EINHELLIG, Frank Arnold, 1938-

EFFECTS OF SCOPOLETIN ON GROWTH, CO_2 EXCHANGE RATES, AND CONCENTRATION OF SCOPOLETIN, SCOPOLIN, AND CHLOROGENIC ACIDS IN TOBACCO, SUNFLOWER, AND PIGWEED.

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EINHELLIG, Frank Arnold, 1938-

The University of Oklahoma, Ph.D., 1969
Botany

University Microfilms, Inc., Ann Arbor, Michigan

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

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A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

FRANK ARNOLD EINHELLIG

Norman, Oklahoma

1969

EFFECTS OF SCOPOLETIN ON GROWTH, CO_2 EXCHANGE RATES, AND CONCENTRATION OF SCOPOLETIN, SCOPOLIN, AND CHLOROGENIC ACIDS IN TOBACCO, SUNFLOWER, AND PIGWEED

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ACKNOWLEDGMENT

I wish to express my sincere thanks to my advisors, Dr. Elroy L. Rice and Dr. Paul G. Risser, for their advice and encouragement during the course of this research. I am also very appreciative of the initial encouragement and help I received from Dr. Lawrence M. Rohrbaugh. The suggestions, advice, and critical reading of the other members of my committee, Dr. Simon H. Wender and Dr. George Goodman, were also a tremendous aid in this work.

I wish to express my thanks to my wife, Trude, and our children, Robert and Richard, for their understanding and sacrifice in the completion of this research.

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CHAPTER I

INTRODUCTION

The presence of scopoletin, 6-methoxy-7-hydroxy coumarin, has been reported in a wide variety of plants. Winkler (1967) listed over 50 species of plants in which scopoletin was identified. They included a range of plant families from Gramineae, such as Avena sativa L. (Goodwin and Kavanagh, 1949), to woody dicots like Prunus domestica L. (Hillis and Swain, 1959) in the Rosaceae. Robinson (1963) stated that scopoletin is the most common coumarin of higher plants.

Dieterman et al. (1964a) found both scopoletin and its glycoside, scopolin, to increase in tobacco plants treated with 2, 4-dichlorophenoxyacetic acid. Scopolin has also been reported to increase in concentration in tobacco and sunflower under stress conditions of UV light (Koeppe, 1968), in nitrogen and boron deficient tobacco (Armstrong, 1968; Watanabe et al., 1961), and

2, 4-dichlorophenoxyacetic acid treated sunflowers (Dieterman et al., 1964b). Best (1944) found accumulations of scopoletin in areas of tobacco plants infected with tomato spotted wilt virus. Scopoletin was abundant in potato tubers infected with leaf roll virus (Andreae, 1948), with greatest fluorescence occurring in cells next to necrotic phloem tissue (Sanford and Grimble, 1944). Sequeira and Kelman (1962) found that treatment of tobacco and tomato cuttings with scopoletin at the levels found in tobacco invaded by Pseudomonas solanacearum (Erw. Smith) Erw. Smith was highly injurious. Goodwin and Kavanagh (1949) reported scopoletin to be at least 20 times greater in mature tissue than in the growing tip of oat roots, and Tryon (1956) found approximately 18 times more scopoletin in differentiating tobacco tissue cultures than those which did not produce organized structures. Therefore, even though hypotheses concerning the function of scopoletin are presently inconclusive, they center around a possible role in growth regulation.

Andreae (1952) and Andreae and Andreae (1953) indicated that scopoletin inhibited the oxidation of indoleacetic acid and suggested it acted as a competitive inhibitor. Sequeira (1964) noted inhibition of indoleacetic acid oxidase which was correlated with an increase in scopoletin in diseased tobacco tissue, but his data indicated non-competitive inhibition. According to

Pollock, Goodwin, and Greene (1954), scopoletin inhibited growth of roots of Phleum pratense L. and Avena sativa L. Kohlmuenzer (1965) reported that scopoletin was an inhibitory portion of the extract from Galium mollugo L. when applied to Helianthus annuus L. However, with a low concentration of scopoletin in tobacco callus tissue culture, Skoog and Montaldi (1961) found some apparent stimulation which was attributed to sparing of indoleacetic acid. Worsham, Klingman, and Moreland (1962) reported that scopoletin at low concentrations activated the germination of seeds of Striga asiatica Kuntze, and Andreae (1952) found a 1 ppm scopoletin solution promoted growth of pea roots.

Little evidence, however, is available concerning the extent and nature of any inhibitory or stimulating action on the whole plant. Therefore, this study was designed to determine the effects of scopoletin on the growth of plants and on amounts of certain phenolics present. Since Sargent and Skoog (1960) found a fairly steady state equilibrium between scopoletin and scopolin in tobacco tissue culture, quantitative studies of these two were logical. It was further proposed that if scopoletin were taken up by plants, it could well be reflected in chlorogenic acid levels since the caffeic acid subunit of chlorogenic acid has been proposed (Steck, 1967) as a possible step in biosynthesis of scopolin in tobacco leaves.

In order to establish some mode of action of scopoletin as a growth regulator, it was suggested that one or both of the major processes of photosynthesis and respiration might be affected. Therefore, experiments were designed to test the hypothesis that scopoletin levels and total plant growth were related in some manner with photosynthesis and respiration rates. The assumption followed that any rate difference and growth change should parallel changes in the plant composition.

CHAPTER II

METHODS AND MATERIALS

Growth Procedure

All experimental plants were germinated and grown under a 16 hour photoperiod in Percival growth chambers with a light intensity of 1000 ft-c as measured by a model 756 Weston Illumination meter with a quartz filter. The light/dark temperatures were respectively 29/21° C. Plants were germinated in pure quartz sand and watered periodically with Fe-EDTA Hoagland's nutrient solution (Hoagland and Arnon, 1950) and distilled water. Seedlings were selected for uniformity and transplanted to light free plastic vials containing a 2:5 ratio of nutrient solution to distilled water.

Tobacco plants (Nicotiana tabacum L. var. One Sucker) were transplanted about 6 weeks after germination. After a 9-12 day period of acclimatization, these seedlings were again selected for uniformity before treatment with scopoletin. For all growth experiments scopoletin (Sigma Chemical Company) was made up in concentrations of 10^{-3}M, 5 x 10^{-4}M, 10^{-4}M, 5 x 10^{-5}M, 10^{-5}M, and 10^{-6}M with a 2:5 ratio of nutrient solution to distilled water as the solvent. The

pH was adjusted to 6.0. Fifteen plants were treated at each concentration by transferring them to 40 ml vials containing the scopoletin solution. Fifteen control plants were transferred to vials containing a 2:5 ratio of nutrient solution to distilled water. Tobacco seedlings were placed randomly back in the growth chamber where all required additions of solution were made with the 2:5 ratio of nutrient solution to distilled water. After 11 days, the shoots and roots were separated and oven-dry weights determined after 48 hours at 105° C. In one experimental set, fresh weights were taken before dry weights for a fresh:dry weight comparison.

Sunflower seeds (Helianthus annuus L. var. Russian Mammoth) were soaked 24 hours in distilled water as a prerequisite to germination to obtain a more uniform germination. While in an early two leaf stage, 7 days after germination, sunflower seedlings were transplanted to vials. After 3 days in vials, treatment was made following the procedure used with tobacco except larger vials and 70 ml of solution were used. Other procedures followed those used in tobacco studies. Root separation was made by shaving roots from the hypocotyl and cutting off the main root at the hypocotyl base.

Rough pigweed (Amaranthus retroflexus L.) seedlings were transplanted to nutrient solution 14 days after germination and treated with 40 ml of solution 3 days

later. The difference in seedling size necessitated the different time periods. Pigweed seedlings were grown 14 days before dry weights were taken and no attempt was made to separate shoots and roots or take a fresh:dry weight ratio.

Scopoletin, Scopolin, and Chlorogenic Acid Determinations

Tobacco plants to be analyzed for concentrations of scopoletin, scopolin, and chlorogenic acids were grown as previously described with treatment concentrations of 10^{-4}M and 5×10^{-4}M. Forty plants were used for each treatment concentration and controls. At harvest, plants of each treatment were randomly divided into 10 groups of 4 plants. Each group was harvested as one sample in order to have adequate plant material for chemical analysis. Plants were separated into shoots and roots, fresh weights were taken, and harvest was completed by fixing in boiling isopropyl azeotrope (88% isopropanol, 12% water) for 5 minutes. The fixed plant material was ground in a blender, transferred to a Soxhlet extraction thimble, and then washed 3 times. A 60 ml wash of boiling isopropyl azeotrope:water (1:1, v/v) was followed by 75 ml boiling IMBW (isopropanol:methanol:benzene:water, 2:1:1:1, v/v/v/v) and 60 ml of boiling isopropyl azeotrope. The thimble containing the washed plant material was placed in a Soxhlet extractor for 24 hours extraction with isopropyl

azeotrope and a final 24 hours extraction with isopropanol. Solvents of fixing, washing, and extraction steps were combined and evaporated to dryness *in vacuo*. The residue was brought to 50 ml volume in IMBW in each case.

Analysis of sunflowers for phenolic concentration differed only in plant number. The larger plant size allowed 2 plants instead of 4 to be used for each sample extraction. In addition, because of the variation of cotyledons at this stage and the reported high concentration of chlorogenic acid in cotyledons (Koeppe, 1968), those still remaining on plants at harvest were discarded.

Quantification of scopolin, chlorogenic acid (3-O-caffeoylquinic acid), band 510 (4-O-caffeoylquinic acid), and neochlorogenic acid (5-O-caffeoylquinic acid) followed the methods of Koeppe (1968) which utilized descending, one dimensional paper chromatography with a solvent system of KFW (methylisobutyl ketone:formic acid:water, 14:3:2, v/v/v). The separation procedure involved streaking an aliquot of the sample extract along a 15 cm origin line on Whatman #1 paper (23 x 57 cm) which had previously been washed 20 hours with 5% methanol. After 20 hours development in a non-equilibrated small glass cab, distinct fluorescent bands of the compounds could be observed under UV light. As reported by Koeppe (1968) and Armstrong (1968), no additional fluorescent compounds were found in each band.

Scopoletin was separated from the extract with separate chromatographs using IFW (isopropanol:formic acid:water, 5:4:95, v/v/v) as the solvent system. After 6 hours development, scopoletin, having an R_f of 0.40, was separate from all other fluorescent compounds. Absorption spectra, cochromatography with authentic scopoletin, and chromatography in other solvent systems verified the purity of this band.

The fluorescent bands concerned in both systems were cut out, eluted from the paper for a 20 hour period with 5% methanol, brought to a known volume with 5% methanol, and read photometrically against a blank which had been eluted from paper run through identical procedures. The chlorogenic acids were read on a Beckman DB-G spectrophotometer at a wavelength of 324 mμ. Scopolin and scopoletin were read on a model 110 Turner fluorometer using pyrex cuvettes and a high sensitivity attachment at a setting of 1X. The primary filter was #7-60 with secondary filters #2A plus #48 (Kodak Wratten filter).

Internal standard reference curves (Fig. 1, 2, 3) were prepared by running known amounts of authentic compounds through the chromatographic system used. Following Zucker, Nitsch, and Nitsch (1965), quantification of the three chlorogenic acid isomers was made on the standard curve for chlorogenic acid since the molecular extinction coefficients of the three were assumed to be the same.

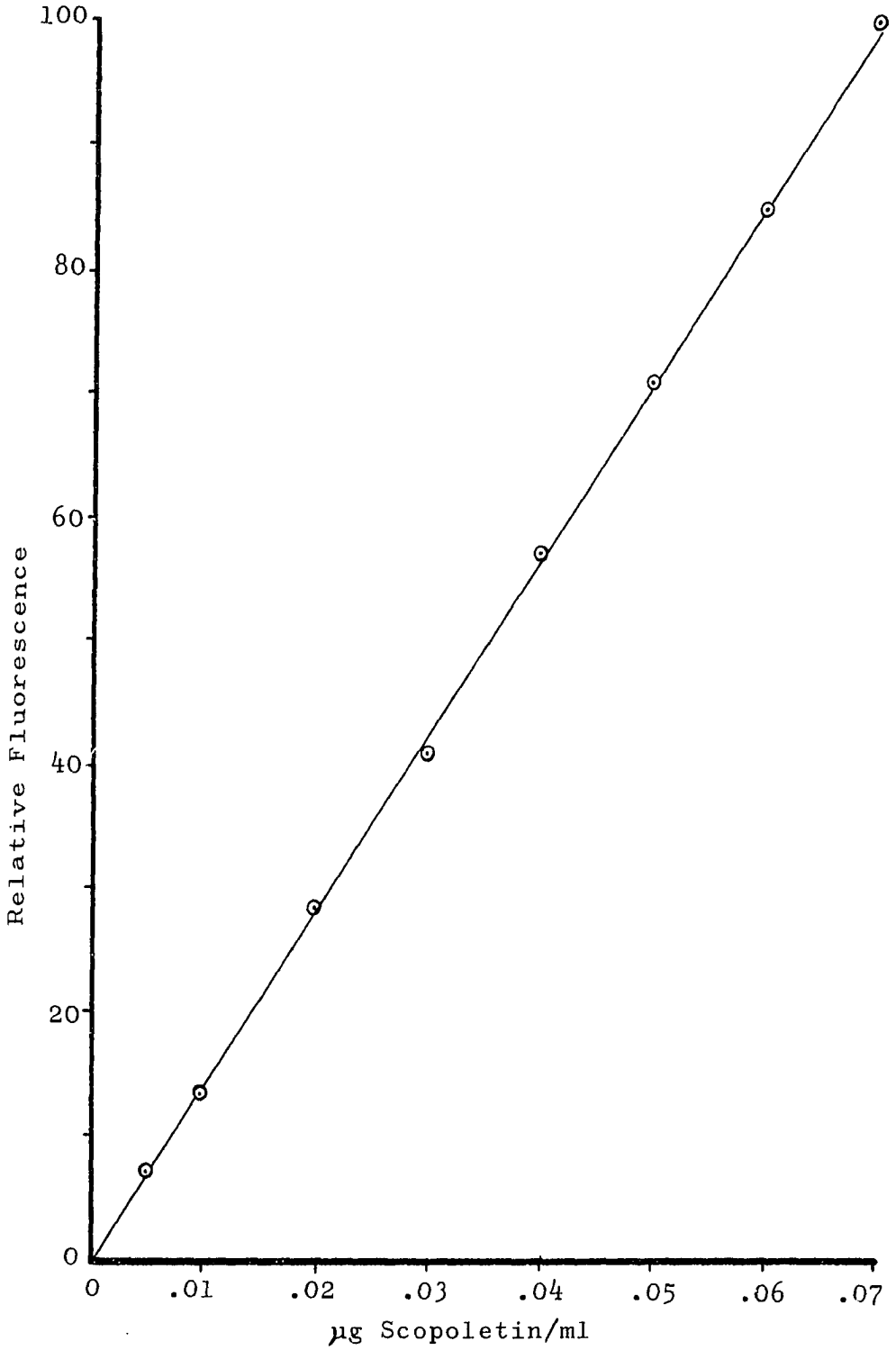

Fig. 1. Relationship of relative fluorescence and scopoletin concentration on the model 110 Turner fluorometer.

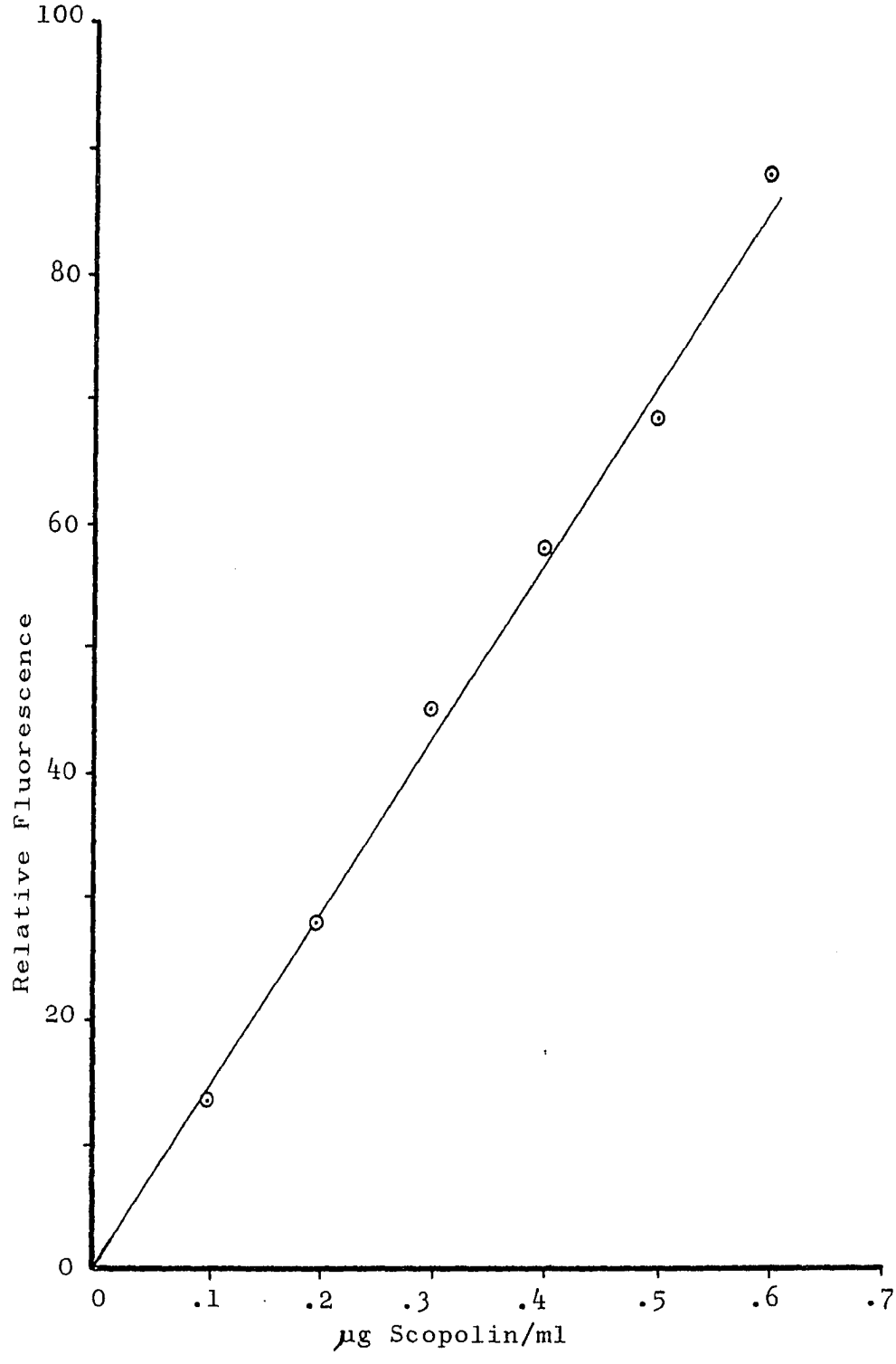

Fig. 2. Relationship of relative fluorescence and scopolin concentration on the model 110 Turner fluorometer.

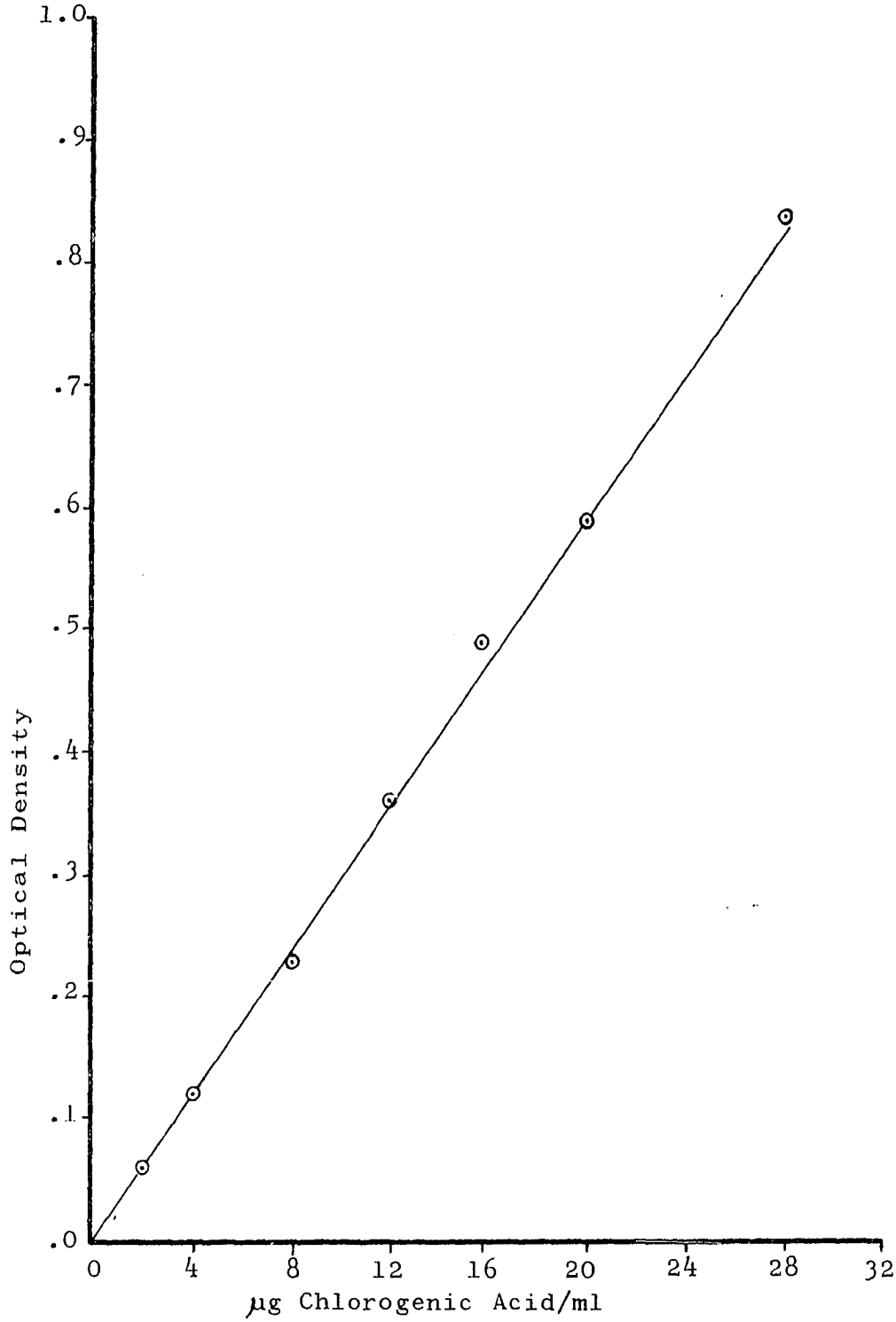

Fig. 3. Relationship of optical density and chlorogenic acid concentration read at 324 mµ on the Beckman DB-G spectrophotometer.

Chlorogenic acid recovery was 58% based on purissimum grade compound from Fluka AG, Buchs, Switzerland. Scopolin synthesized in the laboratory of Dr. S. H. Wender of this campus gave a recovery of 75% while 85% of the scopoletin was recoverable.

Procedures for CO_2 Analyses

A Beckman 215A infrared gas analyzer was used for determination of net photosynthesis and dark respiration. Since experimentation showed daily fluctuation in room atmosphere to be too large to allow meaningful data with an open system, a closed gas circulation was adopted. Pressurization within the analyzer resulted when all circulation was directed through the machine, so a closed system with a by-pass was devised (Fig. 4) whereby only a part of the circulating air was monitored through the analyzer at 500 cc/min. Four 150 w G. E. reflector floods with the light surface 46 cm from the leaf crown provided 1000 ft-c. A 3 cm layer of flowing water filtered heat radiation and controlled the temperature at 25±1° C. Glass chambers of different size were used for tobacco, sunflower, and pigweed to gain maximum sensitivity. Previously described growth steps were followed for control plants, 10^{-3}M, and 5 x 10^{-4}M scopoletin treatments.

Net photosynthesis was measured by taking plants directly from the growth chamber and placing the whole plant and vial in the analysis chamber. After a minimum

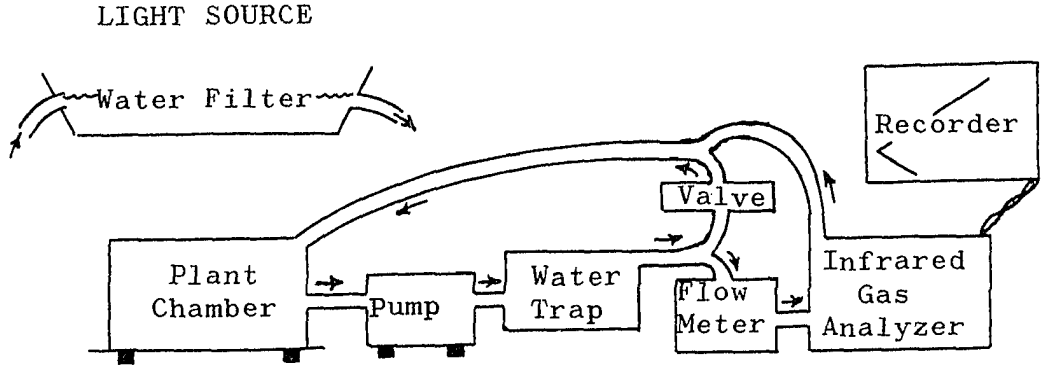

Fig. 4. Flow diagram of CO_2 analysis.

of 15 minutes stabilization time, CO_2 reduction was checked during the next 15 minute interval, or longer. All readings were taken between a CO_2 concentration range of 420 to 275 ppm since a straight line uptake was found within and surpassing this range. Respiration was steady within a few minutes after lights were shut off. Therefore, after 15 minutes dark stabilization, respiration was monitored during the next 15 minute interval. All CO_2 changes were related to the plant leaf area which was established each time by tracing leaves immediately after the analysis and measuring to the nearest 0.1 cm^2 with a model 620005 Keuffel & Esser compensating polar planimeter. Gas volume measurements were corrected for the average barometric pressure and a temperature of 25° C.

For each treatment in tobacco the same four plants were analyzed individually over a 12 day period including monitoring immediately prior to scopoletin treatment. Plants were randomized initially and the order of analyses

kept the same for each daily sequence. A daily series for sunflower was run in the same manner except 8 plants were used for each treatment with 2 run simultaneously at each analysis. Sunflower elongation made light adjustment necessary to keep 1000 ft-c constant at the leaf crown.

Daily analyses were not made for pigweed. Instead, net photosynthesis and dark respiration measurements were taken on control and 10^{-3}M scopoletin treated pigweed by monitoring individually 10 plants of each series on the fifth day after treatment. Similar experiments were also carried out for tobacco and sunflower for purposes of statistical analyses.

CHAPTER III

EXPERIMENTAL RESULTS

Effects of Scopoletin on Seedling Growth

Scopoletin treated tobacco of the 10^{-3}M series showed a mottled, greenish appearance in the roots after a few hours treatment. Pollock et al. (1954) reported similar observations. Within one day, the greenish discoloration was quite distinct and it continued to increase with time. After several days, a very slight root discoloration could be detected in 10^{-4}M treatments, but it was not apparent at lower concentrations. After 3 days treatment with the 10^{-3}M scopoletin concentration, tobacco plants were stunted and showed loss of some leaf turgor. Stunting was apparent in the 5×10^{-4}M scopoletin treated plants by the fifth day. Seedlings of both these treatments checked under UV light showed very substantial fluorescence along the leaf veins by day 5. At harvest, fluorescence was still abundant in old leaves, but not in new leaves. Yellowing of old leaves increased throughout the experiment in the 10^{-3}M series and wilting remained.

In sunflower plants the same general root and leaf fluorescence, and stunting conditions prevailed. Some

leaf twisting but little leaf yellowing was visible, and turgor loss was not comparable to that of tobacco. As in tobacco, plants growing in scopoletin concentrations of 10^{-4}M and lower were indistinguishable in size from the controls. Pigweed seedlings also exhibited symptoms similar to tobacco except the 10^{-3}M scopoletin treated plants were even more severely stunted, and the first leaves were often lost by time of harvest.

The same inhibitory trend was exhibited in all three species with greatly reduced growth in the 10^{-3}M treatments and less reduction, but still a significant retardation, at the 5×10^{-4}M level (Table 1). It was also found that in all cases the 10^{-3}M scopoletin treated plants were inhibited significantly more than the 5×10^{-4}M. Therefore, it appeared that a fairly good correlation existed between growth reduction and scopoletin concentration within this inhibitory range. Sunflower in one experiment appeared to be stimulated in the 10^{-4}M and 5×10^{-5}M series, but this was not reproducible (Table 1, Expt. 2). The fresh:dry weight ratios in tobacco and sunflower showed no significant trends with respect to treatment. The overall mean for this ratio was 13.5 for tobacco and 9.5 for the sunflower.

Only those plant series that showed a growth reduction had an alteration in shoot:root ratio (Table 2). All tobacco experiments had a significantly lower shoot:root

Table 1. Effects of scopoletin on seedling growth.

Species	Expt. No.	Mean oven-dry weights in mg/plant[c] Control	10^{-6}M Scopoletin	10^{-5}M Scopoletin	$5x10^{-5}$M Scopoletin	10^{-4}M Scopoletin	$5x10^{-4}$M Scopoletin	10^{-3}M Scopoletin
Tobacco	1	172.8	171.1	154.7	161.1	158.0	60.2[a]	31.6[a]
	2	147.4	159.8	178.5	156.9	162.9	54.6[a]	31.8[a]
	3	218.2	219.7	217.6	219.0	209.4	138.8[a]	66.0[a]
Sun-flower	1	725.8	672.2	637.7	686.9	703.3	407.8[a]	207.9[a]
	2	768.6	783.3	886.7	939.6[b]	953.1[b]	630.8[a]	407.5[a]
Pigweed	1	130.3	123.5	130.3	102.1	118.2	37.7[a]	7.6[a]
	2	195.5	205.0	196.0	200.5	194.8	78.0[a]	40.0[a]

[a]Weights differ significantly below the 1% level from that of the control and all other treatments.

[b]Weights differ significantly from the control below the 5% level.

[c]Each figure represents the mean of 15 plants.

Table 2. Effects of scopoletin on seedling shoot:root ratio of oven-dry weights.[1]

Species	Expt. No.	Control	Scopoletin Concentration					
			10^{-6}M Scopoletin	10^{-5}M Scopoletin	$5x10^{-5}$M Scopoletin	10^{-4}M Scopoletin	$5x10^{-4}$M Scopoletin	10^{-3}M Scopoletin
Tobacco	1	5.4	5.0	5.0	5.5	5.1	4.0[a]	4.0[a]
	2	4.7	4.6	4.1	4.3	3.8	3.3[a]	3.4[a]
	3	4.1	4.2	4.1	3.9	3.7	3.6[a]	3.1[a]
Sun-flower	1	6.2	6.3	5.5	5.2	5.8	6.3	5.7
	2	4.6	5.1	5.0	4.1	4.5	4.3	2.7[a]

[1]Each entry represents the mean ratio of 15 plants.

[a]Differs significantly below the control at the 5% level, or better.

ratio in 10^{-3}M and 5 x 10^{-4}M groups than in control plants, but these two treatments did not show a significant difference from each other. A reduction of the sunflower shoot: root ratio was evident only in 10^{-3}M treated plants in one experiment (Table 2).

Scopoletin Effects on Scopoletin, Scopolin, and Chlorogenic Acid Content in Tobacco

A 5 x 10^{-4}M series of tobacco plants was analyzed for differences in scopolin, scopoletin, and chlorogenic acids even though there was no evidence of leaf damage since their growth was retarded. A 10^{-4}M scopoletin treatment series was also checked in each case to determine scopoletin, scopolin, and chlorogenic acid alterations that might occur without growth effects. No scopoletin was present in any nutrient solution at the end of the experiments and quantitative analyses of tobacco extracts indicated that scopoletin was taken into the plant (Fig. 5). Although no radioisotope tracer studies were conducted, present radioisotopic tracer studies in this laboratory indicate that scopoletin does enter the plant. The concentration of scopolin changed even more markedly with scopoletin treatment (Fig. 6). The increase in scopolin in relationship to the control plants was greater for the shoot than roots in all treatments and the scopolin: scopoletin ratio increased in the shoots. Scopoletin buildup in the 10^{-4}M treated plants was quite close in the

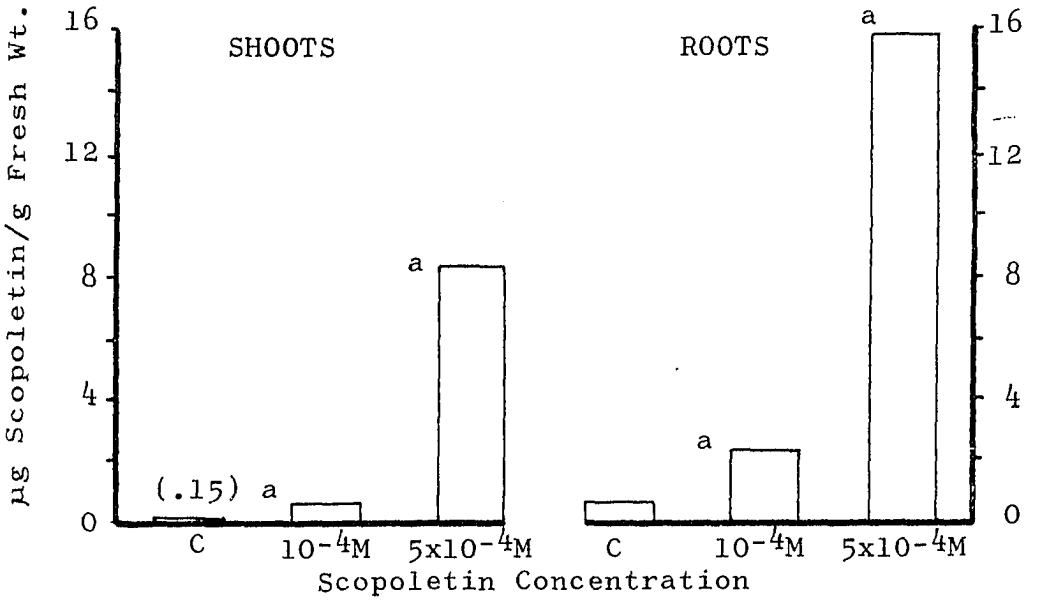

Fig. 5. Effects of scopoletin treatment on concentration of scopoletin in tobacco. Each bar is a mean of 10 samples. C = control. [a]Differs significantly from all others at the 1% level.

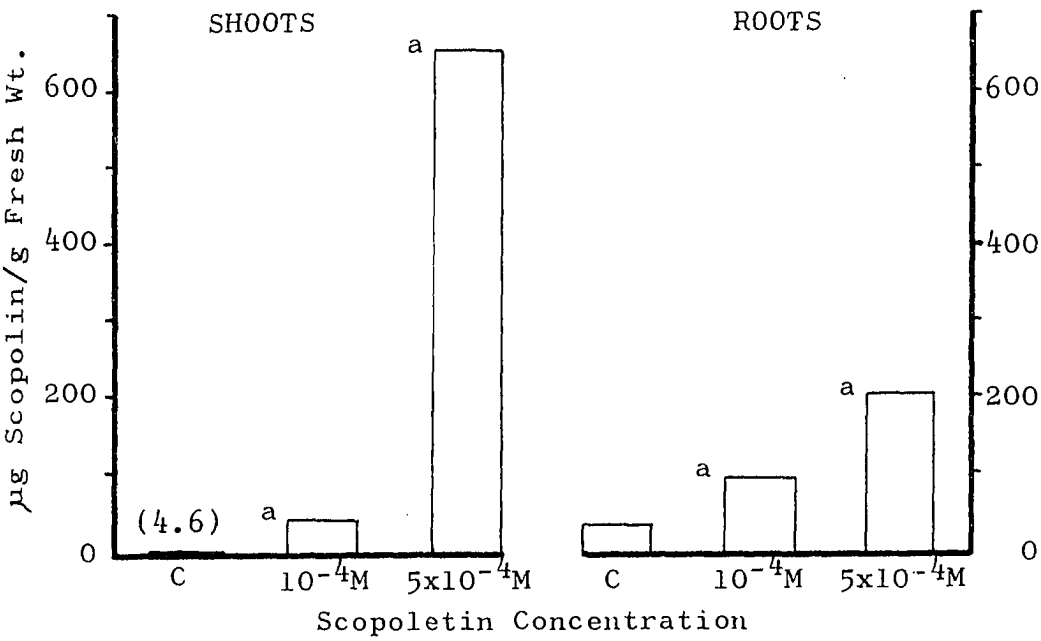

Fig. 6. Effects of scopoletin treatment on concentration of scopolin in tobacco. Each bar is a mean of 10 samples. C = control. [a]Differs significantly from all others at the 1% level.

shoots and roots, whereas, at the 5 x 10^{-4}M level the shoots had a greater increase of scopoletin than roots (Fig. 5).

Chlorogenic acids showed more overall variability than scopolin and scopoletin, and no significant changes in the total chlorogenic acids were observed (Fig. 7). Statistically, the neochlorogenic acid of the 10^{-4}M tobacco shoots differed significantly from the control, but no pattern seemed apparent with respect to the 5 x 10^{-4}M group (Fig. 8). Band 510 and neochlorogenic acid isomers were not detectable on the chromatograms of root extracts by either UV or a color test using ferric chloride-potassium ferricyanide reagents (Smith, 1960). Koeppe (1968) previously reported the absence of these isomers in tobacco roots.

Scopoletin Effects on Scopoletin, Scopolin, and Chlorogenic Acid Content in Sunflowers

Neither scopoletin nor scopolin was detected with UV light on chromatograms of sunflower control roots. In addition, no scopoletin band was visible with the ferric chloride-potassium ferricyanide reagent (Smith, 1960). No band 510 or neochlorogenic acid was detected in any sunflower roots, but only small amounts of roots were analyzed, so it is possible that these compounds are present in trace amounts. Higher concentrations of chlorogenic acids were found in sunflowers than reported

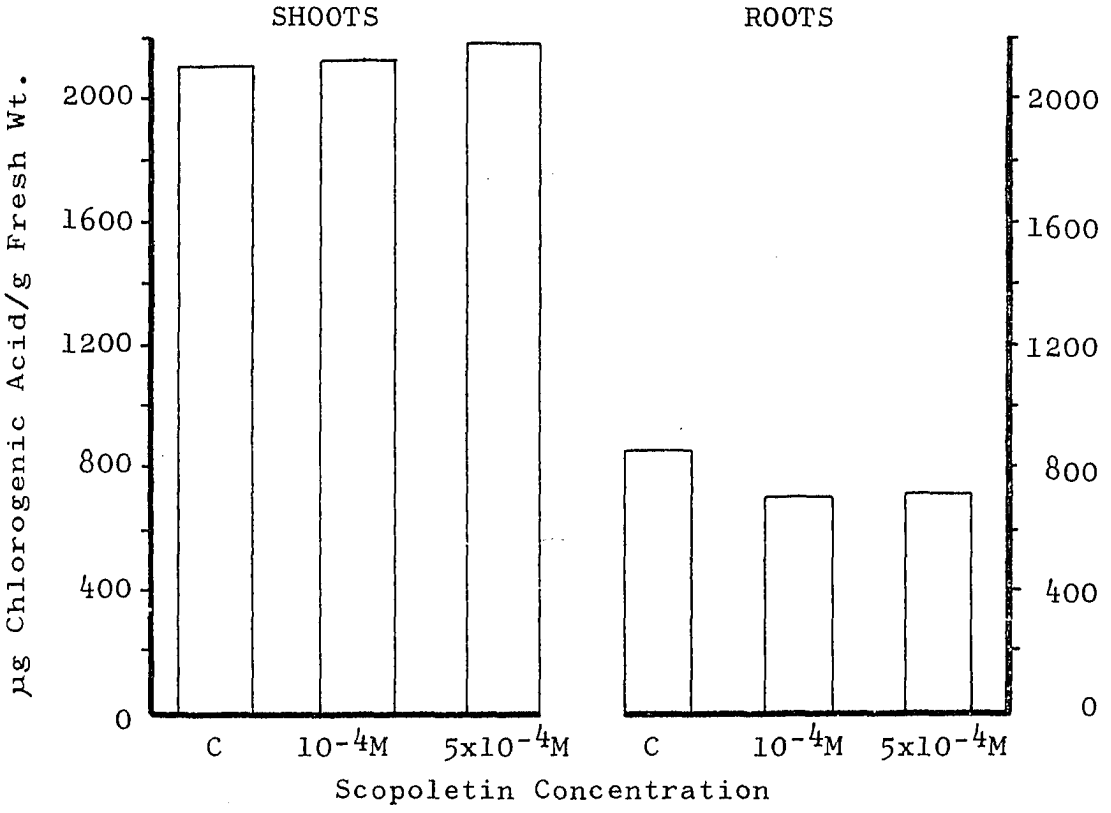

Fig. 7. Effects of scopoletin treatment on concentration of total chlorogenic acid in tobacco. Each bar is a mean of 10 samples.

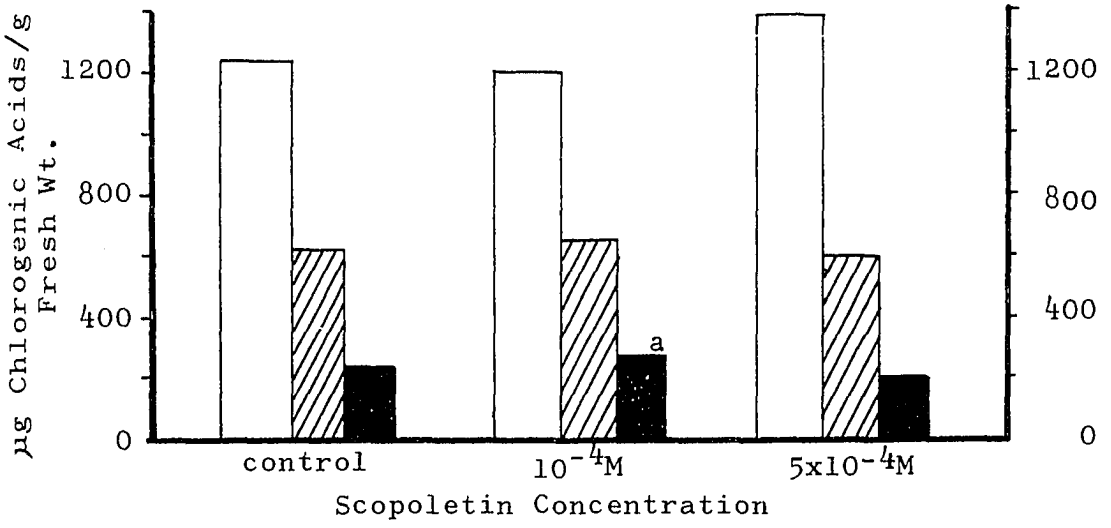

Fig. 8. Effects of scopoletin treatment on concentration of chlorogenic acid isomers in tobacco shoots. Open bar = chlorogenic acid; Slashed bar = band 510; Solid bar = neochlorogenic acid. Each bar is a mean of 10 samples. [a]Differs significantly from the control at the 5% level.

by Koeppe (1968). Since Armstrong (1968) reported a large build up of chlorogenic acids with nitrogen deficiency, the higher concentrations found in these experiments could be due to the use of the dilute nutrient solution instead of the double strength solution used by Koeppe (1968)

The pattern of scopoletin and scopolin accumulation in the sunflower paralleled closely that of tobacco even though the concentrations were different (Fig. 9, 10). For comparison, all scales were kept the same as tobacco. Both compounds showed increases in accordance with increases in treatment concentration (Fig. 9, 10). As in tobacco, the greatest change was the magnitude of the scopolin increase in shoots with the 5×10^{-4}M treatments. Similarly, no scopoletin was found in the treatment solution at harvest and total chlorogenic acids in sunflowers showed no significant changes although there appeared to be a possible shifting of isomer ratios (Fig. 11, 12).

Effects of Scopoletin on Photosynthesis and Respiration in Tobacco, Sunflower and Pigweed

Alteration of light intensity on tobacco seedlings grown under the conditions previously stated showed the light compensation point, using a step-wise reduction in intensity, to be about 100 ft-c. The CO_2 compensation point at 1000 ft-c was about 68 ppm with some variation among plants. Similar figures for sunflower were 150 ft-c, and 70 ppm. During the 12 day check of CO_2 exchange, the

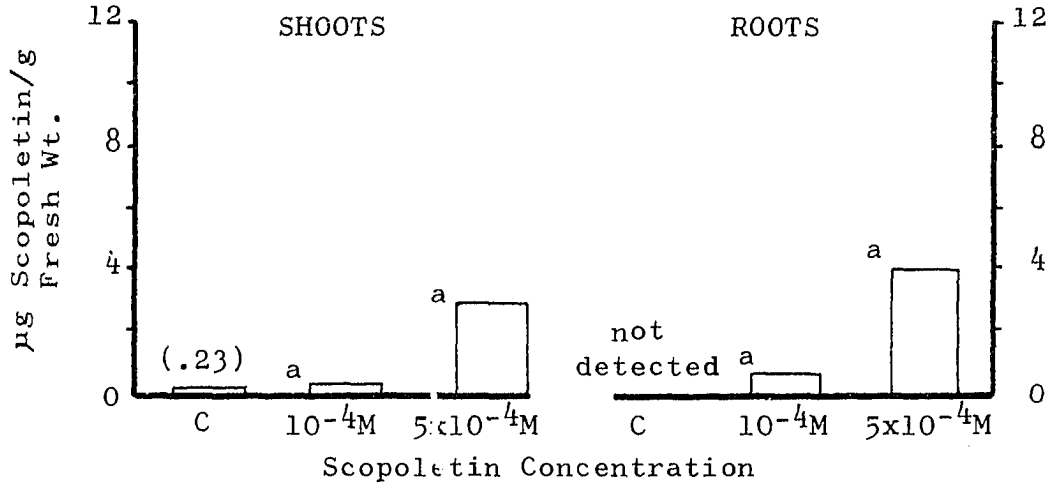

Fig. 9. Effects of scopoletin treatment on concentration of scopoletin in sunflowers. Each bar is a mean of 10 samples. C = control. [a]Differs significantly from all others at the 1% level.

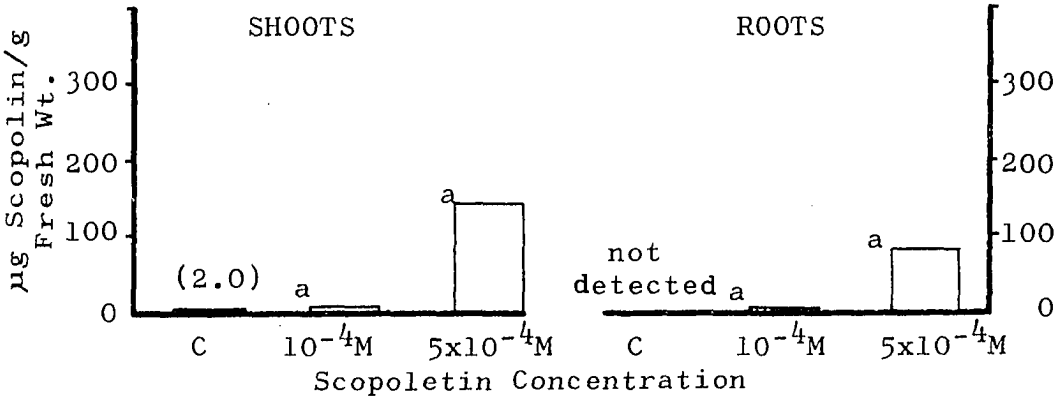

Fig. 10. Effects of scopoletin treatment on concentration of scopolin in sunflowers. Each bar is a mean of 10 samples. C = control. [a]Differs significantly from all others at the 1% level.

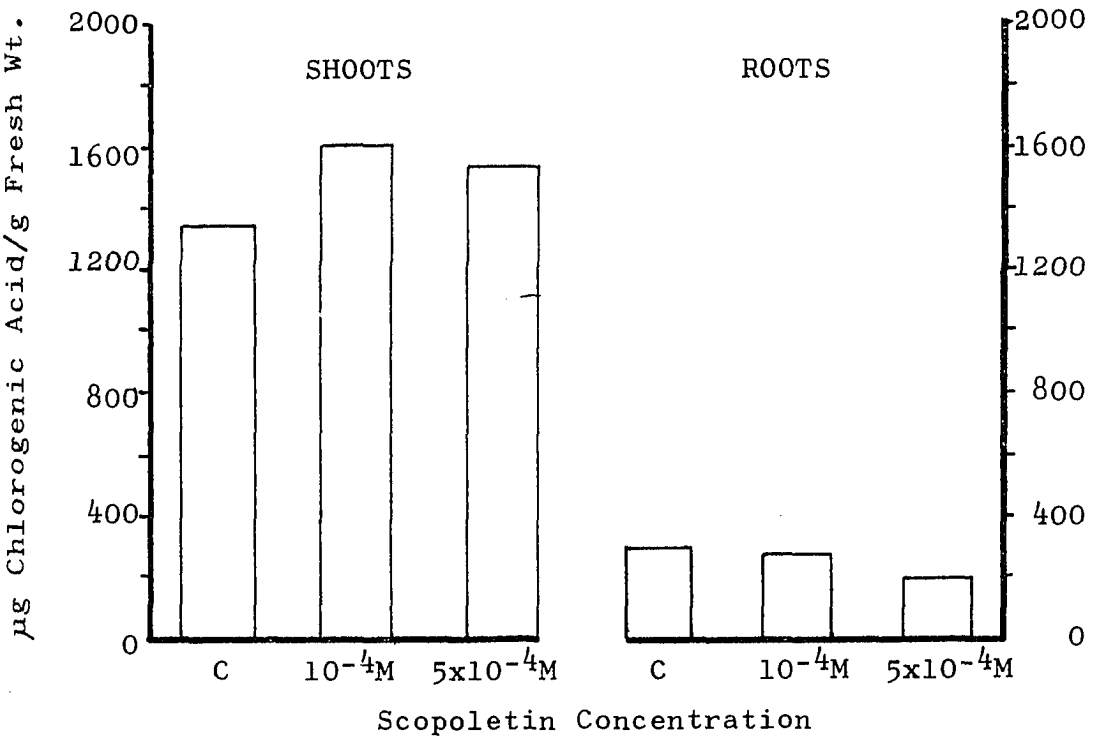

Fig. 11. Effects of scopoletin treatment on concentration of total chlorogenic acid in sunflower. Each bar is a mean of 10 samples.

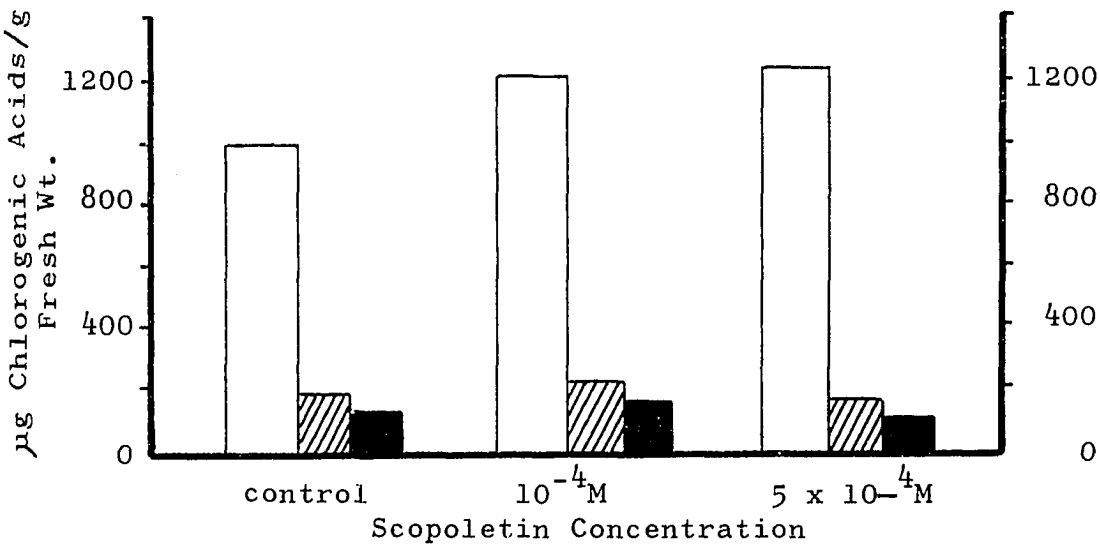

Fig. 12. Effects of scopoletin treatment on concentration of chlorogenic acid isomers in sunflower shoots. Open bar = chlorogenic acid; Slashed bar = band 510; Solid bar = neochlorogenic acid. Each bar is a mean of 10 samples.

rates of net photosynthesis in both tobacco and sunflower controls were reduced in the later days of the experiment (Fig. 13, 15). This might be explained by the reduction in photosynthetic rate found by Elmore, Hesketh, and Muramoto (1967) when mature leaves age. There was little difference between treatment and controls in respiration rates for both species (Fig. 13, 15). Net photosynthesis curves were drawn according to what appeared to be the best fit. Leaf area and respiration curves were plotted from a Fortran IV program using a model outlined by Jameson (1967) and run on the IBM 1130.

It was evident that there was a large reduction in net photosynthesis in tobacco by the second day after treatment in both 10^{-3}M and 5 x 10^{-4}M concentrations (Fig. 13). This reduction reached a low point by day 4 which was followed by a recovery phase. The degree of reduction correlated well with the concentration of scopoletin used in treatment. Correspondingly, leaf area expansion also correlated well with the concentration of scopoletin used for the treatment, with 10^{-3}M scopoletin treated seedlings having no growth during the time of very low photosynthesis (Fig. 14). Since first additions of nutrient solution were made after the analyses on day 5 in the 5 x 10^{-4}M series and after the day 9 analyses in the 10^{-3}M series, recovery of photosynthesis did not directly correlate with these additions. The average

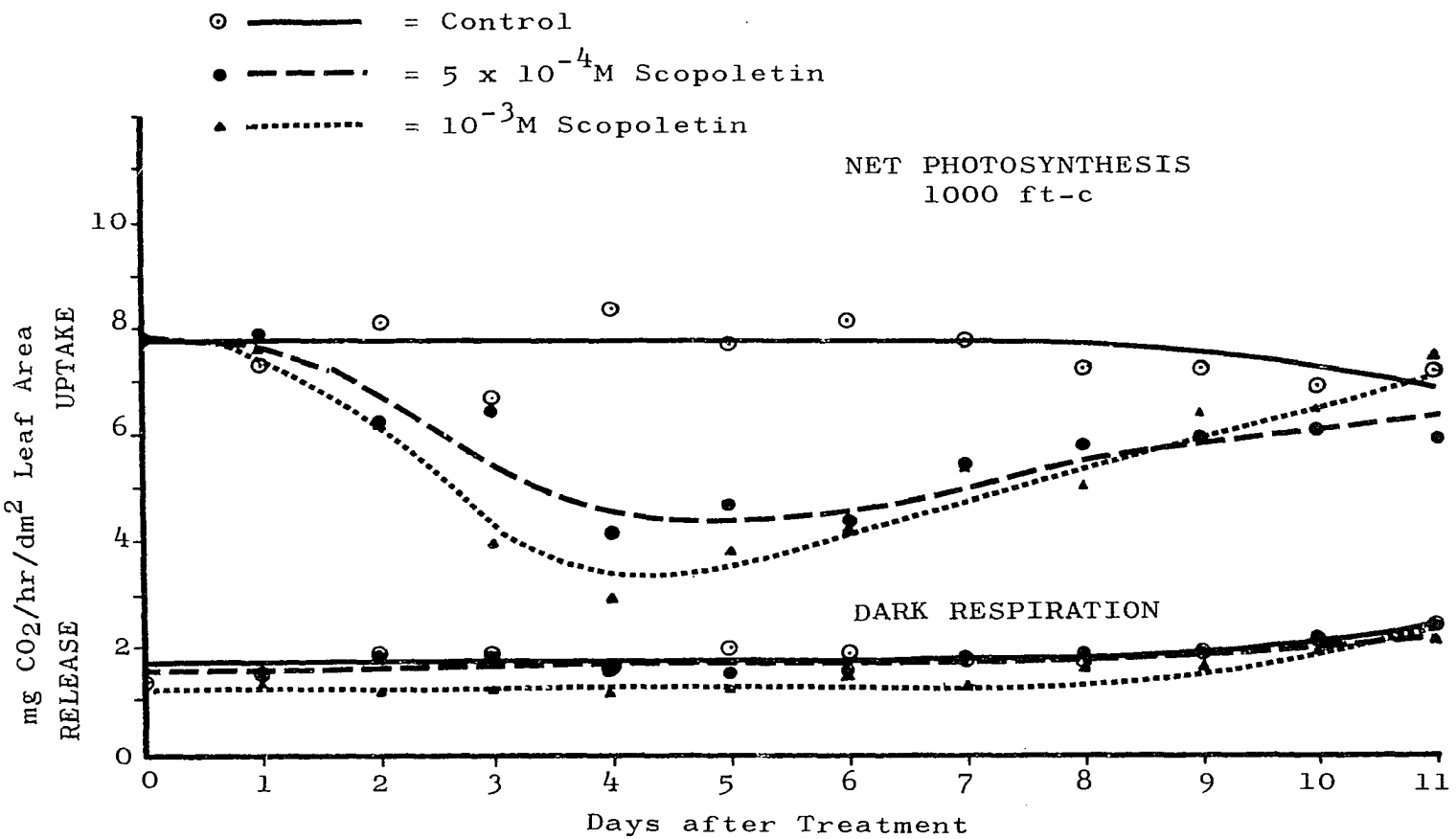

Fig. 13. Effects of scopoletin treatment on net photosynthesis and dark respiration in tobacco seedlings. Each point is a mean of 4 plants.

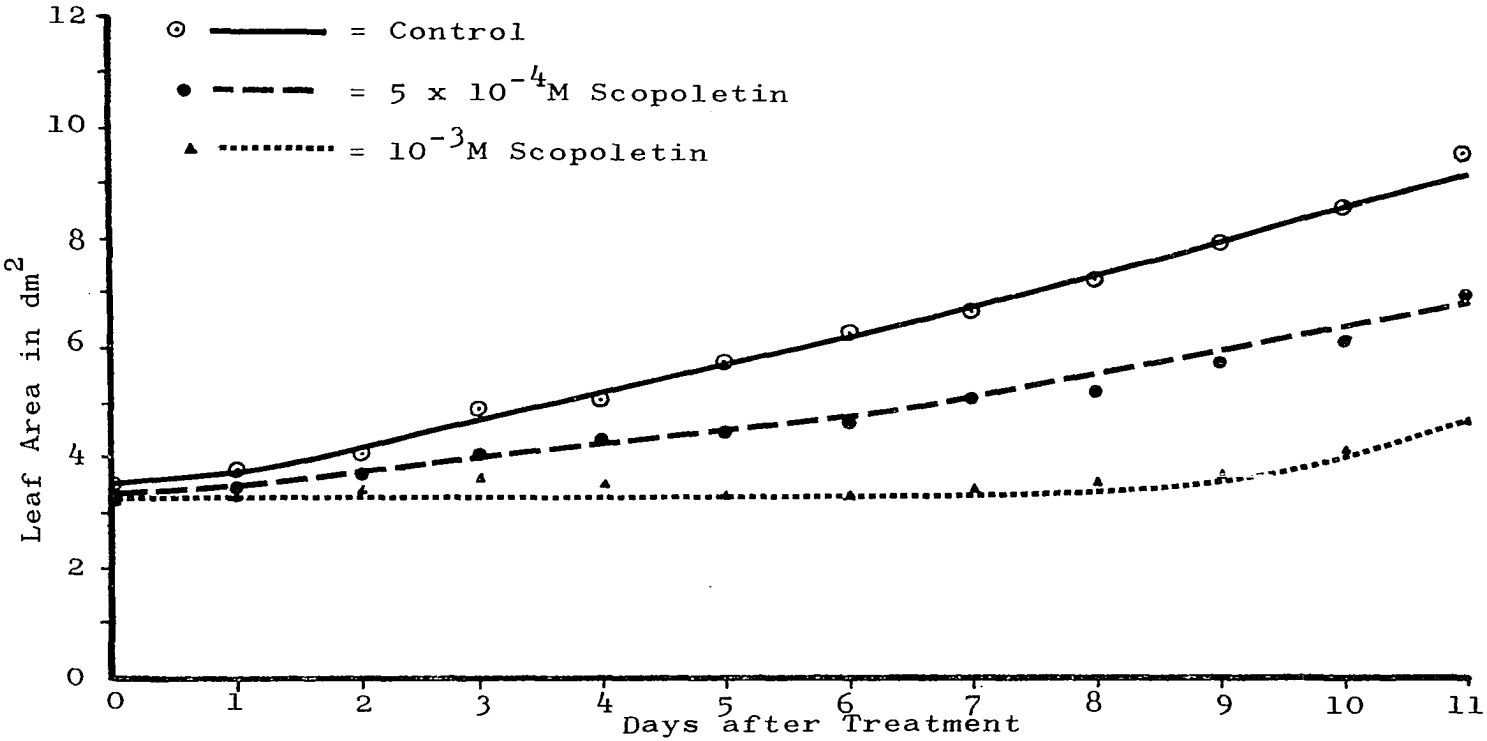

Fig. 14. Effects of scopoletin treatment on total leaf area of tobacco plants. Each point is a mean of 4 plants.

amounts of solution added during the total experiment to the controls, 5 x 10^{-4}M, and 10^{-3}M treated tobacco were respectively: 134 ml, 89 ml, and 47 ml.

Growth in leaf area and net photosynthetic rates in sunflower seedlings were suppressed less than in tobacco, but the repression lasted longer in the 10^{-3}M treatment. Both sunflower treatments showed recovery phases in photosynthesis and leaf expansion similar to those in tobacco (Fig. 15, 16). Additions of nutrient solution were first made to the 5 x 10^{-4}M series after analyses on day 4 and to the 10^{-3}M series after day 6.

Separate experiments involving just controls and a 10^{-3}M concentration of scopoletin confirmed that the photosynthetic rate was significantly reduced in all species, whereas dark respiration was not (Table 3).

Effects of Scopoletin on Amount of CO_2 Fixed in Tobacco and Sunflower

A calculation of CO_2 fixed per hour of illumination in the daily photosynthesis of tobacco and sunflower shows a striking relationship with reduced CO_2 fixation in 10^{-3}M and 5 x 10^{-4}M scopoletin treated plants (Fig. 17, 18). By the end of the experiment the 10^{-3}M scopoletin treated tobacco seedlings fixed only 51% as much CO_2 as the controls and the 5 x 10^{-4}M series fixed 60% (Fig. 17). Similar calculations with sunflowers are comparable. Both experiments correlated well with the fact that dry weights of

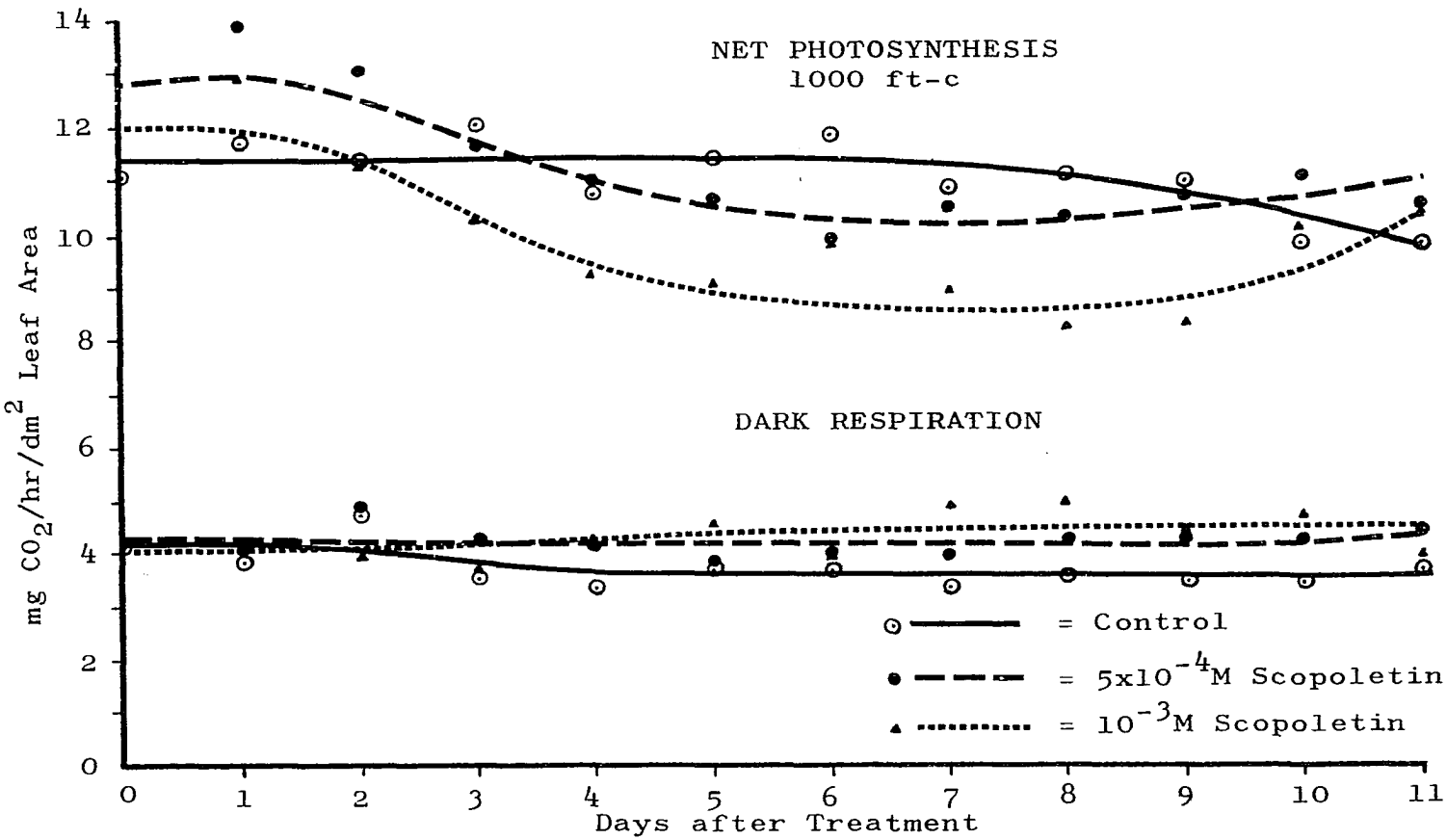

Fig. 15. Effects of scopoletin treatment on net photosynthesis and dark respiration in sunflower seedlings. Each point is a mean of 8 plants.

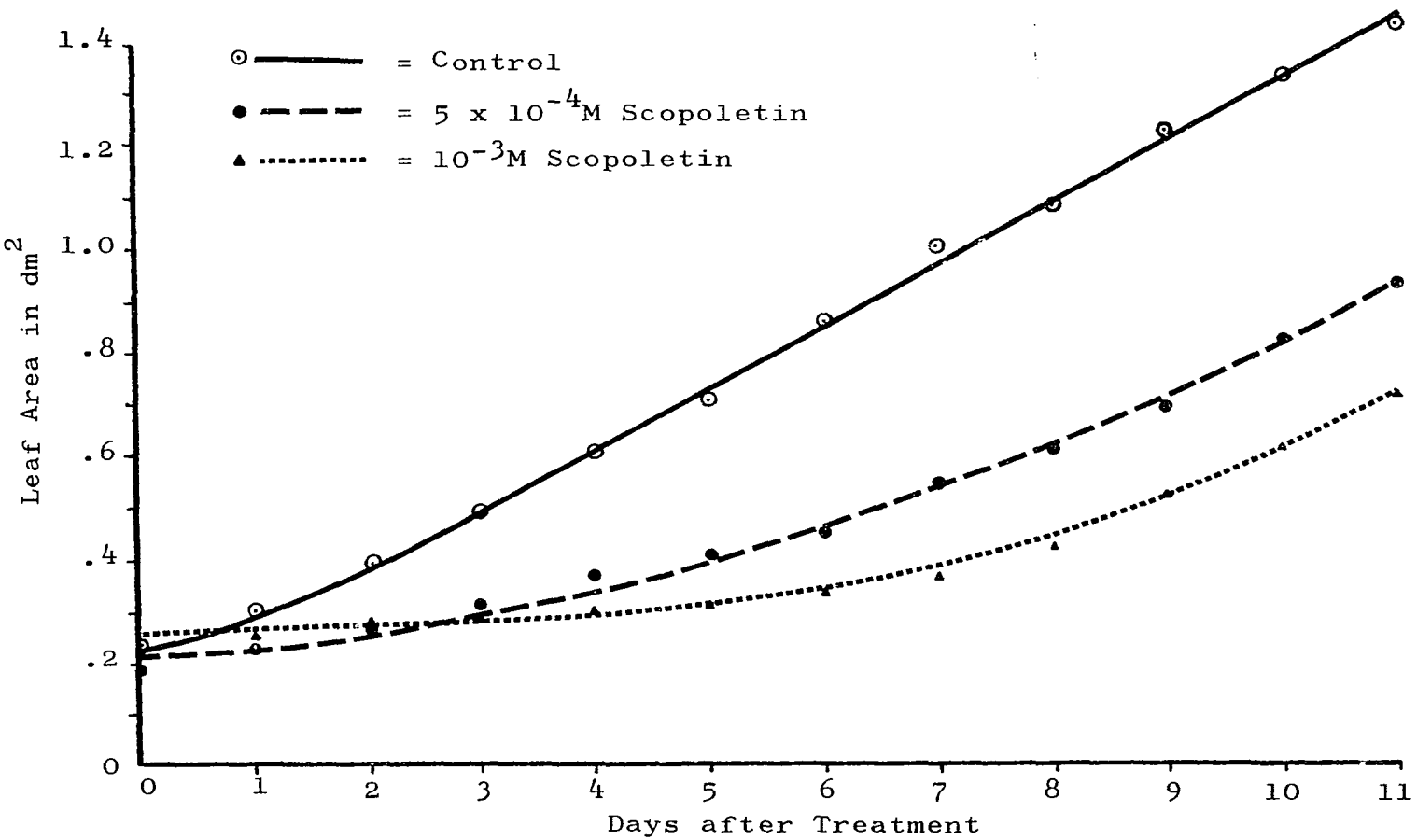

Fig. 16. Effects of scopoletin treatment on total leaf area of sunflower plants. Each point is a mean of 8 plants.

Table 3. Effects of scopoletin on net photosynthesis and dark respiration of whole seedlings after 5 days treatment.

Species	Treatment	Mean rates in CO_2/hr/dm^2 leaf area[b]	
		Photosynthesis 1000 ft-c	Respiration Dark
Tobacco	Control	8.9	2.1
	10^{-3}M Scopoletin	5.7[a]	1.8
Sunflower	Control	11.8	4.4
	10^{-3}M Scopoletin	8.8[a]	4.3
Pigweed	Control	14.4	3.3
	10^{-3}M Scopoletin	6.4[a]	3.0

[a]Rates differed significantly from the control below the 1% level.

[b]Each figure represents the mean of 10 plants.

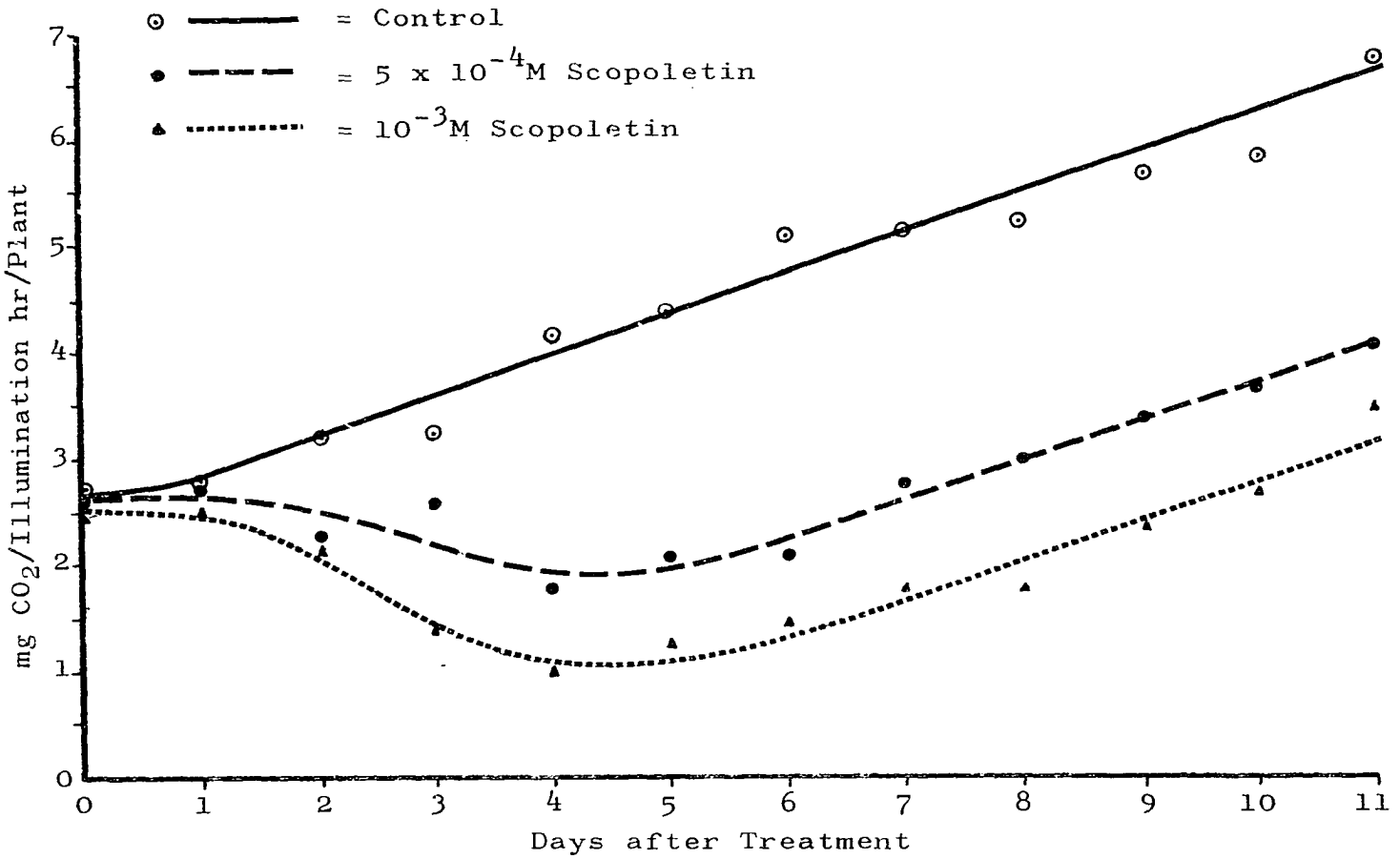

Fig. 17. Effect of scopoletin on CO_2 fixed/illumination hour in tobacco seedlings. Each point computed from mean leaf area and net photosynthesis of 4 plants.

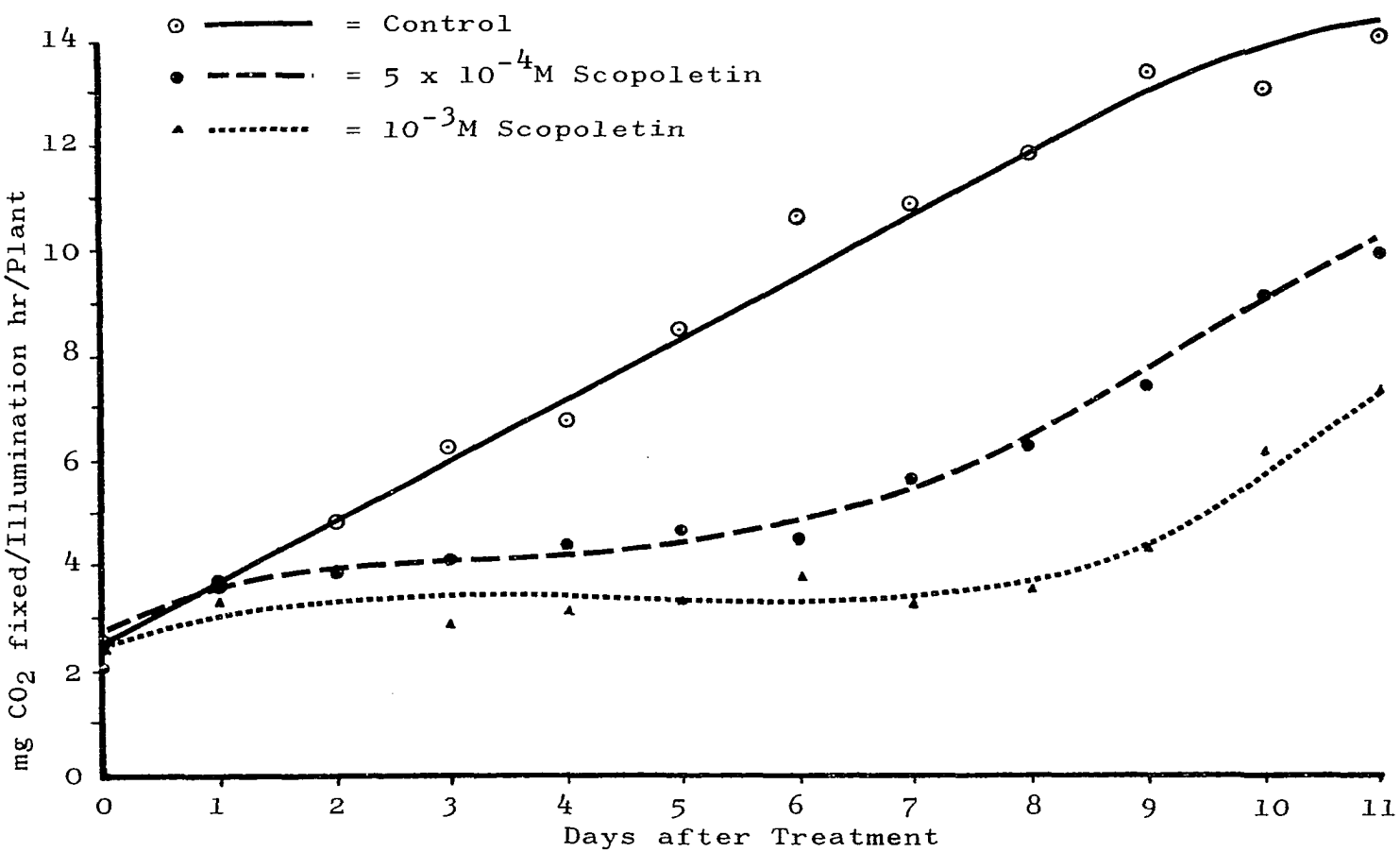

Fig. 18. Effect of scopoletin on CO_2 fixed/illumination hour in sunflower seedlings. Each point computed from mean leaf area and net photosynthesis of 8 plants.

the 5 x 10^{-4}M scopoletin treatments (Table 1) showed an intermediate growth reduction when compared to the 10^{-3}M series and controls.

CHAPTER IV

DISCUSSION

Tobacco, sunflower, and pigweed all exhibited a similar threshold of inhibition at 5 x 10^{-4}M scopoletin while the 10^{-4}M solution caused no inhibition of growth. Nevertheless, tobacco and sunflower plants treated with the 10^{-4}M concentration had a significant increase in the scopoletin and scopolin content. It appeared, therefore, that both tobacco and sunflower seedlings possess a fairly high tolerance to scopoletin. Once this tolerance was exceeded, growth reduction was correlated with build-up of scopoletin and scopolin in the tissue. Sunflower normally has only trace amounts of scopoletin and scopolin and was less affected by scopoletin than tobacco which normally contains these two in larger quantities. Both scopoletin and scopolin showed the greatest increase in concentration in the shoots of tobacco seedlings grown in 5 x 10^{-4}M scopoletin solution, and these plants had a lower shoot: root ratio than the controls. The greater retardation of shoot growth correlated well with the greater increases in scopoletin and scopolin concentration in the shoots.

Several factors suggest that it is scopolin that predominates at sites of accumulation: (1) the ratio of scopolin to scopoletin increased in the shoots of all treated plants analyzed in comparison with controls, (2) tobacco and sunflower shoots had a higher ratio of scopolin to scopoletin than the roots, (3) there was a greater percent increase in scopolin in shoots than roots, and (4) the actual concentration of scopoletin in treated plants was lower in shoots than in roots. The lack of fluorescence in new leaves at the end of the experiments gave some indication that once scopolin accumulates in the leaves it may not be further translocated.

Inhibitory activity of scopoletin on total plant growth correlated well with decreases in net photosynthesis, but no respiration differences were found. Freeland (1949) found that several different growth substances sprayed on bean leaves resulted in photosynthesis alterations which could not be accounted for entirely upon the basis of their effects on respiration. No attempt was made to correlate these photosynthesis differences with growth phenomena. Thomas and Hill (1937) reported SO_2 fed into the air stream above alfalfa plots reduced photosynthesis and respiration to varying degrees depending on concentration. Recovery occurred after treatment. Muller, Lorber, and Haley (1968) reported that cineole, one of the volatile terpenes of Salvia leucophylla Greene, severely retarded

respiration of roots and intact seedlings of germinating herbs. In both these instances reduction in growth was recorded. However, as in the case of the experiments presently reported, it was difficult to determine whether the differences in absolute growth were the result of changes in photosynthesis and/or respiration, or if growth reduction was caused by other factors.

Nevertheless, the effect of scopoletin on net photosynthesis appeared to be related to growth reduction in the present experiments. When the growth of leaf area in tobacco is compared with the corresponding net rate of photosynthesis, it seems plausible to conclude that reduction of photosynthesis caused the reduced growth. By the second day, photosynthesis in 10^{-3}M treated tobacco seedlings was reduced and leaf expansion had stopped. At the lowest point, their photosynthetic rate was only 34% of that of controls. As the rate of photosynthesis increased, the growth rate also increased although it lagged behind photosynthesis. By the end of the experiment, the photosynthetic rate of treated plants was equivalent to that of the controls. It is likely that plant recovery occurred after scopoletin was diluted or metabolized to some level below the toxic level within the tobacco.

If a comparison of the slopes of the leaf area curves is taken as an indication of tobacco growth rate, then at the final stage in the experiment the treated plants were

growing at rates comparable to controls. This was not true during the time of reduced photosynthetic rates. It seems that below a certain critical net photosynthetic rate no growth occurred, but once the rate rose above this point expansion of leaves corresponded somewhat with net photosynthesis. While this might be expected, it is difficult to establish the connection between growth rate and photosynthesis. For example, when comparing different species, Elmore et al. (1967) found that even though one species might have twice the photosynthetic rate of another, they still could have the same relative leaf area growth rate. Muramoto, Hesketh, and El-Sharkawy (1965) found differences in vigor, net assimilation rate of dry matter, and dry weight in varieties of American cotton were not necessarily associated with photosynthetic rate. They did find a good relationship between final leaf area and dry weight which led to the conclusion that differences in rate of leaf area development were associated with differences in rates of dry matter production.

A comparable analysis of sunflowers is not so clear. Growth in leaf area did not lag behind the return of photosynthesis to normal in the 10^{-3}M scopoletin treated seedlings. However, the lowest photosynthetic rate was 74% of that of the controls and these sunflowers, although greatly retarded, never completely stopped growth as in the case of tobacco. Furthermore, the return of leaf

growth rate to normal in 5 x 10^{-4}M treated sunflowers does correlate with an upward trend in photosynthesis. In the 5 x 10^{-4}M scopoletin treatment, however, the present hypothesis does not explain the high photosynthetic rates found during the first two days after treatment. These high rates do indicate that some variation in photosynthesis is likely without good growth correlation.

While there are many factors limiting growth other than the supply of photosynthate, a calculation of CO_2 fixed per hour of illumination in the daily photosynthesis of tobacco and sunflower clearly indicates that plants that were stunted fixed much less CO_2 than controls. Throughout the experiments the 5 x 10^{-4}M scopoletin treated plants fixed more CO_2 than the 10^{-3}M treated seedlings. Since these 5 x 10^{-4}M scopoletin treated plants were intermediate in growth to controls and 10^{-3}M treated plants, this agrees with the hypothesis that inhibition was the result of reduced photosynthesis.

The cause of the reduced photosynthesis is uncertain. It could be through a direct action on some enzyme of the photosynthesis pathway or it could be a more indirect action, such as an effect on stomatal aperture. Stomatal interference is suggested because of the loss of turgor noted in the 10^{-3}M scopoletin treated tobacco. Barrs (1968) found a constant ratio between net photosynthetic rate and transpiration rates and suggested these related

directly to stomatal opening. In contrast, several workers found that when stomatal openings were reduced by chemical antitranspirants, CO_2 exchange was not reduced in the same proportion (Zelitch and Waggoner, 1962; Shimshi, 1963; Slatyer and Bierhuizen, 1964). Furthermore, the fact that the 10^{-3}M series of tobacco exhibited some turgor loss does not necessarily mean that the stomates were closed. El-Sharkawy and Hesketh (1964) occasionally found some visibly wilted cotton and sunflower leaves to have open stomates and photosynthetic rates as high as turgid leaves. Since Zelitch (1967) found that a 10^{-3}M chlorogenic acid solution inhibited stomatal openings 50%, and Floyd and Rice (1967) found that a 0.83×10^{-3}M concentration inhibited growth significantly, studies of the effects of scopoletin on stomatal behavior appear to be desirable.

Regardless of the cause of the reduction in photosynthesis the significance is great with respect to seedling survival. While it is unlikely that scopoletin of the concentration found inhibitory in these tests occurs in the soil, certainly many plants contain scopoletin which will eventually be added to the soil. Börner (1960) reported that scopoletin is lost from certain plants, and unpublished results of Dr. E. L. Rice (personal communication) confirm that scopoletin can escape to the substratum in root exudates and in leachates of certain fallen leaves. Wang, Yang, and Chuang (1967) found that the total

concentration of phenolic acids in many soils was inhibitory to young wheat, corn, and soya bean when applied in nutrient cultures. It is logical, therefore, that scopoletin along with other inhibitory compounds may have a synergistic effect of ecological significance even when it is below the toxicity level itself. Thus scopoletin can be important when considering the capacity of a plant to grow under certain environmental conditions.

CHAPTER V

SUMMARY

In an attempt to establish the effects of scopoletin on growth of tobacco, sunflower, and pigweed; seedlings were treated with scopoletin through a nutrient culture. A threshold level of inhibition was found in all cases between 10^{-4}M and 10^{-3}M concentrations of scopoletin with the former showing no major growth effects, whereas the 10^{-3}M solutions were greatly inhibitory to all three species. All 5 x 10^{-4}M treatments had an intermediate effect on growth.

Analyses of scopoletin, scopolin, and chlorogenic acid concentrations of tobacco and sunflower treated with 10^{-4}M and 5 x 10^{-4}M scopoletin concentrations showed that at both of these levels, scopoletin and scopolin increased significantly in the tissue when compared with the control. The plants treated with the 5 x 10^{-4}M solution had the greatest increase in these compounds. The great increase in scopolin suggested a direct conversion of scopoletin to its glycoside, scopolin, within the plant. Chlorogenic acid levels were not different from controls and the variations in isomers (band 510 and neochlorogenic

acid) were indefinite. The fact that 10^{-4}M plants did increase in scopoletin and scopolin without growth reduction indicated sunflower and tobacco seedlings have some tolerance for abnormal amounts of these constituents, but a level of toxicity is soon reached as shown by growth reduction in 5 x 10^{-4}M treated plants. Further, a reduced shoot:root ratio coincided with a greater build up of scopoletin and scopolin in the shoots than in the roots of inhibited tobacco seedlings.

Respiration rates in treated plants remained unchanged, but CO_2 exchange analyses indicated that a reduced net photosynthetic rate was a contributing factor to reduced growth. Net photosynthesis in 10^{-3}M scopoletin treated tobacco plants was depressed to as low as 34% of that of the controls by the fourth day after treatment. In sunflowers, which normally have very small amounts of scopoletin and scopolin in the tissue, growth retardation was not as pronounced and the lowest photosynthetic rate resulting from treatment was 74% of controls. Reduced growth in leaf area over a 12 day experiment correlated well with the significant reduction in the rate of net photosynthesis in tobacco and a fairly good correlation was found also in sunflower. Amounts of CO_2 fixed/illumination hour in treated plants compared with controls reinforced the conclusion that a reduction in net photosynthesis contributed to plant inhibition in tobacco and

sunflower plants. Limited experiments with pigweed also indicated significantly reduced photosynthesis in the 10^{-3}M scopoletin treated seedlings. Scopoletin could contribute to a synergistic effect causing plant inhibition in the natural environment and therefore be a factor of ecological significance.

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