EFFECT OF AQUEOUS ALCOHOL EXTRACTS OF BUNCH

DISEASED PECAN SHOOTS ON TOMATO SEED

GERMINATION AND A POSSIBLE

CAUSE OF THE DISEASE

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Bunch disease of the pecan, <u>Carya</u> <u>illinoensis</u> (Wang) K. Koch., is
a poorly understood but increasingly serious problem in most of the
commercial pecan production states. While originally bunch disease was
reported as a minor disease occurring primarily in scattered regions of
Louisiana, Mississippi, Oklahoma, and Texas, it has since spread north
and west to become a seriously devitalizing problem of commercial or-
chards in Arkansas, Missouri, and Kansas as well (4, 13).

On pecans, bunch disease is often characterized as having a
"witches' broom" effect or "brooming" of shoots as a result of excessive
forcing of lateral buds. While not uncommon in wild native trees, the
disease most frequently appears on vigorously growing trees in young,
well maintained orchards (4). Bunch disease has commonly been observed
on trees growing under wet, soggy conditions and appears to spread most
rapidly during abnormally wet years (10, 11).

Symptoms of bunch disease include massive lateral bud forcing,
occurring primarily at the tips of branches and, in severely affected
trees, these symptoms will frequently appear along the length of lateral
branches and on the trunk. Bunch disease affected branches usually
foliate 10 to 14 days earlier in the spring than unaffected branches on
the same tree and the leaves produced are thinner, more wavy, and more
flexible than normal leaves (14). On some varieties the leaves are

abnormally broad in relation to their length (12). Leaves of branches affected with bunch disease are smaller, the rachis shorter, and diseased branches frequently defoliate earlier in the fall although the rachis may be retained for an abnormally long period. Terminal dieback is often characteristic of severely affected trees. Catkins of bunch diseased branches are generally smaller than those on unaffected branches, fewer in number, and sometimes "broomed" (4, 21, 26). Severely affected trees do not bear fruit in significant quantities and ultimately heavily affected trees become so seriously devitalized that death of the tree may ensue (14).

The specific causal agent of bunch disease on pecans remains undetermined. While it was originally thought to be a virus and later suspected of being a fungus, the recent discovery of taxonomically unclassified mycoplasmalike organisms (MLO) in the phloem tissue of diseased pecan wood incriminates MLO as the causal agent (4, 11, 13, 21, 26). In any case, it has been considered that investigations into the physiological effects of the disease could possibly provide some insight as to its specific cause and ultimately enable the development of control measures.

The characteristic growth habits of affected branches would indicate the involvement of some form of hormonal imbalance. Death of terminals generally results in lateral bud breaks and is correlated with the loss of auxin induced apical dominance (18). Bunch diseased branches with the terminals still present suggest that the means for auxin production are still present. This implies that auxins themselves are still being produced but that somehow their effects are being subdued or overridden. One hormone known to be capable of overriding the

effect of auxin and thereby stimulating lateral bud development in plants is kinetin (18, 31). Characteristic massive lateral bud development of affected branches coupled with the known interactions and effects of auxins and cytokinins leads to at least two conclusions about bunch disease: (1) that the manifestation of bunch disease is ultimately the result of an absence of inhibitory levels of auxins in the wood or; (2) if present, then the effects of the auxin are possibly being overridden by abnormally high levels of cytokinins.

The object of this study is to analyze aqueous alcohol extracts from bunch diseased pecan wood for the presence of growth promoters or inhibitors and compare these to extracts from nondiseased wood. If their presence is indicated by the bioassay, then qualitative identification of the growth substances (specifically of indole-3-acetic acid and indole butyric acid) will be accomplished by means of thin layer chromatography. An additional investigation will involve the subjection of healthy pecan seedlings to foliar applications and trunk injections with extracted solutions, followed by observation of the seedlings for auxin induced leaf epinasty and/or lateral bud forcing. Finally, phloem tissue from diseased wood will be applied to disease free seedlings in an attempt to transmit the disease to the seedlings.

CHAPTER II

LITERATURE REVIEW

History of Bunch Disease

Bunch disease of pecans was not reported as such until 1932. Previously the disease was confused with pecan rosette, a zinc deficiency problem that manifests itself in much the same way as bunch disease. In 1932, after controls for rosette had become effective, Cole (4) observed trees near Shreveport, Louisiana that exhibited a peculiar witches' broom disorder with symptoms distinct from those of rosette. Due to the characteristic growth habit of trees thus affected, Cole named the disorder "bunch" disease.

The earliest known attempt to determine the specific cause of bunch disease was made by Cole (4). By grafting scions from the diseased cultivar Schley onto both Schley and Stuart understock, Cole found that the disease could be transmitted to Schley but not to Stuart, demonstrating the existence of varietal susceptibility. KenKnight (10) likewise was able to transmit bunch disease to susceptible varieties by grafting and he reported that Parson had done the same.

Neither Cole nor KenKnight was able to identify an organism capable of transmitting the disease from the scion and both concluded that the causal agent was a virus. Cole attempted to substantiate this conclusion by inoculating healthy leaves of the highly susceptible Schley

cultivar with juice extracted from leaves of diseased Schley. He also injected diseased extract into holes bored into unaffected limbs of healthy trees. In both of these experiments, Cole failed to transmit the disease to the healthy trees. Additional work performed by Cole demonstrated that removal of affected branches from diseased trees could free the tree from disease symptoms. KenKnight (14) later pointed out that this finding is not consistent with normal virus etiology since a truly systemic virus usually cannot be eliminated simply by removal of infected parts.

Later work suggested that a fungus might be the causal agent of bunch disease in pecans. KenKnight (11) notes that Parson first suspected this and although he (KenKnight) suspected at first that the disease was caused by a slow moving systemic virus, he later concluded that a fungus was the more likely prospect as a causal agent. Moore (21) isolated five fungus organisms from buds of diseased and nondiseased trees. These included species of Fusicladium, Alternaria, Gloeosprium, Cladosprium, and Taphrina. Of the five, Gloeosprium was the only fungal species isolated from diseased but not from nondiseased wood. KenKnight (12) has noted that the symptoms of bunch disease are quite similar to disease symptoms produced by the fungus Taphrina on oak, alder, and cherry. KenKnight (11) attempted to validate the fungal nature of bunch disease by inoculating young Mahan and seedling pecans with fungi isolated from shoots affected with bunch but, like Cole, was unable to transmit the disease by this method. Nevertheless, KenKnight's initial observations seemed to support the fungal theory of bunch disease. He reported that Parson noted the spread of the disease was most rapid during wet, humid periods (fungus spores would require moisture for

germination) and that each broom appears to be a separate infection (removal of affected limbs can eliminate the disorder). KenKnight also noted that the rate of spread of the disease appeared to be restricted in orchards where fungicides were used to control scab, that the disease was most prevalent in regions of high humidity and high soil moisture, and that no evidence of systemic viral infection could be found. All of these factors support the fungal rather than the viral theory of the causal agent of bunch disease but to date no specific fungus organism has been identified.

The most recent discovery about pecan bunch disease has been made by Seliskar et al. (26) in 1974. Using electron microscope techniques, Seliskar determined a consistent association of mycoplasmalike organisms (MLO) with the phloem tissue of diseased trees. These organisms were found with greatest consistency in the phloem sieve tubes and companion cells of stems and leaves of diseased trees. In general, the more severe the infection the more abundant the MLO. MLO were found with much less frequency in nonsymptomatic tissue of diseased trees and were not found at all in the phloem tissue of nondiseased trees. As Seliskar suggests, the fact that bunch disease can be controlled by pruning, coupled with his discovery of mycoplasmalike organisms in the phloem tissue, strongly implicates a nonsystemic MLO as the causal agent of pecan bunch disease.

Regardless of the specific agent responsible for bunch disease, it was presumed by several workers that the agent is spread from tree to tree by an insect vector. In an attempt to determine the vector, Cole (4) fed pecan aphids (Melanocallis caryaefoliae, Fitch) on diseased leaves and then transferred them to leaves of healthy trees but was

unable to accomplish a disease transmission. In another study, KenKnight (13) reported that only one species of insect, a lantern fly (species unnamed) was found in abundance on bunch diseased branches. The insect was checked as a possible vector but no results (to this writer's knowledge) were reported. KenKnight (11) also transferred various insects (unidentified) collected from diseased trees to young seedlings of the highly susceptible Mahan cultivar but reported negative results.

Attempts to control bunch disease have involved both mechanical methods, such as pruning off affected branches, and chemical methods, such as spraying with various fungicides. KenKnight (11) reported the clearing of an orchard in which 30% of the trees exhibited various degrees of bunch disease. The diseased stumps produced apparently healthy sprouts the following growing season. He also noted that diseased trees cut off at breast height and with all stump branches removed, produced healthy growth the following growing season. Cole (4) also noted that pruning was an effective control method so long as the infection was not too severe and providing that all visible evidences of the disease were removed from the area.

Pruning of diseased trees to chest high stumps followed by dormant spray applications of maneb coupled with injections of various antibiotics resulted in trees with normal growth (12). Spraying of severely bunch diseased Mahan trees with dodine did not induce recovery but did improve nut production on "broomed" shoots. Improved nut production has also been obtained on bunch diseased trees of the Schley cultivar sprayed with Bordeaux mixture and on trees of the Mahan cultivar sprayed with triphenol tin hydroxide (11). KenKnight (10) determined that

dipping of infected scions in dodine was ineffective in disinfecting the scionwood since grafts from treated scions developed bunch disease the second growing season.

Cole (4) noted that when scions from diseased trees of the Schley cultivar were grafted onto the apparently resistant Stuart understock, the resultant growth from the scion was healthy. This suggests that resistance can be imparted to susceptible varieties by using resistant stock.

Similarity to Diseases of Other Genera

There are numerous diseases in other species of plants which are quite similar to bunch disease of pecans. Noted earlier was the similarity of bunch disease to zinc rosette in pecans. Pecan bunch disease is also quite similar in appearance to an apparent MLO induced witches' broom disorder of black locust, Robinia pseudoacacia L. (4, 25). Early foliation is characteristic not only of bunch disease but also of phony peach disease (Nanus mirabilis) and of peach yellows Chlorogenus persicae) (4).

According to KenKnight (13), the only known host of the causal agent of bunch disease are the pecan, and water hickory, C. aquatica (Michx. f.) Nutt. Other species which exhibit disease symptoms resembling those of pecan bunch disease include ironwood, elderberry, overcup oak, green ash, box elder, hackberry, and upland hickory trees. In northeastern United States, shagbark hickory (C. ovata (Mill.) K. Koch) is susceptible to a witches' broom disorder very similar to bunch disease of pecans and the apparent causal agent is the fungus Microstroma juglandis (Bereng.) Sacc. (29).

A disorder of eastern walnuts is known as bunch disease and is also quite similar to pecan bunch disease. The Japanese walnut (Juglans cordiformis Maxim.) is highly susceptible as is the butternut (J. cinerea L.). Black walnut (J. nigra L.) and Persian walnut (J. regia L.) show some resistance to walnut bunch disease. In walnuts, bunch disease is thought to be a viral infection but, as with pecans, it has been noted that many of the symptoms are similar to those induced by certain fungi; primarily those associated with the mildews and leaf curl, e.g., Taphrinas (17, 20, 30). Symptoms of walnut bunch disease similar to pecan bunch disease include dwarfed catkins, early foliation, massive lateral bud forcing, greatly reduced nut production, production of poor quality nuts, and transmissibility through grafting (bark-patch) (7, 17, 21). It has been suggested that bunch disease of walnuts and bunch disease of pecans are caused by the same organism, however, no evidence exists that substantiates such a relationship (14, 17, 21).

KenKnight (12) reported "bunch-like" symptoms occurring on blue beech and box elder growing in wet situations. The etiology suggested a fungus organism as the agent responsible although none could be detected. Observation of "witches' broomed" green ash growing under conditions of high moisture showed that the leaves of diseased branches were retained longer than those of normal branches, while the opposite is true for pecan trees affected with bunch disease. Another "witches' broom" disease that is similar to that which occurs on pecans is a disease which infects the Japanese cherry (Prunus yedoensis Mat.) and is known to be caused by the fungus Exoaseus cerasi (Fickl.) Sadeb. The disease spreads very slowly in affected trees, causes early foliation, and can

be controlled by pruning and burning of the infected wood (24).

Witches' broom of blackberry is also known to be caused by a fungus, specifically by Cercosporella rubi (Wint.) Plakidas (12).

Earlier reference was made to the discovery of mycoplasmalike organisms in the phloem tissue of bunch diseased pecan wood. Seliskar, et al. (25) also detected mycoplasmalike bodies in the phloem tissue of black locust (R. pseudoacacia L.) affected with witches' broom while none were found in the tissues of normal trees. Holmes, et al. (8) found MLO in the phloem tissue of witches' broom affected willow species (Salix rigida Muhl) but not in the phloem tissue of healthy specimens.

As with bunch disease of pecans, witches' broom of willow and black locust are both graft transmissible (8, 25).

Review of Physiology

Necessary to the study of any disease problem is an understanding of its physiological effects. With respect to bunch disease of pecans, physiological information is extremely limited. Previous mention has been made concerning attempts to transmit bunch disease by means of bark grafts (4, 10).

In a preliminary study, Hanna and Hinrichs (6) used a tomato seed bioassay to study auxin levels in the extracts of bunch diseased pecan wood. Their work indicated the presence of a germination inhibitor in bunch diseased wood and that concentrations of the inhibitor were higher in the fall than in the spring. The literature contains many other examples indicating the presence of various growth regulators in plant parts. Rapp and Ziegler (23) isolated a substance identified as abscisic acid from grape vines. Sladky (27) detected the presence of

high concentrations of auxins and gibberellin like compounds in the

youngest bud primordia of walnut (J. regia L.). Goren, et al. (5)

studied seasonal changes in the concentrations of auxins, gibberellins,

and inhibitors in the leaves and bark of Shamouti orange (Citrus

sinensis (L.) Osbeck). Ballard and Lipp (1) determined the presence of

tomato seed germination inhibitors in extracts of Echium plantagineum L.

Heydecker and Joshua (7) found that extracts from dried chili peppers

(Capsicum sp.) contained growth substances which stimulated the germina-

tion of tomato seed as well as seed of onion and carrot. KenKnight (15)

reported the presence of an unidentified germination inhibitor in the

wood and bark of peach (Prunus persica Batsch) and wild Prunus (P.

angustifolia Marsh., P. serotina Ehrh.). Lipe, et al. (16) studied

seasonal fluctuations of endogenous growth substances in pecan fruits.

The literature contains many examples of antagonism between auxins

and kinins, however, no specific information on the involvement, if any,

of these particular growth regulators in the manifestation of pecan

bunch disease is available. Boswell and Storey (2) reported that sprays

of synthetic cytokinin caused axillary buds to break dormancy and

reduced terminal shoot growth when applied to macadamia seedlings

(Macadamia tetraphylla L.) although the suppression of apical dominance

was not as great as when terminals were removed. Milbocker (19) re-

ported the same phenomenon when axillary buds of the poinsettia were

treated with kinetin at one p.p.m. Wickson and Thimann (31), working

with decapitated stem sections of Pisum sativum L., showed that applica-

tions of exogenous auxin (IAA) promoted inhibition of lateral bud devel-

opment while the application of kinetin together with IAA resulted in

the complete loss of the auxin induced apical dominance. These workers

concluded that the customary apical dominance phenomena depends on an interaction between auxin and a kinetin-like substance in the plant rather than on auxin activity alone.

All of these reports lend plausibility to the theory that the manifestation of bunch disease in the shoots of affected branches is the result of an interaction between imbalanced hormones, specifically between IAA and kinetin.

CHAPTER III

MATERIALS AND METHODS

Sample Collection

On February 2, 1975, 150-200 gram samples of one and two year old
wood were collected from each of three native pecan trees exhibiting
varying degrees of effects from bunch disease. The orchard, located at
Ogelsby, Oklahoma, was recovered from wilderness in 1968 and has been
under an intensive management program for seven years. The soil type is
Osage Clay (heavy bottomland). While unobserved originally, occurrences
of the disease have increased steadily during the management period so
that approximately five percent of the trees now exhibit symptoms of
bunch disease. Samples of both one and two year old diseased and
healthy wood were taken from trees that were approximately 30 years of
age. The samples were weighed, bagged, labeled, and packed with dry ice
into an ice chest. Upon returning to Stillwater, the samples were
placed in a freezer and maintained at -18° C.

On February 12, 1975, samples were taken of one and two year old
wood from a heavily affected bunch diseased Western pecan tree (row 5,
tree 1) located at the Oklahoma Pecan Experiment Station, Sparks, Okla-
homa. Samples of one and two year old wood were also collected from a
tree of the Western cultivar apparently unaffected by bunch disease
(row 6, tree 4). These samples were treated as were those collected

13

from the Ogelsby orchard and returned to Stillwater for storage at -18°
C. The trees of the Western cultivar were 23 years of age.

Extraction

Following collection, the materials in each of the 16 samples were
reduced to wood chips by grinding in a very large mill. The smaller
pieces of wood were then ground to a pulp in a Wiley Mill, using a No.
20 mesh screen. To facilitate collection, a vacuum system was attached
to the mill in the manner shown in Figure 11 of the appendix (page 43).
A glycerine coated guaze filter was wired over the mouth of the vacuum
line to prevent loss of the finer particles of the ground fiber. The
mill was thoroughly cleaned between samples as were all glass pipes,
hoses, and flasks. The pulp from each sample was collected in a 500 ml
Erylnmeyer flask and each sample was weighed and subdivided into two
50 gram portions (subsamples). The subsamples were placed into separate
flasks, labeled, and, when time delays were encountered, stored at -18°
C.

To each 50 gram subsample was added 250 ml of 70% ethyl alcohol.
The samples were then extracted at 21° C (room temperature) for 30
minutes, with shaking each ten minutes during this period (four times).
The extract was then filtered from the pulp through Whatman Frit-Fold
Coarse Qualitative Filter Paper (14 cm). The pulp was washed twice with
20 ml of 70% ethanol and the filtrate collected in a clean 250 ml flask.
The extracted solution of each subsample was stored at 4.5° C until all
of the samples had been processed. Each extract was evaporated to dry-
ness (hard, shiny residue) in a water bath at 98° C. The residue was
then cooled to 21° C (room temperature) followed by redilution with

20 ml of distilled water. The stock solutions thus derived were trans-
ferred to 25 ml test tubes, sealed with cork stoppers, and stored at
-30° C until needed.

For experimental purposes, the assumption had to be made that 50
grams of wood pulp occupies the same volume as 50 ml of water. Since
the initial solution is extracted from 50 grams of pulp, evaporated to
dryness, and then rediluted in 20 ml of water, resulting in a dilution
factor of 5:2 (v/v basis), the 20 ml of stock solution can be con-
sidered to be 2.5 times as concentrated as it was in the wood of the
live tree. Maintaining the same dilution factor, two ml of the stock
solution can be diluted with three ml of water to get what would be the
normal concentration of the extracted chemicals in the live wood prior
to extraction. The "normal" stock may then be readily diluted to
desired concentration.

Tomato Seed Bioassay

The objective of the tomato seed bioassay was to compare the hormo-
nal content of extracts of one and two year old bunch diseased and
healthy pecan wood and to determine the relative concentrations of
growth hormones present in the extracts. Relative concentrations were
determined by comparing the effect of progressive dilutions of the stock
solutions on seed germination to the effect of known concentrations of
IAA (indole-3-acetic acid) and of kinetin (6-furfurylaminopurine). The
bioassay was set up in a randomized block design with 27 treatments
replicated four times. There were two subsamples. Data analysis were
accomplished by means of the LSD test.

For the bioassay, each of the stock solutions extracted from both one and two year old wood of diseased and nondiseased growth were diluted to normal, 1-10, 1-50, and 1-100 concentrations. Sixteen separate solutions were thus derived. For reference treatments, solutions of IAA were made up at concentrations of 0 ppm (2.5 cc 95% ethanol + 97.5 cc water), 25, 50, 100, 200, and 400 ppm. Solutions of 10, 20, 40, 80, and 100 ppm kinetin were also made up for each replication. In the second subsample, it was necessary to add IAA concentrations of 800 and 1600 ppm, a 320 ppm solution of kinetin, and controls of distilled water and 92 cc distilled water + 8 cc 95% ethanol. Although these additional treatments were run with the second subsample, they were not incorporated into the randomized block design and therefore were not included as part of the analysis. They were used solely for purposes of comparison.

Subsample number one was begun on May 15, 1975. Tomato seed of the cultivar Nemared were placed in 100 x 15 mm petre dishes (50 seeds/dish) on Sargents No. 500 filter paper. The dishes were then placed on germination trays according to randomized block design and each moistened with 2.5 cc of the respective treatment solution. The treated seeds were then placed in a 32° C germination chamber and given constant light and high humidity. Daily readings on the number of seeds which had germinated in each dish were taken and the results recorded. The second subsample was begun on June 4, 1975 and executed in exactly the same manner as subsample number one. The bioassay test was terminated after 14 days, since the germination rate fell to very low levels by this time. Graphs were prepared based on percent total or cumulative germination per treatment per day.

Qualitative Determination of Indoles

A qualitative analysis for the presence of indole-3-acetic acid and indole butyric acid was accomplished by means of thin layer chromatography. Glass thin layer plates (20 x 20 cm) coated with Silica Gel G were activated by oven drying at 110° C for three hours. Stock solutions of extracts from one year old diseased and nondiseased wood were concentrated to 30% of their original volume by evaporating in a drying oven at 85° C for four to six hours. Loads of approximately 10 micro-liters of the reduced stocks were applied to the plates using micro-pipettes. Standards used included IAA and IBA (unknown concentrations) and IAA at a concentration of 1600 ppm. The plates were developed in a glass tank using the ascending technique and a solvent system consisting of chloroform-95% acetic acid (95:5 v/v). Indoles are colorless and must be detected by chromogenisis, therefore the plates were sprayed with approximately 15 ml of Ehrlich reagent (1% solution of p-dimetyl-aminobenzaldehyde in 95% ethanol) and for five minutes placed in a vessel saturated with hydrochloric acid vapor (3, 28). The chromatography was performed three times to insure accurate results.

Treatment of Seedlings

On June 12, 1975, eight to ten month old container grown Western pecan seedlings (five plants per eight inch container) were obtained and the upper and lower leaf surfaces of ten seedlings were sprayed to the point of run-off with normal concentrations of solutions of extracts from bunch diseased growth. Similarly, ten seedlings were sprayed with the extracts of nondiseased growth (one year old wood) and five

seedlings were left as unsprayed controls. A three ml syringe with a
26 gauge needled was used to inject an additional ten seedlings with the
bunch extract. Likewise, ten seedlings were injected with the extract
of nondiseased wood. The injections were made at a point just below a
node in the terminal portion of the stem, each seedling receiving four
to five injections for a total quantity of .5 ml of the extract. All
plants were grown on in a fiberglass greenhouse with an average
temperature of 21° C and normal daylengths. Daily observations of the
sprayed seedlings were made for leaf deformations (specifically for leaf
epinasty, as this is known to be auxin induced (14)), and for abnormal
bud breaks on the injected seedlings, this being the most prevalent
manifestation of pecan bunch disease.

On September 2, wood from a severely bunch diseased tree of the
Love cultivar was collected from the pecan orchard located at Stillwater.
To insure that phloem tissue would be present, the outer layers of tis-
sue from the bark through the green cambial tissue were scraped off and
collected in a beaker. Anhydrous lanolin was liquified in a water bath
and approximately 15 ml was added to the collected shavings to form a
sticky paste. Ten month old, container grown, Western pecan seedlings
were then wounded around the circumference of the lower trunk by scrap-
ing to the cambial tissue with a sharp knife. The wounds were approx-
imately 1.5 inches long. The bark paste was then applied to the wounds
and the area wrapped with a piece of parafilm loosely tied above and
below the point of application. The seedlings were placed in a fiber-
glass greenhouse, given normal days and an average temperature of 21° C.
The plants were maintained for eight weeks and observed periodically
to determine if abnormal bud breaks were induced (implying

transmissibility of the disease to support the theory that MLO are the causal agent).

In all cases, the seedlings were given a 50/50 mix of peat and perlite and were given the following fertilizer applications per eight inch container: 21.7 gms 14/14/14 Osmocote (based on 1,000 #A), 8 gms single superphosphate (based on 4 lbs./yd^3 soil mix), 2 gms Frit 409 (based on 1 lb./yd^3 soil). These initial applications were followed bimonthly with Peter's 20/20/20 (liquid) and 500 ppm each of N, P_2O_5, and K_2O. The soil mix was also amended with Dolomite (Ca-MgSO$_4$) at 8 lbs./yd^3 to prevent excessive acidity.

CHAPTER IV

RESULTS AND DISCUSSION

Tomato Seed Bioassay

The statistical results of the bioassay are shown in Table I.
Although no statistical correlations could be drawn between the various
IAA and kinetin concentrations and the extracts of bunch diseased and
healthy wood, the analysis did show a significant difference in germina-
tion inhibition by extracts of one year old bunch diseased wood when
compared to the control (2.5 cc 95% ethanol + 97.5 cc water) and to the
extracts of one and two year old healthy and two year old bunch diseased
wood at normal concentrations. In all cases extracts of both bunch dis-
eased and healthy wood at normal concentrations differed significantly
from the control although there was no significant difference between
extracts of one and two year old healthy and two year old bunch diseased
wood. Figure 1 illustrates the relationship between the nondiluted wood
extracts and the ethanol control.

Statistical analysis of the 1-10, 1-50, and 1-100 dilutions of one
and two year old bunch diseased and healthy wood showed no significant
difference in germination inhibition from that imposed by the control
and this finding is further supported by the graphs in Figures 2 through
5.

There was no significant difference in suppression of germination
by IAA concentrations of 25, 50, 100, 200, and 400 ppm when compared to

20

TABLE I

THE EFFECT OF SPECIFIC TREATMENTS ON THE GERMINATION
OF SEEDS OF THE TOMATO CULTIVAR, NEMARED

Treatment	Mean Germination[1]
1. 0 ppm IAA (ethanol control)	45.625^{2}_{de}
2. 25 ppm IAA	46.000_{de}
3. 50 ppm IAA	46.750_{e}
4. 100 ppm IAA	44.875_{de}
5. 200 ppm IAA	44.250_{de}
6. 400 ppm IAA	41.500_{cd}
7. 10 ppm Kinetin	46.875_{e}
8. 20 ppm Kinetin	46.250_{e}
9. 40 ppm Kinetin	44.875_{de}
10. 80 ppm Kinetin	44.875_{de}
11. 160 ppm Kinetin	45.250_{de}
12. One year healthy, 1-0 dilution	37.250_{b}
13. One year healthy, 1-10 dilution	44.500_{de}
14. One year healthy, 1-50 dilution	45.875_{de}
15. One year healthy, 1-100 dilution	43.750_{de}
16. Two year healthy, 1-0 dilution	36.250_{b}
17. Two year healthy, 1-10 dilution	46.250_{e}
18. Two year healthy, 1-50 dilution	47.000_{e}
19. Two year healthy, 1-100 dilution	45.375_{de}
20. One year diseased, 1-0 dilution	25.000_{a}
21. One year diseased, 1-10 dilution	47.125_{e}
22. One year diseased, 1-50 dilution	45.375_{de}

TABLE I (Continued)

Treatment	Mean Germination[1]
23. One year diseased, 1-100 dilution	45.875^2_{de}
24. Two year diseased, 1-0 dilution	37.750_{bc}
25. Two year diseased, 1-10 dilution	46.000_{de}
26. Two year diseased, 1-50 dilution	47.625_{e}
27. Two year diseased, 1-100 dilution	45.875_{de}

[1]50 seeds/replication; two subsamples for each of four replications.

[2]Means followed by the same letter(s) are not significant at the .05 level.

the control although all concentrations inhibited germination to a much less degree than did the extracts of one year old bunch diseased and healthy wood at normal concentrations (Figure 6). Results of the first subsample of the bioassay indicated that the range of neither the IAA nor the kinetin solutions was broad enough to establish a correlation between these concentrations and those of the extracts, therefore additional concentrations of 800 and 1600 ppm IAA and 320 ppm kinetin were employed in the second subsample. Figure 7 compares the effects of the 800 and 1600 ppm IAA solutions to the effects of extracts of one year old diseased wood at normal concentrations. As the graph demonstrates, the level of inhibition imposed by the bunch disease extract approximates that imposed by the concentration of 1600 ppm IAA, suggesting that IAA may be present at a similar rate in the extract.
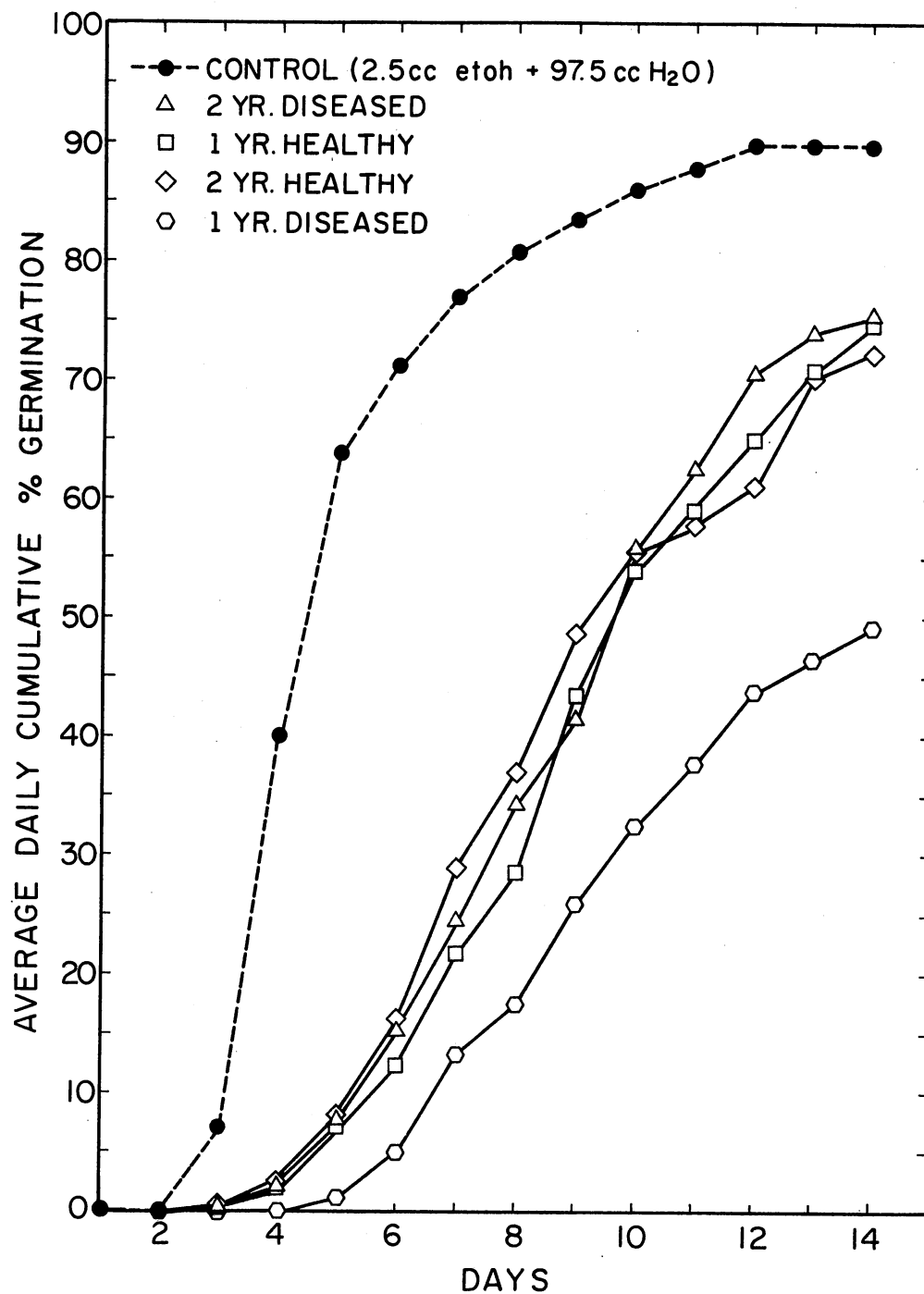
Figure 1. Effect of Normal Concentrations of Extracts
from One and Two Year Old Diseased and
Healthy Pecan Wood on Tomato Seed
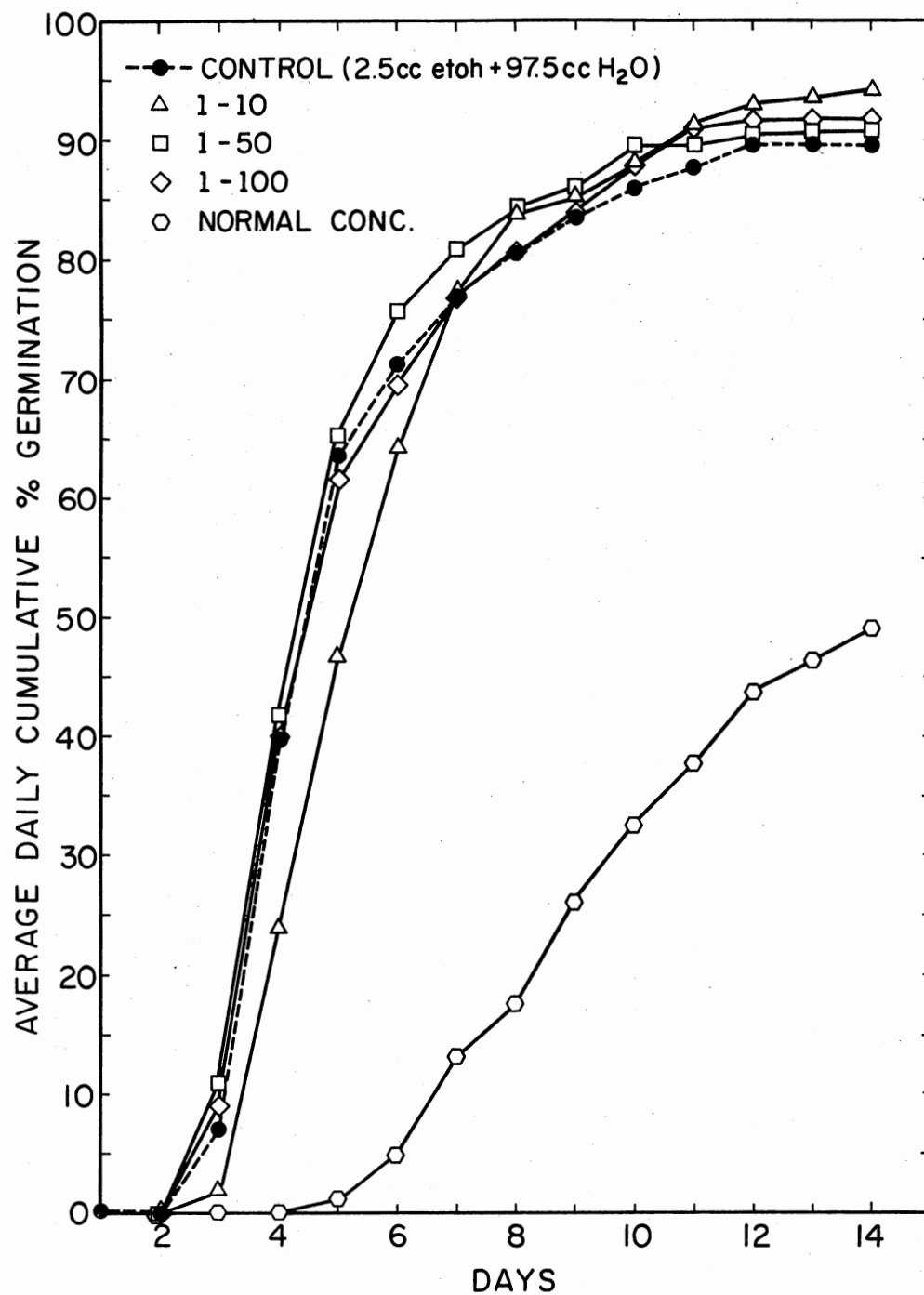Germination

Figure 2.   Effect of Diluted Extracts of One Year Old
            Bunch Diseased Pecan Wood on Tomato Seed
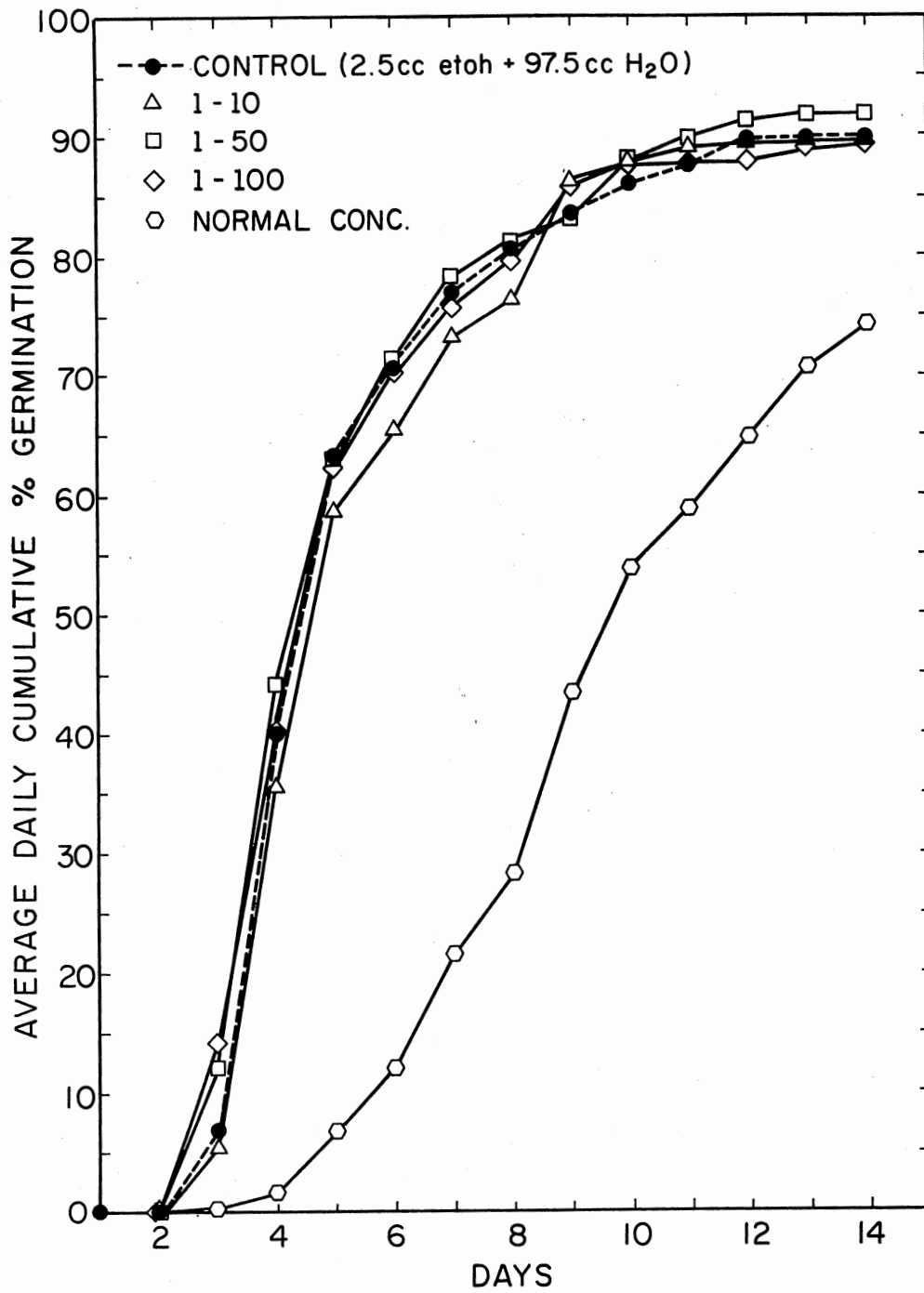   Germination

Figure 3. Effect of Diluted Extracts of One Year Old
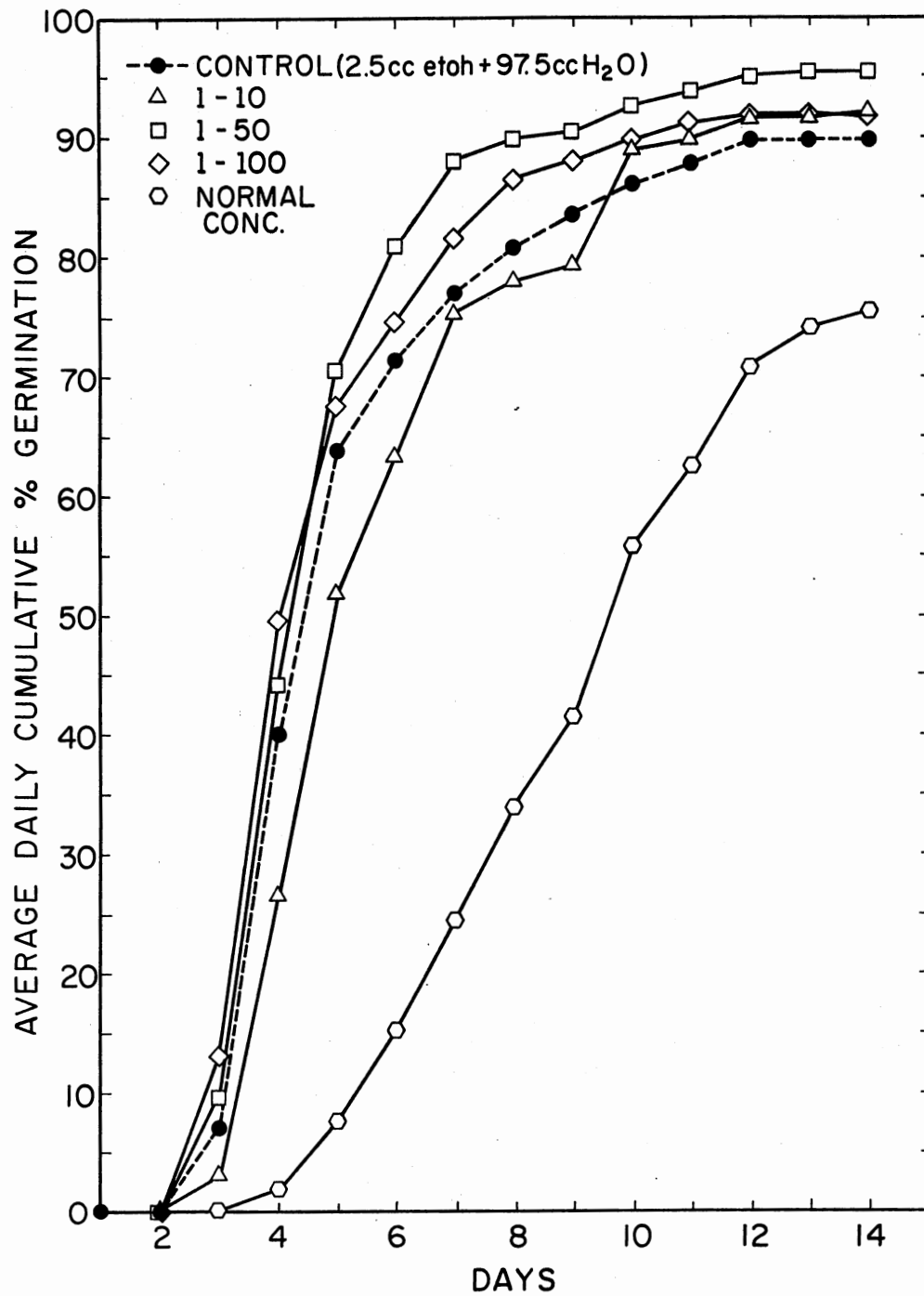Healthy Pecan Wood on Tomato Seed
Germination

Figure 4. Effect of Diluted Extracts of Two Year Old
Bunch Diseased Pecan Wood on Tomato Seed
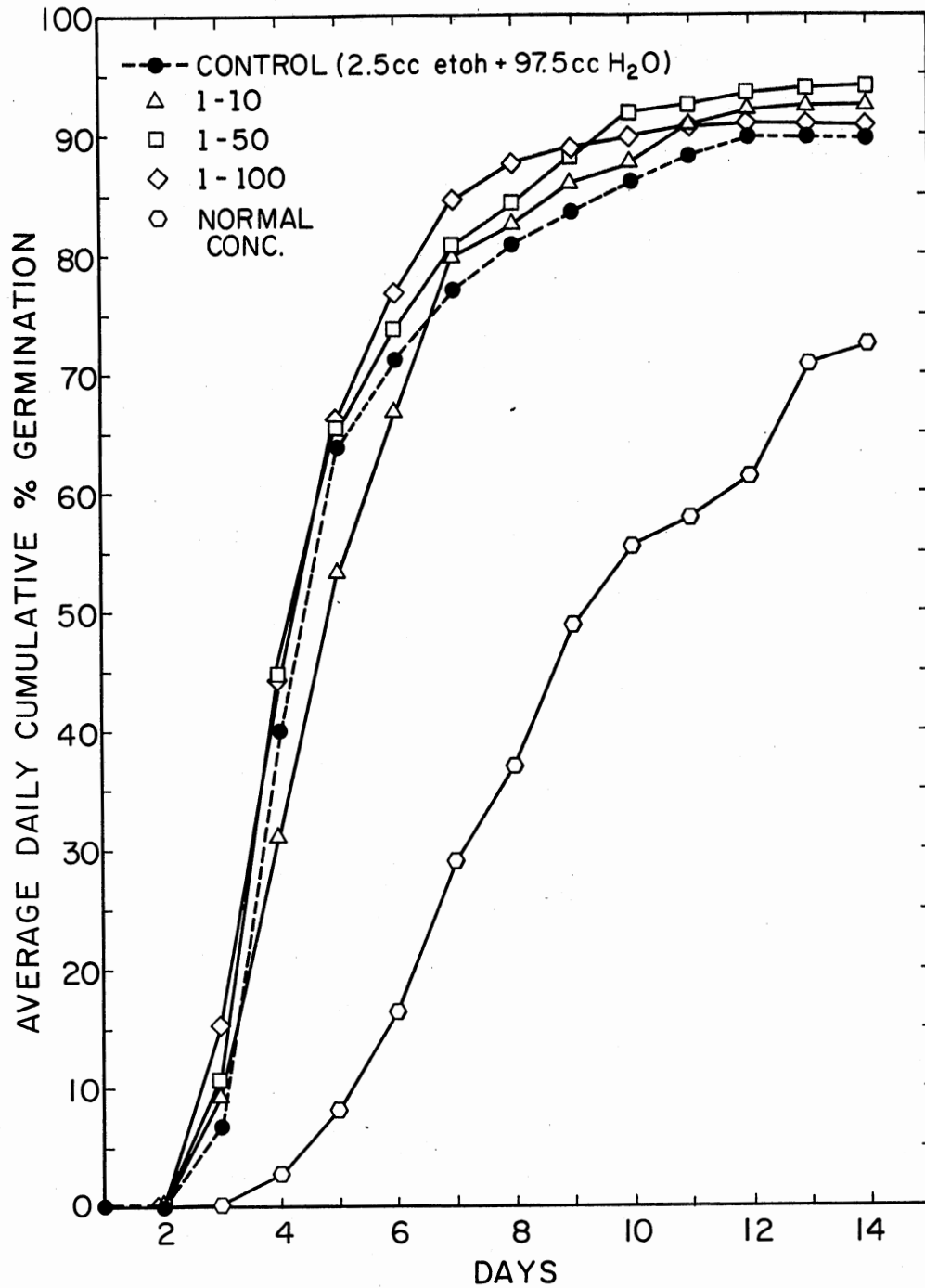Germination

Figure 5.   Effect of Diluted Extracts of Two Year Old
Healthy Pecan Wood on Tomato Seed
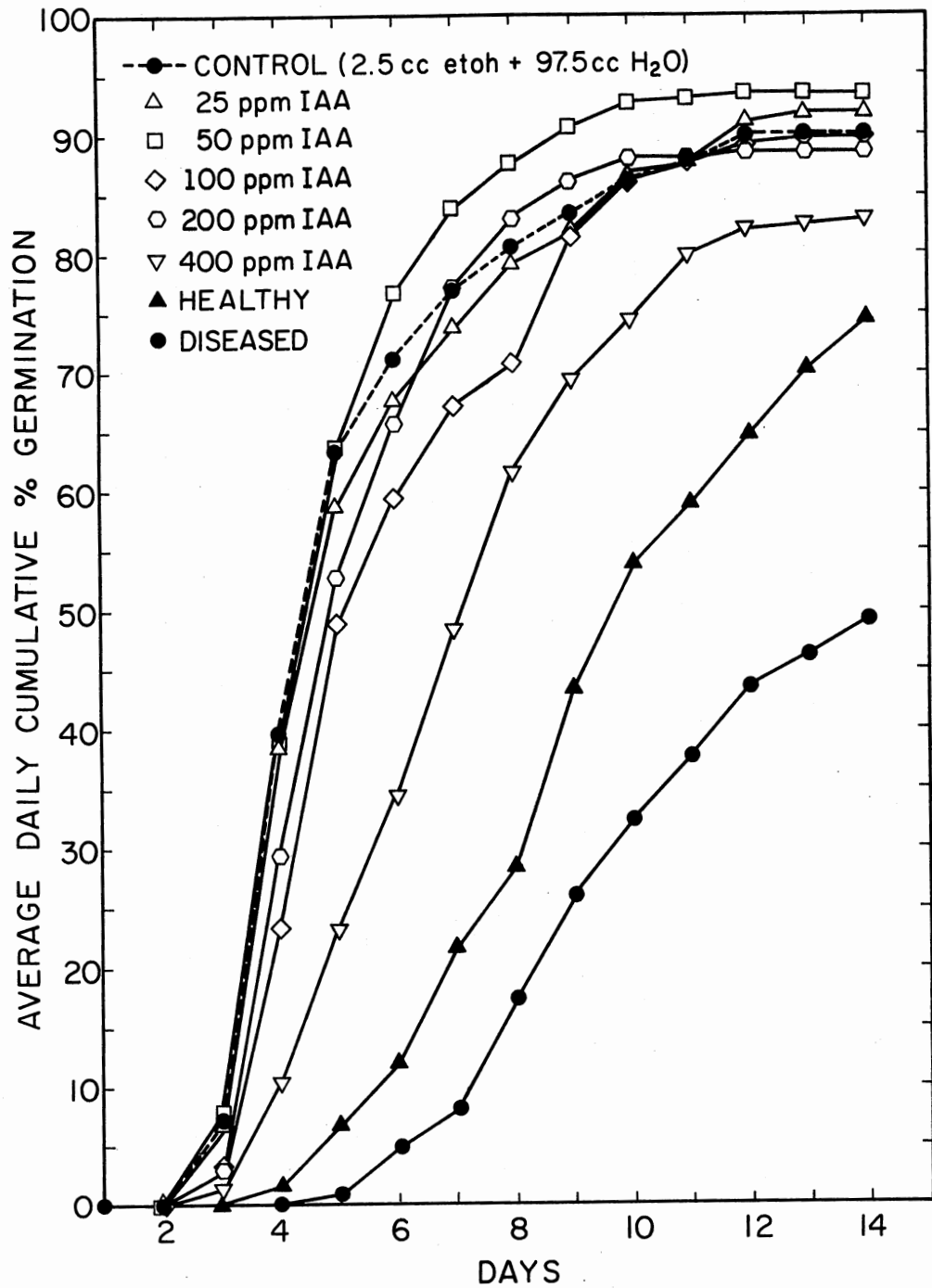Germination

Figure 6.   Comparison of IAA Solutions to Normal
           Concentrations of Extracts of One
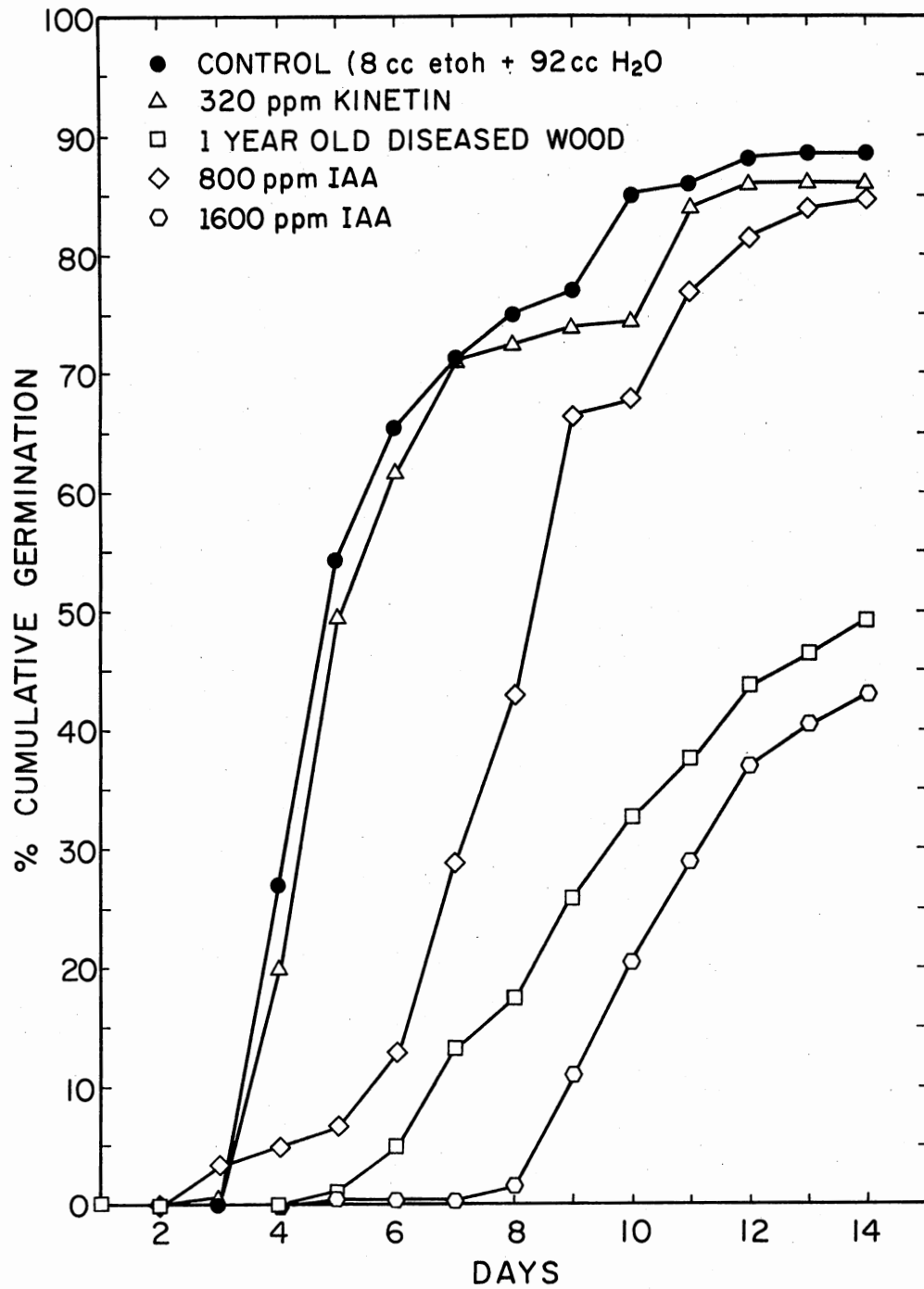   Year Old Diseased and Healthy
           Pecan Wood

Figure 7. Effect of Specific Hormone Concentrations on Tomato Seed Germination

No significant differences were apparent between kinetin concentrations of 10, 20, 40, 80, and 160 ppm when compared to the control (Figure 8) and none of the kinetin rates were shown to be significantly different from any of the dilutions of extracts from either one or two year old diseased or normal wood. Kinetin apparently had no effect on tomato seed germination even at a rate of 320 ppm (Figure 7).

The apparent absence of effect of kinetin may be misleading due to several factors. To obtain the desired solutions of kinetin, it was necessary to first dissolve measured amounts of the hormone in 95% ethanol followed by dilution with enough distilled water to bring the total volume to 100 ml. During the dilution, in order to keep the substance in solution, the flask containing the kinetin had to be heated with hot tap water and sometimes in a water bath. Following storage at 4° C, the kinetin would precipitate out of solution when the container returned to room temperature. After precipitation, heating in a boiling water bath was required to get the kinetin back into solution. The solution then had to be maintained at a high temperature to prevent the hormone from reprecipitating. When the solution was drawn into pipettes and transferred to the petre dishes it probably cooled considerably. It therefore is possible that the kinetin precipitated from solution during the transfer process so that a uniform distribution of the hormone was not obtained in an available form over the seeds. It is also possible that the heating process previously mentioned may have destroyed the kinetin or altered its chemical nature. These factors, coupled with the possibility that tomato seeds may be less sensitive to this particular hormone than to others, suggests that the results obtained in this phase of the bioassay may be misleading.
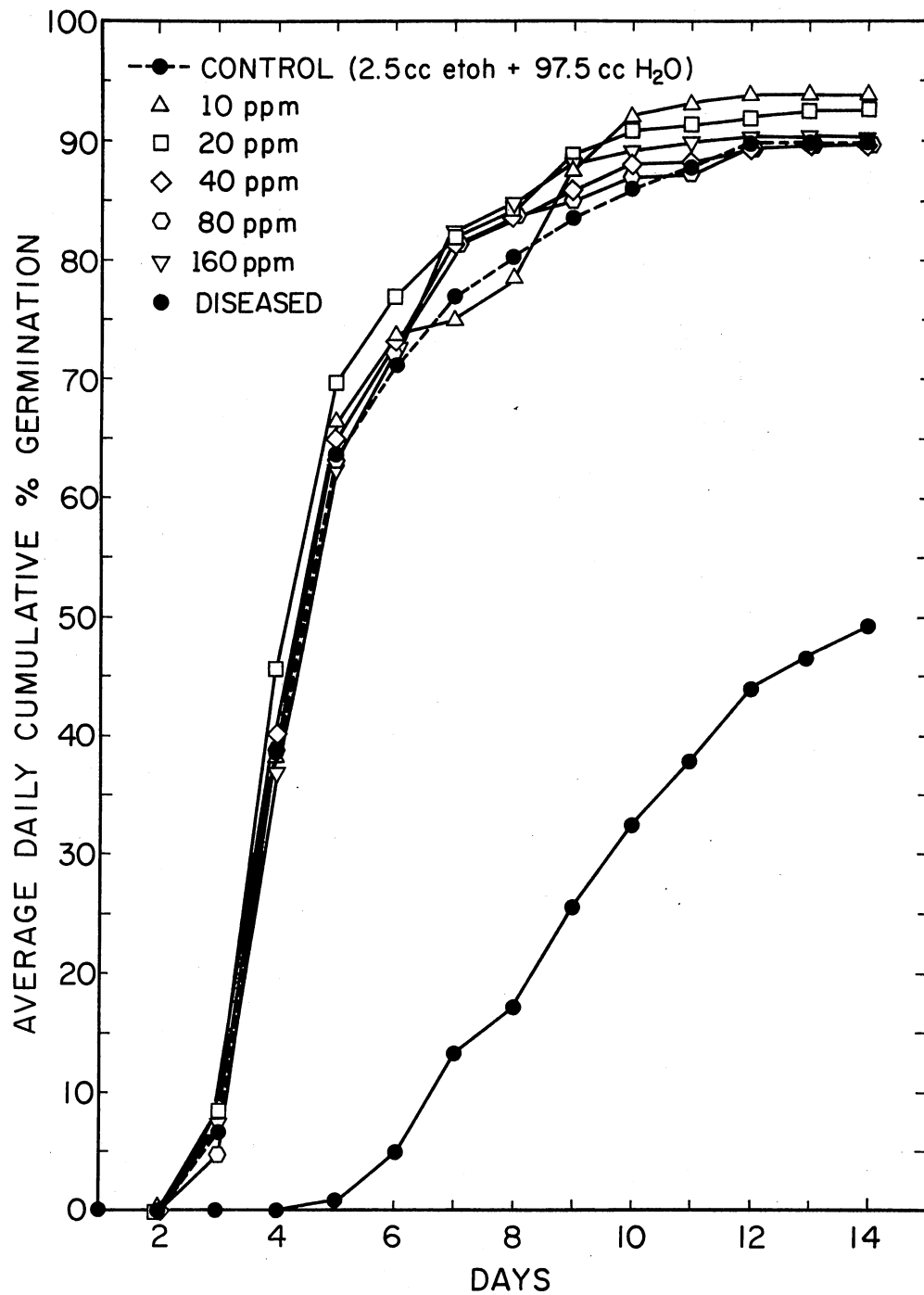
Figure 8.   Comparison of Effects of Kinetin Solutions to
Those of Normal Concentrations of Extracts
from One Year Old Diseased Pecan Wood

The higher hormone concentrations used in the second subsample re-
quired dissolving in a considerably larger amount of alochol than that
used in the original control. Therefore, the effect of the highest
alcohol concentration (8 cc of 95% ethanol in 92 cc water) was compared
to the effect of the 2.5 cc ethanol solution as well as to the effect of
distilled water. Figure 9 shows that the alcohol solutions had no
effect on germination.

Chromatography of the Indoles

The results of the bioassay indicate that kinetin has no strong
effect on germination. This was interpreted to mean that kinetin may or
may not be involved in the manifestation of bunch disease. The bioassay,
however, indicated that an auxin may inhibit germination although the
concentrations required were very high and, thus, auxin may not be in-
volved directly in producing the bunch disease symptoms. The assumption
was made that an auxin such as IAA might be involved and thin layer
chromatography was used in an attempt to substantiate this assumption.

As Figure 8 indicates, the extract from bunch diseased wood produced
inhibition at approximately the same rate as 1600 ppm IAA. Therefore,
to validate the sensitivity of the chromatogram, a known concentration
of 1600 ppm IAA was sampled, the assumption being that if the chromato-
gram indicated the presence of IAA in the 1600 ppm solution, then it
should also be sensitive enough to indicate IAA in the extract if,
indeed, it was present in the extract.

The chromatograms showed that no auxins, specifically IAA or IBA,
were present in detectable amounts in the extracts of either bunch dis-
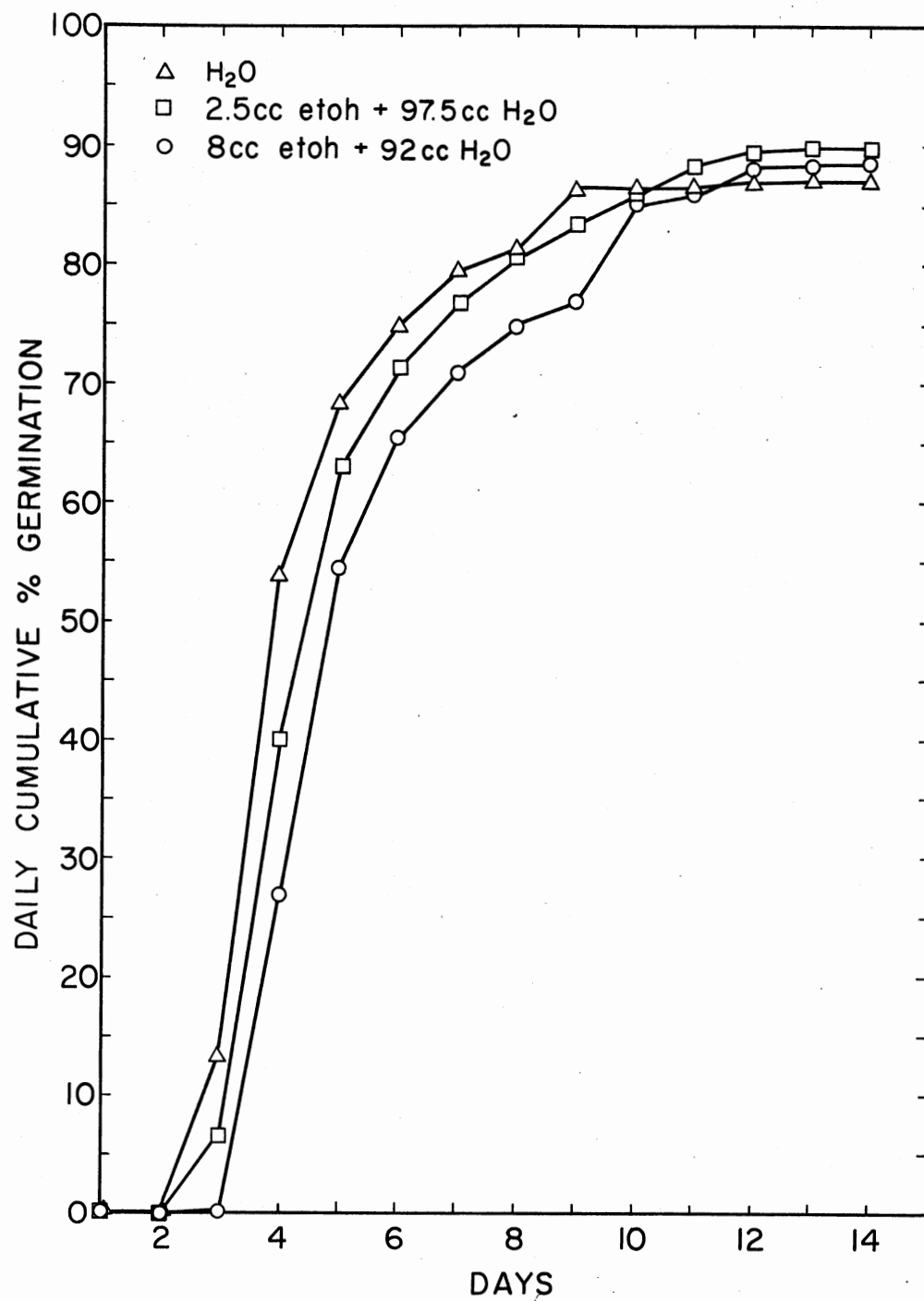eased or normal wood. The appearance of one of the thin layer plates

Figure 9. Comparative Effect of Three Controls on
Tomato Seed Germination

following chromogenisis is duplicated in Figure 10. The results suggest

at least two possible conclusions: (1) that no auxins of any kind are

present in the extracts, thus they play no apparent role in the mani-

festation of bunch disease or (2) that auxins which were present during

the bioassay could have been chemically degraded prior to the chromatog-

raphy. The extracts were frozen, thawed and refrozen several times dur-

ing the course of the study and this process could have caused a

chemical alteration of the hormones and this could, in turn, produce

misleading results on the chromatogram.

<div style="text-align:center">Response of Treated Seedlings</div>

As a further check for the presence of auxins in the bunch diseased

wood extract, Western pecan seedlings were subjected to spray applica-

tions with extracts of diseased and normal wood and observations made

for auxin induced leaf epinasty. While epinastic type distortions were

observed on the seedlings treated with extracts of diseased wood, they

were also observed on those treated with the extract of healthy wood,

and on the controls as well. The seedlings were removed from their

containers and subsequent observation revealed a severely root bound

situation. Root binding produces considerable stress, especially with a

tap rooted species such as the pecan, and ultimately may lead to leaf

distortions. The conclusion drawn was that the observed distortions

were the result of root binding and that the extracts themselves had no

discernible effect on the seedlings.

Injection of seedlings with minute quantities of the extract of

bunch diseased wood produced no apparent differences in leaf and stem
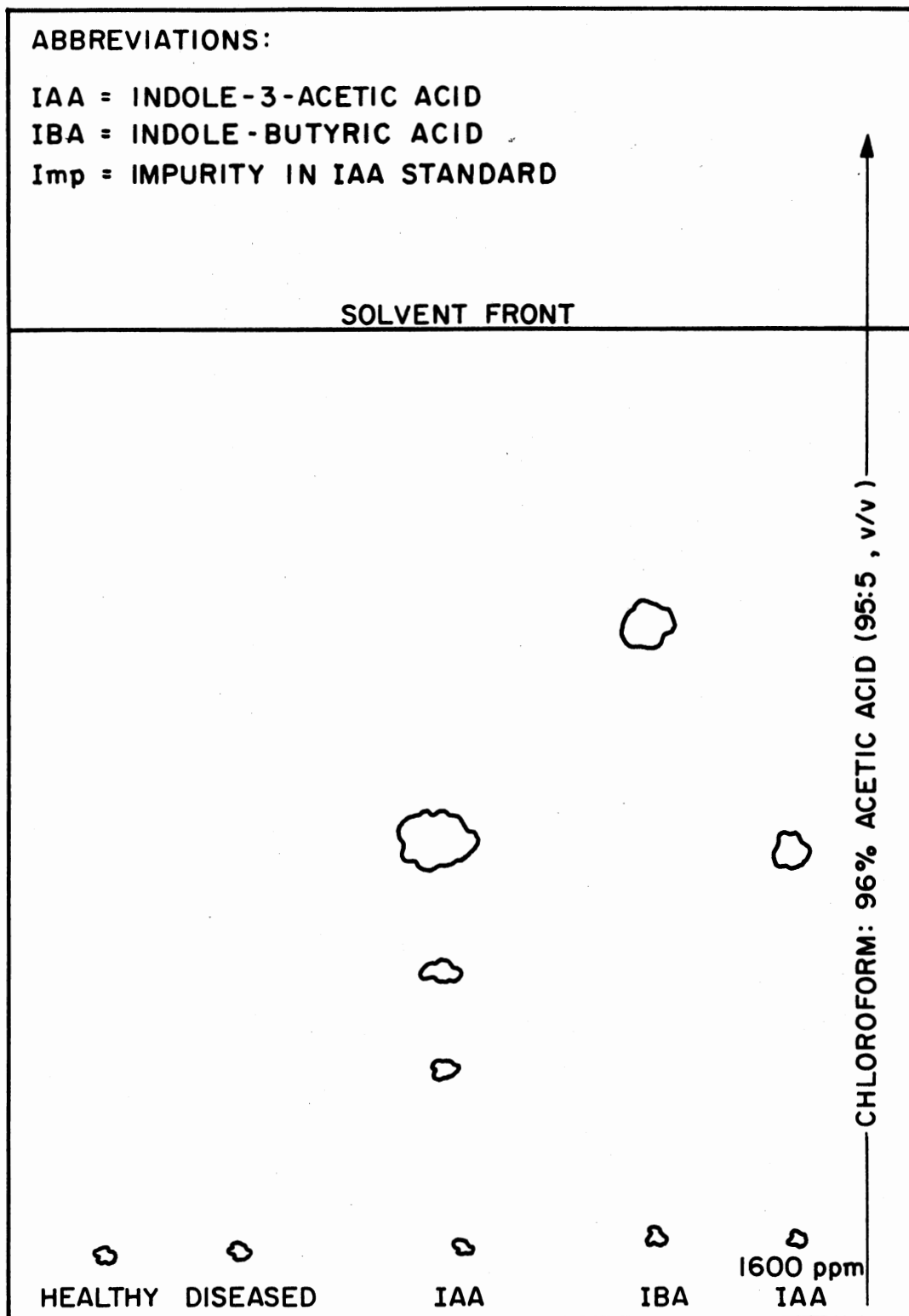
Figure 10.   Thin-Layer Chromatogram of Auxins in Extracts
of Diseased and Healthy Pecan Wood

growth when compared to seedlings treated with extracts of disease free wood.

Application of phloem tissue scrapings obtained from wood of bunch diseased pecans followed by subsequent observation for abnormal bud breaks produced no differences following an eight-week growth and observation period. When the parafilm and lanolin were removed it was noted that many of the treated seedlings had produced an unusual amount of callus tissue around the wounded areas. These calluses were large for the size of the trunk and presented a round, gall-like appearance. Approximately 60% of the treated seedlings exhibited callus tissue of this nature but, because there were no controls the results are not valid and the test needs to be repeated.

CHAPTER V

SUMMARY AND CONCLUSIONS

Bioassay techniques demonstrate that some type of germination inhibitor is present in the extracts of one year old bunch diseased pecan wood and it is available in significantly larger concentrations than are those found in extracts of one and two year old healthy and two year old bunch diseased wood. Although the level of germination inhibition produced by the extract of bunch diseased tissue is approximately the same as that induced by a concentration of 1600 ppm IAA, thin-layer chromatography failed to distinguish the presence of large amounts of IAA in the extracts. Although IAA may or may not be involved in the manifestation of pecan bunch disease, the observed germination suppression must be attributable to other growth substances. Another substance known to inhibit germination is abscisic acid. Some of the symptoms of bunch disease such as rachis retention, leaf chlorosis, and shoot growth retardation, are effects known to be induced by this particular hormone. Such symptoms could then implicate the involvement of abscisic acid in the expression of the disease. Future investigations could pursue this possibility.

While involvement of kinetin cannot be ruled out, nevertheless, the lack of a significant increase in germination rate suggests that this particular hormone is not present in significant quantity in the extracts of either bunch or normal wood or, if present, then the

potential effects of kinetin are either being masked by other factors or the assay system was insensitive to kinetin.

Neither auxin induced leaf epinasty nor excessive lateral bud breaks were observed when seedlings of the Western cultivar either were sprayed with normal concentrations of the extracted solutions or were injected with minute quantities of the solutions. This further suggests that IAA plays no role in the manifestations of bunch disease.

Abnormal callus tissue formation occurred on seedlings treated with phloem tissue of bunch diseased wood but further investigations would be necessary to determine the specific hormone involved. Kinetin is known to be associated with callus tissue formation and therefore future investigations could pursue its potential role in this phenomenon.

A SELECTED BIBLIOGRAPHY

1. Ballard, L. A. T. and A. E. G. Lipp. 1959. Differential Specif-
       icity Exhibited by Two Germination Inhibitors Present in
       Echium plantagineum L. Aust. J. Biol. Sci. 12: 343-347.

2. Boswell, S. B. and W. B. Storey. 1974. Cytokinen-Induced Axillary
       Bud Sprouting in Macadamia. HortScience. 9(2): 115-116.

3. Chan, T. K. 1968. A Biochemical Study of Resistance in Barley to
       the Greenbug, Schizaphis graminum: "Thin-Layer Chromatography
       of the Indoles." (Unpub. Ph.D. dissertation, Oklahoma State
       University.)

4. Cole, J. R. 1937. Bunch Disease of Pecans. Phytopathology.
       27: 604-612.

5. Goren, R., E. E. Goldschmidt and S. P. Monsellis. 1971. Hormonal
       Imbalance in Bark and Leaves of Shamouti Orange Trees (Citrus
       sinensis L., Osbeck) in Relation to Ringing. Journal of
       Horticulture Science. 46: 443-451.

6. Hanna, J. D. and H. A. Hinrichs. 1966. Preliminary Study on
       Growth Inhibiting Materials in the Alcohol-Soluble-Extracts
       Collected in Spring and Fall from Bunch Diseased and Normal
       Pecan Trees. (Unpub. Research, Oklahoma State University.)

7. Heydecker, W. and A. Josshua. 1972. Extract from Capsicum Hastens
       Seed Germination. Commercial Grower. 3999: 239-240.

8. Holmes, F. O., H. Hirumi and K. Maramorosch. 1972. Witches'-Broom
       of Willow: Salix Yellows. Phytopathology. 62: 826-828.

9. Hutchins, L. M. and H. V. Wester. 1947. Graft-Transmissible
       Brooming Disease of Walnut. (Abstract) Phytopathology.
       37: 11.

10. KenKnight, G. E. 1966. Research on Bunch Disease of Pecan. Proc.
       Southeast Pecan Growers Association. 59: 69-71.

11. _____. 1965. Research on Bunch Disease of Pecan. Proc.
       Southeast Pecan Growers Association. 58: 81-87.

12. _____. 1963. Progress Report on Bunch Disease of Pecan and
       Witches' Broom Diseases of Trees in Woods Near Pecan Orchards.
       Proc. Texas Pecan Growers Association. 42: 94-98.

13. _____. 1963. Research on Bunch Disease of Pecans. Proc. Southeast Pecan Growers Association. 56: 43-45.

14. _____. 1962. Bunch Disease of Pecans. Proc. Texas Pecan Growers Association. 41: 67-73.

15. _____. 1952. Germination Inhibitor in Wood and Bark of Peach and Wild Prunus. (Abstract) Phytopathology. 42: 285.

16. Lipe, A. J., P. W. Morgan and J. B. Storey. 1969. Growth Substances and Fruit Shedding in the Pecan, Carya illinoensis. J. Amer. Soc. Hort. Sci. 9(6): 668-671.

17. MacDaniels, L. H. and D. S. Welch. 1964. The Walnut Bunch Disease Problem. Proc. Northern Nut Growers Association. 55: 41-48.

18. Meyer, B. S., D. B. Anderson, R. H. Bohning and D. G. Fratianne. 1973. Introduction to Plant Physiology, D. Van Nostrand Co., Cincinnati, 1973, pp. 513-514.

19. Milbocker, D. C. 1972. Axillary Shoot Stimulation in Poinsettia with Kinetin. HortScience. 7(5): 483-484.

20. Millikan, D. F. 1965. Bunch Disease of Walnut. Proc. Northern Nut Growers Association. 56: 75-76.

21. Moore, W. F. 1966. The Symptomology of Bunch Disease of Pecan and a Comparison of the Fungal Flora of the Buds of Diseased and Healthy Trees. Proc. Southeast Pecan Growers Association. 59: 78-79.

22. Phillips, I. D. J. 1964. Root-Shoot Hormone Relations. I. The Importance of an Aerated Root System in the Regulation of Growth Hormone Levels in the Shoots of Helianthus annus, Annuals of Botany. 28: 17-35.

23. Rapp, A. and A. Ziegler. 1971. Determination of Abscisic Acid in Vines. (Abstract) Vitis. 10(2): 111-119.

24. Rathbun-Gravatt, A. 1927. A Withces' Broom of Introduced Japanese Cherry Trees. Phytopathology. 17: 19-24.

25. Seliskar, C. E., C. L. Wilson and C. E. Bourne. 1972. Mycoplasma-like Bodies Found in the Phloem of Black Locust Affected with Witches' Broom. Phytopathology. 63: 30-34.

26. _____. G. E. KenKnight and C. E. Bourne. 1974. Mycoplasmalike Organisms Associated with Pecan Bunch Disease. Phytopathology. 64: 1269-1272.

27. Sladky, Z. 1972. The Role of Endogenous Growth Regulators in the Differentiation Processes of Walnut (Juglans regia L.). Biologia Planatarum. 14(4): 273-278.

28. Stahl, Egon, ed. 1965. Thin-Layer Chromatography; a Laboratory
         Handbook, by H. R. Bolliger, et al. Springer, Verlag, Berlin,
         New York.

29. Stewart, F. C. 1917. Witches'-Broom on Hickory Trees. Phyto-
         pathology. 9: 185-187.

30. Waite, M. B. 1932. Notes on Some Nut Diseases with Special
         Reference to the Black Walnut. Proc. Northern Nut Growers
         Association. 23: 60-67.

31. Wickson, M. and K. V. Thimann. 1958. The Antagonism of Auxin and
         Kinetin in Apical Dominance. Physiologia Plantarum. 11: 62-
         74. (From Laetsch, W. M. Papers on Plant Growth and Develop-
         ment. Little Brown and Co., Boston. pp. 118-129.)
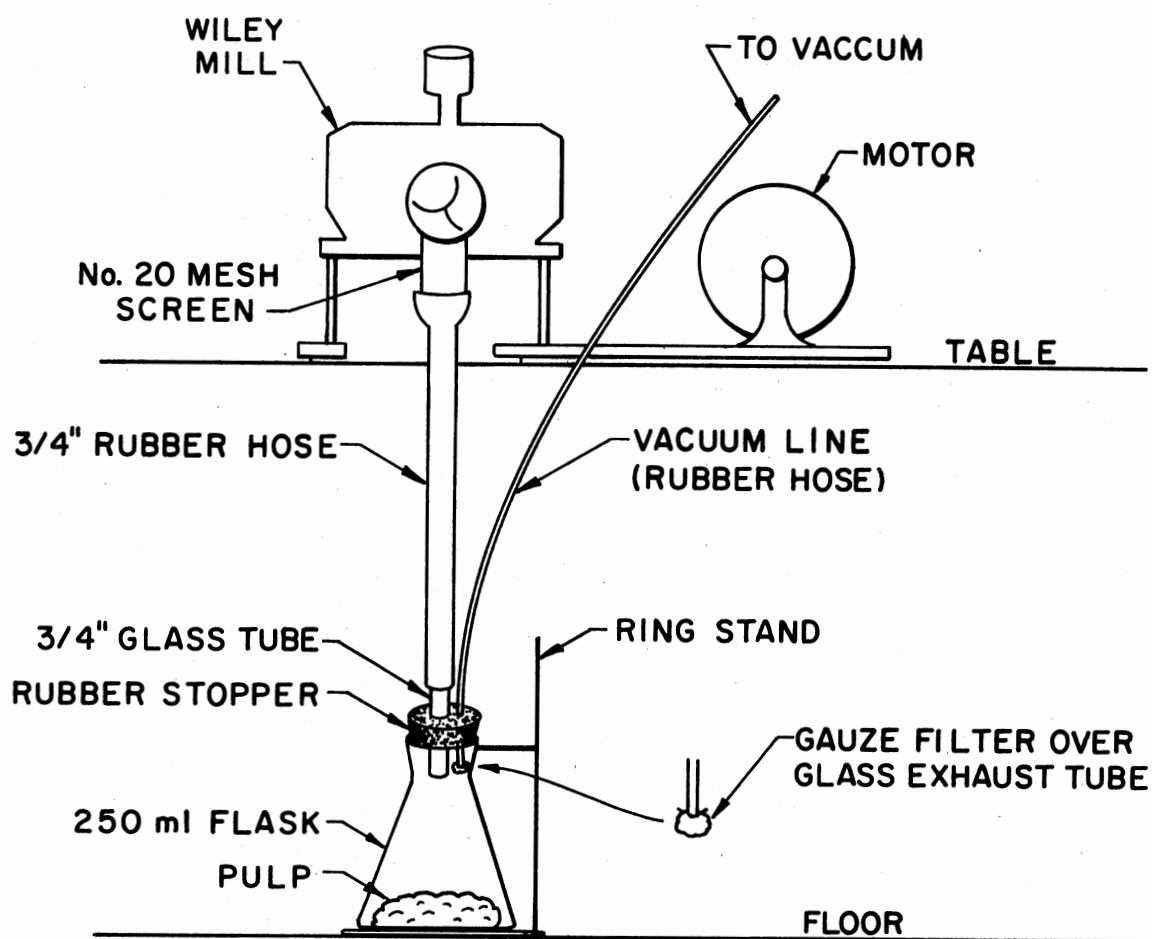
APPENDIXES

Figure 11. Vacuum Method for Collecting Ground Wood Pulp

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VITA

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