THE ACCUMULATION OF GROWTH REGULATORS IN PLANT INFECTED BY *NECTRIA PTEROSPERMI* SAW.*

PETER HAO YU, LUNG-CHI WU, and CHI-CHANG CHEN**

(Received July 30, 1967)

Introduction

The fungus *Nectria pterospermi* induced canker of maple-leaved pterospermum (Chen, 1965). The trunk and branch of the affected plants became malformed, callus accumulated, and the internal tissues tended to be darkened. As the canker spread, the dead bark eventually cracked and sloughed off.

The economic and scientific importance of tree canker were dissussed in a symposium held by the American Phytopathological Society (Zabel, 1964). Most of the literatures on Nectria canker accounted for disease occurence. Only a few dealed with pathogen identification, pathogenicity, epidemiology, and disease control.

In order to understand the disease development of Nectria canker, a quantitative comparison of the auxin contents of ether extracts from newly developed Nectria-affected canker portion and from the corresponding portion of healthy bark as well as the culture filtrate of the causal organism was carried out in the present experiment. A nearly 6-fold increase of indole-3-acetic acid (IAA) in diseased tissue was observed. This fact would strengthen the hypothesis suggested by Berducou (1952) that the pathogenesis of Nectria canker was induced by the production of IAA.

Materials and Methods

1. *Extraction*

Four hundreds grams newly developed bark at the margin of the canker were cut into small pieces and extracted with 2,000 ml 70% methanol for five days at 5°C. The extract was obtained by straining through four layers of cheese cloth, then two layers of filter paper, and concentrated in a rotary

* Research was supported in part by USDA grant No. FG-Ta-103, paper No. 15, Important Epidemic Diseases of Forest Trees in Taiwan, Journal-Series.

** Respectively, Teaching Assistant and Professors of Plant Pathology, Department of Plant Pathology and Entomology, National Taiwan University, Taipei, Taiwan, China.

evaporator at 45°C. The concentrated extract was adjusted to pH 3.2 with 0.1 N HCl, then extract with peroxide-free ethyl ether (Bentley, 1962) for three times with a separatory funnel. Combined ether extracts were evaporated to nearly dry, and then the residue was taken up in a mixture of hexane and acetonitrile (1:1, v/v) to eliminate fats and pigments. The acetonitrile fraction containing auxin was concentrated to 4 ml and stored at 4°C for further use. Bark of the healthy plants collected from the corresponding portion was treated exactly in the same manner mentioned above.

Existence of peroxide in ether was detected by liberation of iodine when 2 ml of sample were shaken with an equal volume of 2% KI solution and a few drops of dilute HCl. Brown coloration or blue coloration of treated sample with starch solution indicated the presence of peroxide. The peroxide was removed by shaking with concentrated ferrous solution which was prepared from 60 grams of crystalline ferrous sulphate, 6 ml of concentrated H_2SO_4 and 110 ml H_2O (Vogel, 1962).

2. *Paper chromatography*

Descending paper chromatography were performed with Whatman No. 1 paper (30 × 28 cm) in a paper chromato-cabinet at 26°C. Two solvent systems were chosen for the development of chromatograms in the present experiments, i. e. Isopropanol: Ammonia (28%): water (8:1:1) (IAW), and 70% ethanol. Fifty micro-liters of the concentrated extracts were applied to each spot. The cabinet was previously saturated with the solvent over night, and developed through a distance of 30 cm, for 9 to 12 hours. Color developments of the chromatograms were either sprayed with Salkowski's reagent or Ehrlich's reagent. They were also examined under a UV lamp or exposed in ammonia vapor to locate the spots.

Salkowski's reagent was prepared by dissolving 2 ml of 0.05 M $FeCl_3$ in 100 ml of 5% $HClO_4$. Pink color indicated the presence of indole-3-acetic acid (IAA) (Sen and Leopold, 1954).

Ehrlich's reagent was prepared by dissolving *p*-dimethylaminobenzaldehyde in 2 N HCl, to make 2 per cent, and then mixed with ethanol (20:80, v/v) (Steelink, 1959). IAA showed a blue-green color.

3. *Bioassay*

Avena coleoptile straight growth test (Nitsch and Nitsch, 1956) was adopted in the present experiments as follows: Unhusked oat seeds, var. Brington, were surface-sterilized with 0.1% $HgCl_2$ for 2 minutes, rinsed in sterile water for 3 times, and soaked in sterile tap water for one hour at room temperature. The treated seeds were laid in parallel rows and pointed in the same direction with embryo up on 1% water agar in a Petri-dish (12 cm

in diameter and 10 cm in height). The seeds were allowed to germinate and grow for 72 hours under a red fluorescent lamp.

When the coleoptiles reached about 1.8 to 2.2 cm in length, coleoptiles of equal length were cut into 4 mm sections, at the level 3 mm below tip. The primary leaf was left inside. These sections were floated for three hours in glass-distilled water containing 1 mg/liter of $MnSO_4 \cdot H_2O$. This step was omitted in the later experiments, since no difference in the data was obtained.

The developed chromatogram was cut into seven strips (4 × 30 cm) as seven spots were previously made in each chromatogram. Each strip was divided into 10 sections, then they were eluted separately in Petri-dish (5 cm in diameter and 1 cm in height). Three to 4 pieces of coleoptile sections were distributed in each dish to determine their activity. Uncontaminated Whatman No. 1 paper (3 × 4 cm) was used as check. They were incubated for 22-23 hours in the dark at 26°C before reading. The length of the sections were measured with a ruler under magnifier.

4. *Spectrophotometry*

Gordon and Weber's (1951) method was adopted in the experiments. To a 1.0 ml aliquot, 2.0 ml of the Gordon and Weber reagent (1.0 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added and read at 530 mμ after 25 minutes.

According to the preliminary experiment on the culture filtrates and the ether extracts, it showed that there was a linear relationship between the quantities of IAA (between 1 to 50 μ g/ml) and the optical density at 530 mμ (E_{530}) by a spectrophotometer of the Unicam SP 500. Color reaction was completed in 60 minutes, though Gordon and Weber (1951) suggested that measurement could be made 25 minutes after addition of color reagent. Thus the reading was made after 60 minutes until the completion of color reaction.

Results

Quantitative analysis of growth regulators in diseased and healthy plants of maple-leaved pterospermum

Although auxin contents in plant tissues were too low to show pink or blue-green color on chromatograms either with Salkowski's or Ehrlich's reagent, they were readily detected by bioassay (avena coleoptile straight growth test) with the same chromatograms. The results are shown in Fig. 1.

The Rf values were matched to the Rf values of authentic IAA, i. e. 0.35 with the solvent system of Isopropanol: ammonia water (28%): water (8:1:1), and 0.81 with 70% aqueous ethanol. The contents of IAA in diseased tissues were apparently higher than that in the healthy ones.

In preparation of standard curve with authentic IAA by avena coleoptile straight growth test, the quantities of IAA were estimated (Fig. 2).

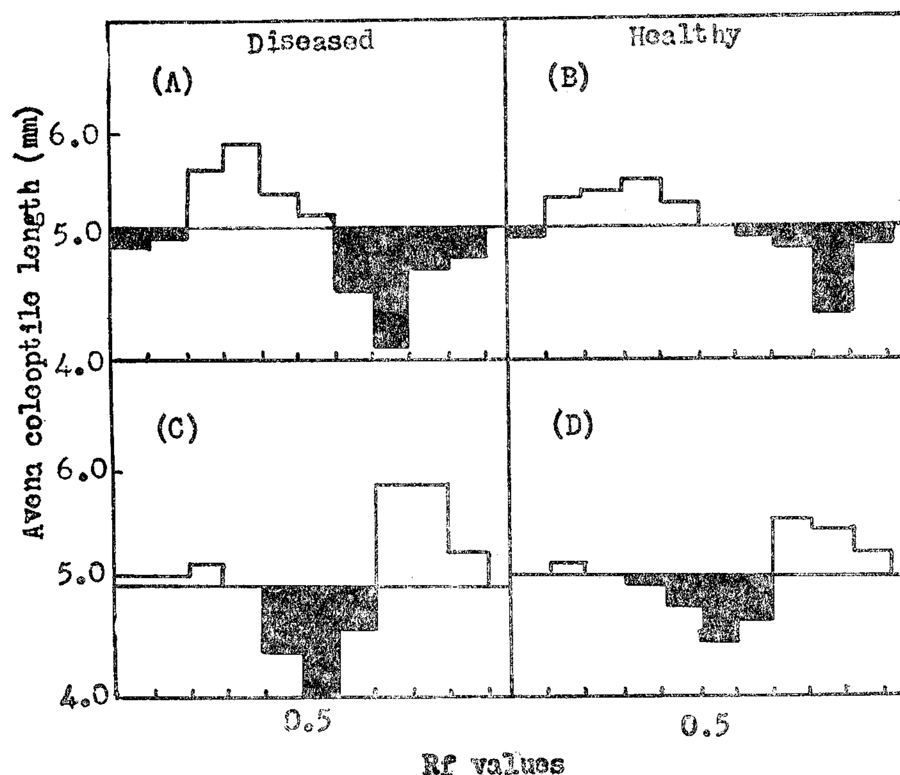

Fig. 1. The growth regulators in diseased and healthy tissues of maple-leaved pterospermum.
Developing solvents: (A) and (B) are Isopropanol: ammonia (28%): water (8: 1: 1); (C) and (D) are 70% aqueous ethanol. The length of control coleoptile are from 5.0 to 5.2 cm.

The elongation ratio of avena coleoptile were 1.5 (L/L_o 6.0/4.0) for diseased, and 1.4 (5.6/4.0) for healthy. Therefore, the log C_{ppm} were extrapolated as -0.8 ($\log C_d$) and -1.6 ($\log C_h$) for diseased and healthy, respectively. They are culculated as follows:

Since, $\log C_d^* = -0.8$

$\log C_h^* = -1.6$

thus, $C_d = 0.140$ *ppm* in the extracts

$C_h = 0.025$ *ppm* in the extracts

$C_d = 0.00140$ *ppm* in the plant tissue

$C_h = 0.00025$ *ppm* in the plant tissue

Therefore the quantities of IAA were 1.4 μg/1000 grams of cankered tissues, and 0.25 μg/1000 grams of healthy tissues. A 6-fold increase of auxin in the diseased tissue was thus obtained.

* The symbols are respectively "*d*", diseased; "*h*", healthy; and "*C*", concentration.

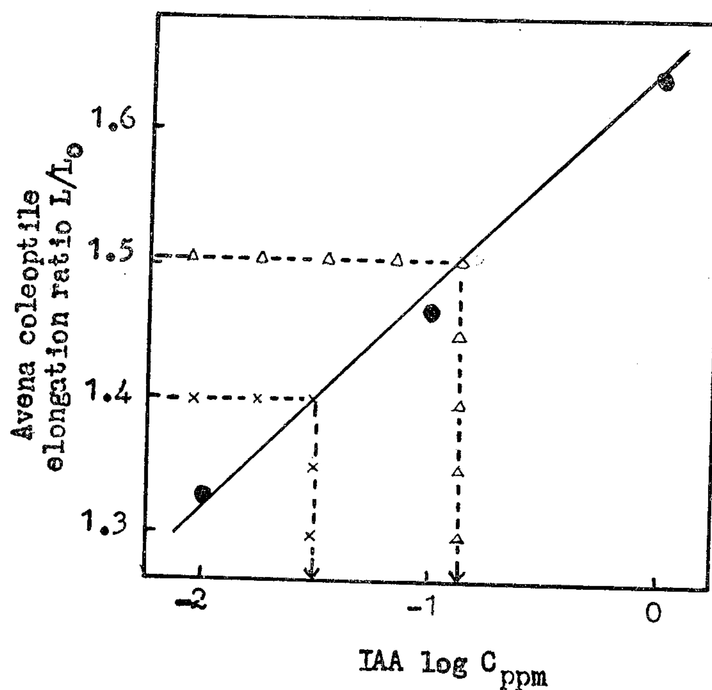

Fig. 2. Determination of IAA contents by extrapolation of the values for the growth of avena coleoptile.
The symbols are expressed respectively, △- -△, diseased; ×- -×, healthy; C, concentration in ppm; L, the length of avena coleoptile growing for 22 hours; L_o, initial length (4 mm).

An unidentified growth inhibitor was also found in the same extracts. Its Rf values were found to be 0.88 (IAW), and 0.53 (70% ethanol). It could be separated easily from IAA by Rf values. The inhibitory effect was quite significant. It is worthy to mention that the inhibitor softened the avena coleoptile texture in addition to the inhibition of coleoptile elongation. On the paper chromatogram, it was characterized as light brown spot with a tail, changed to brown spot with Salkowski's reagent, to yellow color in saturated ammonia vapor, and to yellowish grey under UV light.

The determination of IAA produced by Nectria pterospermi

1. *Bioassay and chromatography of the partially purified IAA of the culture filtrate of Nectria pterospermi*

Following the same method adopted above, it was found that Rf of the partially purified sample material inducing elongation of avena coleoptile agreed with the Rf values of the authentic IAA, 0.35 (IAW), and 0.81 (70%, ethanol) (Fig. 3).

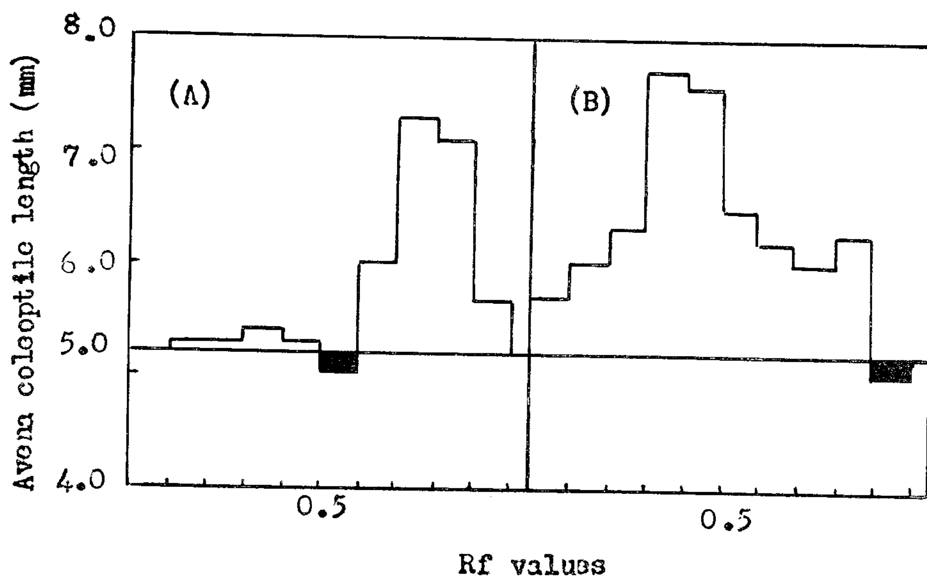

Fig. 3. The auxin contents in the culture filtrate of *Nectria pterospermi.* Developing solvents: (A), 70% ethnol; (B), Isopropanol: ammonia (28%): water (8:1:1) The control coleoptiles were 5.2 cm long at the horizontal line.

The chromatogram sprayed with either Salkowski's or Ehrlich's color reagent appeared a distinct pink and blue-green spot, respectively, at the region corresponded to the Rf values of authentic IAA.

2. *The quantitative analysis of IAA in a 50 days old culture*

a) *Chromatögram spot area method*—

The spots area of 10 μl of concentrated extract was compared with a series of concentrations of authentic IAA (20, 40, 60, 80, 100, and 120 μg) by paper chromatography (Fig. 4). It was shown that the IAA contents of the extracts were within the range of 20—40 μg per 10 μl, and equivalent to 20—40 mg per 1,000 ml of the culture filtrate.

b) *Spectrophotometric method*—

In order to calculate the concentrations of IAA in sample solution of the culture filtrates, a standard curve was prepared with authentic IAA in different concentrations (Fig. 5).

One hundred ml of the culture filtrate were adjusted to pH 3.2 and extracted with peroxide free ether for 3 times. The combined ether extracts were evaporated to nearly dry and dissolved in 1 ml methanol. The methanol extract was streaked on Whatman No. 4 paper (30 × 28 cm), and developed with IAW solvent system. The band corresponding to the Rf value of authentic IAA was cut down and re-extracted with methanol. Methanol was elimininated by reduced pressure at 45°C and the residue was dissolved with

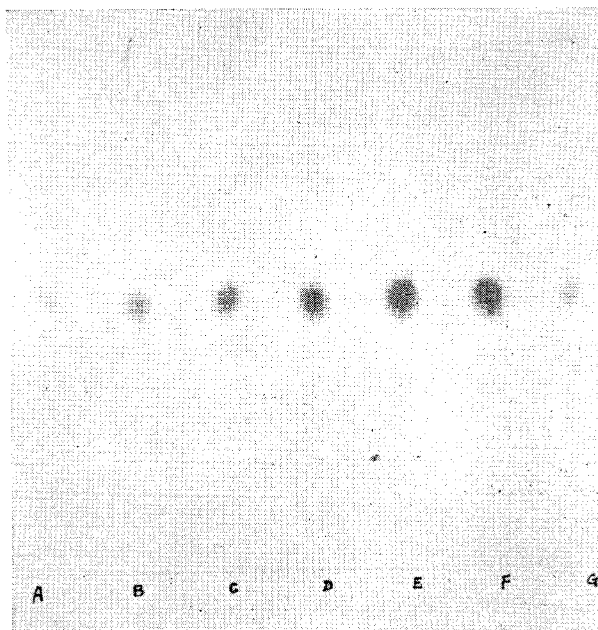

Fig. 4. Determination of IAA content in the 50 days old culture filtrate of *Nectria pterospermi*.
G is the ether extract of sample. A, B, C, D, E, and F, are 20, 40, 60, 80, 100, and 120 μg of authentic IAA, respectively.

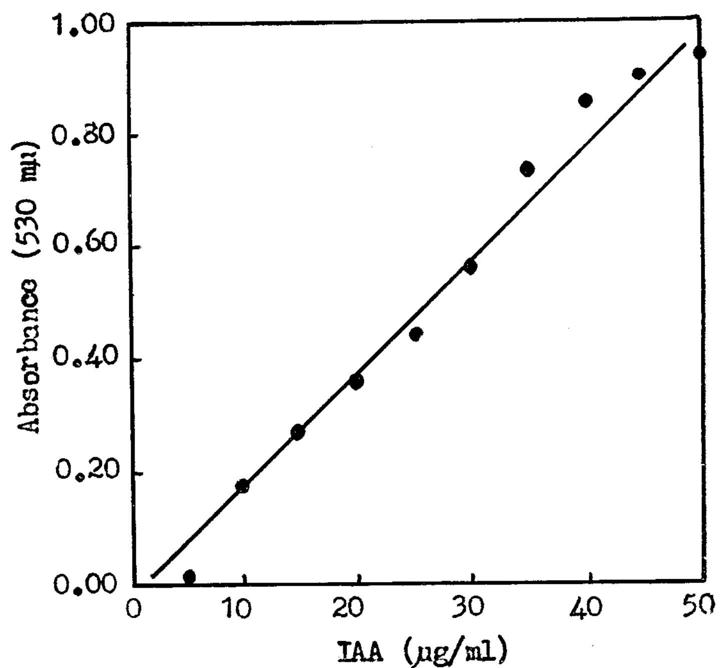

Fig. 5. The standard curve of authentic IAA determined by Gordon and Weber's colorimetric method.

100 ml H_2O. They were kept for determination of IAA by Gordon and Weber's colorimetric method. By the above method, it was clearly shown that one ml of the extract was equivalent to 28 μg of IAA which was measured 0.56 optical density at 530 mμ. Therefore, it was equivalent to 28 mg/1,000 ml of the culture filtrate. The results of the above two methods were reliable.

Discussion

The roles of growth regulators on plant growth and development have been investigated extensively by a large number of plant physiologists, particularly since the discovery by Went (1926) of a quantitative experimental approach. The study on the relationship of plant growth regulators to plant diseases is a new trend in recent years. But, most of the work on this problem has been on disease resistance rather than on the basic aspects of the role of growth regulators in pathogenesis. Sequeira (1963) has given a detail review on these subjects.

The normal plant may be looked as a precisely balanced complex of interrelated reactions that are in a state of dynamic equilibrium. Any deviation in this complex of reactions will tend to disrupt the balanced system and may lead to abnormal, or pathological growth (Braun, 1959). Actually lots of pathogenic organisms do disrupt this complex, i. e. crown gall caused by *Agrobacterium tumefaciens* (Braun, 1962), tobacco and banana wilt by *Pseudomonas solanacearum* (Sequeira and Kelman, 1962), rust of safflower hypocotyls by *Puccinia carthami* (Daly and Inman, 1958), corn tumor by *Ustilago zeae* (Turian and Hamilton, 1960), and rice foolish seedling disease by *Gibberella fujikuroi* (Kurosawa, 1926) etc.

By using histological method, Ashcroft (1934) observed both hypertrophy and hyperplasia of the cambium of black walnut infected by *Nectria galligena*. From this observation, it was convinced that the malformation of the tissues of Nectria canker might be due to the disturbance of hormone balance. Berducou (1952) cultivated the fungi in Knop's medium, IAA was detected in *N. galligena*, but absent in culture of *N. cinnabarina*. She explained that the pathogenesis of Nectria canker was entirely by the production of IAA. However, no further evidence has been made.

In our experiments, a nearly 6-fold increase of auxin in the cankered tissues infected by *Nectria pterospermi* was obtained. And the auxin was identified to be indole-3-acetic acid. This fact would strengthen the hypothesis, viz. the increase of auxin in diseased tissues caused the abnormal proliferation of the cambium and so caused the crack and slough off of the bark. So far, it has not been possible to demonstrate this proliferation of the cambium of maple-leaved pterospermum directly by tissue culture technique. However, it seems

logical to assume that such an increase of IAA plays a vital role during the fungal invasion within the host bark in the present case since the activity of normal cambium of many other wood plants influenced by auxins was already proved (Gautheret, 1955).

The increase of the level of IAA in canker tissue might be attributed to the pathogen though stimulation of auxin production in host plant induced by infection was not ruled out. However, the activity of IAA synthesis by the fungus was very strong provided that tryptophan was present in the culture media. In the plant tissues, a living medium, certain quantities of free amino acids including tryptophan are readily available for the synthesis of IAA by the invading pathogen. If the pathogen can hydrolyze the proteins in the host tissues (many fungi possess proteolytic enzymes), bound tryptophan may become readily available. Actually, *N. galligena* could produce IAA when the fungus was growing on a water suspension of powdered bark of yellow birch (Lortie, 1962). The increase of the unidentified inhibitor was also found, yet, the role of the inhibitor was not known. It may be correlated to the callus formation as reported by Katsumi (1962).

Summary

A quantitative comparison of the auxin content of ether extract from healthy bark and newly developed Nectria-affected canker portion as well as the culture filtrate of *Nectria pterospermi* Saw. has been carried out.

Partially purified extracts were accomplished by chromatography with different solvent systems. The auxin was identified by bioassay (avena straight growth test) and chemical methods. The auxin contents were found to be equivalent to 1.4 μg per Kg of cankered and 0.25 μg per Kg of healthy tissue. A nearly 6-fold increase of auxin was observed in diseased tissue. The Rf values of the auxin were found to be 0.35 [as developed with the solvent system of Isopropanol: ammonia (28%): water (8:1:1)] and 0.81 [as developed with 70% ethanol] which were identical to that of authentic IAA. An unidentified growth inhibitor was also found to be more in the diseased tissue in comparison with that of healthy tissue. Fifty days old culture of *N. pterospermi* produced 28 mg of IAA per liter of culture filtrate.

The main cause of abnormal growth of cambium was attributed to the increase of IAA level in diseased portion and so to cause the crack of the bark, viz. canker. The increase of IAA was suggested to be partly due to the pathogen.

Nectria pterospermi 感染及生長素之聚積

于 浩 吳龍溪 陳其昌

本實驗乃是對由 *Nectria pterospermi* 所引起的白桐癌腫病之新生癌腫組織，及相對部位之健全白桐組織，以及病原菌過濾液等之乙醚抽出物之生長素含量作定量比較分析。乙醚抽出物中之生長素可以濾紙色層分析法，配合燕麥胚軸生長生物檢定法測定。

病組織中，生長素含量約每公斤 1.4 微克，而健全組織中每公斤含 0.25 微克，約有六倍量之增加。此生長素之 Rf 值以 Isopropanol: ammonia (28%): Water (8:1:1) 展開溶媒展開時爲 0.35，以 70% 乙醇展開時爲 0.81，皆與 Indole acetic acid (IAA) 相吻合。另一種尚未確知爲何物之生長抑制物質在病組織中之含量亦高於健全組織。培養 *N. pterospermi* 五十日後之培養過濾液中含 IAA 每公升28毫克。

本病植物體形成層不正常之主要原因可歸之於 IAA 含量增高之故，因此致使樹皮龜裂。IAA 的增加來源至少局部來自病原菌。

Literature Cited

ASHCROFT, J. M. European canker of black walnut and other trees. West Virg. Agr. Expt. Sta. Bull. No. 261, 52 p. 1934.

BENTLEY, J. A. Analysis of plant hormones. Methods of biochemical analysis **9**: 75-125, 1962.

BERDUCOU, J. The role of β-indoleacetic acid in the formation of parasitic canker in plants. C. R. Acad. Sci. Paris. **235**: 309-311, 1952. (Abstr. in Rev. Appl. Mycol. **32**: 196, 1953).

BRAUN, A. C. Growth is affected. 1959. In J. G. Horsfall and A. E. Dimond. (ed.) Plant pathology **1**: 189-248, Academic Press, N. Y.

BRAUN, A. C. Tumor inception and development in the crown gall disease. Ann. Rev. Plant Physiol., **13**: 533-538, 1962.

CHEN, C.-C. Survey of epidemic diseases of forest trees in Taiwan I., Bull. Acad. Sinica **6**: 74-92, 1965.

DALY, J. M. and INMAN, R. E. Changes in auxin levels in safflower hypocotyls infected with *Puccinia carthami*. Phytopath. **48**: 91-97, 1958.

GAUTHERET, R. J. The nutrition of plant tissue culture. Ann. Rev. Plant Physiol. **6**: 433-484, 1955.

GORDON, S. A. and R. A. WEBER. Colorimetric estimation of indoleccetic acid. Plant Physiol. **26**: 192-195, 1951.

KATSUMI, M. The callus-forming effect of antiauxin. Intern. Christ. Univ. Publ. VA I: 10-13, Tokyo, 1962.

KUROSAWA, E. Experimental studies on the secretion of *Fusarium heterosporum* on rice plants. J. Nat. Hist. Soc., Formosa. **16**: 213-227, 1926.

LORTIE, M. The Nectria canker and its incitant. Ph. D. Thesis Univ. Wisconsin. 118 p. 1962. (Cited in M. Lortie. Pathogenesis in cankers caused by *Nectria galligena*. Phytopath. 54: 261-263, 1964).

NITSCH, J. P. and C. NITSCH. Studies on the growth of coleoptile and first internode sections. A new, sensitive, straight-growth test of auxins. Plant physiol. **31**: 94-111, 1956.

SEN, S. P. and LEOPOLD, A. C. Paper chromatography of plant growth regulators and allied compounds. Physiol. Plant. **7**: 98, 1954.

SEQUEIRA, L. Growth regulators in plant diseases. Ann. Rev. Phytopath. **1**: 5-30, 1963.

SEQUEIRA, L. and A. KELMAN. The accumulation of growth substances in plant infected by *Pseudomonas solanacearum*. Phytopath. **52**: 439-448, 1962.

STEELINK, C. Color reactions of certain phenols with Ehrlich's reagent. Nature **184**: 720, 1959.

TURIAN, G. and HAMILTON, R. H. Chemical detection of 3-indolyl acetic acid in *Ustilago zeae* tumors. Biochem. Biophys. Acta. **41**: 148-150, 1960.

VOGEL, A. I. Practical organic chemistry. (3rd ed.) p. 163-165, Longmens, England. 1962.

WENT, F. W. On growth-accelerating substances in the coleoptile of *Avena sativa*. Proc. Ken. Nederl. Akod. Wetensch., Amsterdam. **30**: 10-19, 1926. (Cited in L. J. Audus. Plant growth substances. Leonard Hill, London and Intersciences Publ. Inc. N. Y. 1963).

ZABEL, R. A. Summary with emphasis on trends and needs in research. (in a symposium of tree canker). Phytopath. **54**: 275-278, 1964.