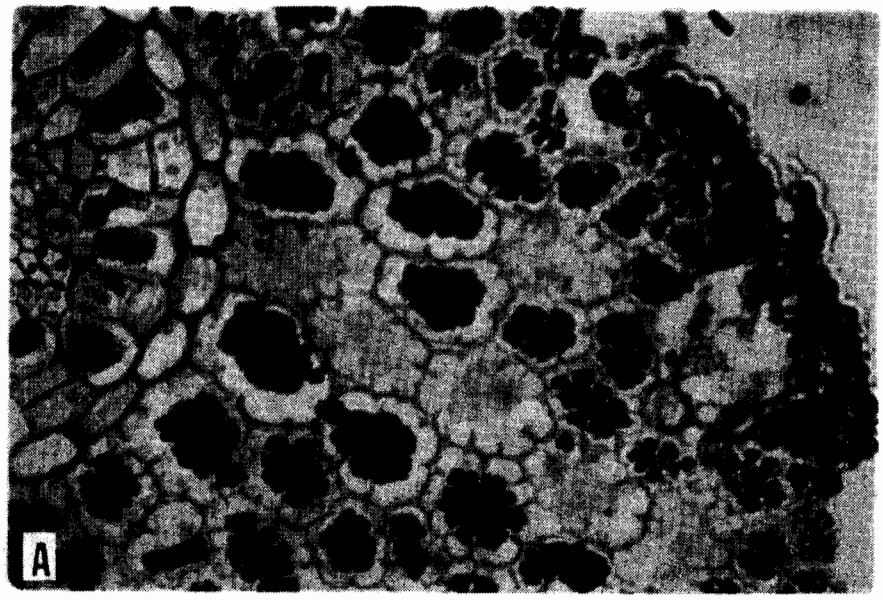
## Morphogenetic Changes in Galls

As mentioned before, high levels of auxin and/or gibberellin will cause cell expansion (hypertrophy) and cell division (hyperplasia). Extensive hypertrophy and hyperplasia occurred in both types of galls (Figures 15 and 17 pages 83, 87). The average mesophyll or parenchyma cell in the round gall increased 13 times in volume and divided 1.5 times more than a cell from the normal needle (Figure 15 page 83) and (Table 5 page 88). However, the results were calculated assuming a spherical cell which only approximates the actual cell shape. The gradual increase in cell size and number from the normal needle to the galled condition can be seen in a longitudinal section of the round gall (Figure 16 page 85). The cross section of a normal needle shows the ring of endodermal cells with casparian strip, transfusion tracheids with bordered pits, and mesophyll cells outside the endodermis (Figure 15 A page 83). On the other hand, the round gall sections had no endodermal cells and the whole vascular bundle is larger and radically different (Figure 17 C page 87). The hypertrophied cells of round galls were often plasmolyzed indicating a lower solute concentration than normal needles. Round galls were

Figure 15. Comparison of cell size in small normal needle and Round Gall.

A. Cross section of small normal needle - 170 x.

B. Cross section of mesophyll tissue of Round Gall - 170 x.


A

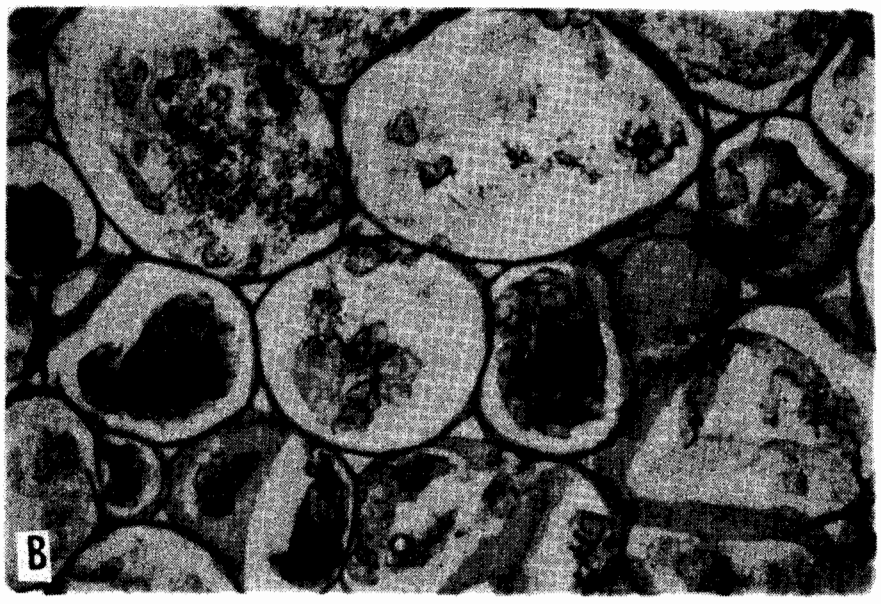

B

Figure 16. Longitudinal section of Round Gall - 40 x.

Figure 17. Comparison of tissues from Round Galls, Stubby Galls, and small normal needles.

A. Cross section of a Round Gall - 40 x.

B. Cross section of a small normal needle - 40 x.

C. Cross section of vascular bundle in a Round Gall - 40 x.

D. Cross section of a Stubby Gall - 40 x.

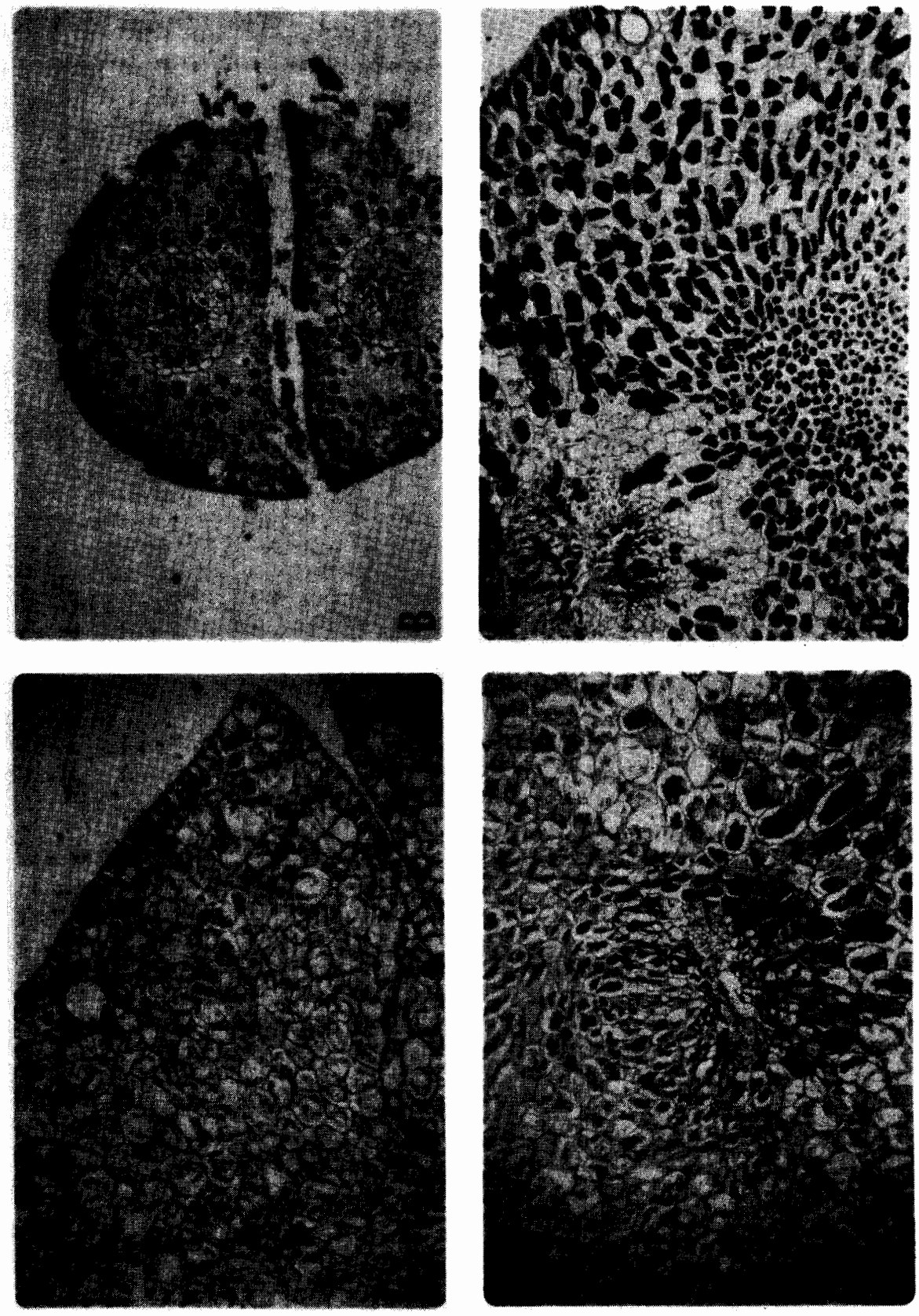


Table 5. Comparison of Cell Size and Number in Small Normal Needle, Round Gall, and Stubby Gall.

| | Small normal needle | Round gall | Stubby gall |
|---|---|---|---|
| # epidermal cells | 151 | 300 | 279 |
| # cells inside endodermis | 402 | 501 | 374 |
| # cells outside endodermis | 558 | 1,527 | 1.906 |
| # cells total | 984 | 2,028 | 2,380 |
| Area outside endodermis (sq. mm) | 0.54 | 8.29 | 4.96 |
| Diameter of average cell outside endodermis (mm) | $3.52 \times 10^{-2}$ | $8.31 \times 10^{-2}$ | $5.76 \times 10^{-2}$ |
| Volume of average cell outside endodermis (cu. mm) | $2.3 \times 10^{-5}$ | $30.1 \times 10^{-5}$ | $10.0 \times 10^{-5}$ |
| X fold increase in volume of average cell outsdie endodermis | 1 | 13.1 | 4.4 |

more watery than normal needles; a condition often observed with tissues exposed to auxin (Sachs 1961, Jablonski and Skoog 1954).

The cellulose content of epidermal cells decreases in round galls as shown in polarized microphotographs (Stamm 1964, Figure 18 D, E page 91). Auxin has been reported to induce several polysaccharidases such as: β-1,3-glucanase, β-1,4-glucanase (cellulase), β-1,6-glucanase, α-1,6-glucanase, and exoglactanases (Cleland 1971). These hydrolase enzymes could weaken the cell walls allowing expansion by decreasing the amounts of cellulose.

The cellular changes in round galls can be explained easily by increased levels of auxin and gibberellin. Cytokinins also may be involved in allowing cell hyperplasia but auxin and gibberellin alone may cause the cell to automatically divide once it becomes too large.

### Other Chemical Changes - Starch and Anthocyanins

Numerous starch grains, probably within chloroplasts, were found throughout the mesophyll of the round gall and stubby gall and were absent in the normal needle. The starch grains have a "x" interference pattern in polarized light due to the concentric layers of starch (Winton 1906, Figure 18 A, B page 91). The starch may provide an extra rich source of food for the midge larvae. Gibberellin in barley seeds causes starch breakdown but in pinyon needles the physiological effects are probably different. The starch grains are

Figure 18. Comparison of small normal needle and Round Gall for starch grains and cellulose in epidermal cells.

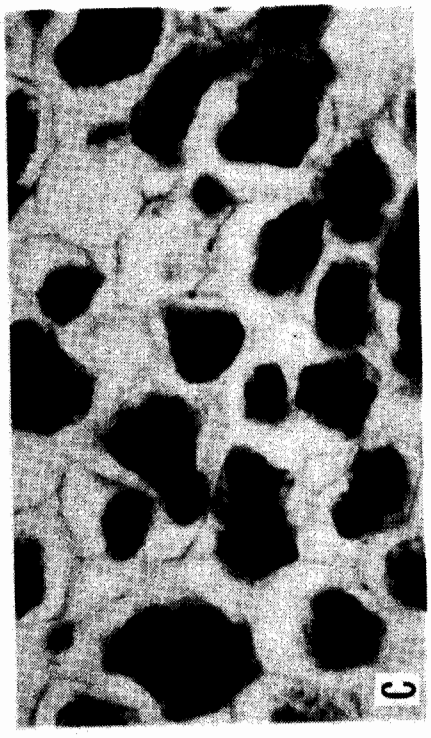
A. Starch grains in Round Gall mesophyll - Polarized light, cross section - 100 x.

B. Starch grains in Round Gall mesophyll - Polarized light, cross section - 400 x.

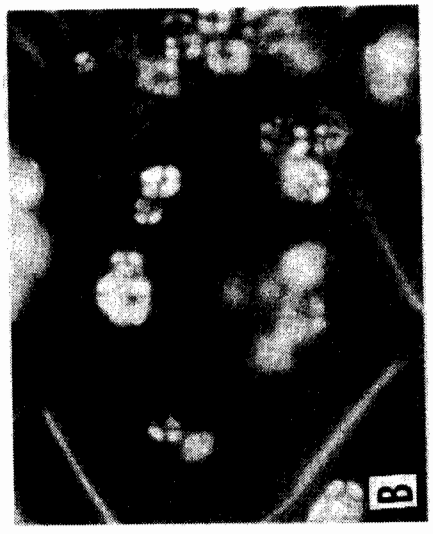
C. Mesophyll cells of Stubby Gall - cross section - 170 x.

D. Cross section of small normal needle - polarized light 100 x (epidermis left).

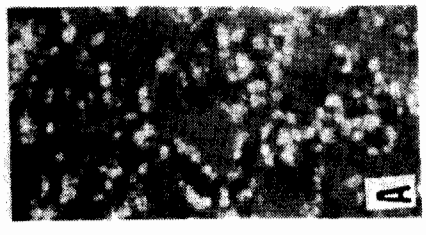
E. Cross section of Round Gall - polarized light - 40 x (epidermis top left).


C


E


B


D


A

probably a secondary response to the galling as the anthocyanins in galls may be.

Anthocyanins were found in round and stubby galls. The red gall pigment turned colorless at pH 3.9 and was yellow-green at pH 7 or more. The pigment had an absorption maximum at 526 ± 2 mm corresponding to a cyanin or peonin derivative (Harborne 1967). Paper chromatography of the gall anthocyanin using several different solvent systems was inconclusive possibly due to displacer compounds but peonidin 3-glucoside, cyanidin 3-galactoside or petunidine 3-glucoside were suggested because of similar $R_f$ values. After acid hydrolysis, the resulting anthocyanidin again was subjected to different paper chromatography systems and it had $R_f$ values almost identical to petunidin. To check these findings pigments in petunia flowers known to be high in anthocyanins were extracted and chromatographed as the galls (Harborne 1967). The petunia flowers had three anthocyanins which all behaved differently than the gall pigment. However, the anthocyanidins (aglycosides of anthocyanin obtained by acid hydrolysis) from the gall and one of the petunia anthocyanins were almost identical. Petunias are reported to have both cyanidin and petunidin (Harborne (1967). Therefore, on the basis of these findings, petunidin 3-glucoside, cyanidin 3-galactoside or a closely related compound may be the anthocyanin in round galls (Figure 2 F page 29 ).

## SUMMARY AND CONCLUSIONS

IAA and gibberellins were found to increase dramatically as the normal needle begins to form a gall. In the still-forming round gall there was from 11 to 27 times more IAA and from 6 to 31 times more gibberellin per gall than in a normal needle. Calculated on tissue weight, round gall tissue had from 2.5 to 6.5 times more IAA and 2 to more than 7 times more GA than normal tissue. The quantities of these growth hormones are not known to be physiologically active in pinyon gall formation although the amounts are within the range of quantities found in other plants. If the gibberellin(s) and auxin(s) are increased due to a secondary response from gall formation, they still could affect the morphogenesis of the gall. The experiments were designed so that the auxin found in pinyon was probably indoleacetic acid but the particular gibberellins involved could not be determined.

The cecidomyiid larvae probably do not secrete IAA but may secrete gibberellins or ecdysone. While a slight gibberellin activity was found in larval extracts, the gibberellin-like activity was believed to be from ecdysone mimicing GA. Differences in peroxidase isozymes were found between galled needles and normal needles. One peroxidase band in the polyacrylamide gel showed an increase in activity in stubby and round galls since it was much darker staining than the same band from normal needles. The increase in this

peroxidase activity could be due to higher levels of auxin or gibberellin in the gall. A second peroxidase band was absent from gall material indicating its inhibition or destruction in gall formation.

One possible mechanism of gall formation may be that the cecidomyiid larvae secrete orthophenols which inhibit the second peroxidase or IAA-oxidase thus allowing auxin to build up. The first peroxidase which was increased in gall tissue must have little specificity for oxidizing IAA or inhibition of the second peroxidase would have no effect on IAA levels. The larvae could also cause the accumulation of gibberellin which then may cause an increase in orthophenols, inhibition of IAA-oxidase, and an increase of IAA concentration.

The round galls senesce and fall off the pinyon tree during the winter following gall growth. Most needles remain living on the tree from four to nine years, therefore, auxin and/or gibberellin may induce senescent changes or change the physiology of the gall to the extent that death results. Auxin will induce ethylene production in many plant tissues. Ethylene then will cause senescence of these plant tissues. Therefore, auxin could gradually build up to concentrations causing cell growth and consequently gall growth and later build up to even higher concentrations inducing ethylene production and senescence. An alternative mechanism could be that abnormal concentrations of IAA together with normal aging cause the galls to senesce.

Cellular changes of hypertrophy and hyperplasia support the theory that auxin and gibberellin cause gall formation. Round gall cells increased in volume about 13 fold from normal needle cells. The amount of cellulose in epidermal cells of round galls and the viscosity of cellular contents decreased showing classical effects of auxin and gibberellin.

Stubby galls were not extracted for the plant growth hormones but the peroxidase and cellular changes resembled that of the round gall. Therefore, various concentrations of auxin and gibberellin may also be involved in stubby gall formation. Other cecidomyiid pinyon galls, such as the spindle gall (Houseweart and Brewer 1972), are probably formed in much the same way as the round gall. However, different hormone concentrations and different times of hormone inducement probably cause the different morphological galls.

The evidence indicates that auxin and gibberellin cause gall formation but the mechanism of auxin induction can only be speculated.

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# APPENDIX

Table 6. Oat Coleoptile Growth in Response to Indoleacetic Acid - Standard Curve Values in Units.

| | Water Control (0 ng IAA per tube) | | 10 ng IAA per tube | | 100 ng IAA per tube | | L. S. D. 5% |
|---|---|---|---|---|---|---|---|
| | Ave. length of 10 col. (units) | s.d. | Ave. length of 10 col. (units) | s.d. | Ave. length of 10 col. (units) | s.d. | |
| | | | July 23, 1972 | | | | |
| Col. A | 68.7 | 3.53 | 75.2 | 1.55 | 94.3 | 4.50 | 3.14 |
| Col. B | 62.6 | 2.01 | 72.6 | 3.31 | 87.6 | 6.43 | 3.98 |
| | | | August 6, 1972 | | | | |
| Col. A | 67.5 | 2.80 | 70.6 | 4.06 | 91.3 | 6.63 | 4.38 |
| Col. B | 61.3 | 2.83 | 76.8 | 7.10 | 84.0 | 4.81 | 4.78 |
| | | | August 13, 1972 | | | | |
| Col. A | 67.8 | 4.57 | 75.6 | 6.54 | 92.1* | 2.56 | 4.57 |
| Col. B | 64.0 | 2.36 | 75.6 | 5.44 | 86.1* | 4.33 | 3.61 |
| | | | Average of 5 Bioassays - Above 3 plus 2 others | | | | |
| | Ave. length of 55 col. | s.d. | Ave. length of 55 col. | s.d. | Ave. length of 60 col. | s.d. | L. S. D. 5% |
| Col. A | 68.20 | 3.98 | 73.85 | 4.88 | 92.08 | 6.16 | 1.895 |
| Col. B | 62.15 | 2.50 | 73.82 | 5.82 | 87.08 | 6.75 | 1.996 |

*Average of 15 coleoptiles

s.d. = standard deviation

L. S. D. = least significant difference

1 unit = 0.1176 mm

Table 7. Oat Coleoptile Growth in Response to Extracts of Small Normal Needles (S. N. ) and Round Galls.

| Sample | ml of pinyon extract | Col. type | Ave. length of 10 col. (units) | Sample | ml of pinyon extract | Col. type | Ave. length of 10 col. (units) |
|---|---|---|---|---|---|---|---|
| | | | July 23, 1972 | | | | |
| | Band 4 | | | | Band 5 | | |
| S. N. | 0.5 | A | 70.6 | S. N. | 0.5 | A | 70.4 |
| S. N. | 1.0 | B | 64.2 | S. N. | 1.0 | B | 63.8 |
| Gall | 0.5 | A | 66.8 | Gall | 0.5 | A | 74.2 |
| Gall | 1.0 | B | 61.2 | Gall | 1.0 | B | 65.0 |
| | Band 6 | | | | Band 7 | | |
| S. N. | 0.5 | A | 72.4 | S. N. | 0.5 | A | 65.2 |
| S. N. | 1.0 | B | 62.6 | S. N. | 1.0 | B | 54.2 |
| Gall | 0.5 | A | 75.4 | Gall | 0.5 | A | 66.2 |
| Gall | 1.0 | A | 66.4 | Gall | 1.0 | B | 60.4 |
| Gall | 1.0 | B | 54.8 | | | | |
| | | | August 6, 1972 | | | | |
| | Band 4 | | | | Band 5 | | |
| S. N. | 15.0 | B | 62.2 | S. N. | 2.0 | A | 66.0 |
| Gall | 1.4 | B | 65.2 | S. N. | 13.0 | B | 59.8 |
| Gall | 8.6 | A | 56.4 | Gall | 1.0 | B | 65.6 |
| | | | | Gall | 9.0 | A | 52.8 |
| | Band 6 | | | | Band 7 | | |
| S. N. | 1.0 | B | 67.2 | S. N. | 1.0 | B | 59.8 |
| S. N. | 9.0 | A | 56.8 | S. N. | 9.0 | A | 54.4 |
| Gall | 1.0 | A | 72.8 | Gall | 1.0 | A | 65.0 |
| Gall | 9.0 | A | 56.8 | Gall | 9.0 | A | 51.4 |
| | | | August 13, 1972 | | | | |
| | Band 4 | | | | Band 5 | | |
| S. N. | 0.5 | B | 58.6 | S. N. | 0.5 | B | 62.4 |
| Gall | 0.5 | B | 60.4 | S. N. | 1.0 | A | 64.0 |
| | | | | Gall | 0.5 | A | 64.8 |
| | | | | Gall | 1.0 | B | 62.2 |
| | Band 6 | | | | Band 7 | | |
| S. N. | 0.3 | A | 71.0 | S. N. | 0.5 | B | 62.6 |
| S. N. | 0.5 | B | 66.2 | S. N. | 1.0 | A | 64.0 |
| S. N. | 0.5 | A | 72.0 | Gall | 0.5 | A | 66.2 |
| S. N. | 1.0 | B | 63.8 | Gall | 1.0 | B | 59.6 |
| Gall | 0.3 | A | 75.6 | | | | |
| Gall | 0.3 | B | 70.2 | | | | |
| Gall | 0.5 | A | 77.8 | | | | |
| Gall | 0.5 | B | 71.2 | | | | |
| Gall | 1.0 | B | 61.8 | | | | |

Unit = 0.1176 mm

Table 8. Inhibition of Growth or α-amylase Release.

| Sample | mls of extract | $R_f$ band | Col. type | α-amylase units or average length 5 col. | Inhibition correction factor |
|---|---|---|---|---|---|
| S.N. | 1.0 | 1 | | 228.1 | 1.00 |
| S.N. | 0.5 | 2 | | 114.0 | 1.60 |
| S.N. | 1.0 | 2 | | 146.3 | |
| S.N. | 1.0 | 2 | | 66.5 | 1.72 |
| Gall | 1.0 | 1 | | 222.2 | 1.00 |
| Gall | 0.5 | 2 | | 95.2 | 1.92 |
| Gall | 1.0 | 2 | | 54.7 | |
| Gall | 1.0 | 2 | | 81.7 | 2.68 |
| S.N. | 1.0 | 4 | B | 76.2 | 1.13 |
| S.N. | 0.5 | 6 | A | 89.4 | 1.03 |
| S.N. | 0.5 | 6 | B | 84.4 | 1.02 |
| S.N. | 1.0 | 6 | A | 80.0 | 1.15 |
| S.N. | 1.0 | 6 | B | 76.0 | 1.13 |
| Gall | 1.0 | 4 | A | 83.6 | 1.10 |
| Gall | 0.5 | 6 | A | 87.4 | 1.05 |
| Gall | 0.5 | 6 | B | 83.2 | 1.03 |
| Gall | 1.0 | 6 | A | 77.2 | 1.19 |
| Gall | 1.0 | 6 | B | 70.0 | 1.23 |

Table 9. ∝-Amylase Release from Barley Half-Seeds in Response to Gibberellin in Extracts of Small Normal Needles (S. N. ) and Round Galls Collected July 23, 1972.

| No. | Sample | mls | $R_f$ Band | O. D. | Units per ml | x dil. | x .2g/weight of 10 seeds | x 5 = | Total ∝-amylase units |
|---|---|---|---|---|---|---|---|---|---|
| Controls | | | | | | | | | |
| 1 | 0 ng | | | .75 | .329 | 1:11 | 1.1905 | | 21.5 |
| 2 | 0 ng | | | .53 | .479 | 1:11 | 1.0840 | | 28.6 |
| 3 | 1 ng GA | | | .55 | .465 | 1:22 | 1.1136 | | 57.0 |
| 4 | 1 ng GA | | | .59 | .433 | 1:22 | 1.2063 | | 57.5 |
| 5 | 10 ng GA | | | .48 | .510 | 1:66 | 1.1912 | | 200.5 |
| 6 | 10 ng GA | | | .58 | .445 | 1:66 | 1.1848 | | 174.0 |
| 7 | 100 ng GA | | | .58 | .445 | 1:99 | 1.1884 | | 262.3 |
| 8 | S. N. | .7 | 0 | .55 | .465 | 1:11 | 1.1062 | | 28.4 |
| 9 | S. N. | .5 | 1 | .90 | .226 | 1:11 | 1.2203 | | 15.2 |
| 10 | S. N. | 1.0 | 1 | .90 | .228 | 1:11 | 1.2555 | | 15.8 |
| 11 | S. N. | .7 | 2 | .75 | .329 | 1:11 | 1.1007 | | 19.9 |
| 12 | S. N. | 1.0 | 2 | .86 | .255 | 1:33 | 1.1488 | | 48.3 |
| 13 | S. N. | .7 | 3 | .91 | .221 | 1:11 | 1.1007 | | 13.4 |
| 14 | S. N. | 1.0 | 3 | .95 | .196 | 1:11 | 1.1312 | | 12.2 |
| 15 | Gall | .5 | 0 | 1.2 | .025 | 1:11 | 1.2107 | | 1.7 |
| 16 | Gall | .5 | 1 | .94 | .198 | 1:11 | 1.2173 | | 13.3 |
| 17 | Gall | 1.0 | 1 | .86 | .255 | 1:11 | 1.1255 | | 15.8 |
| 18 | Gall | .5 | 2 | .76 | .322 | 1:22 | 1.0965 | | 38.9 |
| 19 | Gall | .7 | 2 | .81 | .287 | 1:33 | 1.1640 | | 55.2 |
| 20 | Gall | 1.0 | 2 | .52 | .485 | 1:11 | 1.1655 | | 31.1 |
| 21 | Gall | .7 | 3 | .95 | .196 | 1:11 | 1.1760 | | 12.7 |
| 22 | Gall | 1.0 | 3 | .99 | .166 | 1:11 | 1.1630 | | 10.7 |
| 23 | silica control | | | .96 | .188 | 1:11 | 1.1710 | | 12.1 |
| 24 | silica 10 ng | | | .70 | .363 | 1:66 | 1.1770 | | 141.1 |
| 25 | silica 10 ng | | | .30 | .642 | 1:66 | 1.1869 | | 251.3 |
| 26 | Larvae 1-3 | | | .90 | .228 | 1:33 | 1.1898 | | 44.8 |

Table 10. ∝-Amylase Release from Barley Half-Seeds in Response to Gibberellin in Extracts of Small Normal Needles (S. N. ) and Round Galls Collected August 6, 1972.

| Sample | mls | $R_f$ Band | O. D. | Units per ml | x dil. | x .2g/weight of 10 seeds | x 5 = | Total ∝-amylase units |
|---|---|---|---|---|---|---|---|---|
| Controls | | | | | | | | |
| 0 ng | | | .56 | .485 | 1:11 | 1.1280 | | 28.4 |
| 0 ng | | | .92 | .210 | 1:11 | 1.1765 | | 13.6 |
| 1 ng GA | | | .84 | .266 | 1:55 | 1.2143 | | 89.1 |
| 1 ng GA | | | .44 | .536 | 1:22 | 1.1280 | | 66.6 |
| 10 ng GA | | | .64 | .404 | 1:66 | 1.0971 | | 146.3 |
| 10 ng GA | | | .49 | .504 | 1:66 | 1.2070 | | 200.8 |
| 100 ng GA | | | .66 | .390 | 1:99 | 1.1242 | | 217.0 |
| 100 ng GA | | | .69 | .371 | 1:110 | 1.1442 | | 233.4 |
| S. N. | 2.0 | 1 | .44 | .537 | 1:11 | 1.1891 | | 35.1 |
| S. N. | 13. | 1 | 1.2 | .025 | 1:11 | 1.2020 | | 1.7 |
| S. N. | 2.0 | 2 | .47 | .521 | 1:22 | 1.1450 | | 65.6 |
| S. N. | 13. | 2 | .95 | .196 | 1:11 | 1.1969 | | 12.9 |
| S. N. | 13. | 3 | .31 | .622 | 1:11 | 1.1530 | | 39.4 |
| Gall | 1.0 | 1 | .66 | .390 | 1:22 | 1.1786 | | 50.6 |
| Gall | 9.0 | 1 | 1.1 | .093 | 1:11 | 1.1527 | | 5.9 |
| Gall | 1.0 | 2 | .62 | .418 | 1:55 | 1.1976 | | 137.8 |
| Gall | 9.0 | 2 | 1.2 | .025 | 1:11 | 1.1648 | | 1.6 |
| Gall | 9.0 | 3 | .82 | .279 | 1:11 | 1.1154 | | 17.1 |
| Silica control | | | .46 | .525 | 1:11 | 1.1947 | | 34.5 |

Table 11. $\alpha$-Amylase Release from Barley Half-Seeds in Response to Gibberellin in Extracts of Small Normal Needles (S. N. ) and Round Galls Collected August 13, 1972.

| Sample | mls | $R_f$ Band | O.D. | Units per ml | x dil. | x .2g/weight of 10 seeds | x 5 = | Total $\alpha$-amylase units |
|---|---|---|---|---|---|---|---|---|
| Controls | | | | | | | | |
| 0 ng | | | .95 | .196 | 1:11 | 1.1581 | | 12.5 |
| 0 ng | | | .77 | .317 | 1:11 | 1.1312 | | 19.7 |
| 1 ng GA | | | .64 | .404 | 1:22 | 1.1287 | | 50.2 |
| 1 ng GA | | | .15 | .735 | 1:22 | 1.2055 | | 97.5 |
| 10 ng GA | | | .63 | .412 | 1:66 | 1.0858 | | 147.5 |
| 10 ng GA | | | .36 | .593 | 1:66 | 1.1136 | | 218.0 |
| 100 ng GA | | | .40 | .570 | 1:99 | 1.0805 | | 304.9 |
| S. N. | 1.0 | 1 | .10 | .769 | 1:11 | 1.0695 | | 45.2 |
| S. N. | 0.5 | 2 | .67 | .383 | 1:11 | 1.1105 | | 23.4 |
| S. N. | 1.0 | 2 | .71 | .357 | 1:11 | 1.0633 | | 41.7 |
| S. N. | 1.0 | 2 | .88 | .242 | 1:11 | 1.1876 | | 15.8 |
| S. N. | 1.0 | 3 | .83 | .271 | 1:11 | 1.1480 | | 17.1 |
| S. N. | 1.0 | 1 | .39 | .575 | 1:66 | 1.2019 | | 228.1* |
| S. N. | 0.5 | 2 | .53 | .479 | 1:44 | 1.0810 | | 114.0* |
| S. N. | 1.0 | 2 | .35 | .600 | 1:44 | 1.1080 | | 146.3* |
| S. N. | 1.0 | 2 | .46 | .527 | 1:22 | 1.1470 | | 66.5* |
| Gall | 1.0 | 1 | .70 | .363 | 1:11 | 1.1460 | | 22.9 |
| Gall | 0.5 | 2 | .83 | .275 | 1:33 | 1.1574 | | 52.5 |
| Gall | 0.5 | 2 | .79 | .302 | 1:33 | 1.0980 | | 54.6 |
| Gall | 1.0 | 2 | .89 | .235 | 1:33 | 1.2055 | | 46.7 |
| Gall | 1.0 | 3 | .81 | .287 | 1:11 | 1.1510 | | 18.2 |
| Gall | 1.0 | 1 | .38 | .580 | 1:66 | 1.1610 | | 222.2* |
| Gall | 0.5 | 2 | .69 | .371 | 1:44 | 1.1670 | | 95.2* |
| Gall | 1.0 | 2 | .90 | .228 | 1:44 | 1.0887 | | 54.7* |
| Gall | 1.0 | 2 | .78 | .308 | 1:44 | 1.2040 | | 81.7* |
| Sugar Beet | 1.0 | 1-3 | .70 | .363 | 1:22 | 1.1448 | | 45.7 |
| Larvae | 6.0 | 1-3 | .54 | .471 | 1:22 | 1.1357 | | 58.8 |

*10 ng GA added