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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

EFFECTS OF ABSCISIC ACID ON PHENOLIC CONTENT AND LIGNIN BIOSYNTHESIS IN TOBACCO TISSUE CULTURE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

HSIEN-CHI LI

Norman, Oklahoma

1969

EFFECTS OF ABSCISIC ACID ON PHENOLIC CONTENT AND LIGNIN BIOSYNTHESIS IN TOBACCO TISSUE CULTURE

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EFFECTS OF ABSCISIC ACID ON PHENOLIC CONTENT AND LIGNIN BIOSYNTHESIS IN TOBACCO TISSUE CULTURE

CHAPTER I

INTRODUCTION

Abscisic acid (ABA), formerly called abscisin II or dormin (3), was first identified by Addicott and his co-workers (2, 38) in extracts from young cotton fruits. The following year, Cornforth and his associates (14) succeeded in artificially synthesizing the hormone and describing its stereochemical structure. The chemical structure was determined as 3-methyl-5-(-1-hydroxy-4-oxo-2,6,6-trimethyl-2-cyclohexene-1-yl)-cis,trans-2,4-pentadienoic acid by Addicott et al. (4) and Ohkuma et al. (39). These investigators used spectropolarimetry to identify ABA in extracts of avocado, birch, cabbage, lemon, potato, and sycamore. Milborrow (35) extended this list, and recent reports indicate that ABA has been extracted and identified from 23 species of higher plants (2, 14, 16, 17, 25, 26, 35, 38, 42) (Table 1).

Not only is ABA a natural plant component and naturally occurring substance, but it is also effective in both promotive and inhibitory growth regulation, acting as a hormone when applied to an intact plant (35, 36, 40). The exogenous application of ABA has been

TABLE 1

PLANT SPECIES IN WHICH ABSCISIC ACID HAS BEEN IDENTIFIED

Species	Organ	References
Acer pseudoplatanus (sycamore)	bud, bark, leaves, twigs, wood, roots	(15, 35, 43)
Agropyron repens (couch grass)	leaves, rhizome	(35)
Betula pubescens (birch)	leaves	(16, 35)
Brassica oleracea (cabbage)	leaves of the heart	(16, 35)
Castanea sativa (sweet chestnut)	leaves	(35)
Citrus medica (lemon)	fruit	(16, 25, 35)
Cocos nucifera (coconut)	liquid endosperm	(35)
Fragaria ananassa (strawberry)	leaves	(24)
Fraxinus excelsior (ash)	leaves, buds	(35)
Dioscorea batatas (yam)	tubers	(26)
Gossypium hirsutum (cotton)	fruit	(2)
Lupinus luteus (yellow lupine)	fruit	(17, 44)

TABLE 1 - Continued

Species	Organ	References
Persea gratissima (avocado)	fruit, seed	(16, 35)
Phaseolus vulgaris (French bean)	shoot	(35)
Prunus persica (peach)	leaves	(32, 35)
Prunus sp. (plum)	twigs	(35)
Pteridium aquilinum (bracken)	rhizome	(35)
Pyrus malus (apple)	fruit, leaves	(42)
Rosa canina (wild rose)	leaves, stems, pseudocarp, achenes	(35)
Tilia platyphylla (linden)	fruit, seed	(35)
Solanum tuberosum (potato)	skin and pulp of tuber	(16, 35)
Zea mays (maize)	fruit	(35)

reported to promote abscission of leaves and fruits (1, 2, 6, 21, 33), induce seed dormancy (14, 20, 32, 51, 55), and El-Antably (21) indicated that ABA may possibly play a part in the regulation of bud dormancy. Sondheimer and Galson (50) reported inhibition of chlorophyll synthesis by ABA in excised embryos of ash seeds.

Addicott et al. (2) reported that ABA was inactive as an auxin in the growth of Avena coleoptiles; it reduced coleoptile growth to 60% of that induced by indole-3-acetic acid (IAA) alone. Later they found ABA inactive as a gibberellin in the growth of dwarf maize or dwarf peas (4). Chrispeels and Varner (12) found that gibberellic acid-induced synthesis of alpha-amylase and amino acid release in barley aleurone layers can be inhibited by ABA. It appears that ABA can counteract the stimulatory action of IAA and gibberellic acid, GA_3 (2).

Walton and Sondheimer (57) reported that ABA stimulates both development and retention of phenylalanine ammonia-lyase activity. Furthermore, El-Antably (21) and Evans (22) found that ABA inhibited flower induction in the long day species (Betula pubescens, Acer pseudoplatanus, Ribes nigrum, and Lolium temulentum), and it could promote flowering in the short-day plant (Pharbitis nil) under long-day conditions.

The knowledge of the physiological and biochemical effect of ABA on higher plants and their metabolism is not yet well known and is open to experimentation.

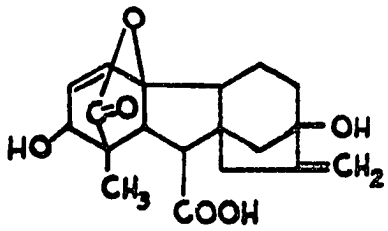
Numerous reports in the literature state that scopolin, scopoletin, chlorogenic acid and lignin are widely distributed in plants (5, 19, 30, 55, 58). Only a few quantitative studies of effects of growth-regulators on phenolic compounds have been reported.

Skoog and Montaldi (49), and Sargent and Skoog (46, 47) studied the auxin-kinetin interaction regulating scopolin and scopoletin

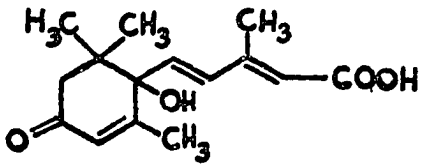
levels in tobacco tissue culture. They found that tobacco callus tissue releases a blue-fluorescent material into the medium, and identified the main part of the material as scopoletin. In healthy tissue the scopolin-scopoletin ratio was very high, about 13:1, and this high ratio was dependent upon the presence of kinetin. A high concentration of IAA, on the other hand, accelerated the degradation of scopolin to scopoletin.

Koblitz (28, 29) reported that kinetin and gibberellic acid induced lignin formation in tissue culture. Furthermore, Bergmann (7, 8) indicated that the levels of scopolin, scopoletin, chlorogenic acid, and lignin in tobacco callus tissue increased after the addition of kinetin.

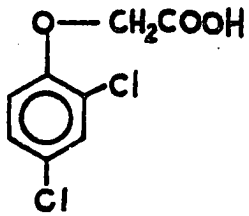
Tissue culture methods offer certain advantages for testing the effects of growth-regulators on plant metabolism. They allow more precise control over experimental conditions such as temperature, light, pH, time, and the chemical environment. They are usually more sensitive to changes in the chemical environment and allow accurate observation at the cellular level. Although some suggestions have been made as to the physiological and biochemical significance of ABA, no study has yet been made to determine whether this compound acts as a growth regulatory substance in tissue culture. Therefore, it seemed of interest to utilize the tissue culture technique to determine whether this compound would regulate the biosynthesis of lignin and other phenolic compounds. Structural formulas of pertinent growth substances and phenolic compounds are shown in Figures 1 and 2.



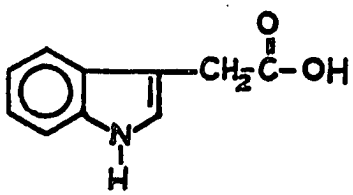
GIBBERELLIC ACID (GA_3)



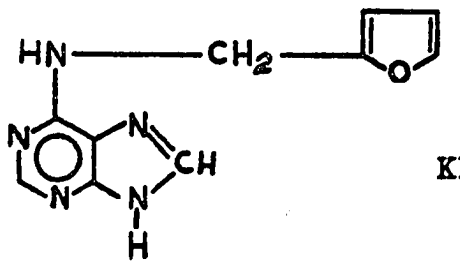
ABSCISIC ACID



2,4-DICHLOROPHENOXYACETIC ACID

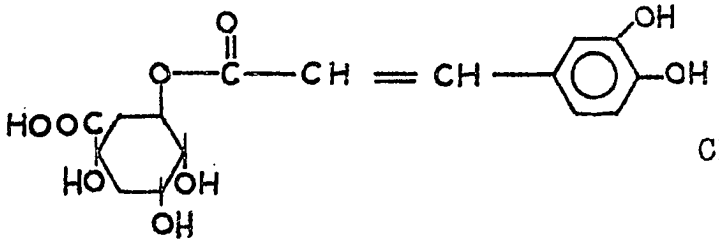


INDOLE-3-ACETIC ACID

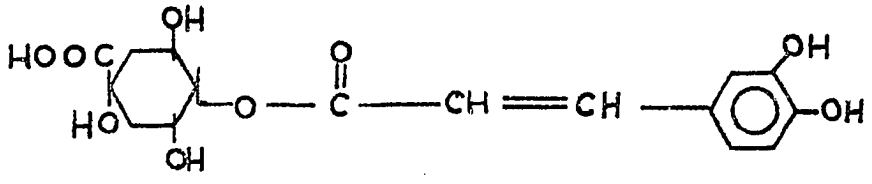


KINETIN

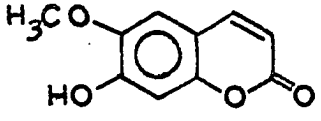
Fig. 1. Chemical structures of growth regulatory substances pertinent to this study.



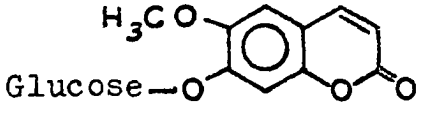
CHLOROGENIC ACID



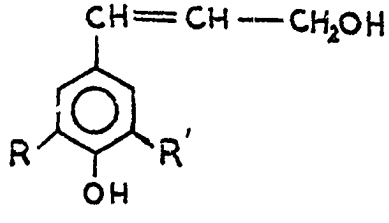
BAND 510



SCOPOLETIN



SCOPOLIN



LIGNIN MONOMERS

Fig. 2. Structural formulas of phenolic compounds pertinent to this study.

CHAPTER II

MATERIALS AND METHODS

Stock callus tissue from cultivated tobacco, Nicotiana tabaccum L., variety Wisconsin number 38, was obtained from Dr. Folke Skoog of the University of Wisconsin and this tissue was used throughout these studies. Abscisic acid (SD. 16108 code 1-6-0-0) was obtained from Shell Development Company, Modesto, California, through the courtesy of Dr. R. Blondeau and Dr. J. van Overbeek.

The stock tobacco callus tissues were subcultured on a basal medium of the following composition (mg/l): NH_4NO_3, 1650; KNO_3, 1900; H_3BO_3, 6.2; KH_2PO_4, 170.0; KI, 0.83; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CoCl_2 \cdot 6H_2O$, 0.025; $CaCl_2 \cdot 2H_2O$, 440; $MgSO_4 \cdot 4H_2O$, 22.3; $ZnSO_4 \cdot 7H_2O$, 8.6; $CuSO_4 \cdot 5H_2O$, 0.025; disodium-ferrous-ethylenediamine tetra-acetate, 37.3; $FeSO_4 \cdot 7H_2O$, 27.8; thiamine-HCl, 0.4; myoinositol, 100; sucrose, 30,000; IAA, 2.0; and kinetin, 0.2. The pH of the culture was adjusted with NaOH to initial values of 5.8-6.0 (before adding 1% of bacto-agar). The medium was placed in 125 ml Erlenmeyer flasks, 50 ml/flask, after which the flasks were stoppered with cotton plugs and autoclaved at 15 lbs steam pressure for 15 minutes.

Transplants were made by placing three pieces of the stock

callus of approximately equal size into each flask. The culture flasks were maintained 3-4 weeks in a continuous weak light at constant temperature (26° C).

In experimental work, pieces of stock tissue 4-5 mm in diameter and weighing approximately 30-40 mg were transplanted into a basal medium supplemented with a series of different concentrations of ABA and gibberellic acid (GA_3), separately, or in combinations of ABA + GA_3. Four separate cultures were run at each concentration of each growth regulator. The culture flasks were kept in weak, diffused light at 26° C until harvested five weeks later. At that time weights were determined, and tissues were extracted according to a procedure devised by Koeppe (30). These extraction procedures used for isolating lignin, scopolin, scopoletin and chlorogenic acid from tobacco callus tissue are summarized in the chart shown in Figure 3.

Tissues were boiled in a minimal amount of isopropyl azeotrope (88% isopropanol in water) for 5 minutes in order to inactivate all enzymes. The tissue was then ground in a blender for two minutes, and transferred to a Soxhlet extraction thimble. Next, the tissue was washed with approximately four times (ml) its fresh weight (g) with boiling isopropanol-water (1:1, v/v), five times its fresh weight with boiling IBMW (isopropanol-benzene-methanol-water; 2:1:1:1, v/v/v/v), and four times its fresh weight with boiling isopropyl azeotrope. The tissue remaining in the thimble was extracted for 24 hours in a Soxhlet extractor with isopropyl azeotrope, then for a second 24 hours with isopropanol.

All washings and extracts were combined and evaporated to

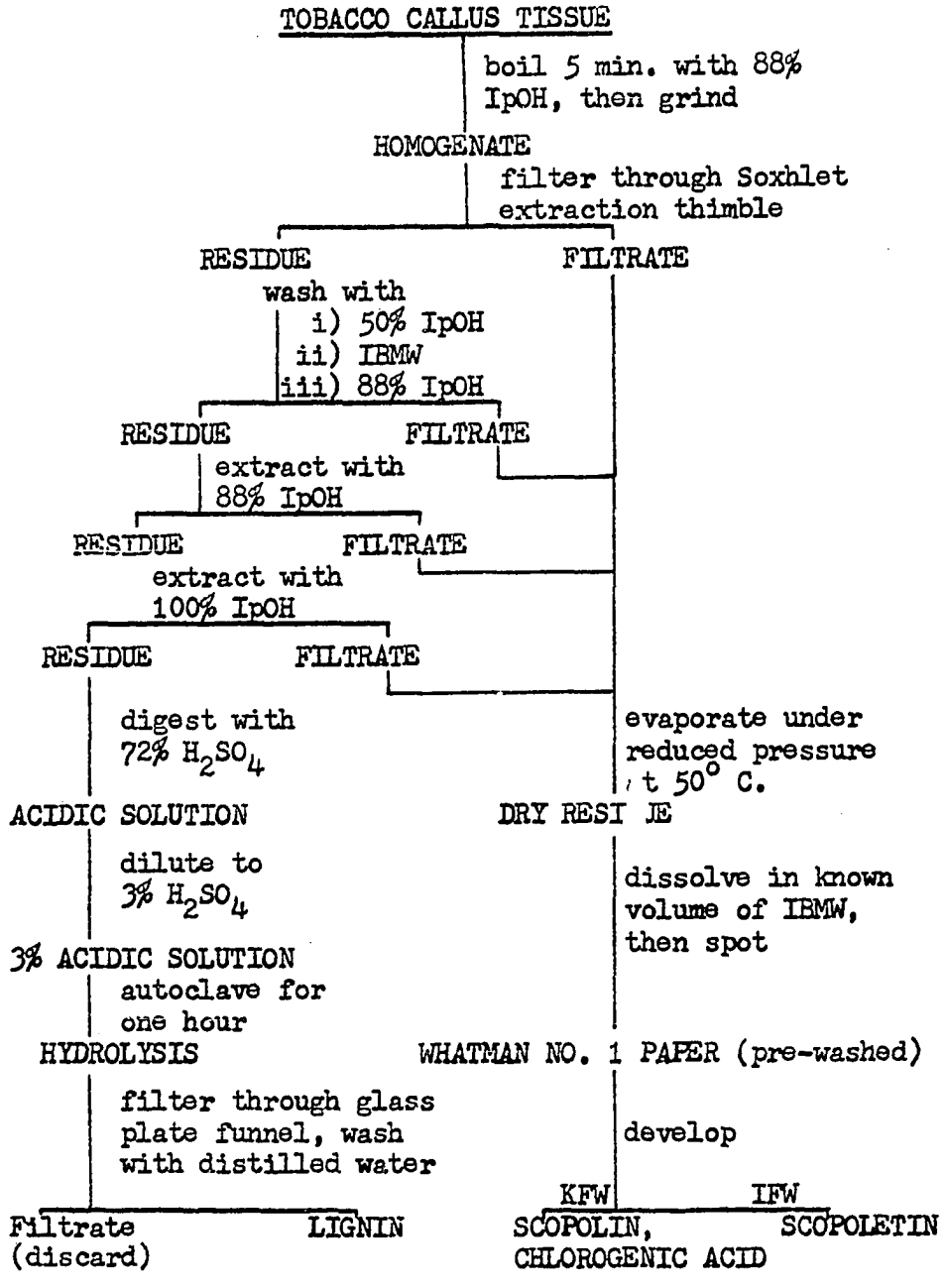


Fig. 3. Extraction procedure for isolating lignin and phenolic compounds from tobacco callus. IpOH = isopropanol, IBMW = isopropanol-benzene-methanol-water, IFW = isopropanol-formic acid-water, and KFW = methyl isobutyl ketone-formic acid-water. See text for ratios of solvents in various solvent systems.

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were read at a wavelength of 324 mμ on a Beckman DB-G spectrophotometer. A standard reference curve (Fig. 4) for chlorogenic acid was prepared using known quantities of synthetic compounds which were carried through similar chromatographic steps.

B 510 has an absorption spectrum and extinction coefficient identical to chlorogenic acid (5, 30), so the standard curve of chlorogenic acid was also used for the quantitation of Band 510.

Scopolin and scopoletin were quantitated with a Model 110 Turner fluorometer using pyrex cuvettes and a high sensitivity attachment at an instrument setting of 1x. A primary filter # 7-60, and a secondary filter # 2A plus # 48 (Kodak Wratten filter) were utilized (58). Standard reference curves for scopolin (Fig. 5) and scopoletin (Fig. 6) were prepared by the same method as described for chlorogenic acid.

The white residue of tissue remaining in the Soxhlet thimbles after extraction was dried in an oven at 60° C for 24 hours. The thimbles were cooled in a desiccator and weighed. Ten ml aliquots of the sample extract were also oven dried. The weight of the dry matter per ml in the aliquot multiplied by the volume of the sample (ml) was added to the weight of the white residue to obtain the total dry weight of the sample.

An aliquot of the dried, isopropanol extracted tissues, was transferred to a 125 ml Erlenmeyer flask. Then 20 ml of 72% sulfuric acid solution were added to the flask with constant shaking at room temperature for 24 hours.

During the digestion period, the white powder of the tissue

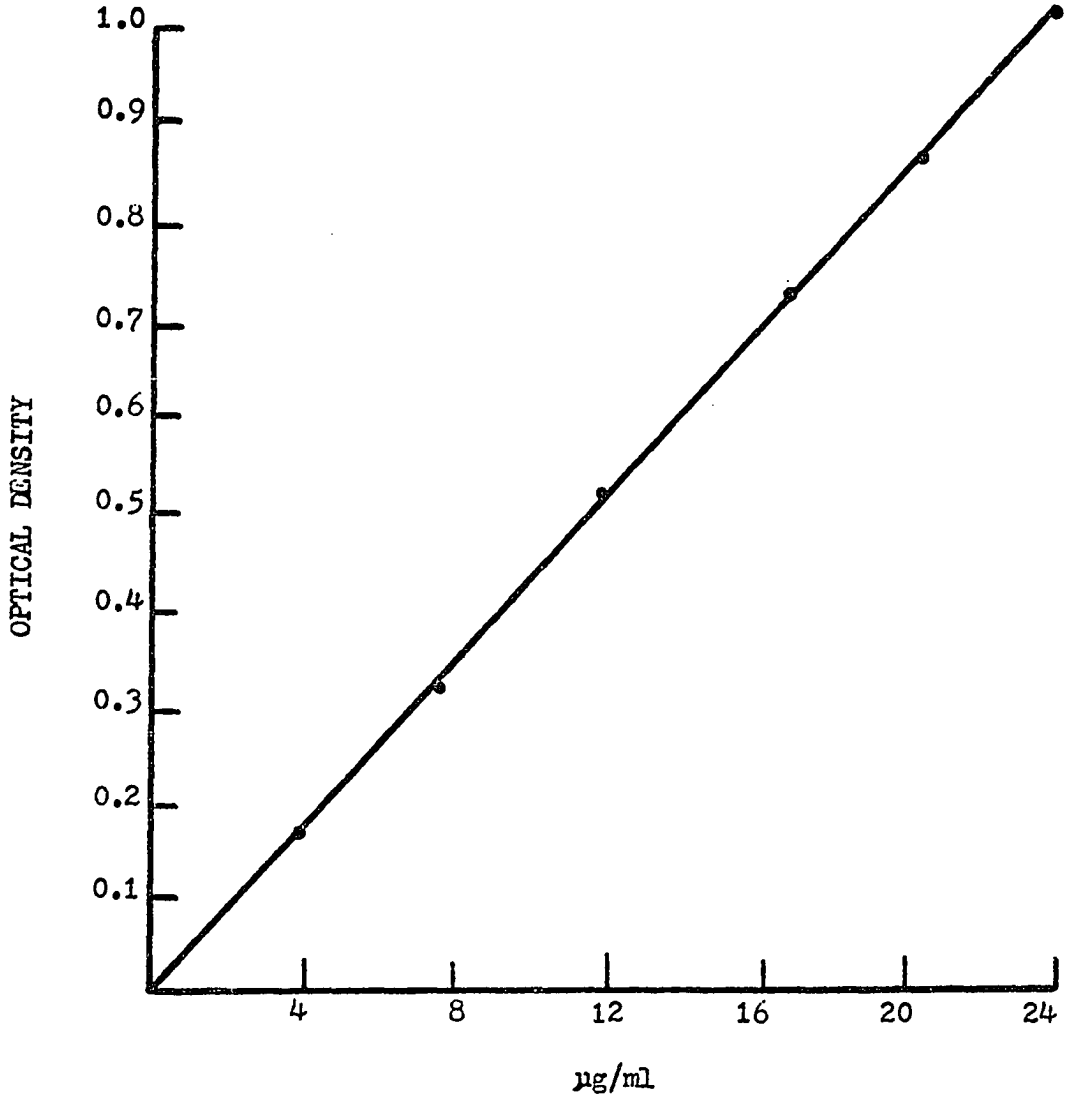


Fig. 4. Relationship of optical density to chlorogenic acid concentration.

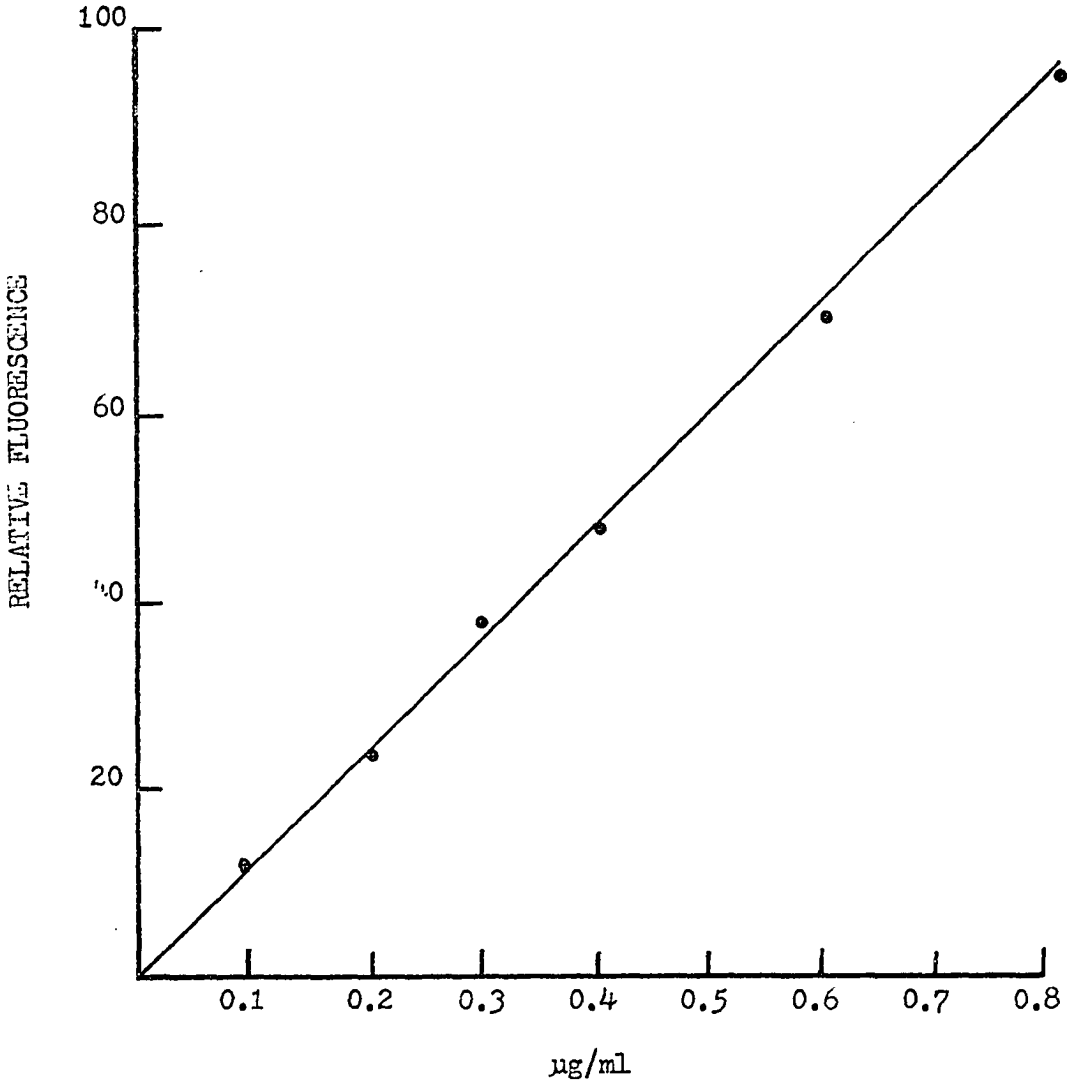


Fig. 5. Relationship of per cent fluorescence to scopolin concentration on the Model 110 Turner fluorometer.

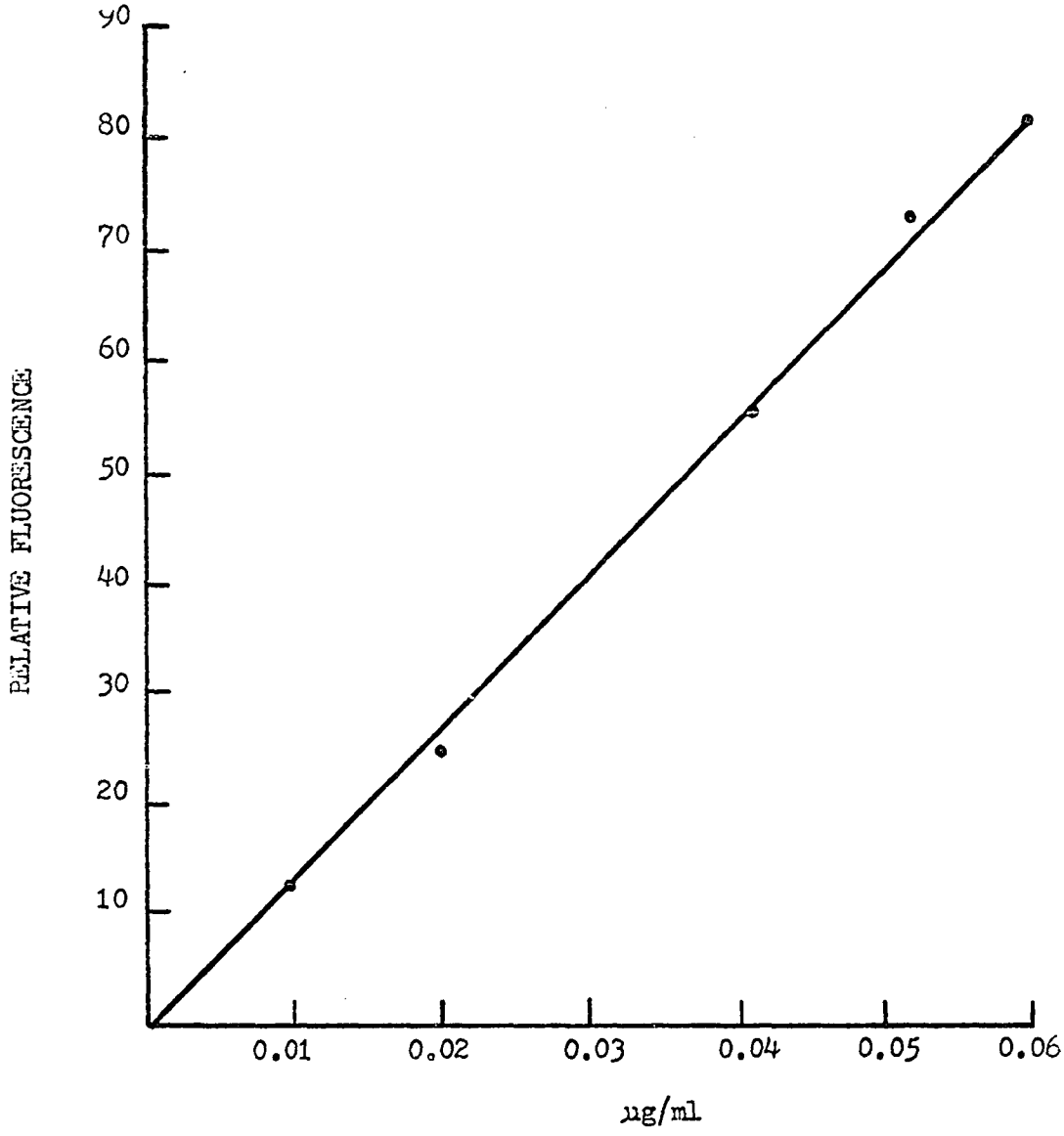


Fig. 6. Relationship of relative fluorescence and scopoletin concentration on the Model 110 Turner fluorometer.

changed from light brownish to black in color. After 24 hours, the mixture was transferred into a one-liter flask and diluted to a 3% concentration of acid with distilled water. Each flask was covered with a 250 ml beaker. The mixture was autoclaved for one hour. After overnight cooling, the hydrolyzed material was filtered through a pre-weighed glass plate funnel and washed with distilled water until free of acid. The resulting isolated Klason lignin was then weighed.

CHAPTER III

EXPERIMENTAL RESULTS

Effects of Abscisic Acid on Callus Growth, Lignin, Chlorogenic Acid, Scopolin, and Scopoletin Content

Tobacco callus cultures were treated with ABA at five different levels, with four replicate flasks at each treatment level. Concentrations of ABA of 0.1, 1.0, 2.0, and 5.0 mg/l significantly decreased callus growth, but the 0.01 mg/l concentration did not. The dry weight decrease closely paralleled that of fresh weight (Fig. 7).

The ABA caused an inhibition of proliferation accompanied by a decrease in total chlorogenic acids and lignin content (Fig. 8).

The amounts of scopolin and scopoletin in the callus tissue varied greatly depending on the concentration of ABA in the medium (Table 2, Fig. 9). The 0.01 mg/l concentration had no significant effect on the scopolin content, but it significantly reduced the scopoletin concentration. An increase in ABA to 0.1 mg/l resulted in a significant decrease in scopolin content with a concomitant increase in scopoletin content. In contrast, a 1.0 mg/l concentration of ABA resulted in a reversal of the effects produced by the 0.1

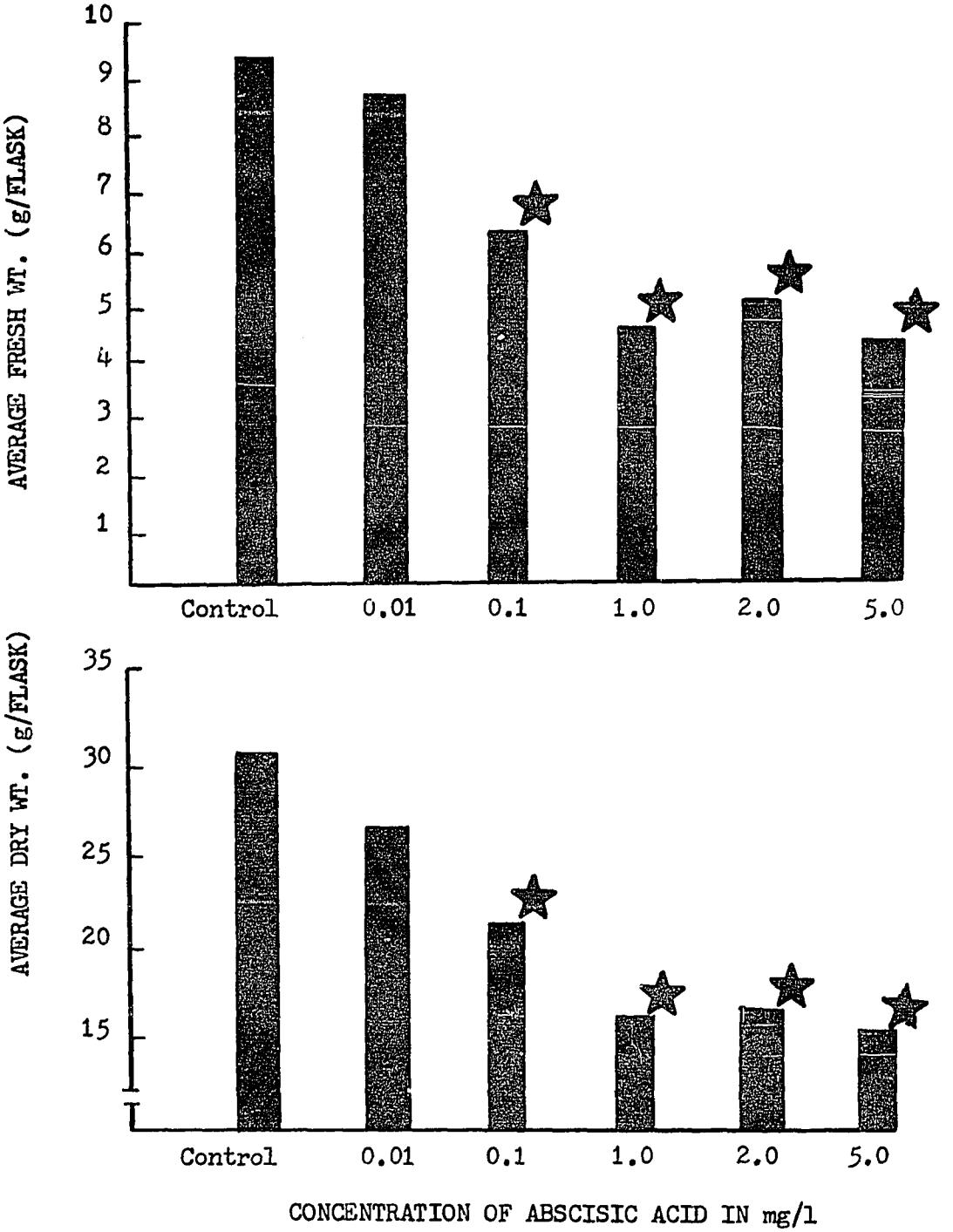


Fig. 7. Effects of ABA on fresh weight and dry weight. Each bar represents mean of four replicates. Star = difference from control significant at 5% level or better.

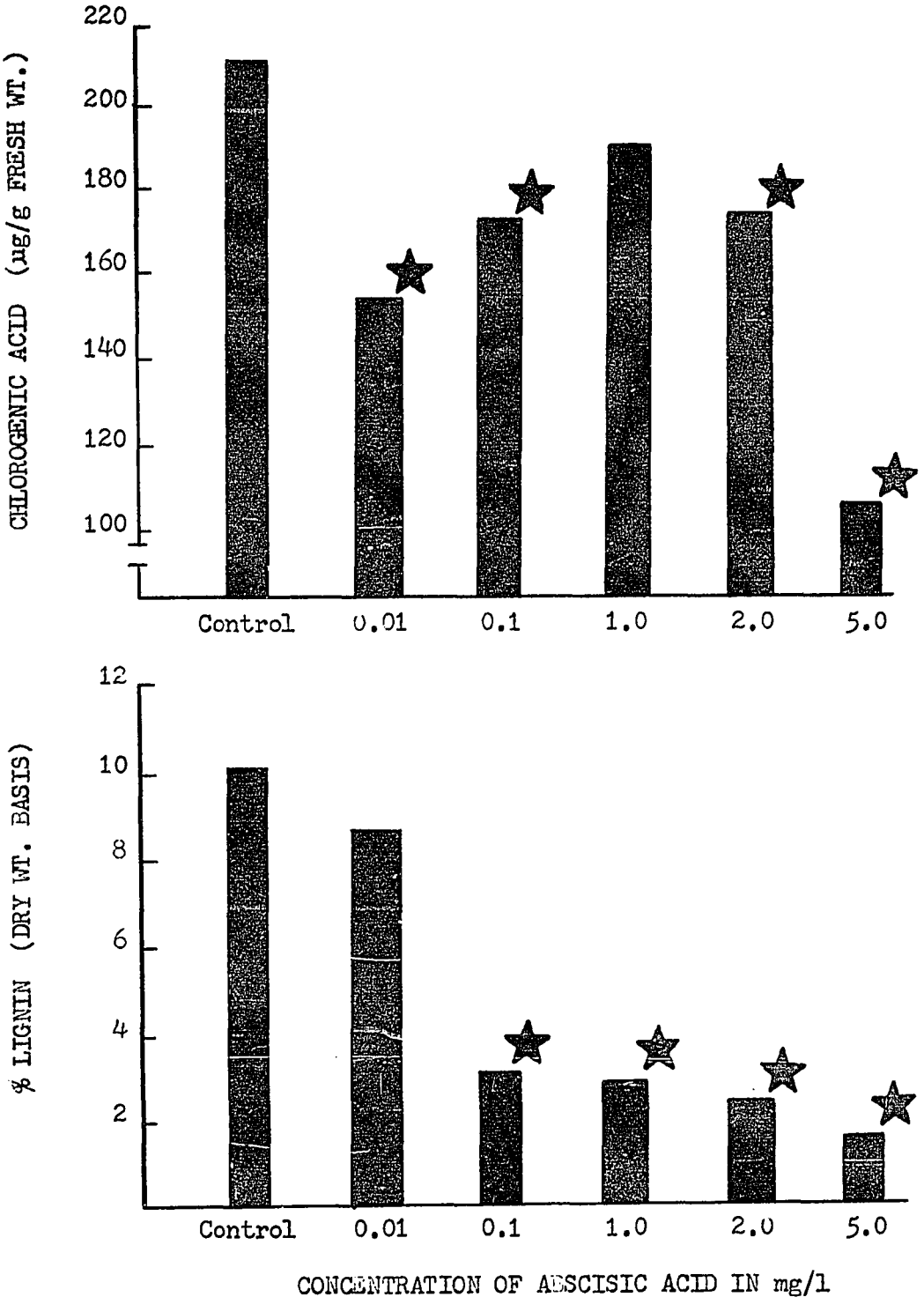


Fig. 8. Effects of ABA on total chlorogenic acids and lignin content. See Fig. 7 for symbols.

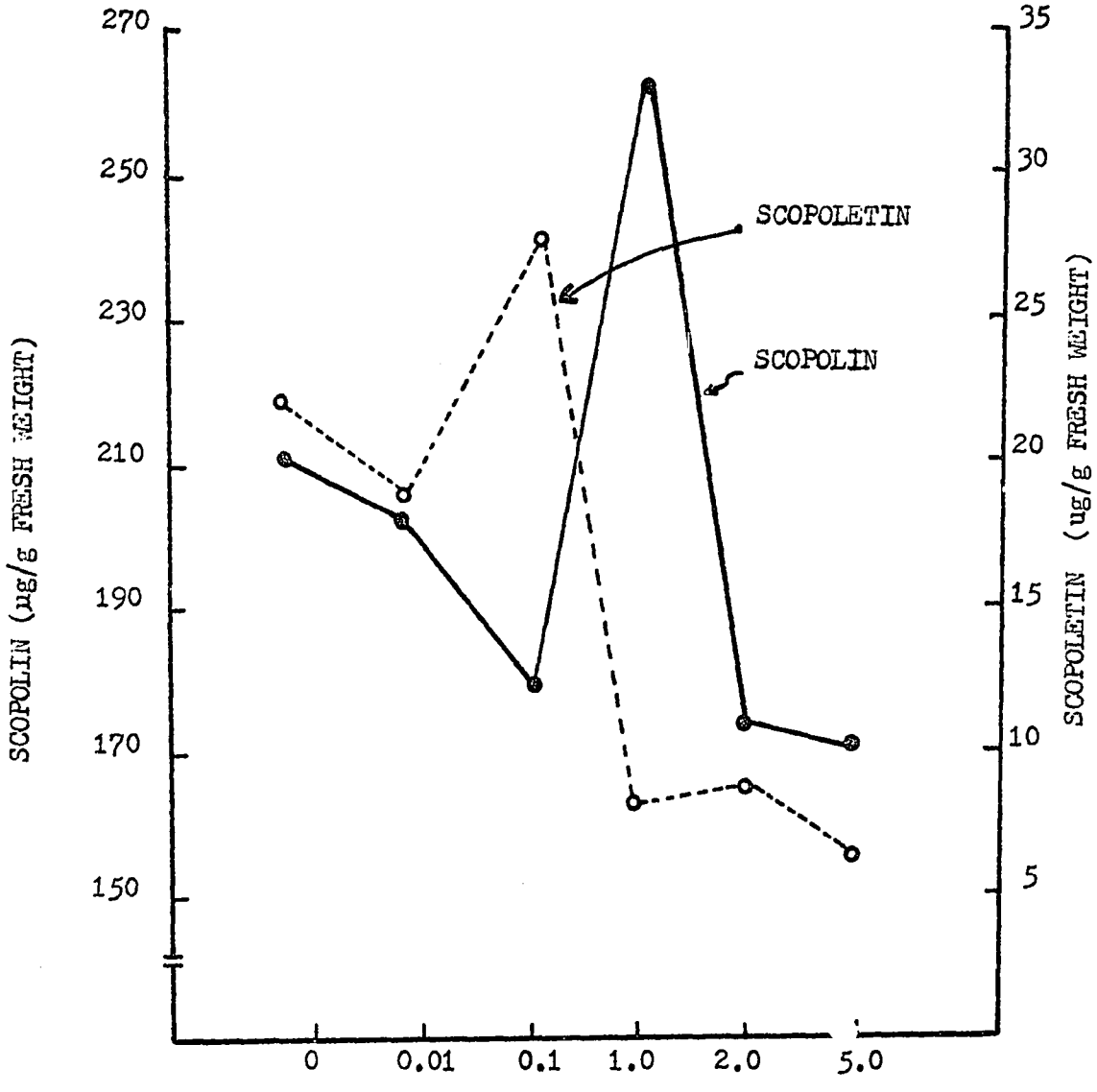


Fig. 9. Effects of ABA on scopolin and scopoletin content of tobacco tissue callus.

mg/l concentration. Further increases in ABA (2.0 and 5.0 mg/l) caused both the scopolin and scopoletin contents to decrease significantly below those in the control.

TABLE 2

EFFECTS OF ABA ON SCOPOLIN AND SCOPOLETIN CONCENTRATION. EACH FIGURE REPRESENTS MEAN OF FOUR REPLICATES WITH STANDARD ERROR

Treatment, mg/l	Scopolin, µg/g fresh wt.	Scopoletin, µg/g fresh wt.
Control	215.3 ± 1.44	23.77 ± 1.82
0.01	206.6 ± 4.7	19.63 ± 2.41*
0.1	199.6 ± 3.49*	29.33 ± 2.93*
1.0	259.9 ± 1.97*	10.55 ± 2.39*
2.0	177.0 ± 2.11*	11.63 ± 3.92*
5.0	170.0 ± 1.88*	7.69 ± 1.96*

*Difference from control significant at 5% level or better.

Effects of Gibberellic Acid on Growth, Lignin, Chlorogenic Acid, Scopolin and Scopoletin Content of Tobacco Callus

GA_3 had a pronounced stimulatory effect on growth of tobacco callus with a 0.1 mg/l concentration increasing the total fresh weight to about 3 times that of the control (Fig. 10). However, the higher concentration of GA_3 (10.0 mg/l) caused an inhibition of callus growth.

The low concentration of GA_3 caused a decrease in amount of total chlorogenic acids, but the higher concentration of GA_3 increased the pool of chlorogenic acids present (Fig. 11). A slightly greater

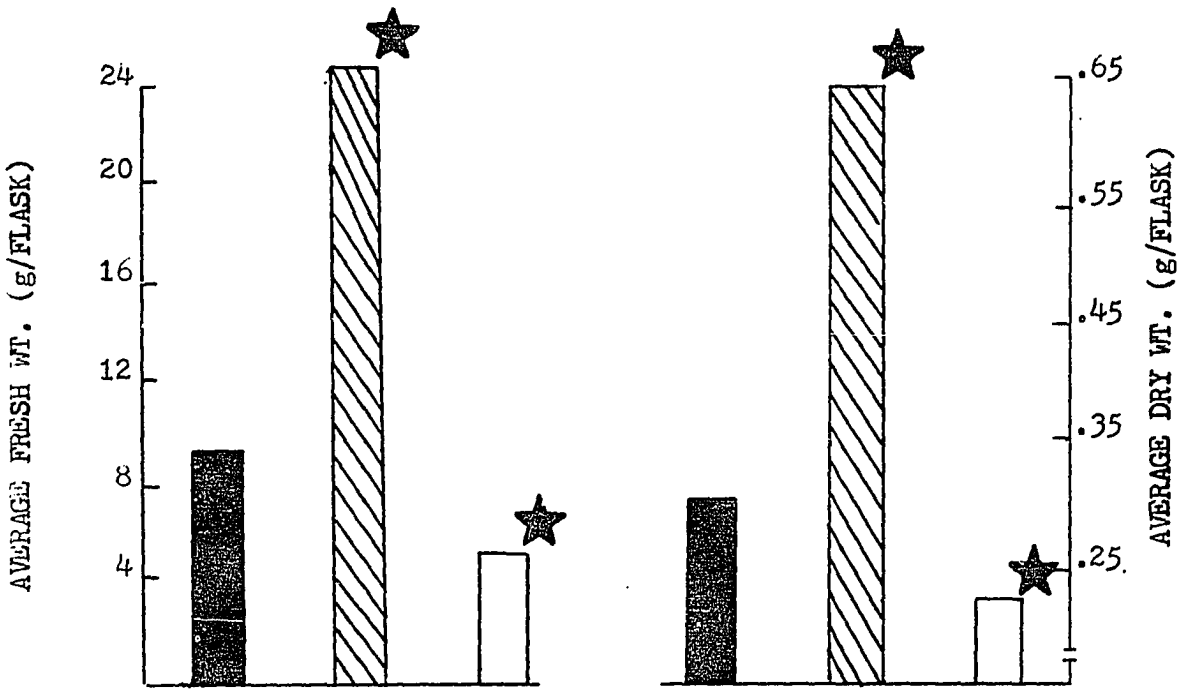


Fig. 10. Effects of GA_3 treatment on fresh weight and dry weight of tobacco callus. Each bar represents mean of 4 replicates. Solid bar = control containing basal medium only, slashed bar = basal medium + 0.1 mg/l GA_3, open bar = basal medium + 10.0 mg/l GA_3. Star = significant difference from control at 5% level or better.

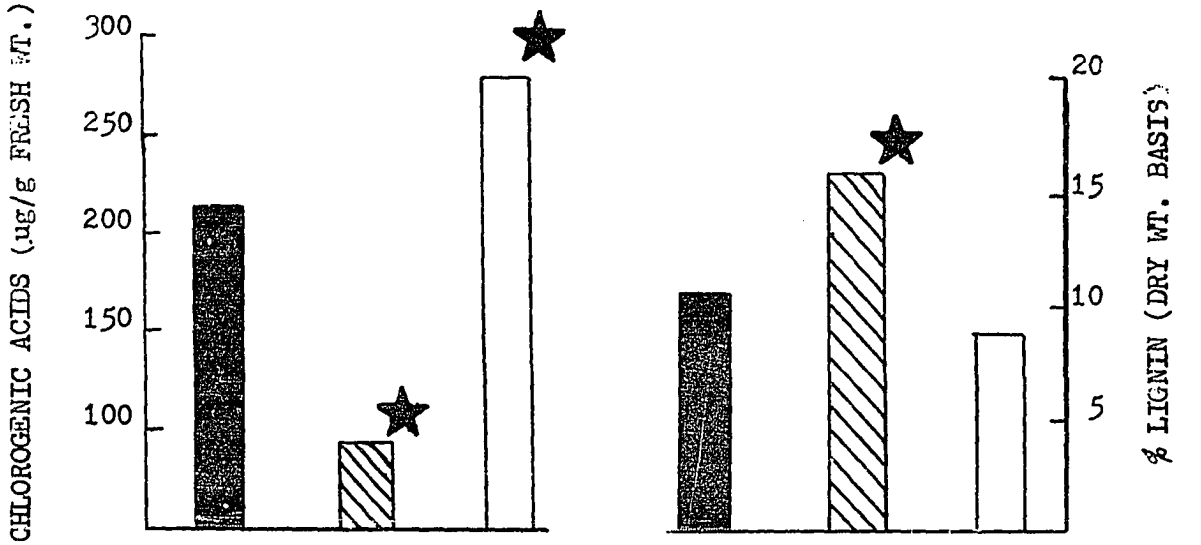


Fig. 11. Effects of GA_3 treatment on total chlorogenic acids and lignin content. Each bar represents mean of 4 replicates. Symbols as in Fig. 10.

content of lignin was found in tobacco callus after treatment with the low concentration of GA_3, but the higher GA_3 treatment caused no statistically significant change in lignin when compared with the controls (Fig. 11). There was a slight indication of decreased lignin synthesis with the heavier treatment.

The low level of GA_3 caused a significant reduction in scopolin compared with the amount in control tissue, whereas the higher GA_3 concentration caused a significant increase in scopolin (Fig. 12). Scopoletin production was increased significantly by both low and high GA_3 concentrations (Fig. 12).

Effects of GA_3 on ABA-Induced Inhibition of Callus Growth, Chlorogenic Acid and Lignin Content

As stated above, the lower concentration of GA_3 added to the basal medium enhanced the growth of tobacco callus. The inhibitory effects on growth of 0.1 and 1.0 mg/l concentrations of ABA were overcome by 0.1 mg/l of GA_3, but the inhibition resulting from 2.0 mg/l of ABA was not reversed by the low concentration of GA_3 (Fig. 13). The 0.01 mg/l concentration of ABA did not significantly affect growth of the callus (Fig. 7), and the low concentration of GA_3 did not change that result (Fig. 13). The inhibition of callus growth by all concentrations of ABA employed was overcome by addition of the higher concentration of GA_3.

Reversal of the inhibition of chlorogenic acid and lignin synthesis was attempted by the addition of GA_3 at two different levels (0.1 and 10.0 mg/l). GA_3 at a concentration of 0.1 mg/l

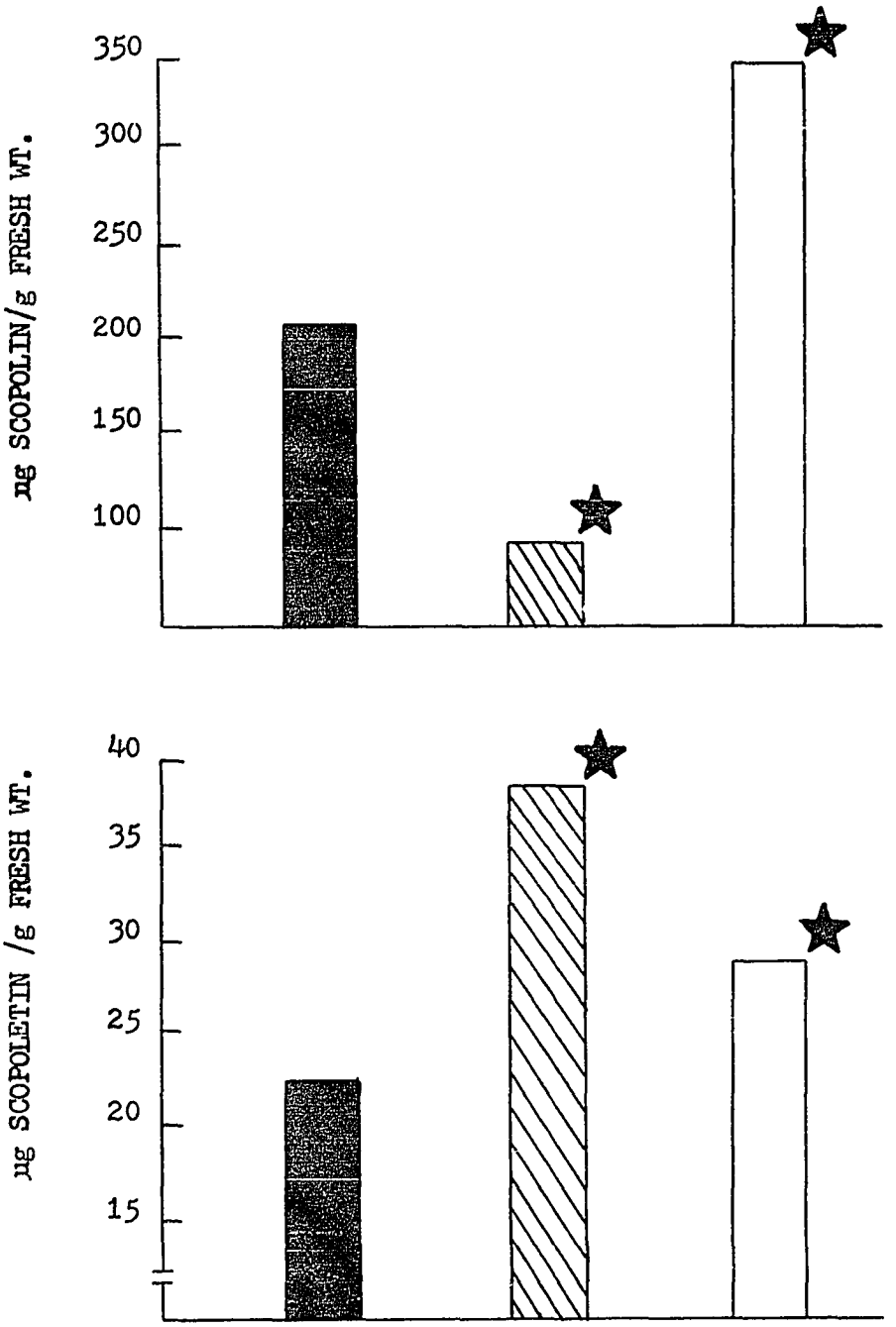


Fig. 12. Effects of GA_3 treatment on scopolin and scopoletin content of tobacco callus. Each bar represents mean of four replicates. Symbols as in Fig. 10.

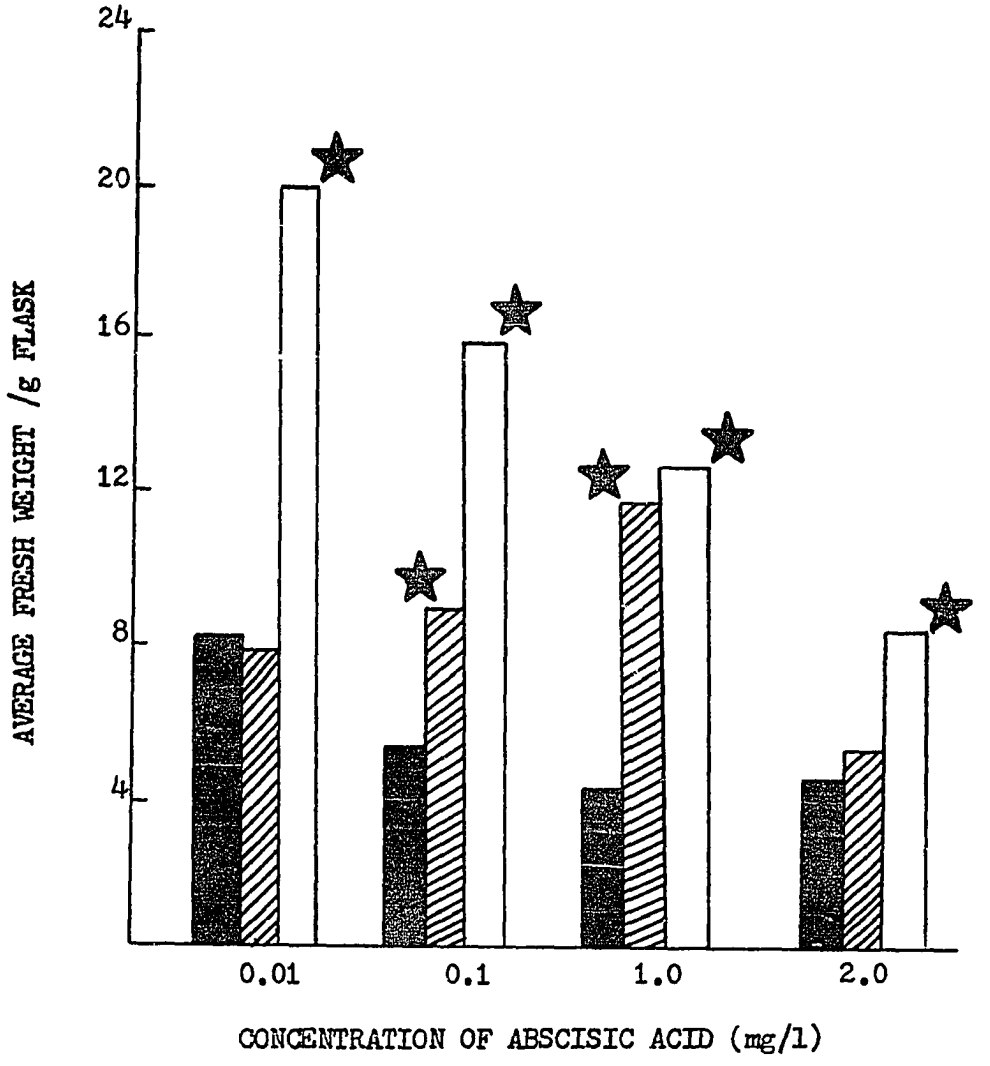


Fig. 13. Comparative growth effects of ABA-GA_3 interaction in tobacco callus tissue. Each bar represents mean of 4 replicates. Solid bar = control containing basal medium + indicated amount of ABA, slashed bar = basal medium + 0.1 mg/l GA_3 + indicated amount of ABA, open bar = basal medium + 10.0 mg/l GA_3 + indicated amount of ABA, solid star = ABA-induced inhibition was significantly reversed by addition of GA_3 at 5% level or better.

completely reversed the inhibition of chlorogenic acid synthesis resulting from the 0.01 mg/l concentration of ABA (Fig. 14). However, GA_3 was not effective in reversing the ABA inhibition of chlorogenic acid synthesis in any other test (Fig. 14). In fact, the combinations of 10.0 mg/l GA_3 and ABA in concentrations of 0.1 to 2.0 mg/l reduced the amount of chlorogenic acids more than the different concentrations of ABA alone.

It was previously stated that there was a reduction of lignin content in ABA-treated callus (Fig. 8). In contrast to this, the lower concentration of GA_3 (0.1 mg/l) significantly increased the lignification of tobacco callus (Fig. 11). It appeared logical, therefore, that GA_3 might reverse the inhibitory effects of ABA on lignin synthesis. This was found to be true because both the low and high concentrations of GA_3 were effective in overcoming ABA inhibition of lignification at all concentrations of ABA tested (Fig. 15). It should be recalled that the 0.01 mg/l concentration of ABA did not affect lignin synthesis significantly (Fig. 8).

Effects of GA_3 on Abscisic Acid-Induced Promotion and Inhibition of Scopolin and Scopoletin Content

In a previous section, it was stated that tobacco callus treated with increasing levels of ABA exhibited an inhibition-promotion of scopolin and scopoletin content. Tissues treated with high concentrations of GA_3 resulted in increases in scopolin and scopoletin accumulation (Fig. 12). The lower concentration of GA_3 caused a dramatic decrease in the scopolin content but an increase in

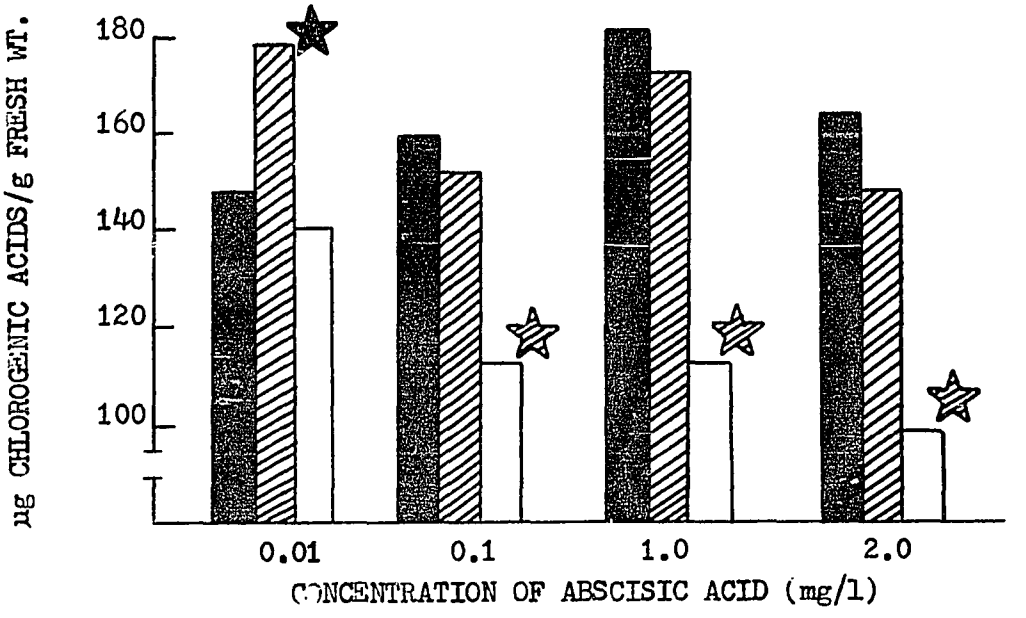


Fig. 14. Effects of ABA-GA_3 interaction on chlorogenic acids content. See Fig. 13 for symbols. Slashed star = ABA-induced inhibition was not reversed by addition of GA_3 but amount of chlorogenic acids was significantly different from appropriate control at 5% level or better.

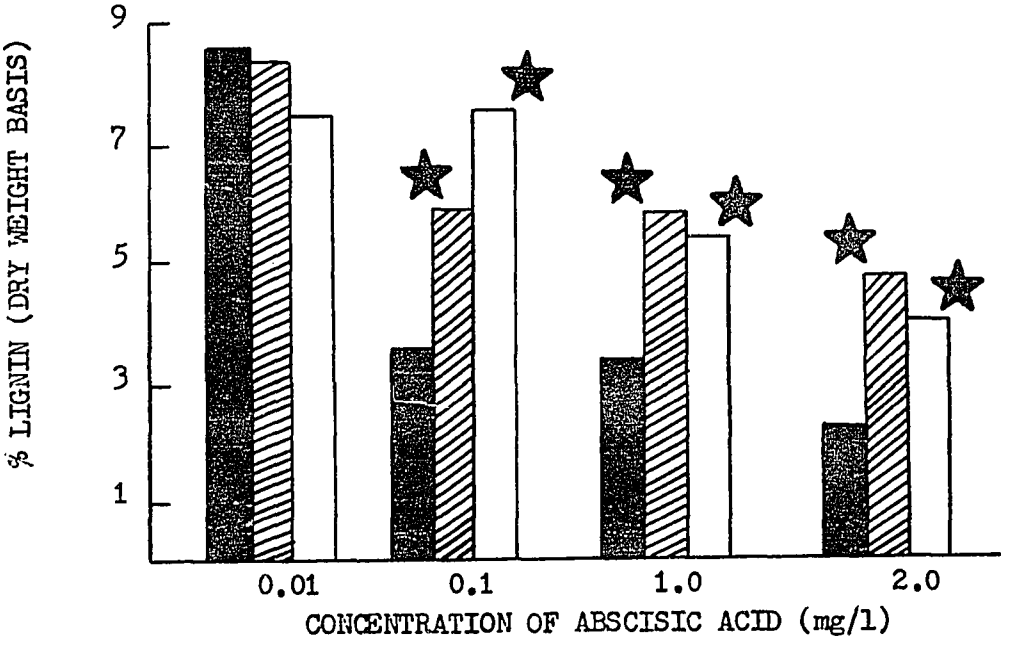


Fig. 15. Effects of ABA-GA_3 interaction on lignin content. Each bar represents mean of 4 replicates. See Fig. 13 for symbols.

scopoletin. Neither of the GA_3 treatments was able to overcome the ABA inhibition of scopolin production (Fig. 16). In fact, ABA + GA_3 were more inhibitory than ABA alone, and GA_3 completely reversed the promotion of scopolin production resulting from 0.1 mg/l of ABA (Fig. 9, 16). GA_3 at 0.1 and 10.0 mg/l were able to reverse the inhibition of scopoletin production by ABA at 1.0 and 0.01 mg/l respectively (Fig. 17). Both concentrations of GA_3 reversed the stimulatory effect of the 0.1 mg/l concentration of ABA on scopoletin production.

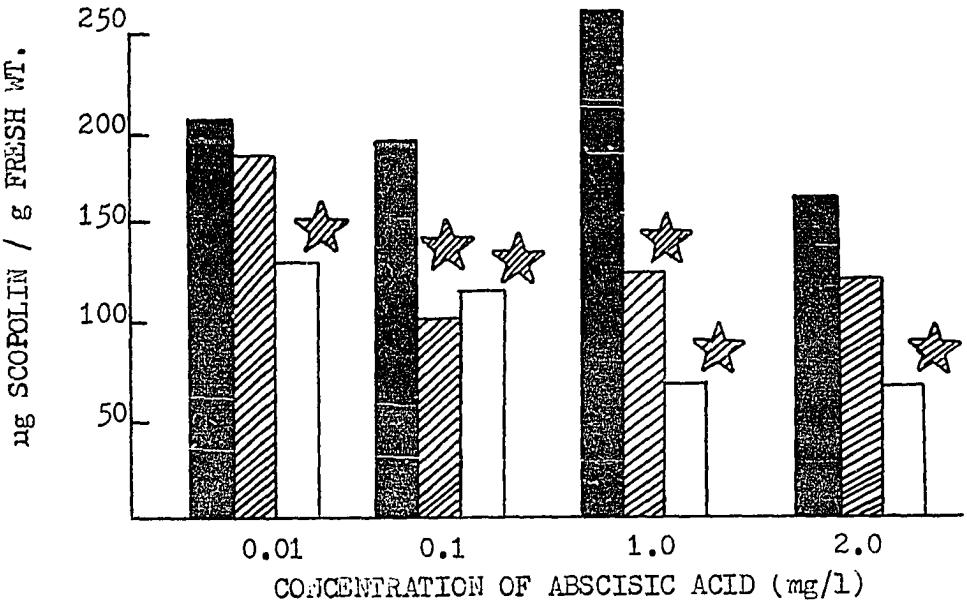


Fig. 16. Effects of ABA-GA_3 interaction on scopolin content. Each bar represents mean of 4 replicates. Symbols as Fig. 13. Slashed star = ABA-induced inhibition was not reversed by addition of GA_3 but amount was significantly different from appropriate control at 5% level.

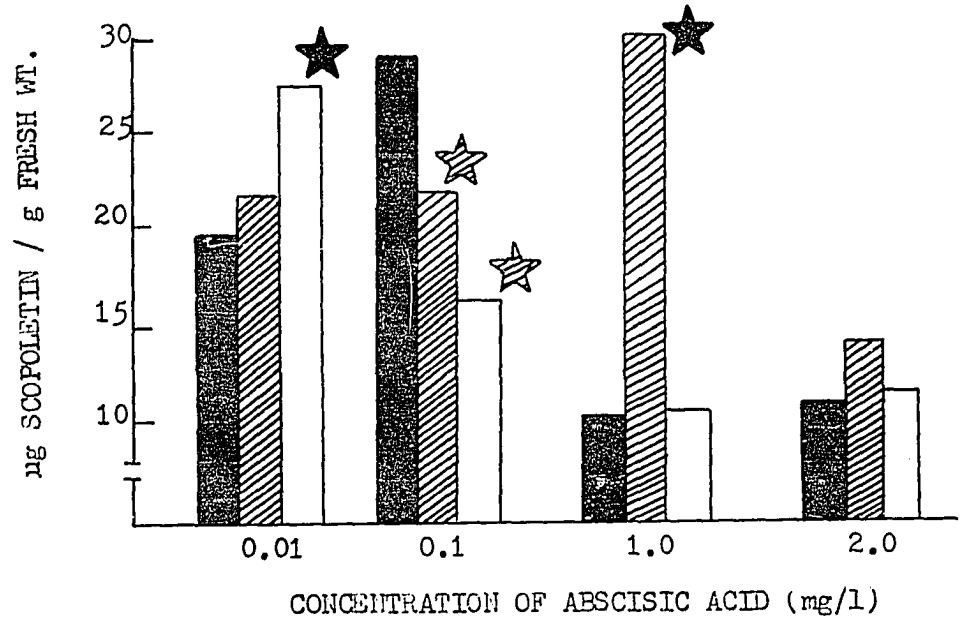


Fig. 17. Effects of ABA-GA_3 interaction on scopoletin content. Each bar represents mean of 4 replicates.
Symbols as in Fig. 13, 14.

CHAPTER IV

DISCUSSION

The present results provide additional knowledge of some of the physiological properties of ABA, particularly of its inhibitory effects in tissue culture on growth, lignin content, and phenolic content.

These experiments have shown that there is a relation between growth and lignin production. ABA decreased the growth rate and the amount of lignin produced. On the other hand, low concentrations of GA_3 intensified growth and increased the elaboration of lignin, whereas high concentrations of GA_3 had the reverse effect.

A postulated metabolic pathway of conversion of phenylalanine to chlorogenic acid, scopolin, scopoletin, and lignin is presented in Figure 18 (10, 23, 53, 54, 55). It appears that many enzymes are involved in the biosynthesis of lignin and phenolic compounds. Koukol and Conn (31) identified the enzyme phenylalanine ammonia-lyase, which converts phenylalanine to cinnamic acid, and this enzyme may be able to regulate lignin synthesis (52, 60).

Steck (53, 54), using $^{14}CO_2$, found that ferulic acid could be incorporated into fiber components through a feruloylglucose intermediate or into scopolin through an ester of ferulic acid

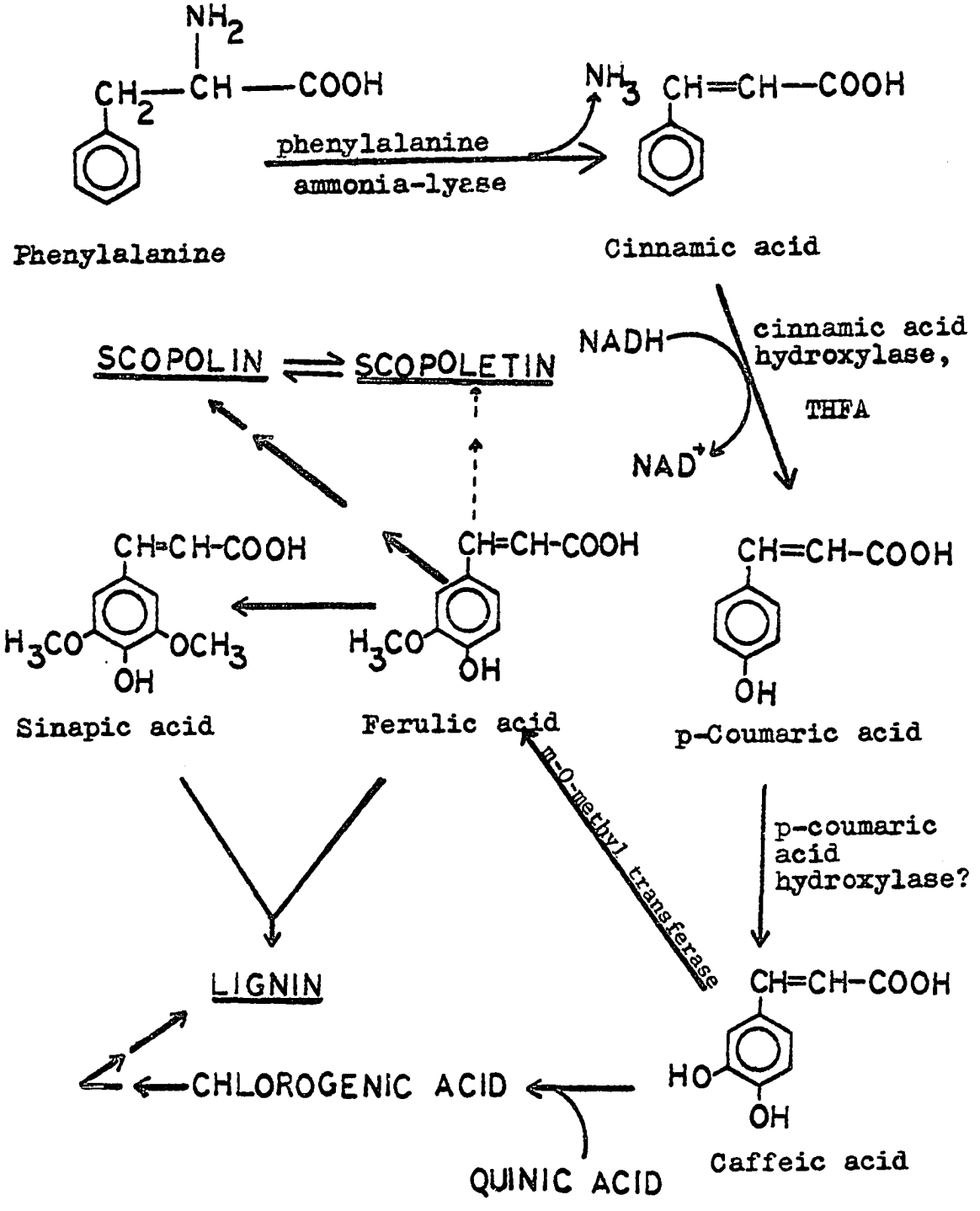


Fig. 18. A proposed metabolic conversion of phenylalanine to lignin and other phenolic compounds.

glucoside, in tobacco. Gamborg (23) proposed a side branch from caffeic acid through ferulic acid in the formation of lignin. Recently, Brown (10) confirmed the enzymes which are involved in lignin synthesis.

The promotion of phenylalanine ammonia-lyase activity by ABA was reported by Walton and Sondheimer (57), and this enzyme was implicated as the one controlling agent in lignification (11, 59). It follows from this that an increase in phenylalanine ammonia-lyase activity could lead to an increase in the synthesis of lignin and phenolic compounds. However, the present experiments suggest a significant decrease in chlorogenic acid and lignin content due to the effect of ABA in tissue culture. Van Overbeek (40) stated that inhibition caused by ABA is the result of an allosteric inhibition of an enzyme. This statement and the results of present data indicate that the activity of enzymes which are involved in the biosynthesis of lignin could be partially blocked somewhere between caffeic acid and chlorogenic acid or between ferulic acid and lignin by ABA. According to the proposed scheme, the decrease in total chlorogenic acids may have caused the decrease in lignification.

Dutta and McIlrath (19) found that peroxidase activity of boron-deficient tissue was consistently and significantly lower than that of tissues receiving boron in the nutrient medium and they proposed that the lower peroxidase activity may account in part for the lower degree of lignification of boron-deficient plants. Later, Stafford (52) reported that ferulic acid induced lignin accumulation in Phleum pratense and Sorghum vulgare. She also pointed out that caffeic

acid can serve as an inhibitor of peroxidase. Therefore, the decreases in chlorogenic acid and lignin in tobacco callus may be, at least in part, due to the accumulation of caffeic acid. Although there is no direct evidence of accumulation of caffeic acid and ferulic acid in the present data, the data do indicate that scopolin and scopoletin increase due to the effects of certain concentrations of ABA. These increases in scopolin and scopoletin might be due to accumulations of caffeic acid and ferulic acid.

Another interesting aspect of the quantitative studies on the tobacco callus was the variation in accumulation of scopolin and scopoletin depending on the ABA concentration in the medium. Scopolin increased in amount whereas scopoletin decreased in amount in an ABA concentration of 1.0 mg/l. It is possible that this concentration of ABA inhibited the conversion of scopolin to scopoletin (Fig. 9, 18), resulting in the scopolin accumulation. However, with an ABA concentration of 0.1 mg/l, the scopolin content was decreased and there was an increased scopoletin accumulation. It may be that the ABA was not concentrated sufficiently at this level to inhibit the conversion of scopolin to scopoletin.

The present data show that callus tissue treated with a low concentration of GA_3 resulted in an increase in callus growth, lignin, and scopoletin content. Tissues treated with a high concentration of GA_3 showed an increase in chlorogenic acid, scopolin, and scopoletin content. Morel (37) reported that Jerusalem artichoke parenchyma cultivated in a medium containing naphthalene-acetic acid plus gibberellin produced a high amount of lignin. Cheng and Marsh (11) found that GA_3

enhanced lignification and increased phenylalanine ammonia-lyase activity in dwarf pea. Therefore, the GA_3-induced formation of lignin and phenolic compounds is apparently due to the increased activity of the phenylalanine ammonia-lyase which produces cinnamic acid from phenylalanine.

Tobacco tissue cannot proliferate *in vitro* unless the basal medium contains an auxin, such as IAA, and a kinetin as shown by Skoog and Miller (48). This study has shown a similar response in tobacco callus tissue; however, the response was greater when tobacco callus was cultured with auxin and kinetin in the presence of GA_3. When GA_3 is applied to a responsive tissue culture system, it apparently brings about specific metabolic changes which help the synthesis of lignin and phenolic compounds.

The possible importance of ABA in the regulation of plant growth has led to numerous studies on its interaction with promotive hormones. Khan (27) and Sankhla and Sankhla (45) found that the inhibitory effect of ABA in lettuce seed germination could not be reversed by increasing the concentration of GA_3, instead it could be reversed only by kinetin. However, gibberellic acids have been found to overcome the effect of abscisic acid in inhibition in many cases. Examples are evident in the germination of seeds of *Fraxinus* sp. (50); *Corylus avellana* (9); and production of alpha-amylase by barley grains (12). Recently, Mercer and Pughe (34) found that the gibberellic acid content of ABA-treated maize tissue was lower than normal and they suggested that ABA may serve as an inhibitor of gibberellin biosynthesis.

The results observed in the present investigation indicate that tissues treated with low GA_3 will overcome ABA inhibition of growth, chlorogenic acid, and lignin biosynthesis to some extent. High GA_3 will only overcome inhibition of growth and lignin formation. Both low and high GA_3 partially overcome ABA inhibition of scopoletin synthesis. It is not clear how a combination of GA_3 and the growth media brings about the reversal of ABA inhibition of growth, chlorogenic acid and lignin synthesis. It is possible that GA_3 stimulates the synthesis of some specific enzymes which are inhibited by ABA. An equally possible and perhaps more likely explanation of the observed effects of ABA-GA_3 interaction on growth and the synthesis of lignin and phenolic compounds is that ABA might act as a GA_3 antagonist *in vivo*.

CHAPTER V

SUMMARY

Experiments were performed to determine the effects of ABA and GA_3, separately, and in combination of these two plant hormones on growth, and the lignin, chlorogenic acid, scopolin and scopoletin content of tobacco callus.

A significant depression of callus growth resulted from low concentrations of ABA added to Skoog's medium. Low concentrations also inhibited proliferation and decreased the chlorogenic acid and lignin content of the callus. The amounts of scopolin and scopoletin in the tissue varied with the concentration of ABA in the medium with some concentrations of ABA increasing the amounts of those compounds and others decreasing the amounts.

GA_3 stimulated callus growth in a low concentration (0.1 mg/l) and inhibited growth at a high concentration (10.0 mg/l). Both levels of GA_3 increased scopoletin accumulation in tobacco callus. A high concentration of GA_3 increased the accumulation of scopolin and chlorogenic acids in the callus tissue, whereas a low concentration of GA_3 decreased the amounts of these two phenolic compounds. In comparison with the control, lignin synthesis was stimulated by a low GA_3 concentration, but a high GA_3 concentration did not have a

significant effect.

Both low and high concentrations of GA_3 overcame ABA inhibition of growth and lignin synthesis, and partially reversed ABA inhibition of scopoletin production. However, GA_3 did not reverse the inhibitory effect of ABA on scopolin production, but it did reverse the promotional effect of a 1.0 mg/l concentration of ABA on scopolin production. The low concentration of GA_3 overcame the inhibition of chlorogenic acid production resulting from a 0.01 mg/l concentration of ABA, but this was the only reversal of chlorogenic acid inhibition resulting from addition of GA_3 to the medium.

Some possible reasons for the observed effects of ABA and GA_3 on growth and biosynthesis of lignin and certain phenolic compounds in tobaccus callus tissue were discussed.

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