THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

# QUANTITATIVE DETERMINATION OF SCOPOLETIN, SCOPOLIN, AND RUTIN IN TOBACCO PLANTS TREATED WITH 2,4-DICHLOROPHENOXYACETIC ACID

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

VIRGIL A. THIESFELD

Norman, Oklahoma

1965

QUANTITATIVE DETERMINATION OF SCOPOLETIN, SCOPOLIN, AND RUTIN IN TOBACCO PLANTS TREATED WITH 2,4-DICHLOROPHENOXYACETIC ACID

APPROVED BY

Lawrence M. Rohrbaugh

Elroy L. Rice

Virgil H. Anderson

Harrison Bolas

Boyd E. Anderegg

DISSERTATION COMMITTEE

## ACKNOWLEDGMENT

I wish to express my sincere appreciation to Dr. L. M. Rohrbaugh and Dr. S. H. Wender for suggesting this problem, for providing a research assistantship with funds made available to them, and for their guidance and patience during the course of this research. I also wish to thank the National Science Foundation for providing the funds to make this research possible.

I wish to thank the faculty and staff of the Department of Botany and Microbiology and the Department of Chemistry. I also wish to thank my fellow graduate students for their guidance, cooperation, and assistance.

TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

Figure | Page

# QUANTITATIVE DETERMINATION OF SCOPOLETIN, SCOPOLIN, AND RUTIN IN TOBACCO PLANTS TREATED WITH 2,4-DICHLOROPHENOXYACETIC ACID

## CHAPTER I

## INTRODUCTION

There are numerous reports in the literature that various disturbances of the metabolism of plant tissues result in the accumulation of a blue-fluorescent material.

For example, Best (4) identified the blue-fluorescing scopoletin (6-methoxy-7-hydroxycoumarin) in tobacco infected with the virus of spotted tomato wilt. Andreae (1) identified scopoletin in potato tubers infected with leaf roll virus. He found no scopoletin in healthy plants or in the unrolled leaves of diseased plants, but did find some in the rolled leaves of diseased plants. No accumulation of scopoletin was found in potato tubers infected with mild mosaic, rugose mosaic, or spindle tuber viruses. Hughes and Swain (9) reported that potato tubers infected with Phytophthora

infestans produced scopolin (the 7-glucoside of scopoletin).

Watanabe et al. (14) have shown that boron deficiency resulted in a twenty-fold increase of scopolin in tobacco leaves in comparison with the leaves of control plants.

Dieterman et al. (6) reported that scopolin and scopoletin increased significantly in sunflower plants treated with 2,4-dichlorophenoxyacetic acid (2,4-D). Johnson and Fults (10) reported that tobacco plants treated with the sodium salt of 2,4-D showed a brilliant bluish-white fluorescence of the stems, nodes, veins, and roots that was many times brighter than in the untreated controls. They identified scopoletin as one of the compounds producing this fluorescence. They suggested that scopoletin appears to be a normal metabolite and that in some way it may act at low concentrations to decrease the effect of the natural auxin in cell elongation, and at high concentrations to accentuate the effect of the auxin. This would account in part for high concentrations of 2,4-D causing abnormal growth followed by death. Andrea (1) found that scopoletin would stimulate or inhibit the growth of cress and pea roots depending on its concentration. Andrea (2) later reported that scopoletin inhibited indoleacetic acid oxidase, and Andrea and Andrea (3) then suggested that when scopoletin was able to promote

growth, it was because it had a sparing effect on the endogenous auxin.

Skoog and Montaldi (12) studied the auxin-kinetin interaction regulating scopolin-scopoletin levels in tobacco tissue cultures. They reported that the cultures release a blue-fluorescent material into the medium, and they identified the main part of the material as scopoletin. The amount of fluorescent material released varied with the age of the tissue and the type of medium. The addition of indoleacetic acid (IAA) to the medium increased the amount of scopoletin released, and at a toxic level of IAA the amount released rose markedly. They found that the addition of kinetin (6-furfurylaminopurine) prevented IAA from causing a release of scopoletin, unless the IAA reached a toxic level.

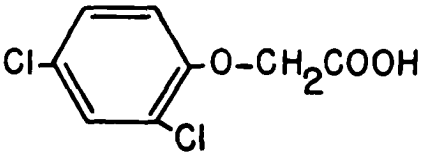
Sargent and Skoog (11) concluded that the pith and callus tissues of tobacco require kinetin to form scopolin and that the auxin-kinetin ratio regulates the equilibrium between scopolin and scopoletin.

Rutin (3-rhamnoglucoside of 3,3',4',5,7-pentahydroxyflavone) has been shown by Watanabe et al. (14) to be present in lower concentration in boron deficient tobacco leaves than in control leaves. Van Bragt et al. (13) reported that leaves of tomato plants treated with 2,4-D contain much less

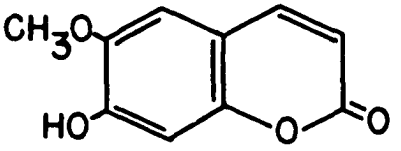
rutin than do control leaves.

This paper deals with the development of a quantitative method for the estimation of scopoletin and the subsequent use of this and other methods to determine quantitatively the amount of change in the concentrations of scopoletin, scopolin, and rutin in 2,4-D-treated tobacco plants as a function of time. The quantitative method for estimating scopolin as reported by Dieterman et al. (5) was modified so that both scopoletin and scopolin could be taken from the chromatogram on which the plant extract had been streaked.

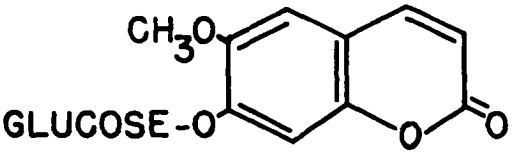
2,4-Dichlorophenoxyacetic acid



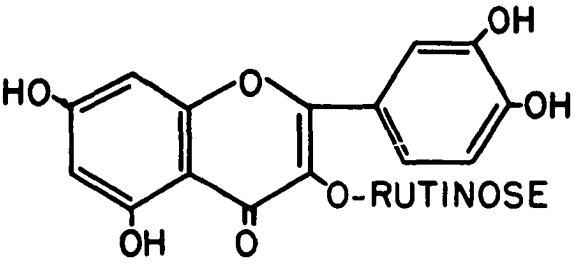

Scopoletin




Scopolin




Rutin




Rutinose

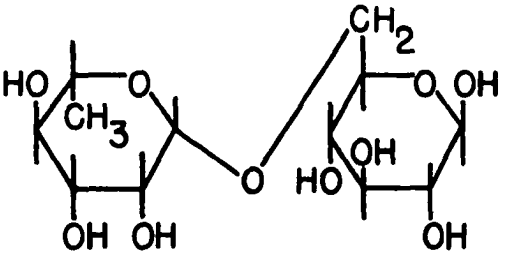



Figure 1. Structural formulas of some compounds pertinent to this study.

# CHAPTER II

# EXPERIMENTAL PROCEDURES AND RESULTS

## Intact Plants

Tobacco plants (Nicotiana tabacum, One-Sucker variety) were grown in the greenhouse under long (16 hour) photoperiods until they were about 50 cm in height. This required from 3 to 4 months of growth. At the start of an experiment the test plants were sprayed thoroughly on the stems and the upper and lower surfaces of the leaves with a solution of the sodium salt of 2,4-D. The spray solution was made by dissolving 1.00 g of 2,4-D in 800 ml of water (pH was adjusted to 7 with NaOH), adding 5 g of Carbowax 1500, and adjusting the volume to 1 liter. Plants treated with 2,4-D were harvested at 4, 8, and 16 hours and 1, 2, 4, 8, and 16 days after spraying. Control plants were harvested only at the beginning and end of an experiment. The harvested plants were divided into leaves, stems, and roots, and each fraction was weighed and then killed in boiling

isopropanol. After maceration in a Waring Blendor, each sample was extracted in a Soxhlet extractor with aqueous 85% isopropanol for 24 hours. For the rutin analysis the extracts were concentrated in vacuo using a rotary evaporator.

For the quantitative determination of scopoletin and scopolin a modification of the method of Dieterman et al. (5) was used. This method utilizes descending paper chromatography in the purification steps. An aliquot of the isopropanol-water extract was streaked on 9 1/8 x 22 1/2 inch sheets of S. and S. no. 589, red ribbon chromatography paper and developed in n-butanol-acetic acid-water (6:1:2 v/v/v). The resulting scopolin ($R_f$, approximately 0.57) and scopoletin ($R_f$, approximately 0.85) areas were cut out, and each was handled separately in the subsequent steps. Scopolin was purified on Whatman no. 1 chromatography paper and scopoletin on S. and S. no. 589 paper. Each cut out zone was sewn onto individual sheets of previously unused paper and chromatographed with isopropanol-formic acid-water (5:0.1:95 v/v/v). The scopolin zone was cut out after development, and sewn onto a third sheet of paper and chromatographed again, using n-butanol-benzene-pyridine-water (5:1:3:3 v/v/v/y). The scopoletin zone was cut out after development, and sewn onto another sheet of paper and chromatographed again, using

benzene-acetic acid-water (125:72:3 v/v/v). The use of these three solvent systems has produced chromatographically pure scopolin and scopoletin. The scopolin was then eluted with water, and scopoletin was eluted with aqueous 50% methanol. The eluates were brought to volume, and their relative intensities of fluorescence were used to determine the amounts of scopolin and scopoletin present by referring to the internal standard reference curves. The standard curves were determined by using known weights of scopolin and scopoletin that were carried through the same procedure as described for the unknowns. The determinations were made in duplicate, and the results are shown in figures 2 through 9. The points on the graphs of sets one, two, and three represent two, four, and eight plants, respectively.

The actual amounts of scopoletin and scopolin differed considerably in the three experiments. It is perhaps easier to note the effects of 2,4-D treatment by comparing the ratios of the amounts of scopoletin and scopolin in the treated plants to the amounts of these substances in the control plants for each of these experiments. These ratios are shown in table I (page 20).

The isopropanol-water extracts were quantitatively analyzed for rutin, using the method of Van Bragt et al. (13).

This method utilizes descending paper chromatography in the purification steps. An aliquot of the extract was spotted on Whatman no. 1 chromatography paper; development was two-dimensional using n-butanol-acetic acid-water (6:1:2 v/v/v) as the first solvent and 2% acetic acid as the second solvent. The rutin zone was cut out, cut into about 50 small pieces, and put in methanol. This mixture was shaken for 1 hour and then filtered through pyrex wool. One-tenth milliliter of methanolic 1% aluminum chloride solution was added to the filtrate which was then brought to volume and allowed to stand for 1 hour for color development. The relative intensity of the rutin-aluminum chloride mixture was determined with a Turner fluorometer. The concentrations of the unknowns were then determined from an internal standard reference curve that was obtained by carrying known weights of rutin through the same procedure as described for the unknowns. The rutin used for determination of the standard curve was purified by and obtained from Dunlap and Wender (7). The results of these quantitative determinations, which were carried out in duplicate, are shown in figures 10 through 13 (pages 21-24).

## Stem Slices and Homogenates

Stem sections from eight plants were cut into slices 2 mm or less in thickness, and weighed. The slices were then

bathed in the appropriate solutions in petri dishes. The test solution contained 3.5 g of $KH_2PO_4$, 3.5 g of $K_2HPO_4$, 10.3 g of sucrose, and 0.10 g of 2,4-D in 1 liter of water. This solution had a pH of 6.95. The control solution was the same, except that it did not contain any 2,4-D. The stem sections for homogenation were weighed, macerated in a Waring Blendor, and the homogenate placed in petri dishes in the appropriate solutions prepared in the same way as were those used for the stem slices. To insure the possibility of aerobic respiration, care was taken to be sure that the stem slices were but about one-half covered with the solution. The homogenates, due to the small size of the particles, were almost completely submerged throughout the duration of the experiment.

Samples were taken from the 2,4-D solutions after 2, 4, 8, 18, and 48 hours and from the control solutions after 2 and 48 hours, at which time the contents of the petri dishes were quantitatively transferred to boiling isopropanol. The slices were macerated in a Waring Blendor, and from this point on all samples were analyzed for scopoletin, scopolin, and rutin in the same manner as those from intact plants as previously described.

The results of the quantitative determinations on the

extracts of the stem slices and homogenates, which were carried out in duplicate, are shown in figures 14 and 15 (pages 25 and 26).

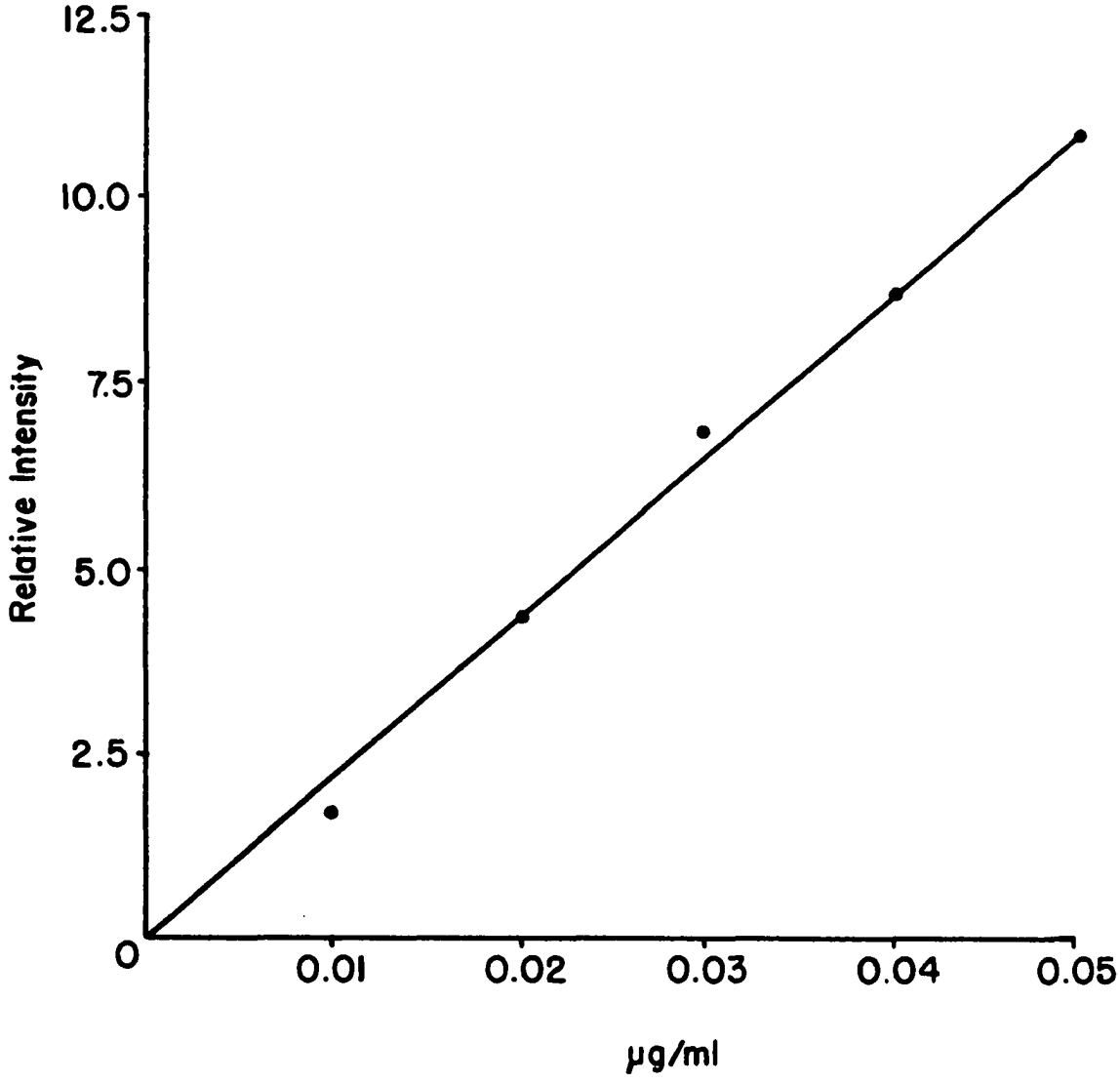



Figure 2. Relationship of relative fluorescence intensity to scopoletin concentration. Instrument, Aminco-Bowman spectrophotofluorometer. Photomultiplier, RCA 1P21 tube. Slit arrangement, 5. Light source, Xenon arc lamp. Meter multiplier setting, 0.3 and 0.1. Sensitivity setting, 0. Sample, aqueous 50% methyl alcohol from paper chromatogram. Activation wavelength, 365 mµ. Fluorescence wavelength, 468 mµ.

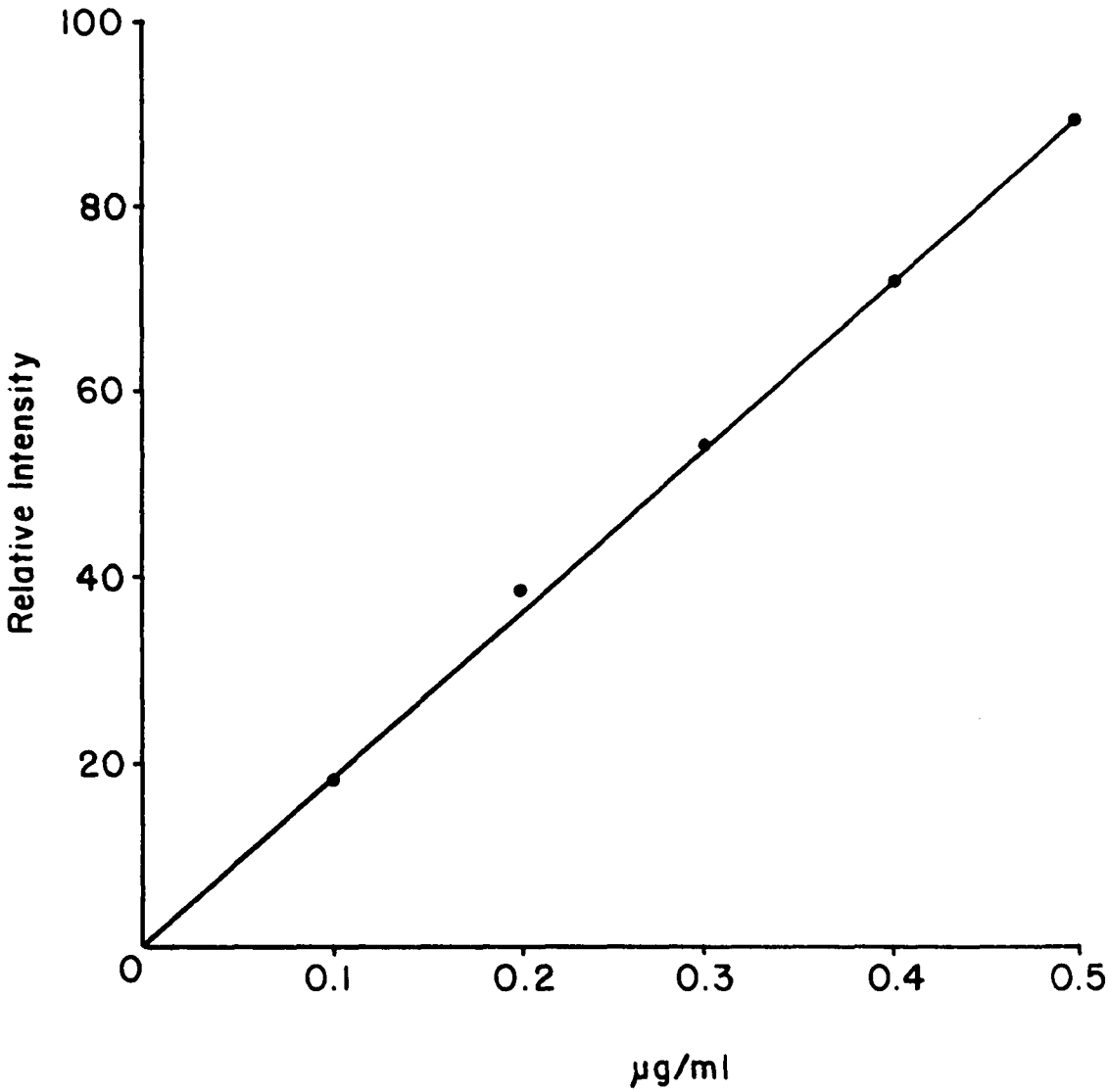



Figure 3. Relationship of relative fluorescence intensity to scopolin concentration. Instrument, Aminco-Bowman spectrophotofluorometer. Photomultiplier, RCA 1P21 tube. Slit arrangement, 5. Light source, Xenon arc lamp. Meter multiplier setting, 1. Sensitivity setting, 40. Sample, aqueous solution of scopolin from paper chromatogram. Activation wavelength, 360 mµ. Fluorescence wavelength, 445 mµ.

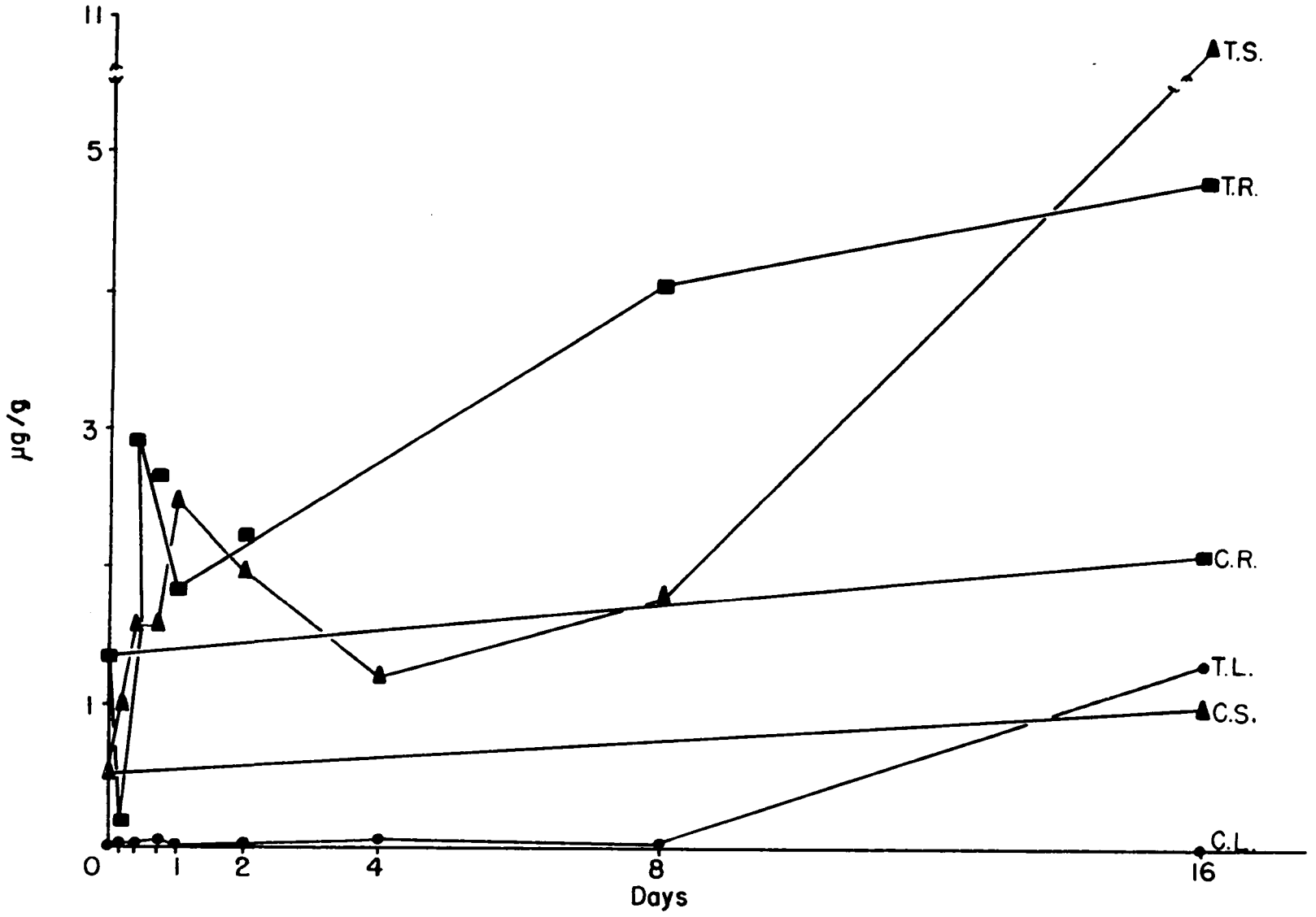



Figure 4. Scopoletin content in micrograms per gram fresh weight in leaves, stems, and roots of tobacco plants sprayed with 2,4-D solution and in similar parts of untreated plants (set 1). Legend: T.L. = treated leaf; T.S. = treated stem; T.R. = treated root; C.L. = control leaf; C.S. = control stem; C.R. = control root.

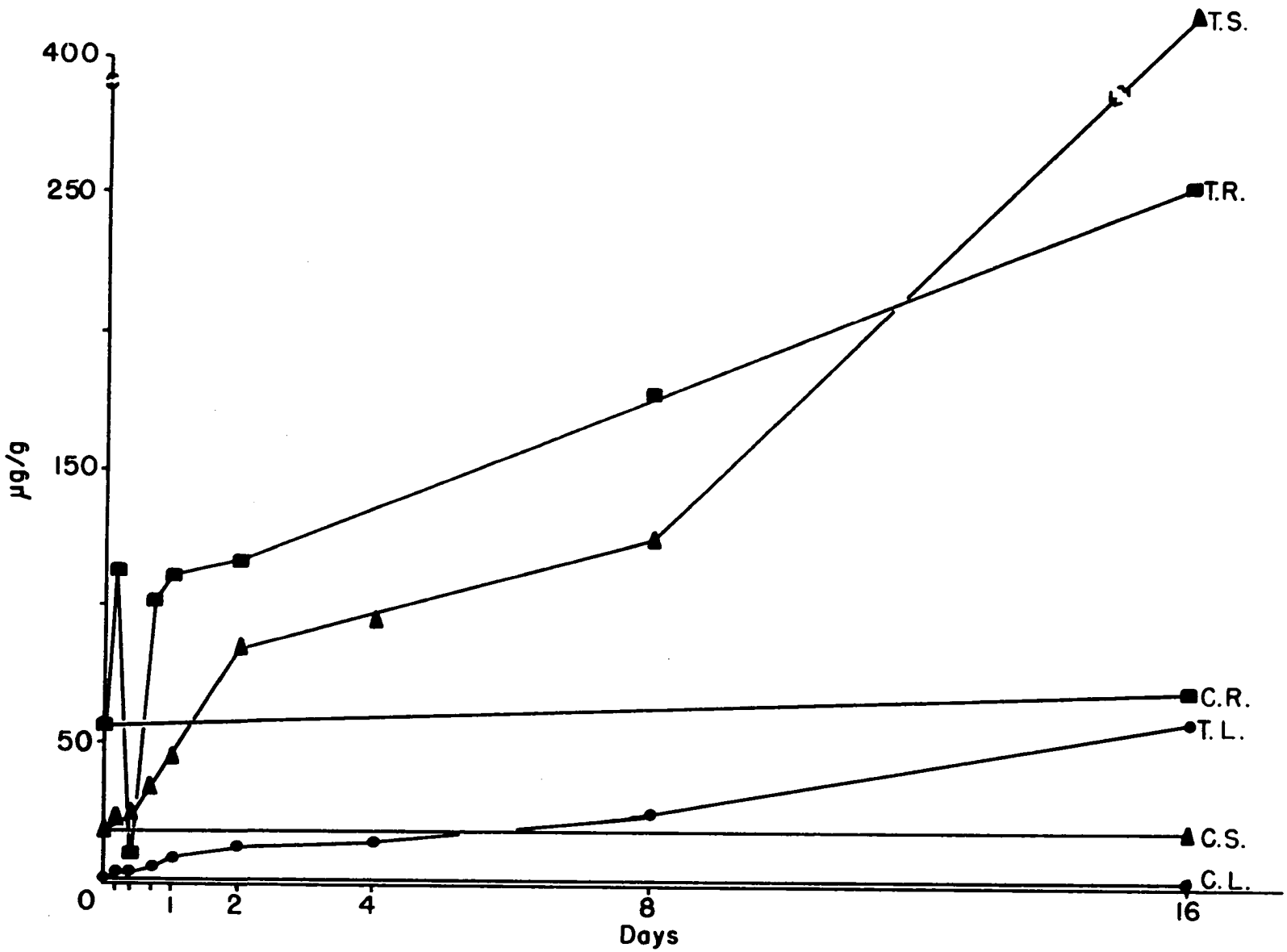



Figure 5. Scopolin content in micrograms per gram fresh weight in leaves, stems, and roots of tobacco plants sprayed with 2,4-D solution and in similar parts of untreated plants (set 1). See fig. 4 for legend.

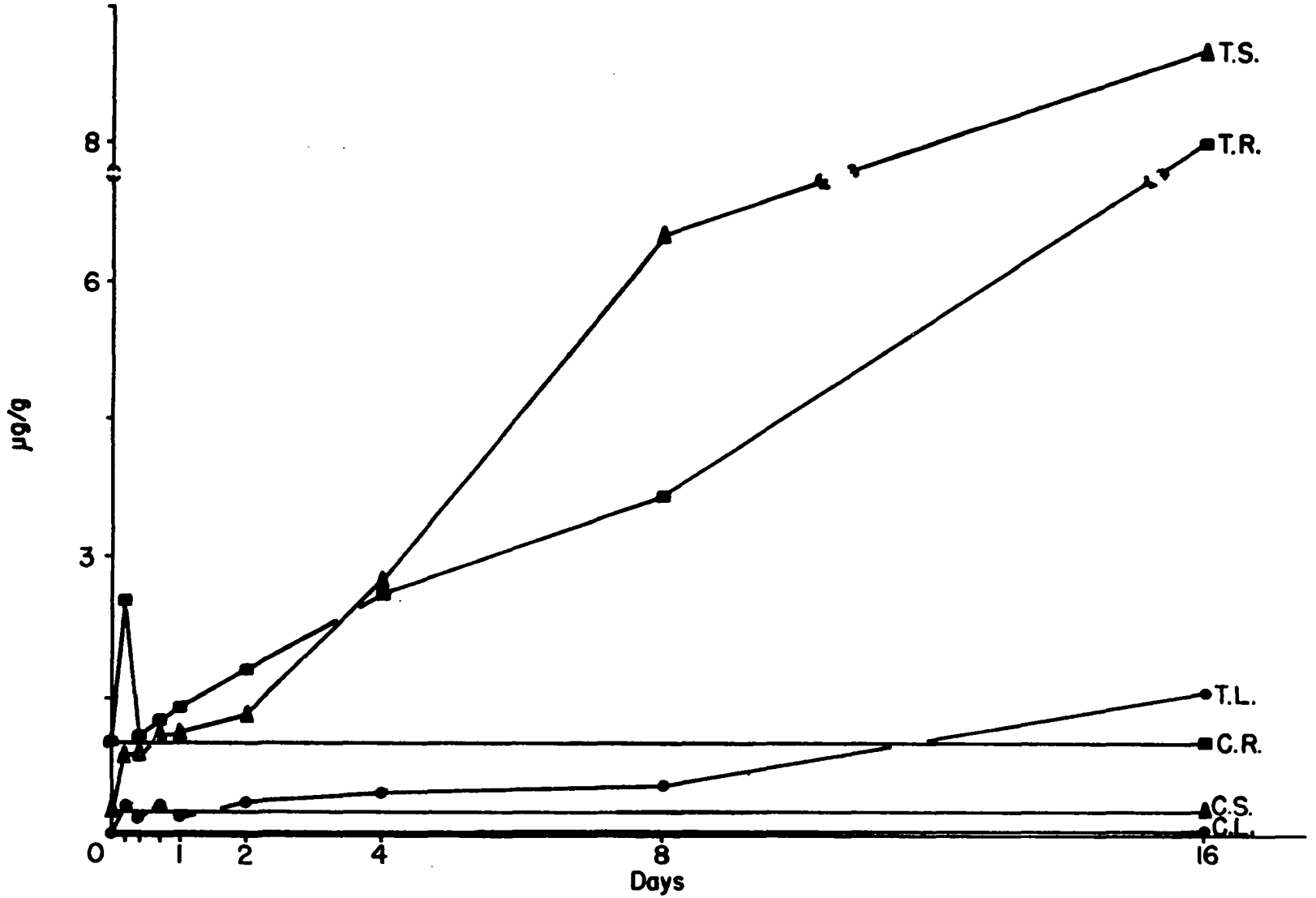



Figure 6. Scopoletin content in micrograms per gram fresh weight in leaves, stems, and roots of tobacco plants sprayed with 2,4-D solution and in similar parts of untreated plants (set 2). See fig. 4 for legend.

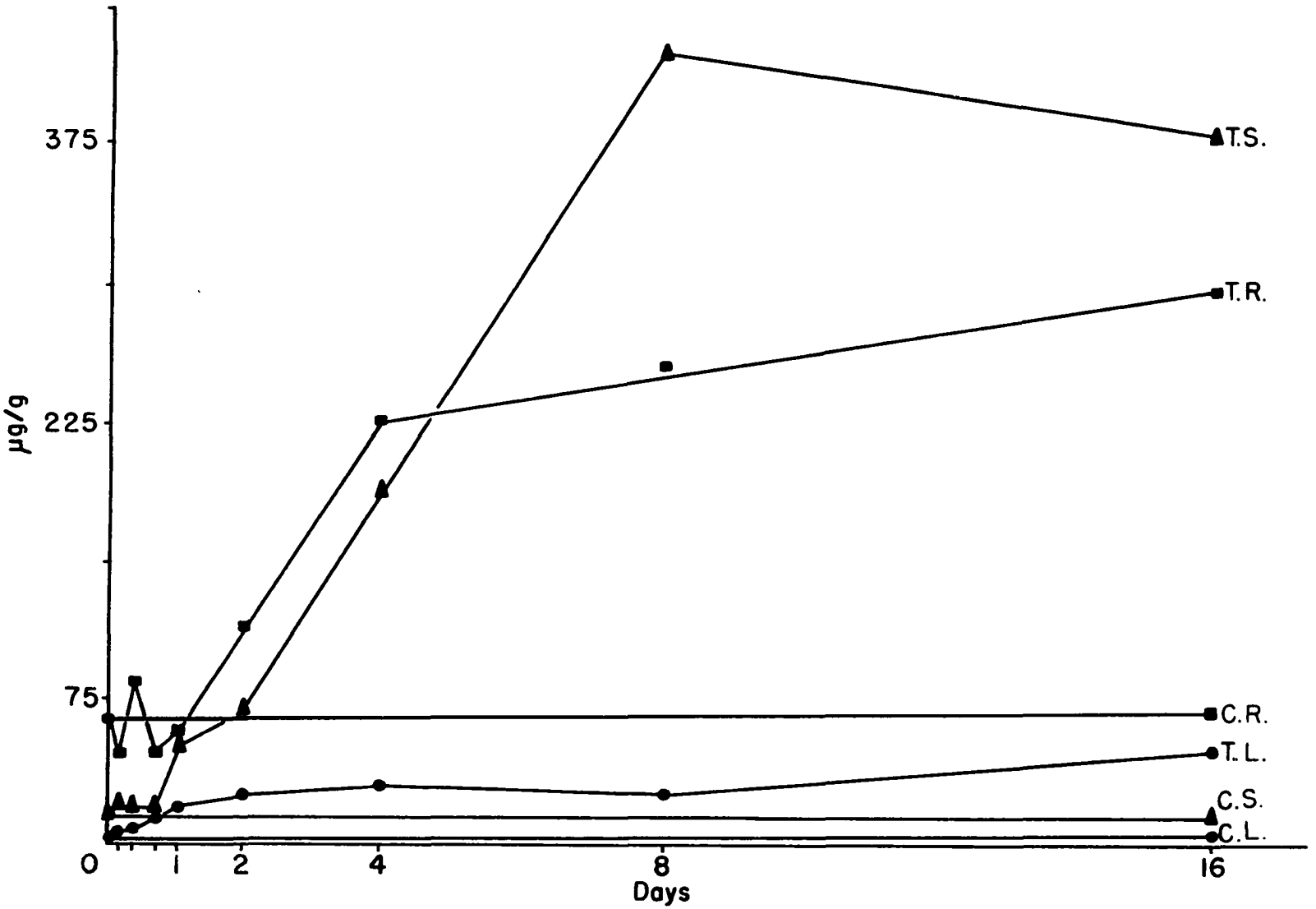



Figure 7. Scopolin content in micrograms per gram fresh weight in leaves, stems, and roots of tobacco plants sprayed with 2,4-D solution and in similar parts of untreated plants (set 2). See fig. 4 for legend.

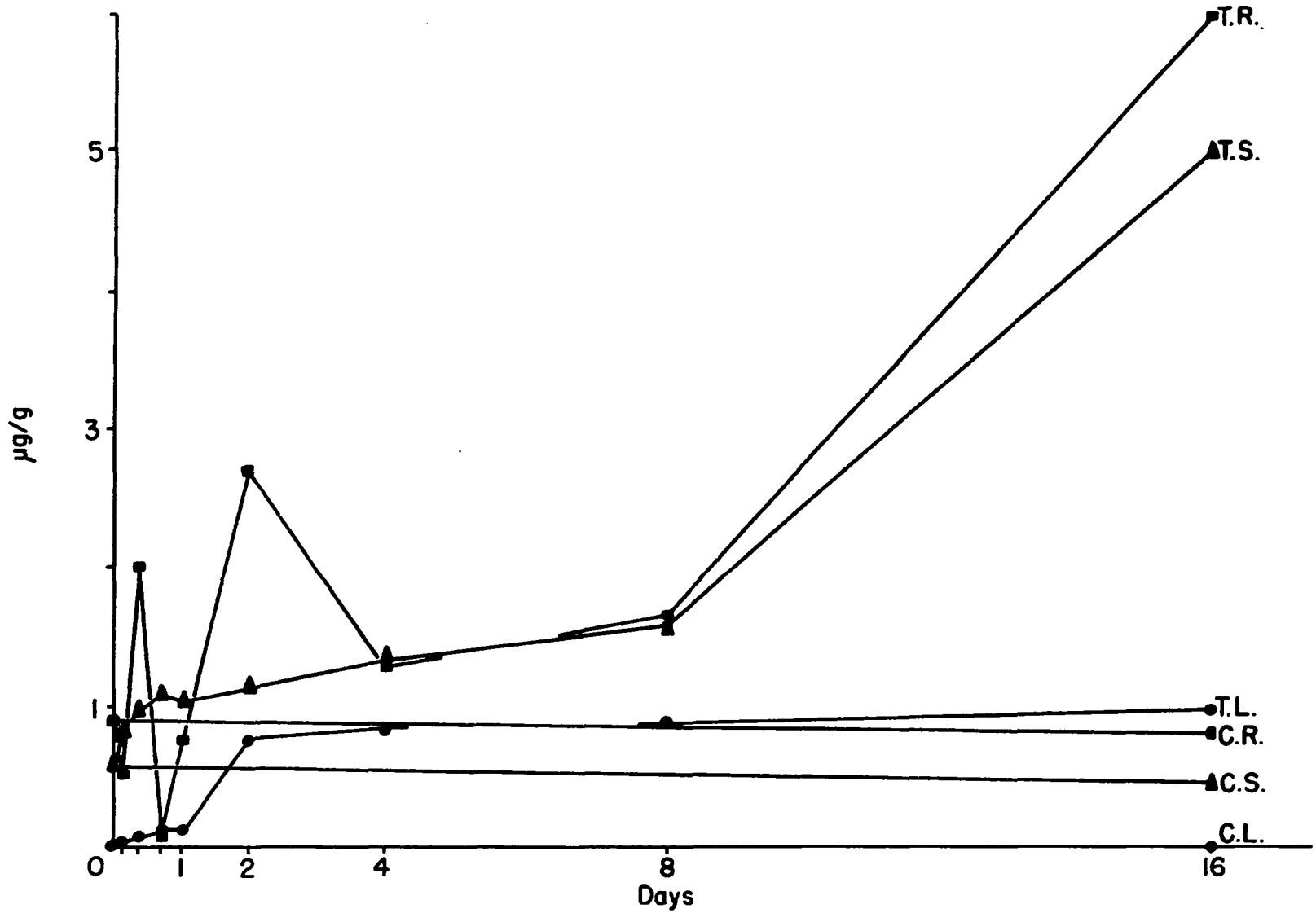



Figure 8. Scopoletin content in micrograms per gram fresh weight in leaves, stems, and roots of tobacco plants sprayed with 2,4-D solution and in similar parts of untreated plants (set 3). See fig. 4 for legend.

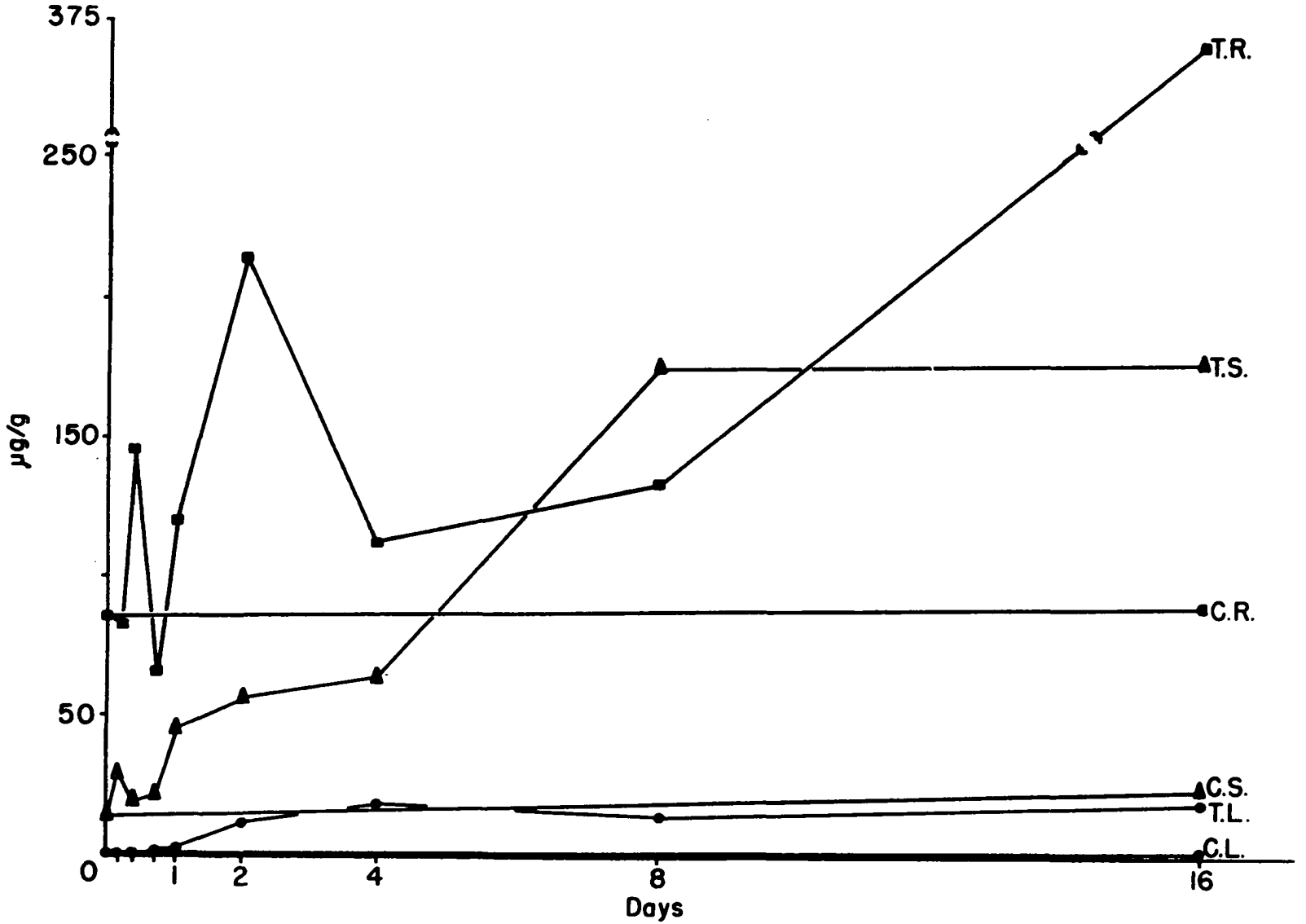



Figure 9. Scopolin content in micrograms per gram fresh weight in leaves, stems, and roots of tobacco plants sprayed with 2,4-D solution and in similar parts of untreated plants (set 3). See fig. 4 for legend.

TABLE I

The ratios of the amounts of scopoletin and scopolin in 2,4-D-treated tobacco plants to the amounts of scopoletin and scopolin in untreated plants on the 16th day after treatment.

| | Scopoletin $\frac{\text{treated}}{\text{untreated}}$ | Scopolin $\frac{\text{treated}}{\text{untreated}}$ |
|---|---|---|
| Set 1 | | |
| Leaf | 16.7 | 20.8 |
| Stem | 10.2 | 22.0 |
| Root | 2.2 | 3.9 |
| Set 2 | | |
| Leaf | 19.3 | 9.2 |
| Stem | 36.0 | 24.8 |
| Root | 8.0 | 4.1 |
| Set 3 | | |
| Leaf | 32.7 | 15.2 |
| Stem | 11.6 | 7.7 |
| Root | 7.5 | 4.1 |

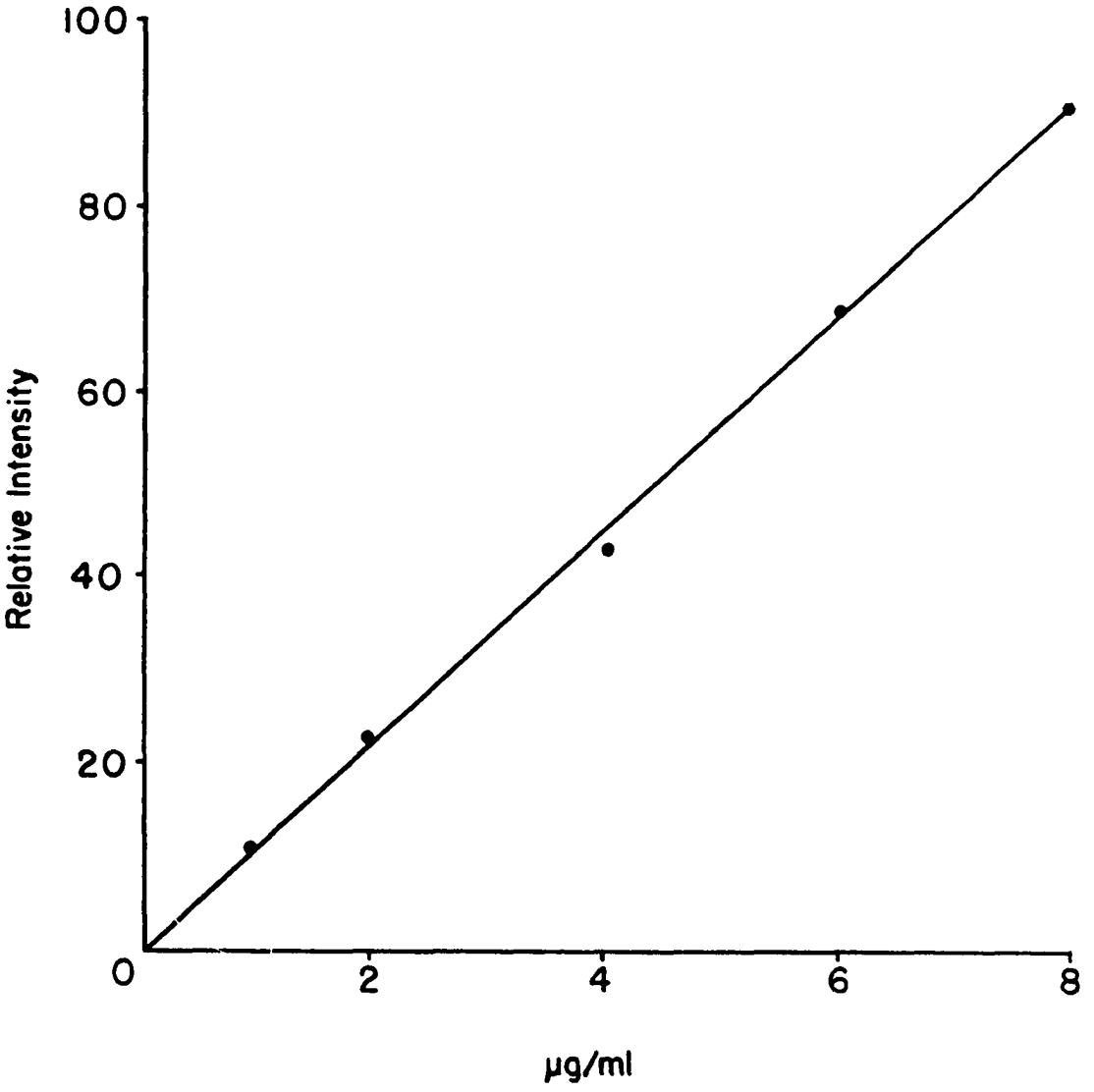



Figure 10. Relationship of relative fluorescence intensity to rutin concentration. Instrument, Turner model 110 fluorometer. Slit arrangement, 3X. Sensitivity setting, 6. Sample, methyl alcohol solution of rutin from paper chromatogram. Activation wavelength, 436 mµ. Activation filters, 47B plus 2A. Fluorescence wavelength, 525 mµ. Fluorescence filter, 2A12.

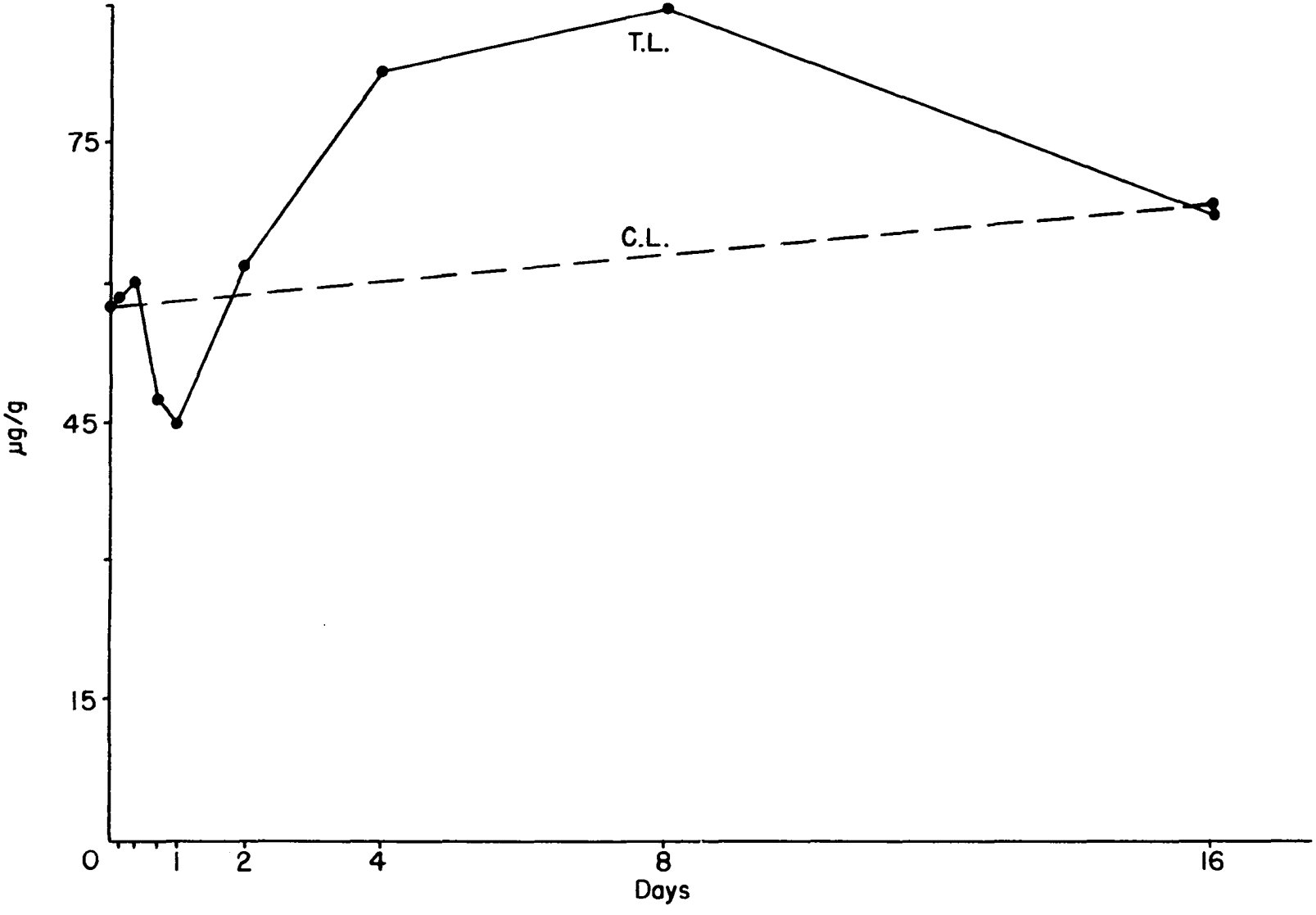



Figure 11. Rutin content in micrograms per gram fresh weight in leaves of tobacco plants sprayed with 2,4-D solution and in leaves of untreated plants (set 1). See fig. 4 for legend.

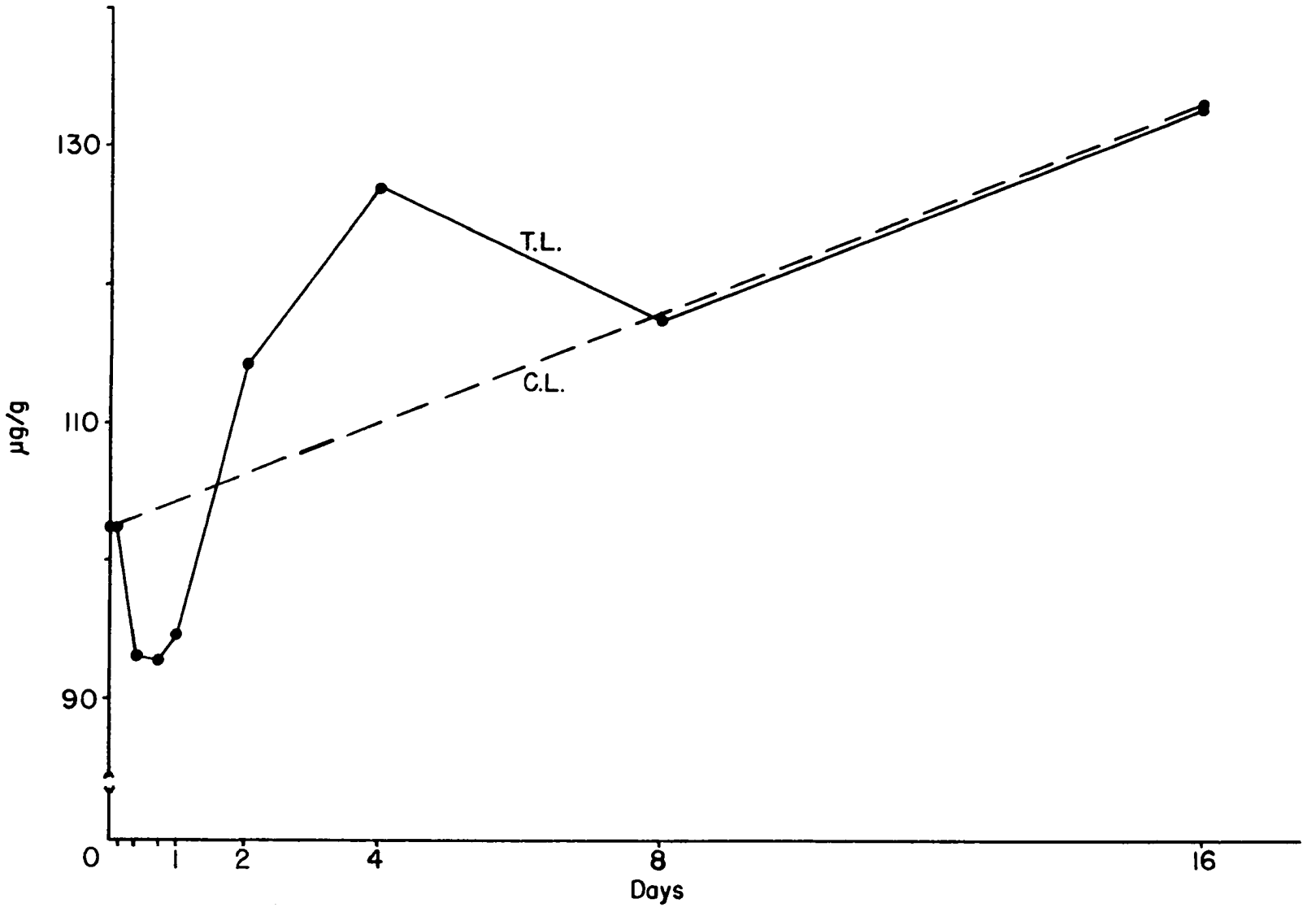



Figure 12. Rutin content in micrograms per gram fresh weight in leaves of tobacco plants sprayed with 2,4-D solution and in leaves of untreated plants (set 2). See fig. 4 for legend.

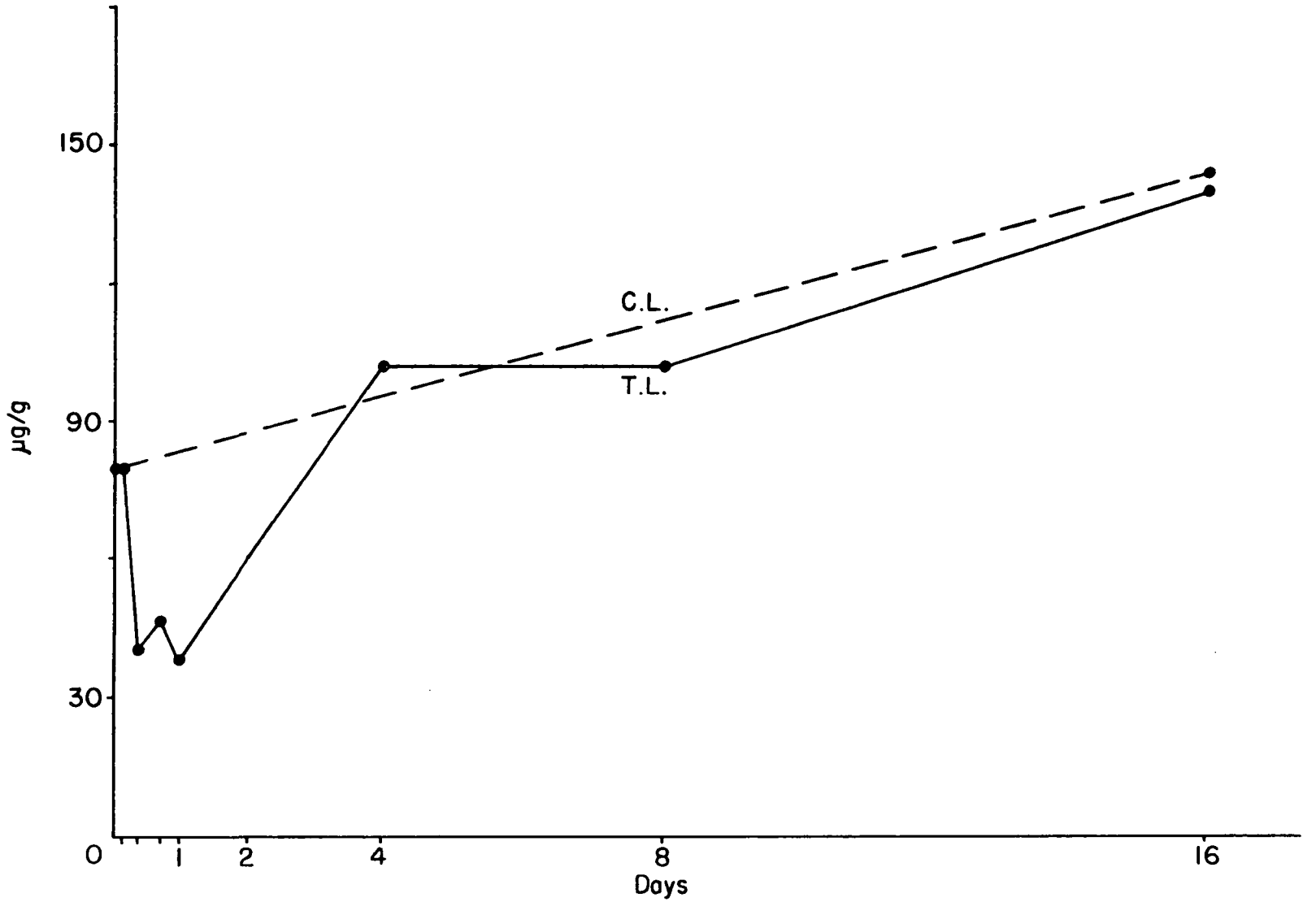



Figure 13. Rutin content in micrograms per gram fresh weight in leaves of tobacco plants sprayed with 2,4-D solution and in leaves of untreated plants (set 3). See fig. 4 for legend.

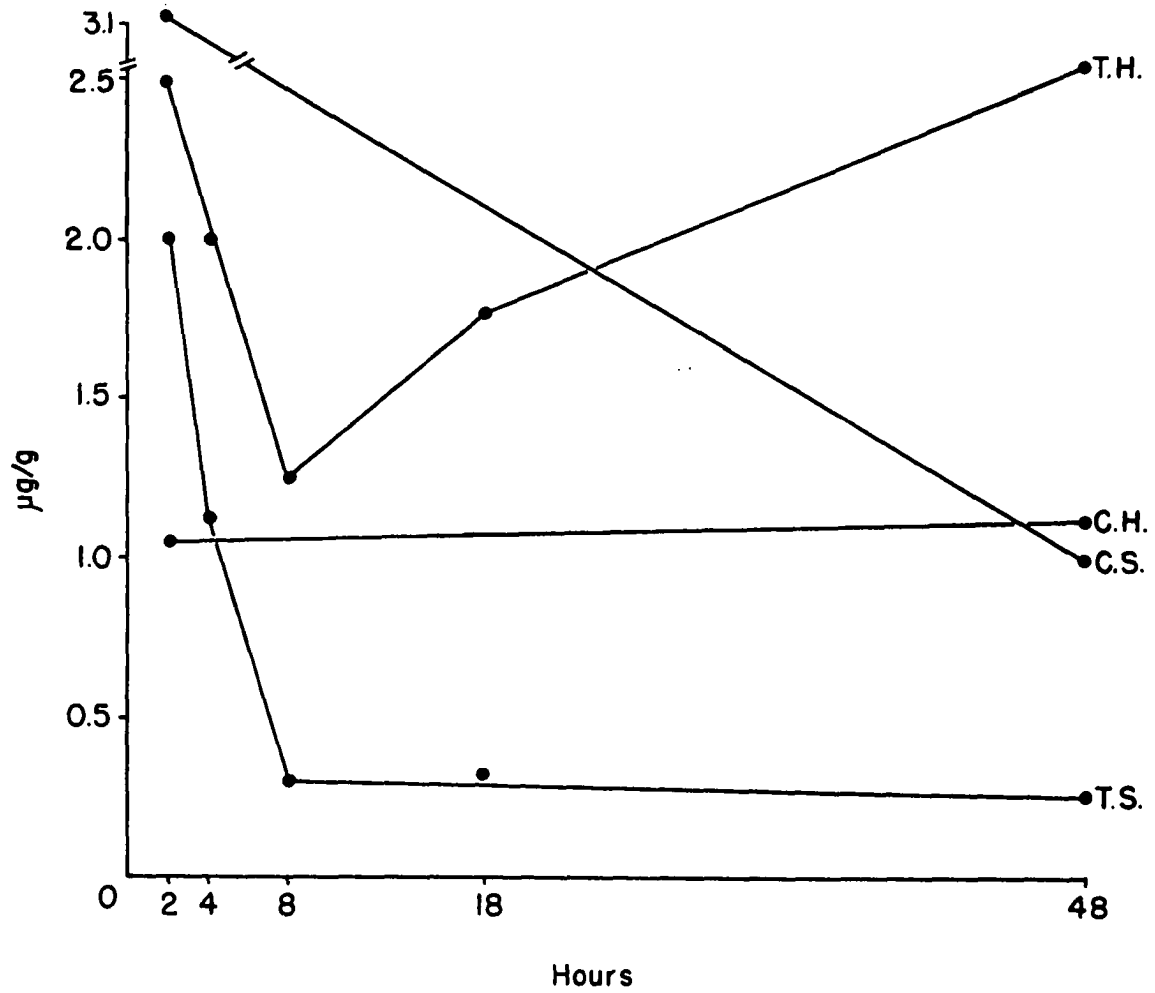



Figure 14. Scopoletin content in micrograms per gram fresh weight of tobacco stem slices and homogenates treated with 2,4-D solution and in untreated slices and homogenates. Legend: T.S. = treated slice; T.H. = treated homogenate; C.S. = control slice; C.H. = control homogenate.

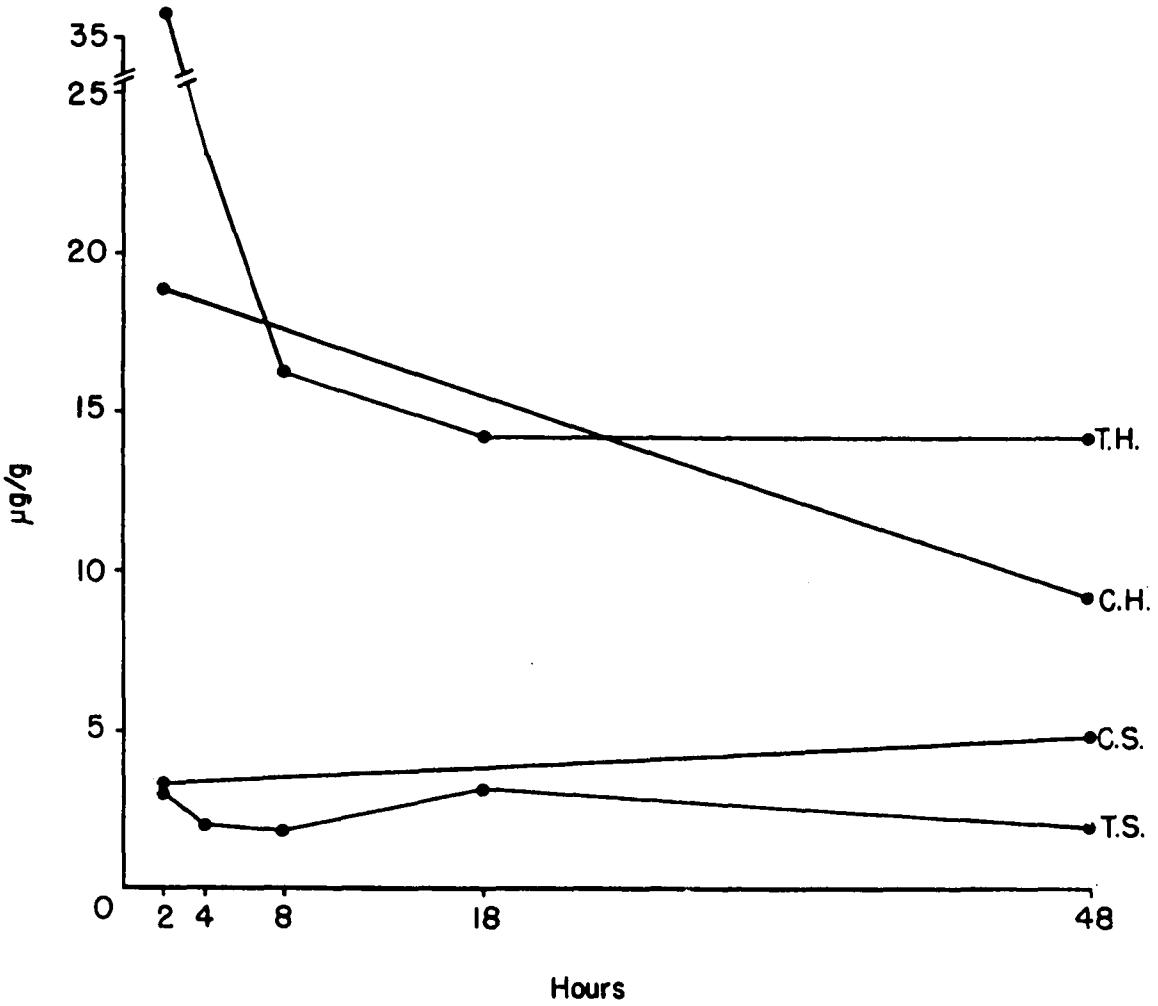



Figure 15. Scopolin content in micrograms per gram fresh weight of tobacco stem slices and homogenates treated with 2,4-D solution and in untreated slices and homogenates. See fig. 14 for legend.

# CHAPTER III

## DISCUSSION

The results given in figures 4-9, figures 11-15, and the ratios listed in table I allow several conclusions. In all of the control plants, the amounts of scopoletin and scopolin were highest in the roots, lower in the stems, and lowest in the leaves. The amount of scopoletin in the leaves of control plants was indeed very low.

In all cases the spraying of the tops of intact tobacco plants with the sodium salt of 2,4-D in the concentration used resulted in an increase in the amounts of scopoletin and scopolin present in leaves, stems, and roots. The amounts of scopoletin and scopolin present in roots of treated plants had large values in micrograms in comparison to those in leaves of treated plants. However, as is demonstrated in table I, the roots of treated plants had a smaller percent increase in these compounds over the amounts in the roots of controls than did the leaves or stems. Except for scopolin

in the stems of sets 2 and 3 (figs. 7 and 9), the increase of scopoletin and scopolin continued for the entire 16 days of the experiments. The accumulation of scopolin, in micrograms, was much larger than that of scopoletin.

Studies with stem slices and homogenates indicate that since accumulation of scopoletin and scopolin did not occur here as it had in intact plants, these substances may not be produced in the stem as a result of application of 2,4-D. The variability in the amounts of both scopoletin and scopolin in the root samples (from intact plants) throughout the first day after treatment indicates that the 2,4-D may cause the root to produce more scopoletin and scopolin which may then be translocated to the rest of the plant. There is no evidence against regarding the leaf as the reaction site, except that the low concentrations of scopoletin and scopolin present in the leaves makes this seem unlikely.

No rutin was found in the roots or stems of intact plants, either treated or untreated, or in stem slices or homogenates, either treated or untreated. This is not to say that there is no rutin present, but that in the small amounts of tissue examined none could be found. Van Bragt et al. (13) were not able to find any rutin in the roots of tomato plants.

The rutin content of treated plants 16 days after treatment was very close to that of the untreated controls. Figures 11, 12, and 13 show that during the first day after being sprayed with 2,4-D, the amount of rutin decreased. This may have resulted from the 2,4-D treatment.

In figures 4-9 and 11-13, the lines connecting the amounts of scopoletin and scopolin in the control plants are drawn as straight lines although no determinations were made at other time periods. In figures 11, 12, and 13 the lines connecting the amounts of rutin in the control plants are drawn as broken lines because there is evidence that rutin accumulation is not linear in some other plants. Griffith et al. (8) reported that the accumulation of rutin in buckwheat may reach a maximum and then decrease depending on the age of the plant and the time of planting. However, since the tobacco plants used in this study were grown under long photoperiods, the time of planting should not have had much effect on the rutin content.

# CHAPTER IV

## SUMMARY

Three sets of tobacco plants between 3 and 4 months old were sprayed with a solution containing the sodium salt of 2,4-dichlorophenoxyacetic acid (2,4-D). Samples were harvested at 4, 8, and 16 hours and 1, 2, 4, 8, and 16 days after spraying; divided into leaves, stems, and roots; weighed; and killed in boiling isopropanol. After maceration in a Waring Blendor, they were extracted with aqueous 85% isopropanol in a Soxhlet extractor for 24 hours. The extracts were analyzed quantitatively for scopoletin, scopolin, and rutin. Descending paper chromatography was used to purify these compounds for the quantitative determinations. A new quantitative method for scopoletin analysis is described.

The results show that treated and untreated tobacco plants contained both scopoletin and scopolin in the leaves, stems, and roots. The amounts of scopoletin and scopolin

present in untreated plants were highest in the roots, lower in the stems, and lowest in the leaves. Treatment with 2,4-D resulted in a large increase of these compounds in the leaves, stems, and roots. This increase continued with time.

Stem slices and homogenates treated with 2,4-D did not accumulate scopoletin and scopolin as did the treated intact plants. It is suggested that the 2,4-D may cause the root to produce more scopoletin and scopolin which may then be translocated to the rest of the plant.

The leaves of treated and untreated tobacco plants contain rutin, but none was found in either treated or untreated stems or roots. The amount of rutin present 16 days after treatment with 2,4-D approximated that present in the untreated controls. There was a decrease in rutin content in the first day after treatment which may indicate a reaction to treatment with 2,4-D.

## LITERATURE CITED


1. Andreae, W. A. 1948. The isolation of a blue fluorescent compound, scopoletin, from Green Mountain potato tubers, infected with leaf roll virus. Canad. J. Res. C 26: 31-34.

2. Andreae, W. A. 1952. Effect of scopoletin on indoleacetic acid metabolism. Nature 170: 83-84.

3. Andreae, W. A. and S. R. Andreae. 1953. Studies on indoleacetic acid metabolism. I. The effect of methyl umbelliferone, maleic hydrazide and 2,4-D on indoleacetic acid oxidation. Canad. J. Bot. 31: 426-437.

4. Best, R. J. 1944. Studies on a fluorescent substance present in plants. II. Isolation of the substance in a pure state and its identification as 6-methoxy-7-hydroxy 1:2 benzo-pyrone. Australian J. Exptl. Biol. Med. Sci. 22: 251-255.

5. Dieterman, L. J., C.-Y. Lin, L. Rohrbaugh, V. Thiesfeld, and S. H. Wender. 1964. Identification and quantitative determination of scopolin and scopoletin in tobacco plants treated with 2,4-dichlorophenoxyacetic acid. Anal. Biochem. 9: 139-145.

6. Dieterman, L. J , C.-Y. Lin, L. M. Rohrbaugh, and S. H. Wender. 1964. Accumulation of ayapin and scopolin in sunflower plants treated with 2,4-dichlorophenoxyacetic acid. Arch. Biochem. Biophys. 106: 275-279.

7. Dunlap, W. J. and S. H. Wender. 1961. Identification of minor flavonoid impurities found in commercial rutin. Anal. Biochem. 2: 317-323.

8. Griffith, J. Q., Jr., C. F. Krewson, and J. Naghski. 1955. Rutin and Related Flavonoids. Mack Publishing Co., Easton, Pa. p. 37-39.

9. Hughes, J. C. and T. Swain. 1960. Scopolin production in potato tubers infected with Phytophthora infestans. Phytopathology. 50: 398-400.

10. Johnson, M. and J. L. Fults. 1950. A cumulative fluorescent chemical found in certain plants treated with 2,4-D identified as scopoletin (6-methoxy-7-hydroxy 1:2 benzo-pyrone). Colo. Wyo. Acad. Sci. 4: 61.

11. Sargent, J. A. and F. Skoog. 1960. Effects of indoleacetic acid and kinetin on scopoletin-scopolin levels in relation to growth of tobacco tissues in vitro. Plant Physiol. 35: 935-941.

12. Skoog, F. and E. Montaldi. 1961. Auxin-kinetin interaction regulating the scopoletin and scopolin levels in tobacco tissue cultures. Proc. Nat. Acad. Sci. 47: 36-49.

13. Van Bragt, J., L. M. Rohrbaugh, and S. H. Wender. The effect of 2,4-dichlorophenoxyacetic acid on the rutin content of tomato plants. Phytochemistry. In Press.

14. Watanabe, R., W. J. McIlrath, J. Skok, W. Chorney, and S. H. Wender. 1961. Accumulation of scopoletin glucoside in boron-deficient tobacco leaves. Arch. Biochem. Biophys. 94: 241-243.