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THE EFFECT OF AGE AND VARYING UV INTENSITIES ON CHLOROGENIC ACID AND SCOPOLIN CONCENTRATION IN TOBACCO AND SUNFLOWER

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BY

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THE EFFECT OF AGE AND VARYING UV INTENSITIES ON CHLOROGENIC ACID AND SCOPOLIN CONCENTRATION IN TOBACCO AND SUNFLOWER

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THE EFFECT OF AGE AND VARYING UV INTENSITIES ON CHLOROGENIC ACID AND SCOPOLIN CONCENTRATION IN TOBACCO AND SUNFLOWER

CHAPTER I

INTRODUCTION

Recent comprehensive reports indicate that scopolin (scopoletin-7-glucoside) and chlorogenic acid (3-O-caffeoylquinic acid) are widely distributed among the higher plants (40,53). However, relatively few studies have been done that involve quantitative analyses of these compounds within various parts of the plant when it is subjected to changing environmental conditions. Best (3) reported on the quantitative distribution of scopoletin (6-methoxy-7-hydroxycoumarin) in tobacco, and later Goodwin and Pollock (16) reported on the distribution of scopoletin in a growing Avena root. Although quantitative information on these compounds is relatively sparse, both scopoletin and scopolin have been quantitated in sunflower and tobacco plants treated with 2,4-dichlorophenoxyacetic acid, in tobacco treated with maleic hydrazide, and in boron-deficient sunflower and

tobacco (12,13,48,49,53). Quantitation of scopoletin and scopolin was accomplished by paper chromatography and followed by a determination of fluorescence (12, 13,39). Winkler recently reported good results using thin layer chromatography for the separation (53). Chlorogenic acid, on the other hand, has received continuous attention since its discovery by Robiquet and Boutron (34) in 1837. Reports of the isolation and quantitative determination of chlorogenic acid in various plant parts are numerous (40). Band 510 (4-O-caffeoylquinic acid) and neochlorogenic acid (5-O-caffeoylquinic acid) however, have been quantitated less frequently (19,56). Isolation has been achieved through a variety of methods including column chromatography, countercurrent distribution, paper chromatography, gas chromatography, and others (9,19,50,51,56). Quantitative analyses of chlorogenic acid are usually done by spectrophotometry (55,56).

There are many reports of the physiological effects of scopoletin, its glycoside scopolin, and chlorogenic acid. In general, it has been concluded that these compounds have a synergistic effect on IAA action, probably acting to suppress the IAA oxidase system (21,32,35,46). Similar environmental conditions produce differing accumulations of these compounds in different plants, thus leading to the hypothesis that their function may vary

(47,56). It has also been reported that ferulic acid and chlorogenic acid may play a significant role in lignin synthesis (42,44,45). The preliminary finding of Shiroya, Shiroya and Hattori that chlorogenic acid was highest in concentration in the youngest tissue supports the idea that possibly chlorogenic acid plays a dual role; that of metabolic regulator in young tissue, and of lignin precursor in older tissue. The quantitation of scopolin and CGA (chlorogenic acid), along with its isomers, B510 (band 510) and neoCGA (neochlorogenic acid), thus seems to be of physiological significance in understanding their role in the plant. In addition to the physiological importance placed on these compounds, it was shown by Rice (1,33) that chlorogenic acid, along with other phenolic compounds, has a significant phytotoxic affect. These discoveries place further importance on a knowledge of the conditions affecting the concentration of scopolin and chlorogenic acid.

The effects of UV radiation on plants have been studied considerably since Siemens (37) and Deheran (11) in the 1880's demonstrated that UV light from carbon arc light caused injuries to plants. Generally the effects of UV light can be divided into three categories: injurious, inhibitory, and stimulatory, depending on the wave length and intensity of the UV treatment. Injurious

symptoms include stunted growth, smaller than normal leaves, thickened stems, a bronzed shiny appearance of the epidermis, and a general browning of the leaf surface which is probably due to the formation of corky and woody cells immediately beneath the dead epidermal cells (24). UV inhibition of plant growth occurs naturally in high mountains and resembles that caused by low temperatures, apparently being due to a lower growth rate (5,6, 10,14,30). It has been shown by many workers that the stimulation of higher plants occurs only with low dosages (5,14,15,54). In general these stimulations are restricted to field conditions with the natural UV of the sun (24). Lockhardt and Brodfuhrer stated that most injury and inhibition occur with UV wavelengths below 280-290 mμ, while stimulations are observed principally at longer wavelengths (24).

The fact that appropriate dosages of UV light cause both stimulatory and stress conditions in plants, and the discovery that increased stress conditions cause accumulations of scopolin and scopoletin, made it seem desirable to study the effects of UV light on the concentration of certain phenolics in test species. Appropriate methods were developed and experiments were designed, therefore, to determine the effects of UV light and age on the CGA, B510, neoCGA, and scopolin content of tobacco and sunflower plants.

CHAPTER II

MATERIALS AND METHODS

All plants were grown in Percival growth chambers under 16 hour light periods. The light intensity for the UV experiments was 700-900 ft-c, while for the plants used in the tobacco age studies it was 1000-1200 ft-c. Plants were carefully selected for uniformity at the beginning of treatment, grown in pure quartz sand, and watered with a Fe-EDTA double strength Hoagland's nutrient solution (21). The double strength nutrient solution was found necessary since the tobacco plants developed a severe chlorosis as they matured on the regular solution. The light period temperature was maintained at 32°C, while the temperature during the dark period was 15.5°.

UV light was supplied from a GE germicidal lamp #G30T8 suspended above the plants on one side of the growth chamber (control plants were isolated in a separate chamber under identical conditions, but without the UV source). When the lamp was covered by 10 layers of window screen the differential UV effect produced was as indicated in figure 1. While most of the energy radiated by this lamp is in the range of 240-260 mμ, it also

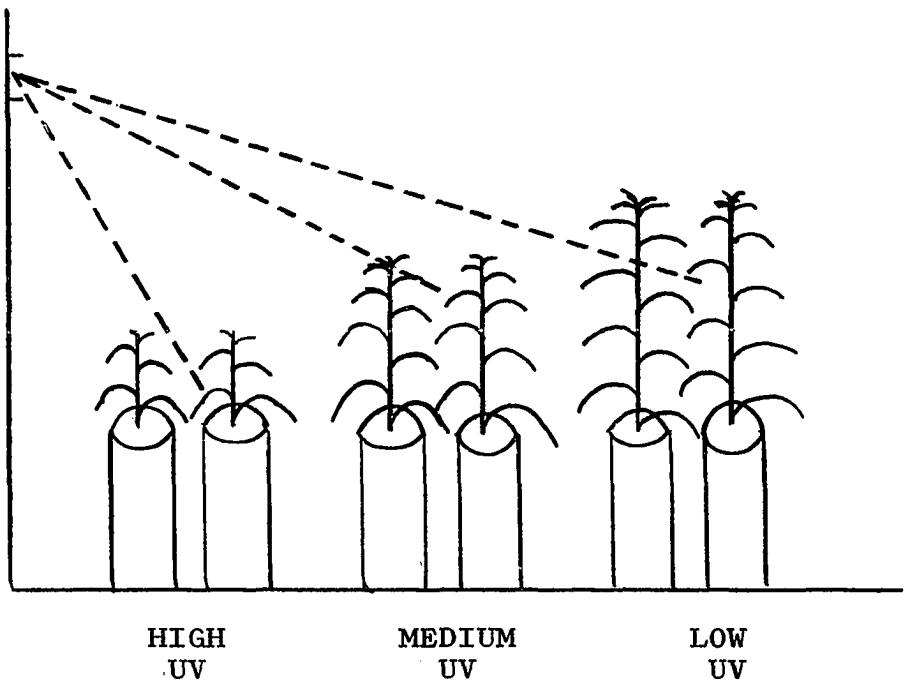

Fig. 1. Experimental UV treatment set up. Intensities of UV as follows: 1) high UV, 5-8 mw/ft^2, 2) medium UV, 4-5 mw/ft^2, and 3) low UV, 1-1.5 mw/ft^2.

irradiates to a limited extent in the middle UV range (280-320 mμ) supplying some of the irradiation of this range that is lacking in the fluorescent tubes used in these chambers but present under natural conditions. UV treatment of the tobacco plants (Nicotiana tabacum L. var. One Sucker) was initiated when the plants were approximately 80 days old and was maintained for a period of 21 days. Treatment lasted for 8 hours each day, being administered in the center of the 16 hour light period. Sunflower plants (Helianthus annuus L. var. Russian Mammoth) received the same UV treatment, but it was

initiated 7 days after planting.

After treatment, plants were harvested, weighed, and fixed in boiling isopropyl azeotrope (88% isopropanol: 12% water, w/w). The fixed plant matter was next ground thoroughly in a blendor and then transferred to a Soxhlet extraction thimble. After washing with 4 times the wet weight of boiling isopropanol:water (1:1, v/v), 5 times the wet weight of boiling isopropanol:methanol:benzene: water (2:1:1:1, v/v/v/v), and 4 times the wet weight of boiling isopropyl azeotrope, the plant matter was placed in a Soxhlet extractor and extracted for 24 hours with isopropyl azeotrope, and then for another 24 hours with isopropanol. The resulting solvent mixtures were combined, evaporated to dryness <u>in vacuo</u>, and then brought back to a known concentration with IBMW (isopropanol: methanol:benzene:water, 2:1:1:1, v/v/v/v), the concentration of plant matter to solvent always exceeding a 3:1 ratio (grams fresh weight:IBMW). The compounds which have been quantitated and reported here have varying degrees of stability in this IBMW solution. Scopolin did not change in concentration over a period of 1 year and the chlorogenic acids in sunflower stem and root samples showed no change. In tobacco extracts the chlorogenic acids showed a concentration loss of 2-3% during the first 5 months after extraction, but in the next 7 months showed a decrease of up to 40% of the original

concentrations which were determined immediately after extraction. In the old leaf extracts of sunflower the change of the chlorogenic acids was the most pronounced, reaching 55% after 1 year. While there was a chemical change in the chlorogenic acids, there was apparently no isomerization since the ratios of the three isomers remained constant. Because of this problem, all quantitative results reported here were obtained within a period of only minimal change. All tobacco samples were quantitated within 3-4 months after extraction and sunflower samples, except stems and roots, were quantitated within 1 month after extraction.

Separation of the phenolic compounds to be quantitated, scopolin, CGA, B510 and neoCGA, was accomplished through one dimensional descending paper chromatography. Whatman #1 paper (9¼ X 22 in) was washed with approximately 50 ml methanol:water (5:95, v/v). Within 2-4 hours after the washed paper was dried, an aliquot of the sample extract was streaked along a six inch strip on the paper, and developed for 20 hours using KFW (methylisobutyl ketone:formic acid:water, 14:3:2, v/v/v) as the solvent. The chromatocab used was a small glass model and was not equilibrated at the start. Larger cabs would probably need to be equilibrated. After drying, bands were noted under UV light (no ammonia). The bands of the four compounds involved were very distinct

(Fig. 2) and readily separable from other fluorescent compounds. Positive identification of the compounds was made by co-chromatography with known compounds in different solvent systems. In addition, the strips developed in KFW with the authentic compounds were cut off,

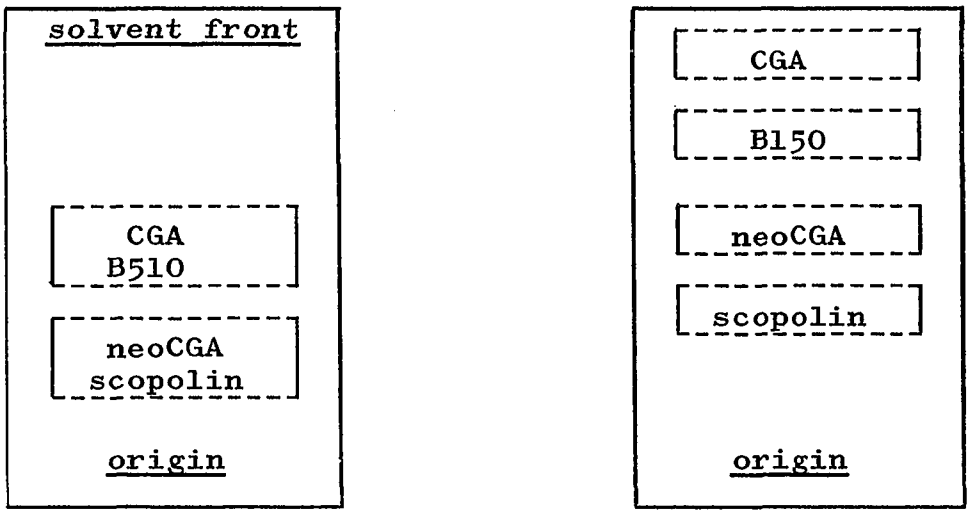

Fig. 2. Separation by paper chromatography of chlorogenic acid (CGA), band 510 (B510), neochlorogenic acid (neoCGA) and scopolin. Left with solvent front still on paper, right after 20 hours. Solvent KFW.

sewn to other papers and developed in other solvent systems. In every instance the sample compound corresponded with the known, further indicating the purity of the fluorescent spot. Absorption spectra were made of all the knowns and unknowns, and the designated compounds were found to have spectra identical with their corresponding reference. Tests were not made for compounds such as sugars and amino acids since they are not fluorescent and would not interfere with the fluorescent and

absorption quantitative techniques used, even though they may have been present.

The bands found under UV were cut out and eluted from the paper in descending fashion with 5-8 ml of 5% methanol-water. The strips were eluted for at least 12 hours. I found through constant checking of the eluted strips with ammonia that loads of chlorogenic acid up to 600 µg/paper were completely eluted with 8 ml of the 5% methanol-water. Scopolin was totally eluted in all concentrations encountered. Following elution the eluates were brought to a known volume and read against a blank which had been carried through the identical procedure. The chlorogenic acids were read at a wavelength of 330 mµ on a Hitachi-Perkin-Elmer Model 139 spectrophotometer. A standard reference curve (Fig. 3) for chlorogenic acid was prepared by carrying the known quantities through all the chromatographic steps used for the unknowns. Since the extinction coefficients and absorption spectra of all three chlorogenic isomers are assumed to be identical, it is possible to use the standard curve of chlorogenic acid for the quantitation of all three isomers (56). Percent recovery of the CGA was between 83-86%, with higher concentrations giving higher recovery percentages. Scopolin was quantitated with a model 110 Turner fluorometer using pyrex cuvettes and a high sensitivity attachment at an instrument setting of 1X. The following filters were used:

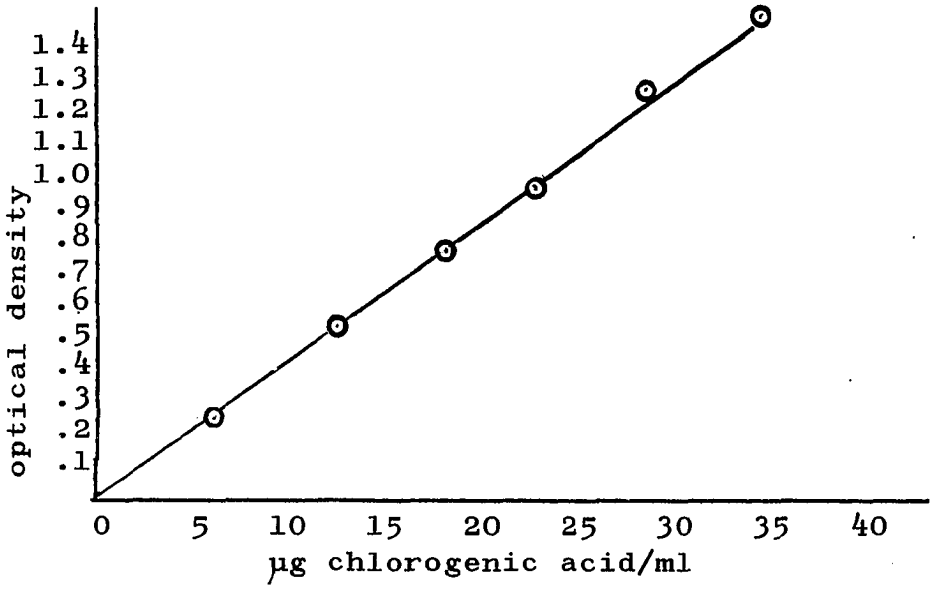

Fig. 3. Relationship of optical density and chlorogenic acid concentration. Instrument, Hitachi-Perkin-Elmer 139 spectrophotometer.

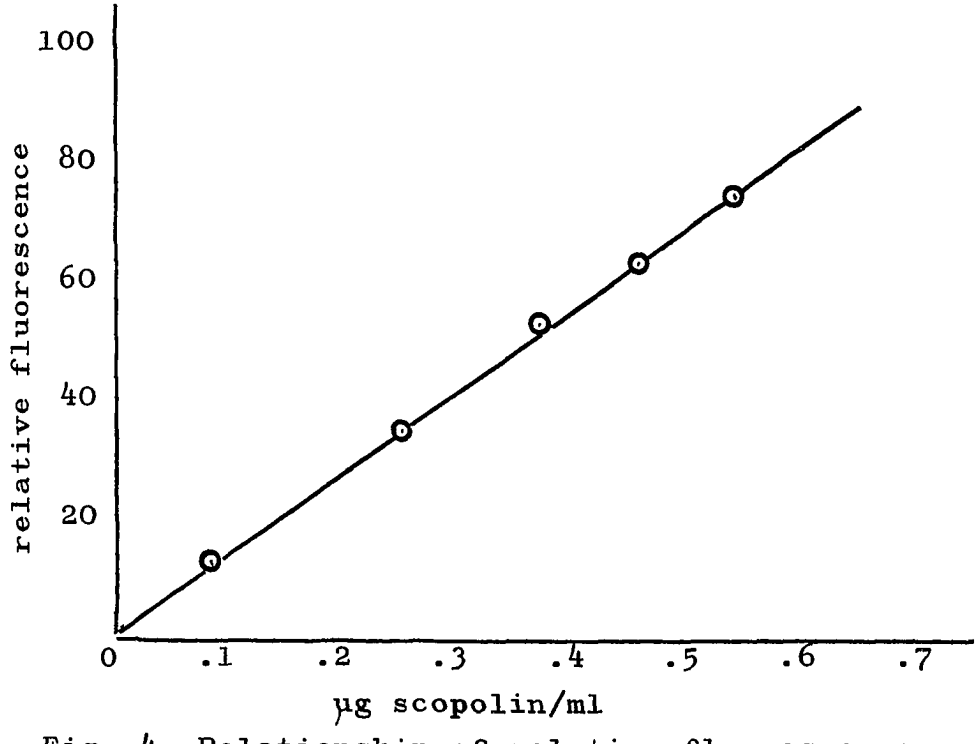

Fig. 4. Relationship of relative fluorescence and scopolin concentration. Instrument, Turner Model 110 fluorometer.

primary filter #7-60, and secondary filter #2A plus #48 (Kodak Wratten filter). A standard reference curve for scopolin was prepared (Fig. 4) in a similar manner as described for chlorogenic acid. The average recovery for scopolin was 80%. More detailed considerations in the quantitation of scopolin and scopoletin were discussed by Winkler (53). All data reported in the remainder of this paper are the averages of at least two quantitative determinations, even though replicates consistently fell within less than a 5% error range.

CHAPTER III

RESULTS

Effects of age on phenolic concentration in tobacco. Plants used in this series of determinations were divided into 2 groups of 2 plants each. The first 6 leaves longer than 2 cm were removed from the plants, extracted, and quantitated as individual leaves numbered 1-6, with 6 being the oldest of the leaves. There was a decrease expressed as µg/g fresh weight in the concentration of CGA, B510, and scopolin as the leaf got larger and further from the apex (Fig. 5 & 6). The total amount of these compounds in the leaf increased with age, even though their concentration decreased (Fig. 7a, 7b). The percent of CGA decreased with age of the leaf, neoCGA increased, and B510 increased at first and then leveled off (Fig. 8). The stems were divided into three 12 cm sections beginning 4 cm down from the apex. Each section was divided into pith and the zone outside of the pith. Again, there was a decrease in CGA with increasing distance from the apex, or meristematic region of the plant (Tables 1a, 1b). Scopolin, on the other hand, generally showed an inverse relationship to this. Whereas the

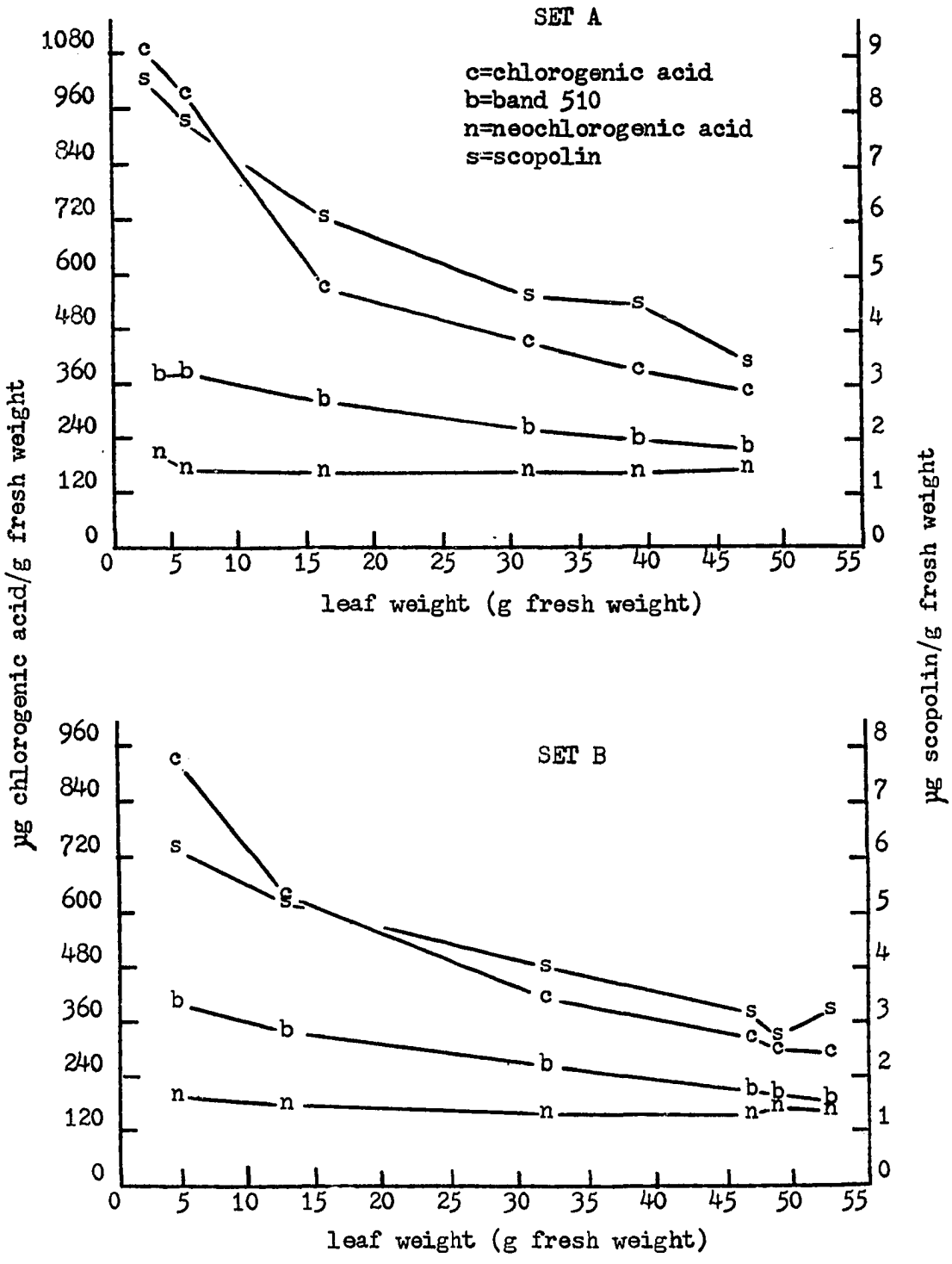

Fig. 5. The relationship of CGA, B510, neoCGA, and scopolin concentration and age of tobacco leaves.

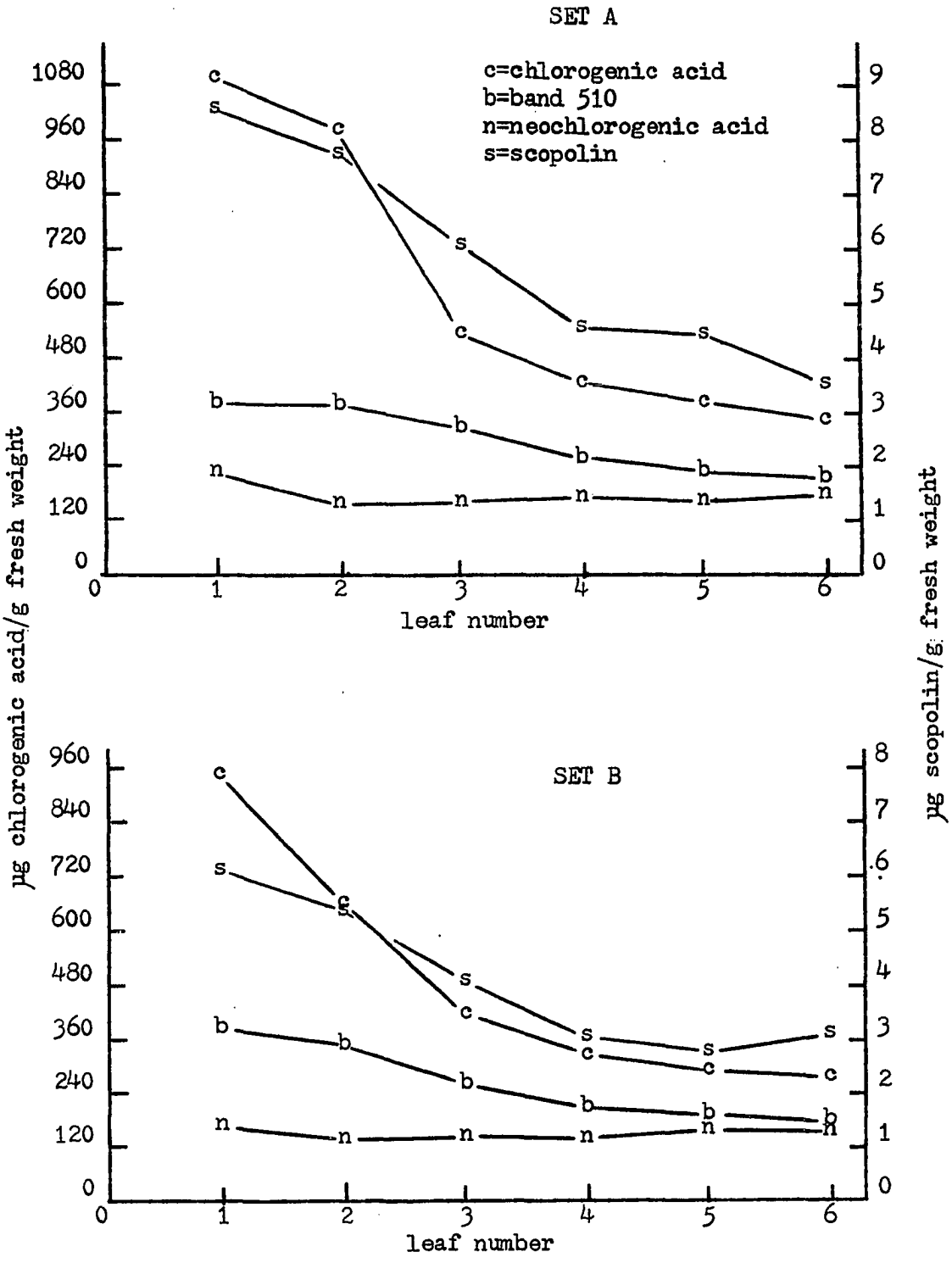

Fig. 6. The relationship of CGA, B510, neoCGA, and scopolin concentration and the position of tobacco leaves over 2 cm from the apex.

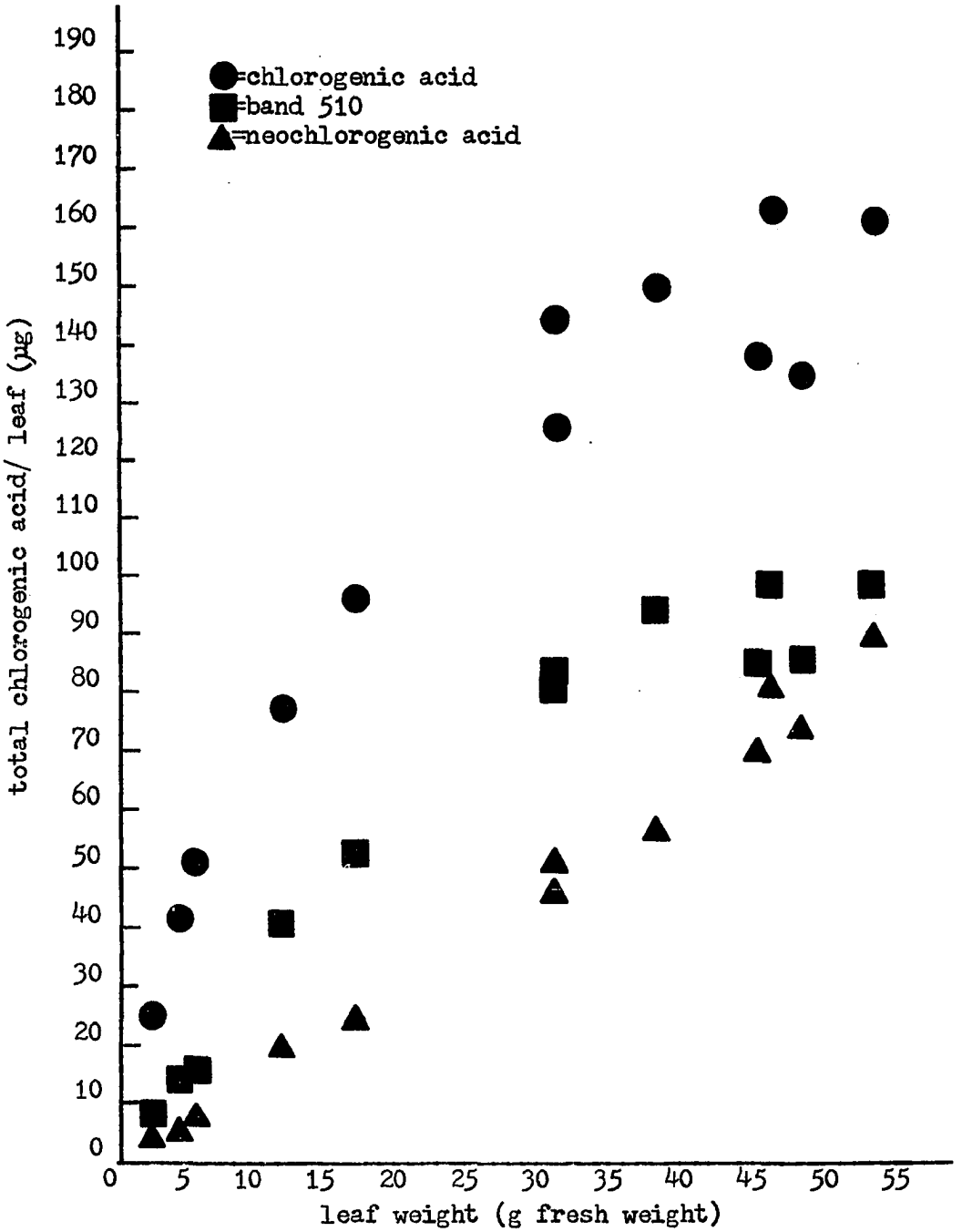

Fig. 7a. The amount of the isomers of chlorogenic acid compared with the age of tobacco leaves (expressed as g fresh weight). Data from both set A and set B. Amounts of CGA X 100.

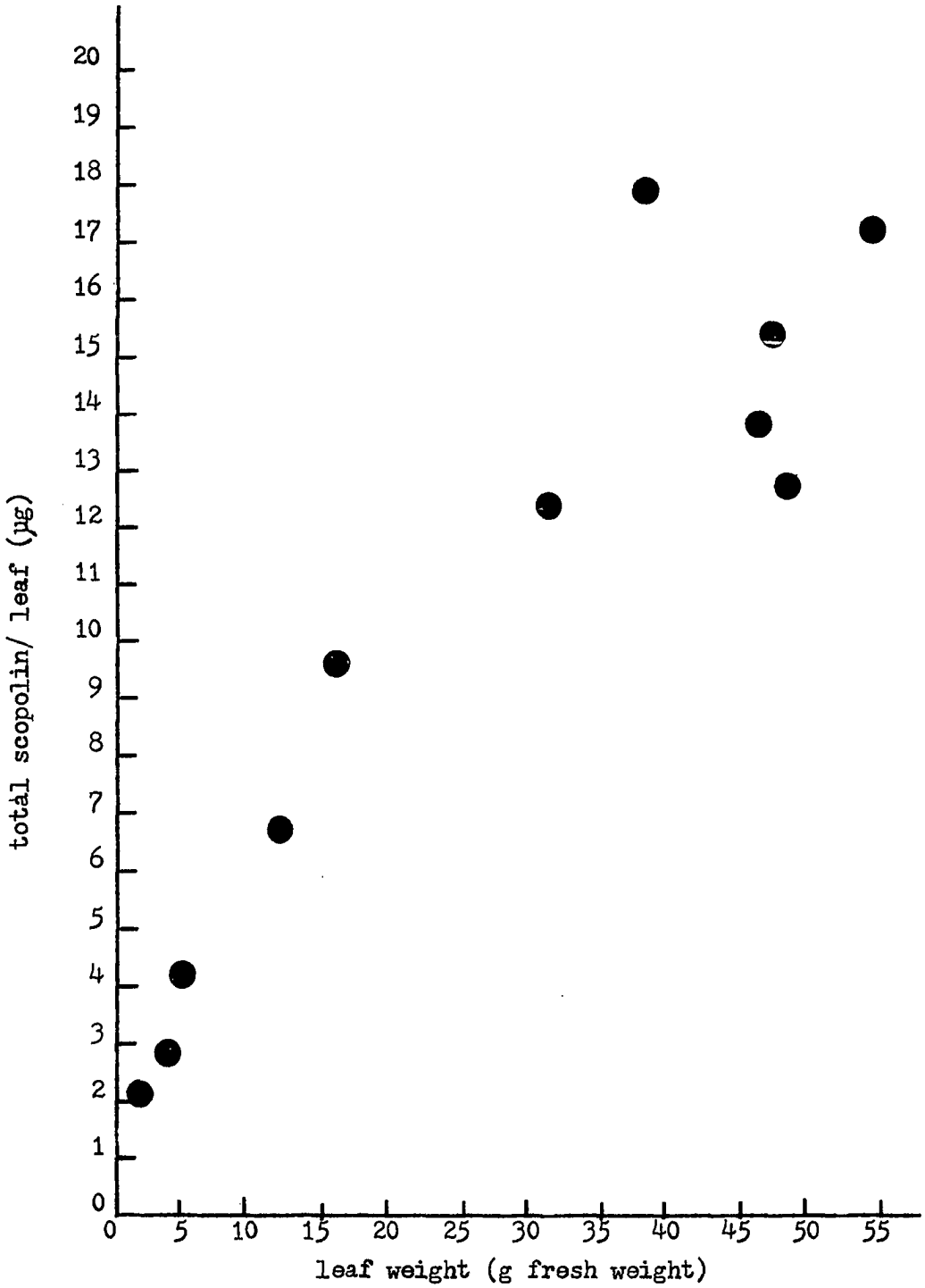

Fig. 7b. The amount of scopolin in tobacco leaves compared with age (expressed as g fresh weight). Data from both set A and set B. Amounts of scopolin X 10.

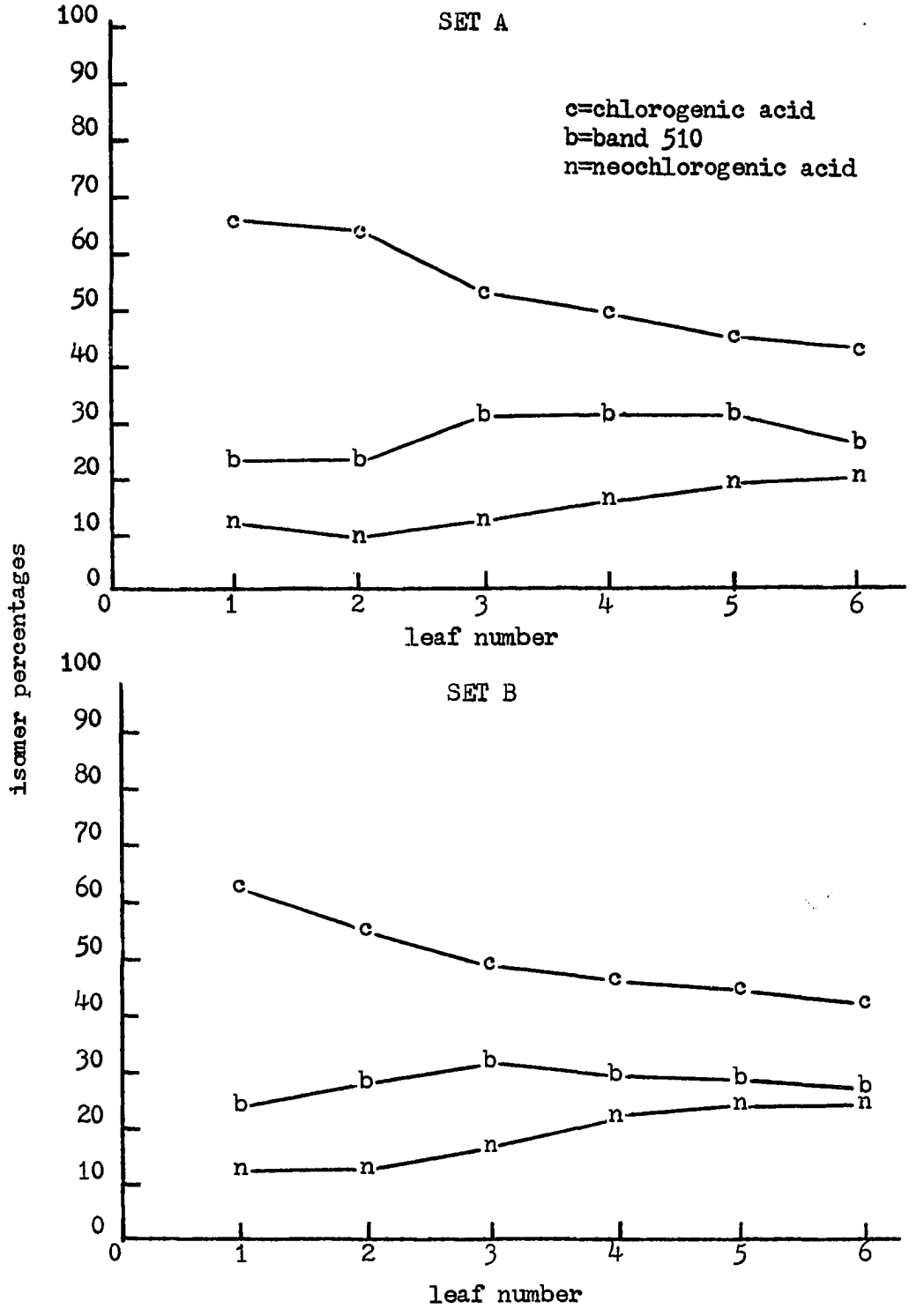

Fig. 8. The relationship of the percentages of CGA, B510 and neoCGA and the position of tobacco leaves over 2 cm from the apex.

Table 1a. The concentration (µg/g fresh weight) of chlorogenic acid in stem sections when the pith is separated from the remainder of the stem.

Section	Set A	Set B
Top pith	102	104
Middle pith	46	24
Bottom pith	37	49
Top external	366	243
Middle external	162	166
Bottom external	99	171

Table 1b. The concentration (µg/g fresh weight) of scopolin in stem sections when the pith is separated from the remainder of the stem.

Section	Set A	Set B
Top pith	2.5	2.0
Middle pith	1.2	1.2
Bottom pith	2.6	19.2
Top external	7.9	7.6
Middle external	9.2	8.7
Bottom external	18.6	53.0

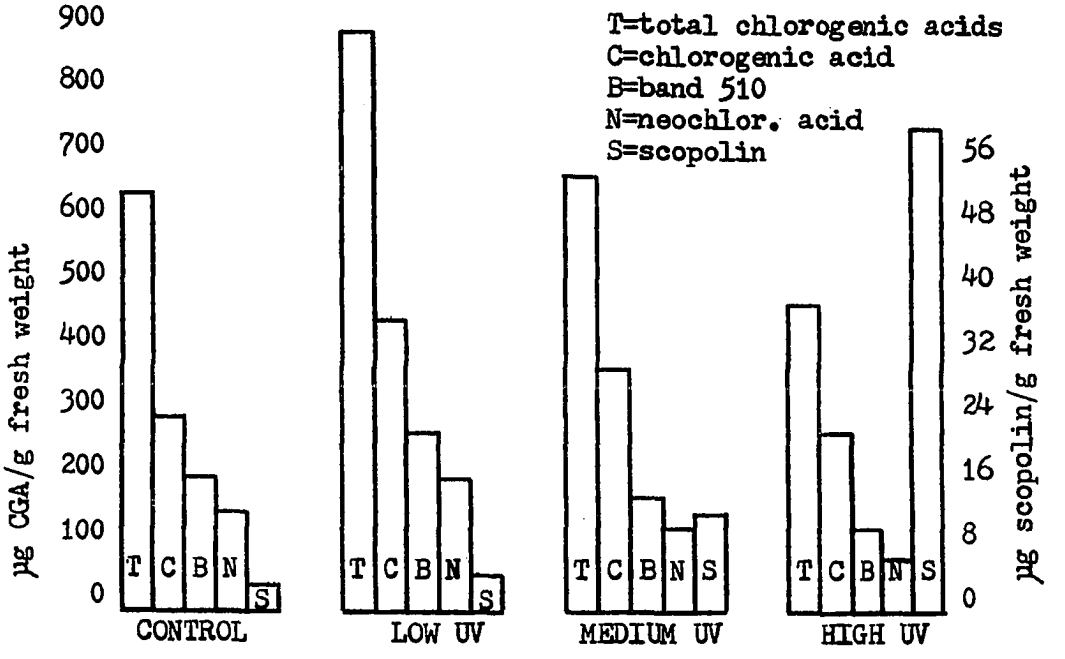

Fig. 9. The conc. of CGA, its isomers, and scopolin in the old leaves (longer than 20 cm) of control and UV treated tobacco plants.

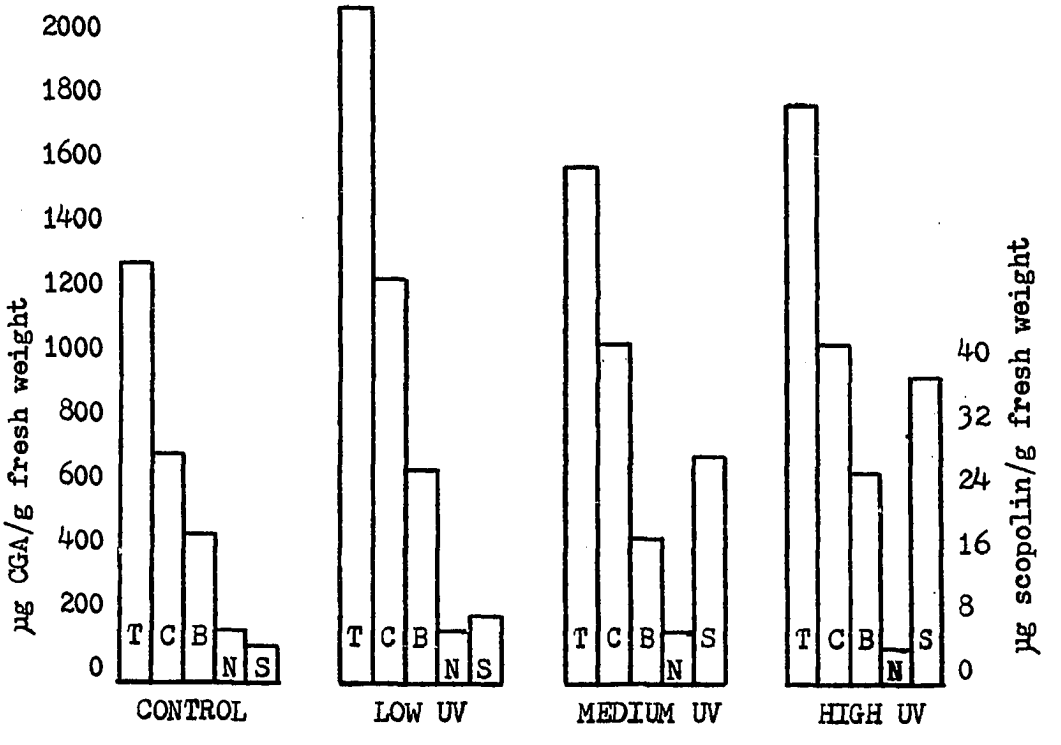

Fig. 10. The conc. of CGA, its isomers, and scopolin in the young leaves (less than 20 cm long) of control and UV treated tobacco plants (see figure 9 for abbreviations of compounds).

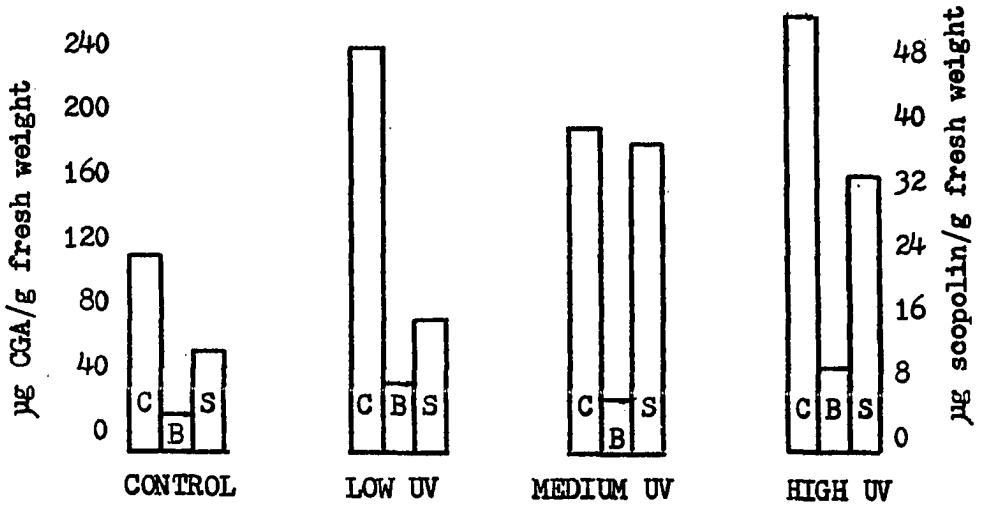

Fig. 11. The conc. of CGA, B510, and scopolin in the stems of control and UV treated tobacco (see figure 9 for abbreviations of compounds).

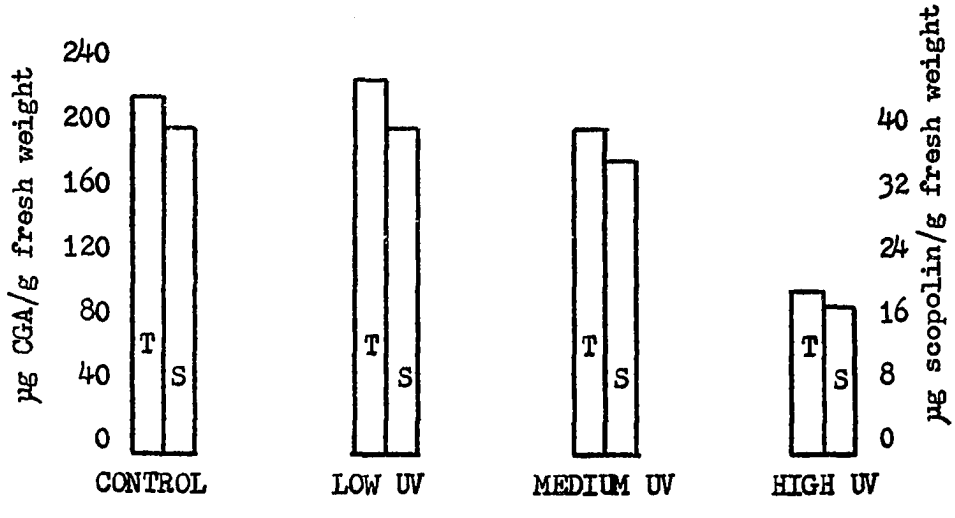

Fig. 12. The conc. of CGA and scopolin in the roots of control and UV treated tobacco (see figure 9 for abbreviations of compounds).

Table 2. Percentages of chlorogenic acid, band 510, and neochlorogenic acid in tobacco leaves of control and UV treated plants.

Treatment	%CGA	%B510	%neoCGA
Control old leaves	46.2	30.8	23.0
Low UV old leaves	49.2	28.9	21.9
Med UV old leaves	53.9	26.7	19.3
High UV old leaves	59.3	25.0	15.7
Control young leaves	53.9	33.7	12.4
Low UV young leaves	59.7	32.3	8.0
Med UV young leaves	63.8	27.0	9.2
High UV young leaves	59.0	35.1	5.9

tobacco leaves had large percentages of B510 and neoCGA, only a trace of B510 and no neoCGA wereobserved in the stems.

UV effects on tobacco. Measurements showed that the four plants subjected to the low UV treatment were consistently taller than the control or higher UV plants, and the leaves were larger. Growth of the plants was positively correlated with total chlorogenic acid concentration of old leaves (Fig. 9). Under high UV the plants became stunted and the leaves became bronze colored in 2-5 days. However, the leaves remained turgid and did not dry out.

The concentrations of CGA, B510, and neoCGA were

greatest under the low UV conditions in both old and young leaves, and CGA was highest in concentration in roots and stems. Scopolin however, generally increased in concentration with increased UV (corresponding to increased stress) in those plant parts exposed to the UV. The roots contained almost a constant 5:1 ratio of CGA to scopolin with the greatest concentration of both at the low UV level.

UV effects on sunflower correlated with age. Sunflower plants were harvested 8, 15, 24, and 37 days after the start of the UV treatments. The numbers of plants included in the samples taken on these dates were as follows per harvest; 1) 16-35 plants, 2) 17-20 plants, 3) 5 plants, and 4) 5 plants. The effects of the UV treatment were virtually the same morphologically as those indicated earlier for tobacco. As previously indicated, treatment was initiated earlier in the sunflower plants than it was for the tobacco, and the high UV treated plants appeared even more stunted. The increase in height and leaf size was again noted in sunflower under the low UV conditions as it was in the tobacco experiment.

The concentration of scopolin in the plant parts exposed to UV was found to increase as it did in tobacco, with increasing UV (Fig. 13, 14). Unlike tobacco however, scopolin was normally present in sunflower at a

level that was minimal for quantitative studies and did not occur in roots in detectable amounts at all. The only appreciable accumulation of scopolin occurred in the leaves which were most exposed to the UV conditions (Fig. 13-17). The production of scopolin appeared to be rapid, occurring within the first 2 weeks of treatment, and then falling off subsequent to this.

The cotyledons remained on better than 75% of the high UV treated plants throughout the duration of the experiment, but dried up and abscised within 25 days after planting in the other treatments and the control plants. A high concentration of the chlorogenic acids occurred in the cotyledons, but decreased with increasing age of the plants (Fig. 17). Prior to abscission the cotyledon CGA concentration was least in the low and medium UV treated plants (Fig. 17). There was, however, no detectable scopolin in any of the treatments, including the high UV. It is interesting to note that the stems also showed the same falling off pattern as the cotyledons (Fig. 15).

In contrast with tobacco leaves which had the highest chlorogenic acid content in the low UV treated leaves, sunflower leaves had the lowest chlorogenic content in low UV and the content increased with increasing UV intensity (Fig. 9, 10, 13, 14). The control leaves of sunflower had a chlorogenic acid concentration

intermediate between those of the low UV and medium UV treated leaves. It was obvious that the concentration of chlorogenic acids in sunflower leaves was a function of age of the plant as well as UV intensity (Fig. 13, 14). Up to 24 days after planting, little, if any CGA was present in the leaves of control and low UV plants, with only small amounts in the medium and high UV treated plants. Sunflower stems and roots, unlike tobacco, showed little correlation with UV conditions or age (Fig. 11, 12, 15, 16). Generally, the stems showed a decreased concentration of the chlorogenic acids after the first harvest, with a possible later increase with age.

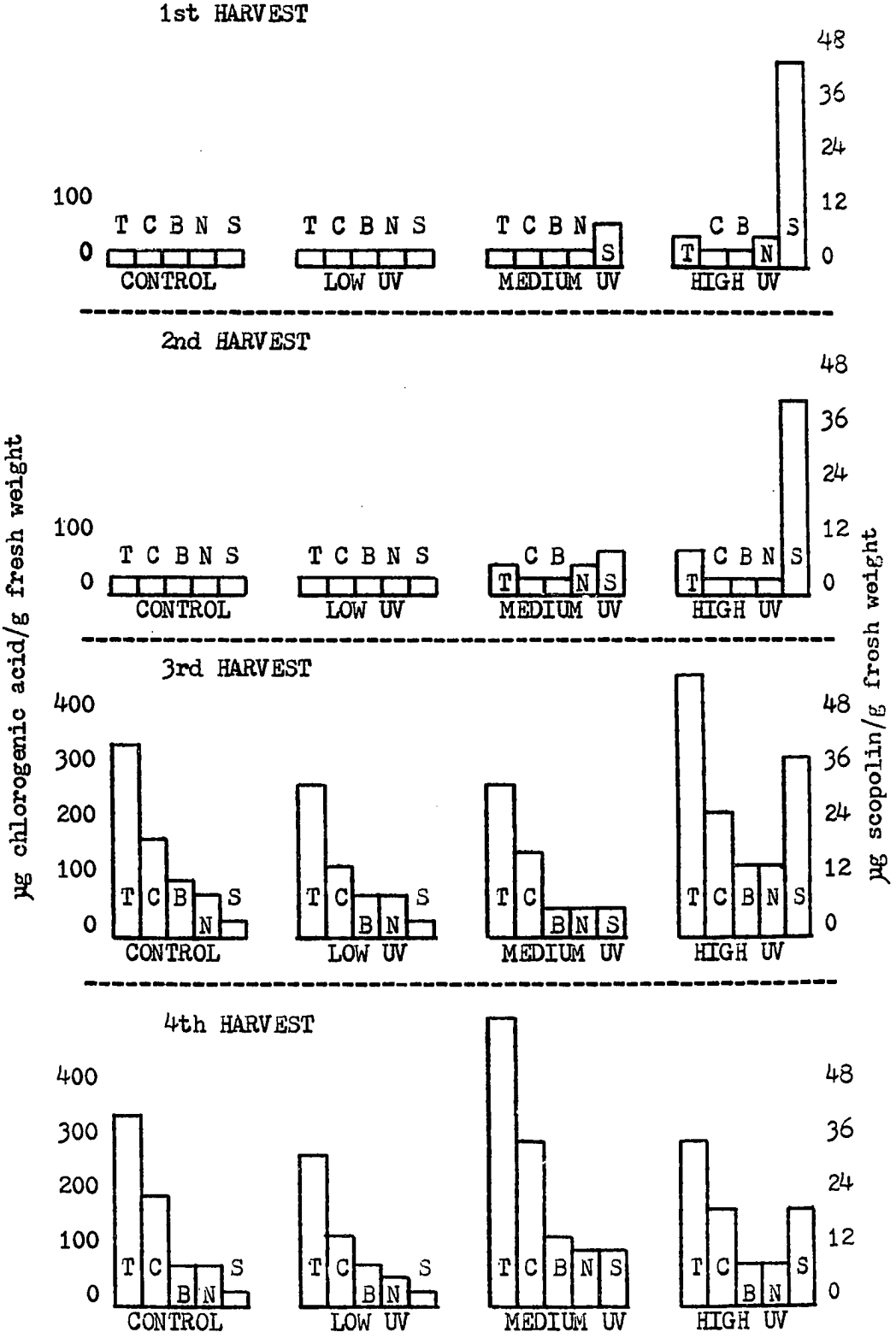

Fig. 13. The conc. of CGA, B510, neoCGA, and scopolin in the young leaves of sunflower (see figure 9 for abbreviations of compounds). Symbols out of boxes represent only traces.

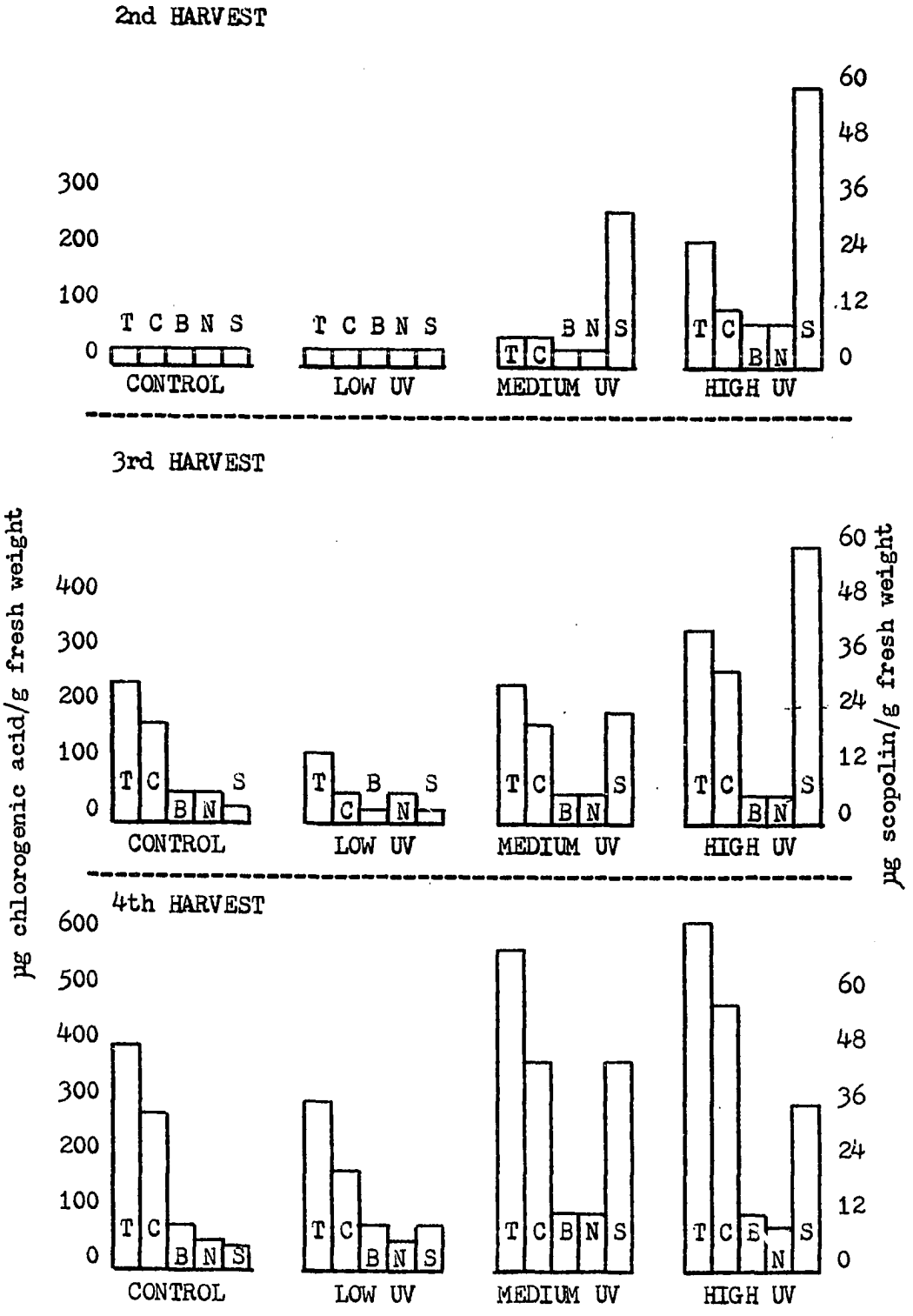

Fig. 14. The conc. of CGA, B510, neoCGA, and scopolin in the old leaves (those discernably separate from the apex) of sunflower (see figure 9 for abbreviations of compounds). Symbols out of boxes represent only traces.

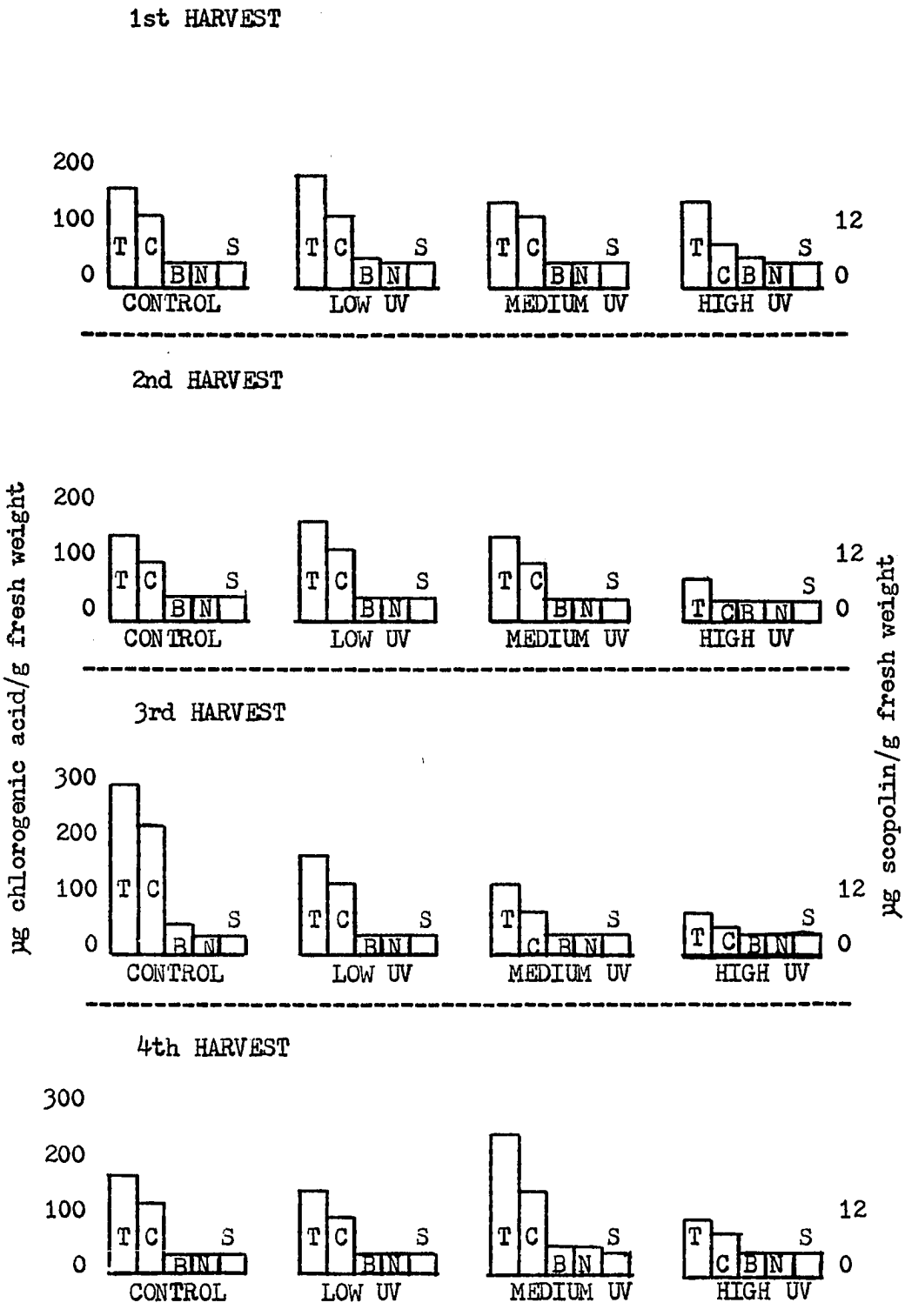

Fig. 15. The conc. of CGA, B510, neoCGA, and scopolin in th stems of sunflower (see figure for abbreviations of compounds). Symbols out of boxes represent only traces.

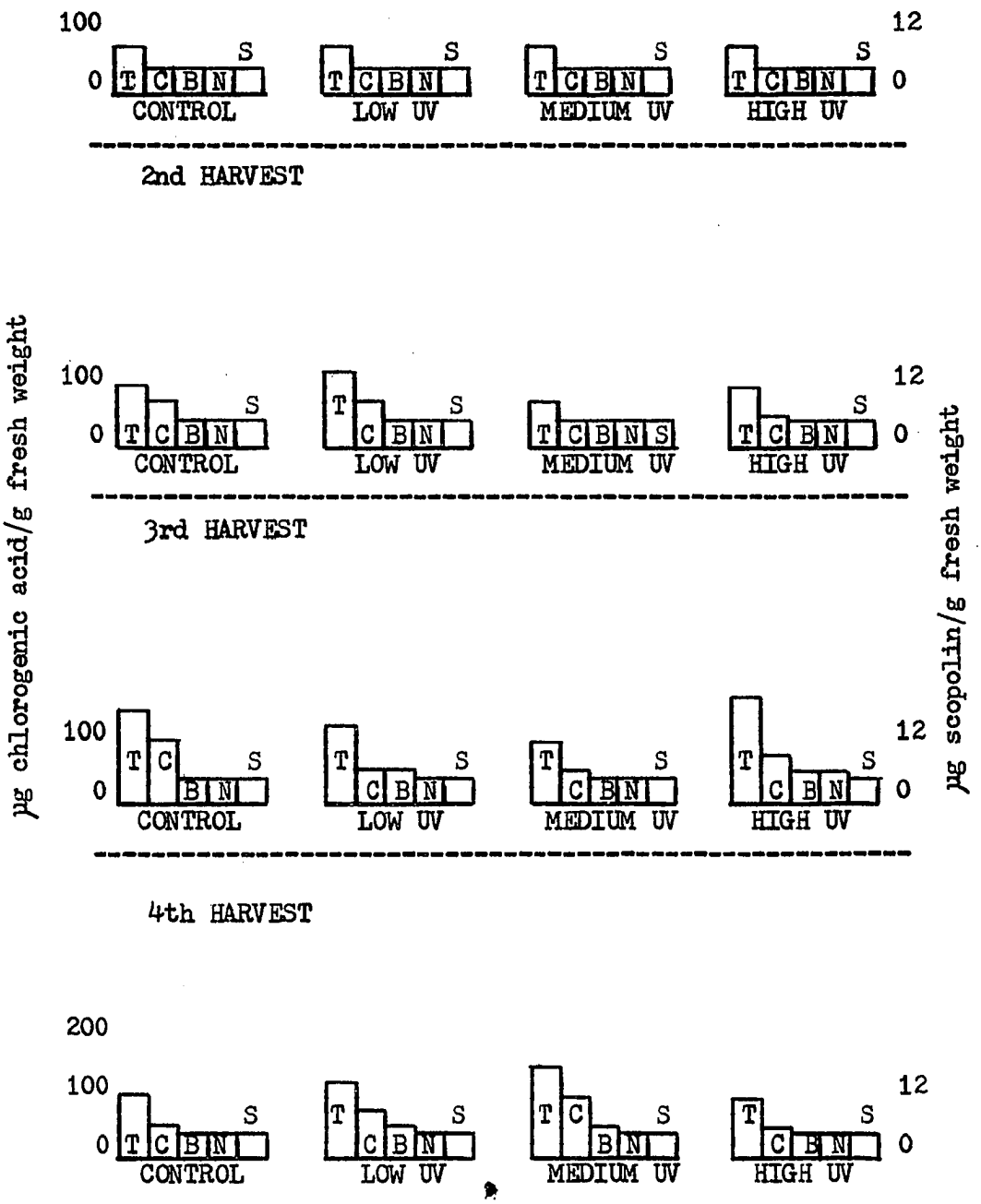

Fig. 16. The conc. of CGA, B510, neoCGA, and scopolin in the roots of sunflower (see figure 9 for abbreviations of compounds). Symbols out of boxes represent only traces.

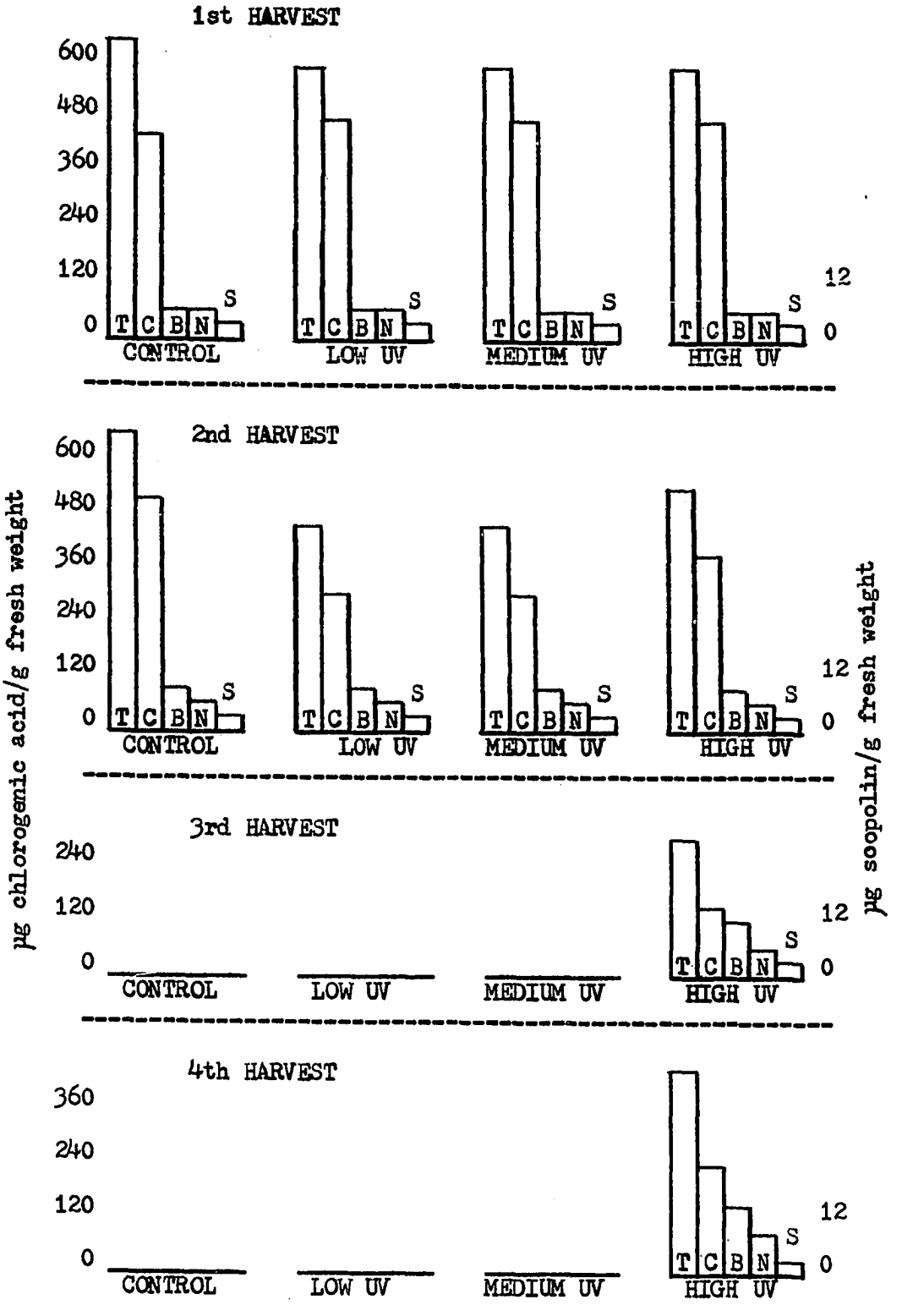

Fig. 17. The conc. of CGA, B510, neoCGA, and scopolin in the cotyledons of sunflower (see figure 9 for abbreviations of compounds). Symbols out of boxes represent only traces. Only high UV plants retained cotyledons at the time of 3rd and 4th harvests.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Shiroya et al. (36) reported a decrease in the concentration of chlorogenic acid in the leaves of tobacco plants from 600 μg/g fresh weight in young leaves to 260 μg/g fresh weight in "low adult leaves." Present results substantiate that finding. The higher concentrations of chlorogenic acid obtained here probably resulted from a more exhaustive extraction procedure. A significant expansion of the work of Shiroya et al. was the discovery that the 4-O and 5-O isomers of chlorogenic acid show significant changes in concentration with leaf age. This is similar to the increase of neoCGA in Theobroma cacao reported by Griffiths (17). This isomerization with age might be associated with Taylor's discovery that in Xanthium the 3-5 dicaffeoylquinic acid is formed from chlorogenic acid (44), and with Taylor and Zucker's finding that isochlorogenic acid and chlorogenic acid are in a constant state of turnover (45). These findings might implicate the metabolic changes of chlorogenic acid in some manner to lignin synthesis. The fact that the extract of roots of tobacco have little, if any, of the

isomers present, indicates that this isomerization is real and not an artifact of the extraction and quantitation procedure. However, the almost total lack of the isomers in the lower lignified region of the stem further complicates the picture. It might be implied from Steck's (43) work that scopolin could be produced in tobacco as a metabolic end product when fiber production was inhibited, thus providing an explanation for the accumulation of scopolin in the lower stem regions. However, the decrease of scopolin in the older leaves either repudiates this hypothesis, or, with the increase of the isomers, implicates a slightly different mechanism of lignin synthesis. This scopolin accumulation could also be the result of translocation from the roots where the scopolin concentration is considerably greater.

Another explanation for the decrease in CGA concentration with increased age of the tissue might lie in its relation to meristematic growth. Zucker and Ahrens (55) found a gradient of CGA from tip to base of tobacco leaves, but tobacco is not the only plant to show such a gradient. Griffiths (17) reported a decrease in chlorogenic and p-coumaroylquinic acids during growth of young leaves of Theobroma cacao. Hamidi and Wanner (18) reported a decrease in concentration and total amount of CGA in the coffee plant. Morgan (28) reported a decrease in total phenolic concentration of older leaves

when compared with younger leaves of cotton plants, and he found this decrease correlated with a corresponding increase in IAA oxidase. Boll (4) reported a similar increase in IAA oxidase activity basipetally in pea stems. Kerstetter and Keitt (23) reported a huge increase in the rate at which old tobacco pith decarboxylates IAA when compared with younger pith sections of the same plants (a decrease in CGA concentration in old pith sections of tobacco was found in the present project). Tomaszewski and Thimann (46) presented good evidence that polyphenols synergize IAA, inducing growth by counteracting IAA decarboxylation. This general idea is supported by many workers including Rabin and Klein (32), Henderson and Nitsch (21), Sondheimer and Griffin (41), and others.

It is possible that the role of the chlorogenic acids in tobacco may be dual, first as a metabolic inhibitor in young tissues and then as precursors to lignin in older tissue. The data presented here however, indicate that older leaves do contain significantly greater total amounts of the chlorogenic acids, probably continuing to produce them in lesser amounts as the leaf matures. The active translocation of the chlorogenic acids seems negligible, except in the precursor form, as determined by Taylor and Zucker (45).

The role of chlorogenic acid in the sunflower seems to be somewhat different from that in tobacco. The high

amounts present in the cotyledons of sunflower are similar to the accumulation found in lettuce cotyledons by Butler (7). He found that by separating the cotyledons from the seedlings in extraction it was possible to show that the chlorogenic acid remained in the cotyledons and little, if any, was translocated into the roots. This also appears to be the case with sunflower. Butler saw this apparent lack of utilization as an indication that CGA may be an end-product that accumulates in the seed, but which serves no metabolic function. The general accumulation of CGA in sunflower leaves and UV treated stems with age might indicate a similar role to that hypothesized for lettuce. It might also be that in these areas of accumulation there is little lignification occurring, whereas in the sunflower stems where little accumulation occurs, there is considerable lignification. The higher UV treated stems did show a much weaker structure, possibly owing to the lack of lignin, and thereby accounting for the accumulation of CGA in the later weeks of the experiment.

Lott (25) reported that UV light can cause a maximum increase in the total chlorogenic acid content of tobacco plants of 79% in open air plants, and 550% in greenhouse grown plants, based on dry weight determinations. His work was done using column chromatography and no mention was made of the isomers. Present results substantiate his findings, even though a different variety of tobacco

was used. No gain approaching 550% was found, however. It is interesting that the isomers in both old and young leaves generally remain in the same relative proportions, even though the total CGA content changes with the various treatments. This is comparable to the changes observed by Zucker, Nitsch and Nitsch (56) with photoperiod changes in tobacco. In the present project the total concentration of the chlorogenic acids changed significantly, but the ratio of the isomers generally remained about the same.

Since the growth of the plants correlated well with the chlorogenic acid concentration, it seems logical to ascribe this increased growth to the effect of the chlorogenic acids in inhibiting IAA oxidase, thus allowing more IAA to be present and thereby stimulating growth. On the other hand, the build up of the chlorogenic acids under low UV conditions may have been due to greater lignification, thus the need for a larger chlorogenic acid precursor pool.

Previously reported stress conditions in tobacco caused by 2,4-D treatment, maleic hydrazide treatment, and boron deficiency all caused large accumulations of scopolin in the treated or deficient plants (12, 13, 48, 49, 53). The accumulation of scopolin in the high UV plants of these experiments is consistent with the idea that increasing stress conditions cause a corresponding increase in scopolin concentration in tobacco. Further

experiments now completed indicate a similar increase in scopolin concentration in tobacco plants treated with lethal doses of ionizing radiation, in plants receiving a cold treatment during the light period, and in plants made potassium and nitrogen deficient (2). Implications from Steck's (43) work indicated that under stress conditions ferulic acid could be blocked from going to the formation of fibers and that scopolin could be produced in larger amounts and accumulate. This might well be the case, in that increasing stress conditions probably lead to a decrease in lignification.

In previous work involving accumulation of scopolin a prime site of such accumulation was in the roots of the treated plant. Since in the present stressed high UV plant roots no such accumulation occurs, it might be hypothesized that scopolin only accumulates *in situ* at the point of stress. Clearly, the effects of 2,4-D and maleic hydrazide treatment can occur in the roots since these herbicides are readily translocated throughout the plant. Thus it may be that scopolin is not translocated throughout the plant from the stress area, but rather accumulates *in situ* in the stressed organ. Scopoletin, however, may prove to be translocatable as indicated by experiments presently being conducted by Mr. Frank Einhellig in this laboratory. The amounts of scopoletin present were found to be minute in the

stressed UV plants and it could be that previous reports of scopoletin accumulation were the result of hydrolysis of scopolin during extraction, and that scopoletin does not naturally occur in significant quantities within the plant.

Another interesting aspect of the quantitative studies conducted on the tobacco roots was the very close correlation of chlorogenic acid to scopolin concentration (very close to 5:1 in all treatments). This fact, the lack of either neoCGA or B510 in the roots, and the knowledge that both of these compounds can influence IAA concentration (35), readily leads to the hypothesis that such a ratio could be critical to the maintenance of an IAA concentration conducive to growth.

It is possible that the influence of UV radiation on plant growth may be due to a direct effect of UV on IAA production or accumulation. Skoog (38) reported the direct inactivation of IAA by ionizing radiation. Hare and Kersten (20) reported an *in vitro* inactivation of IAA by UV radiation, and Popp and McIlvaine (31) clearly demonstrated that UV can reduce the endogenous auxin level in plants. This is not to say however, that the destruction observed by Popp and McIlvaine might not have been due to the stimulation, or repression, of intermediate compounds such as the phenolics mentioned here.

The data obtained from the sunflower UV experiments

show both direct, and inverse, relationships to the tobacco data. First, the accumulation of scopolin with higher UV treatments in leaves is consistent with the tobacco data. The fact that there is an early accumulation and then a falling off with age is somewhat in conflict with reports from other stress experiments in tobacco where there was a continuing increase of scopolin with increasing age of the plant after treatment. This is not necessarily surprising since scopolin does not occur naturally in sunflower in the appreciable quantities that it does in tobacco, the sunflowers received a longer dosage of UV, and the period of treatment occurred much earlier in their physiological lives. There is good evidence in sunflower also, that scopolin is not translocated, as both stems and roots contained no detectable scopolin, even when the leaves contained as much as 60 μg/g fresh weight. It is surprising that in the cotyledons (which remained on the plant) and the stems of the high UV treated plants, only traces of scopolin were found. Since these parts of the plant received high amounts of UV, it might be expected that they would produce significant quantities of scopolin.

The sunflower chlorogenic acid data, when compared to that obtained from the tobacco experiments, indicate quite possibly a different role for these compounds. There was apparently little difference in CGA concentration

between the young and old leaves (except in the high UV treated plants), and these phenolics were not present at all in detectable amounts during the first three weeks of the plant's very rapid growth. This suggests that they might be involved more prominently in lignification than in growth regulation. If this is so, the high concentration of chlorogenic acids in the cotyledons is hard to explain. The accumulation of CGA in the stems of the higher UV treated plants where lignification was obviously lacking, may implicate CGA further in the lignification process. The increased concentrations of the chlorogenic acids in the older leaves of sunflower in contrast to the reduced amounts found in the tobacco plants, suggests that the roles of these compounds are different in the two species.

The work reported here adds impetus to the knowledge that changing environmental conditions do affect the phenolic concentration of plants. This, and the increasing knowledge that many of these phenolic compounds have a phytotoxic effect on many higher plants and microorganisms, possibly indicates the importance of phenolic compounds in mediating the effects of environmental conditions.

Martin (26) pointed out that under favorable conditions the excretion of scopoletin is very low from intact roots, but that this excretion is increased under

more and more unfavorable conditions. The increase found in scopolin concentration under stress UV conditions is consistent with Martin's work and may correlate with the release of scopoletin from the plant. Little free scopoletin was found in the plant extracts, but this does not exclude the possibility that scopolin could be hydrolyzed and scopoletin released from the roots. Since both scopoletin and scopolin have been found to be effective germination inhibitors and growth substances, this might place them in a key role in the allelopathic relations of plants. Wilson's (52) discovery that scopolin is leached from the leaves of native sunflower may indicate another mode of release from the plant.

In addition to affecting IAA oxidase, Sondheimer (40) found that chlorogenic acid is a strong inhibitor of several enzyme systems. Rice (30) saw this as a possible chief role of this compound in the inhibition of seed germination, and growth of higher plants, bacteria, and fungi. Wilson (52) found greater allelopathic effects from soil near sunflower plants in October than in July, and hypothesized that possibly the only way chlorogenic acid can be released from sunflower is through decomposition. Therefore, the accumulation of chlorogenic acid by sunflower with age might cause its allelopathic effects in the initial weed stage of succession in abandoned fields in Oklahoma to be greater.

The role of UV light in the growth of plants has been shown to be significant, probably as a limiting factor in alpine, and northern taiga and tundra regions. The phenolic concentration changes observed with changes in UV intensity could affect the ability of plants to survive in these regions through either changes as metabolic regulators, or as phytotoxins. Tests for allelopathic effects produced by plants grown from seed in glass houses may be unreliable because of the low UV light intensity under such conditions which could produce an altered phenolic content. Light intensity may also be lowered in glass houses and Armstrong (2) reported a substantial increase in the chlorogenic acid concentration with increased light intensity.

CHAPTER V

SUMMARY

A simple and reliable method for the quantitation of chlorogenic acid, band 510, neochlorogenic acid, and scopolin on a fresh weight basis was developed utilizing one-dimensional paper chromatography. Age studies indicated a decrease in chlorogenic acid concentration with increased age in tobacco leaves and stems. While scopolin concentration also decreased in leaves with increased age, it increased in concentration in the bottom section of tobacco stems. The concentration of chlorogenic acid increased in sunflower with increased age of leaves, but only varied slightly in the remainder of the plant. An increase in scopolin concentration was found in sunflower leaves, and tobacco leaves and stems with increased UV radiation. Chlorogenic acid concentration was higher in tobacco roots, stems and leaves of plants treated at low UV intensities than in comparable plants grown under control and higher UV conditions. Similar UV experiments indicated the chlorogenic acid concentration of sunflower leaves to be least under low UV conditions, with comparable plants of control and higher UV treatments having higher

concentrations. Chlorogenic acid was found to vary little in stems and roots of sunflower under all UV conditions. Band 510 and neochlorogenic acid were found in both tobacco and sunflower, but in much higher concentrations in tobacco. Both isomers were found in most parts of the test plants except tobacco stems and roots. Changes in isomer ratios were not pronounced in UV treated plants, but did generally change with age of tobacco leaves. Hypotheses are presented which indicate the possible significance of phenolic compounds as lignin precursors and in mediating environmental conditions through effects on internal regulatory mechanisms. Internal concentrations of these compounds are possibly correlated with their release from the plant, and subsequently with allelopathic relations among plants.

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