

ALLELOPATHIC EFFECTS OF HELIANTHUS ANNUUS:
A QUANTITATIVE SEQUENTIAL ANALYSIS OF EXTRACTED
CHLOROGENIC ACID

A THESIS

Submitted to

the Department of Biology

Kansas State Teachers College, Emporia, Kansas

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by

Susan B. Vuturo

May, 1971

Th

Robert L. Parenti
Approved for Major Department

Lawrence B. Boyer
Approved for Graduate Council

311015 2

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to Dr. Robert L. Parenti for his encouragement and patience during the course of this investigation. I would also like to thank the members of my graduate committee, Dr. Richard P. Keeling and Dr. James S. Wilson, for their aid during the stages of research and for their assistance in the improvement of this manuscript. Finally, I wish to thank Mr. John L. Koch, whose advice on the preparation of "known" chlorogenic acid dilutions was sincerely appreciated.

TABLE OF CONTENTS

	PAGE
LIST OF TABLES	v
LIST OF FIGURES	vi
INTRODUCTION	1
MATERIALS AND METHODS	4
RESULTS AND DISCUSSION	10
SUMMARY	19
LITERATURE CITED	20

LIST OF TABLES

TABLE		PAGE
I.	Chlorogenic acid "known" dilutions and absorbance values	8
II.	Chlorogenic acid in <u>Helianthus annuus</u> : "unknown" concentrations and absorbance at 330 nM	11
III.	Inhibitory effects of <u>Helianthus annuus</u> extract on <u>Psyllium ovata</u> seed germination.	16

LIST OF FIGURES

FIGURE		PAGE
1.	Chlorogenic acid "known" dilutions and absorbance values	9
2.	Chlorogenic acid in <u>Helianthus annuus</u> : "unknown" concentrations and absorbance at 330 nM	12
3.	Inhibitory effects of <u>Helianthus annuus</u> extract on <u>Psyllium ovata</u> seed germination: total % germination .	17
4.	Inhibitory effects of <u>Helianthus annuus</u> extract on <u>Psyllium ovata</u> seed germination .	18

INTRODUCTION

Booth (1941) described succession in abandoned fields in central Oklahoma and southeastern Kansas as a four-stage process. Stage 1, the weed stage, commonly lasts for two to three years. An important pioneer species of Stage 1, Helianthus annuus, has been found to play an allelopathic role in the self-elimination of the weed stage and its subsequent transition to the annual grass stage of succession (Wilson and Rice, 1968). The phytotoxic effects of H. annuus have been attributed primarily to concentrations of chlorogenic acid, scopolin, and isochlorogenic acid present in all parts of the plant. Although chlorogenic and isochlorogenic acids have not been detected in leaf leachates and root exudates of H. annuus, it has been postulated that phytotoxins present in soil extracts from areas of H. annuus growth are by-products of chlorogenic and isochlorogenic acids (Rice, 1965; Zane, 1963; Sondheimer, 1960; Wilson and Rice, 1968).

Since its discovery in 1837, chlorogenic acid has been the subject of much investigation. Many qualitative reports of the detectable presence and isolation of chlorogenic acid from a variety of plants are available. Quantitative analyses of

chlorogenic acid concentrations are now possible with the use of spectrophotometric equipment (Koepe and Rohrbaugh, 1968).

Chlorogenic acid has been found to have a synergistic effect on indole-acetic-acid (IAA) action due to its competitive inhibition of IAA oxidase (Rabin and Klein, 1957). This phenolic compound has also been found to be an inhibitor of several enzyme systems (Sondheimer, 1964). Rice (1965) has postulated that inhibition of enzyme systems may be the chief mode of action of chlorogenic acid in inhibition of seed germination and growth of associated soil bacteria and fungi.

Because the accumulation of chlorogenic acid in a plant would enhance that plant's allelopathic aspects, a knowledge of the conditions which induce changes in the concentration of chlorogenic acid is desirable. Recent investigation indicates that changing environmental conditions affect the concentration of phenolics in H. annuus (Koepe and Rohrbaugh, 1968). Preliminary research indicates plant age to be another controlling factor of chlorogenic acid concentration.

The following research was initiated to gather quantitative data on the concentration of chlorogenic acid in the native sunflower, Helianthus annuus, of ages varying from 2 weeks to 16 weeks. I hypothesized

that a high concentration of chlorogenic acid in younger plants might account, at least partially, for the rapid establishment of H. annuus due to an initial germination inhibition of associated plant species. A high concentration of this phenolic compound in older plants might also play an important role in the accumulation of phytotoxic compounds and their by-products in the soil. Wilson and Rice, 1968, found that sufficient concentration of these phytotoxins in the soil lead to the eventual self-elimination of sunflower clones and a transition to the annual grass stage of succession.

It was hoped that this study might also indicate the relative competitiveness of H. annuus during the various stages of a single growing season and perhaps display a correlation between the concentration of chlorogenic acid and the inhibition of assay seed germination.

MATERIALS AND METHODS

From April 25, 1970, to July 25, 1970, intact Helianthus annuus plants were collected at 2-week intervals from a roadside site located 1 mile east of Ross Natural History Reservation, Lyon County, Kansas. The sunflower clone at this location was the source of all plants used in extract preparation.

Preparation of extracts: Extracts for inhibition of seed germination

H. annuus extract was prepared by grinding 10 g. fresh plant material in 100 ml. of distilled water for 15 minutes. The mixture was then allowed to stand for 20 minutes before filtration through Whatman No. 1 filter paper in a Buchner funnel. The volume of the filtrate was brought back to 100 ml. with distilled water. Fresh extract was used for all tests.

Preparation of extracts: Extracts for chromatographic assays

Chromatography extracts were prepared in the same manner as extracts for germination inhibition tests, except that absolute methanol was used in place of distilled water. 200 ml. extract were prepared for each bi-weekly test group. After filtration, the filtrate was brought back to original volume and was passed

through a cellulose membrane filter (Metricel, GA-6 grid, 0.45 micron pore size, 1 inch diameter, Gelman Instrument Company, Scientific Products--F2932) to eliminate fungal contaminants. The extract was then flash evaporated (Buchler flash evaporator) to 20 ml. and stored at 4°C. until used.

Assays of plant extract as an inhibitor of seed germination

Tests using Psyllium ovata (Turttox Products) seeds as bioassay organisms were conducted to determine the relative inhibitory effects of the bi-weekly extracts. 100 seeds were placed on germination discs in Petri plates. The 25 seeds per germination disc were saturated with 6 ml. of a 5:1 ratio of distilled water to fresh plant extract. 100 control Psyllium seeds were placed in the same manner on discs saturated with 6 ml. distilled water. The plates were incubated at 27°C. in darkness for the duration of the test. Germinating seeds were counted and removed at the 24th, 36th, and 48th hour from the beginning of each incubation test.

Chromatographic and spectrophotometric analysis of plant extracts

Chromatograms were prepared from bi-weekly methanol extracts for chromatography. 10 ml. of flash-evaporated

extract (called "unknown" extract) were applied to each Whatman 3 MM paper. 2 chromatograms were prepared from each 20 ml. bi-weekly extract. The descending paper chromatograms were then developed in n-butanol: acetic acid: distilled water (63: 10: 27, called B.A.W.) for 14 hours. The chlorogenic acid band was identified as a light blue band under short (2537 Å) and long (3360 Å) ultra-violet light, as a yellow-green band after exposure to ammonia vapors, and as a band with an average R_f value of 0.55 (Parenti and Rice, 1969). The chlorogenic acid band was cut from each test paper and eluted from the paper in absolute methanol for 80 hours. The 2 eluates per test were combined, flash-evaporated to 20 ml., and stored at 4°C. until used.

Chromatograms of known chlorogenic acid (called "known") and a control chromatogram were prepared in the dilution series found in Table 1. All "knowns" were dissolved in absolute methanol. The control was absolute methanol only. 5 ml. of each dilution and the control were applied to Whatman 3 MM paper. These chromatograms were developed in B.A.W., eluted in absolute methanol, and flash-evaporated in the same manner as the previously described "unknown" extract chromatograms.

All eluates, "known," "unknown," and control, were analyzed with the spectrophotometer (Hitachi-Perkin-Elmer, Model 139) at 330 nm and density absorbance

readings were obtained. "Knowns" and the control were read at a 1:10 ("known": absolute methanol) dilution; "unknowns" were read at a 1:100 ("unknown": absolute methanol) dilution. The absorbance values for "known" concentrations are found in Table I.

TABLE I. Chlorogenic acid "known" dilutions and absorbance values.

Dilutions : mg chlorogenic acid per ml, absolute methanol	Absorbance at 330 nM: 1 : 100 dilution ("known; absolute methanol)
2.0	+1.9
1.0	+1.2
.5	+ .470
.25	+ .270
.125	+ .134
.0625	+ .052
.03125	+ .027
.015625	+ .010
.0078125	+ .005
.00390625	- .010
Control	0.000

Absorbance at 330 nM

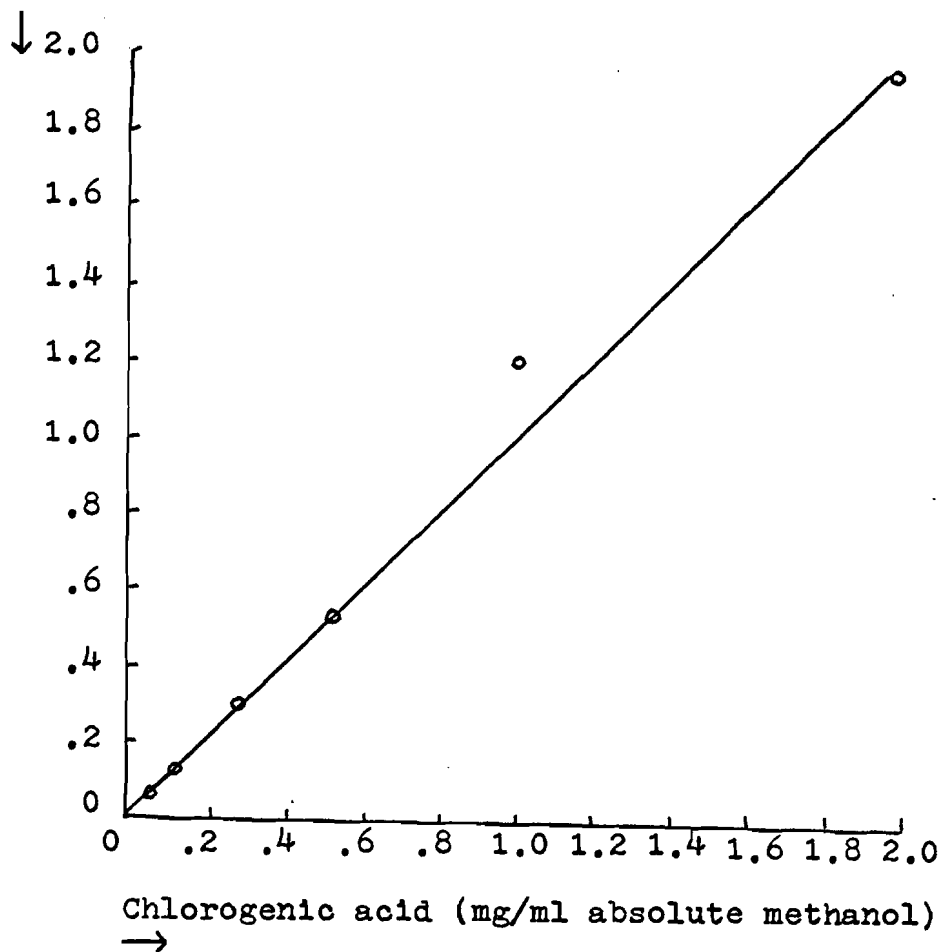


Figure 1. Chlorogenic acid "known" dilutions and absorbance values.

RESULTS AND DISCUSSION

Quantitative results

Quantitative analyses of chlorogenic acid were made on Helianthus annuus extracts from plants 2 to 16 weeks of age. The concentration of chlorogenic acid per gram weight of H. annuus was highest in 2-week old plants. Concentration decreased until the eighth week and then increased substantially to the tenth week. The chlorogenic acid content became static from the tenth to the fourteenth week. Between the fourteenth and sixteenth weeks, the concentration of chlorogenic acid again increased (Table II and Figure 4).

The appearance of chlorogenic acid during the breaking of dormancy in the fruits of the native sunflower is of particular interest. Lane (1965) reported the absence of detectable chlorogenic acid in dry fruits of H. annuus. He first detected the presence of this phenolic compound in sunflower fruits during the period between the sixteenth and thirty-second days of moist, cold stratification of the fruits. Lane suggested that the chlorogenic acid that appeared during the stratification process might act to inhibit IAA oxidase and increase the amount of IAA in the

TABLE II. Chlorogenic acid in Helianthus annuus:
 "unknown" concentrations and absorbance at 330 m μ .

Age of plant (in weeks)	mg chlorogenic acid per g. plant material	Absorbance at 330 m μ . 1 : 100 dilution ("unknown" ; absolute methanol)
2	3.720	1.750
4	1.616	.780
6	.692	.331
8	.012	.080
10	1.932	.930
12	2.004	.951
14	1.960	.940
16	3.172	1.510
Control	0.000	0.000

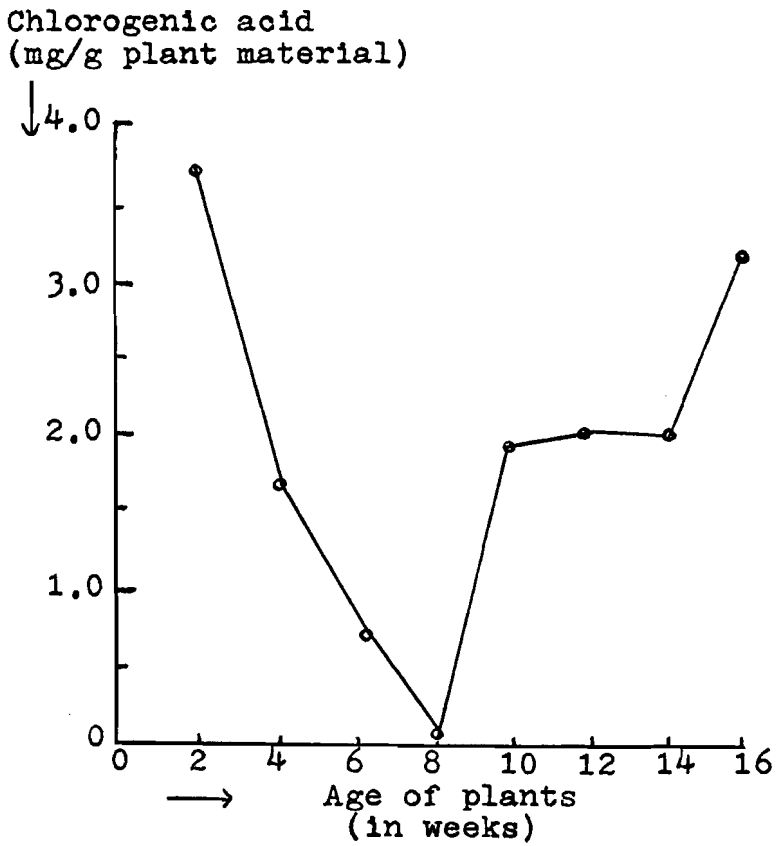


Figure 2. Chlorogenic acid in Helianthus annuus:
"unknown" concentrations and absorbance at 330 nm.

embryo plant. With an increase in IAA, there is an increase of embryonic growth. In conjunction with its synergistic qualities, chlorogenic acid has been shown to be an effective inhibitor of the growth of nitrogen-fixing and nitrifying bacteria (Rice, 1965). The two above-mentioned characteristics of chlorogenic acid would be advantageous factors in the early establishment of young H. annuus plants. Rapid early growth due to an increase in the IAA content of the plants would give H. annuus seedlings priority in the competition for light among surrounding species. A low concentration of soil nitrates tolerated by H. annuus would not enhance the growth of competing species.

The decrease of chlorogenic acid concentration observed from the second to the eighth week may be in part due to a dilution factor caused by increased tissue and reduced, if not arrested, production of chlorogenic acid in the sunflower at this time.

The moderate peak in chlorogenic acid concentration at the tenth week of growth coincides with the development of flower buds on the plants. At this stage, the plant has completed the majority of its tissue development and is beginning to develop the precursive organs of fruit production. Perhaps a relatively high level of chlorogenic acid at this stage enhances the growth

and development of the flowers and future fruits.

Phenolic compounds in mature plants eventually return to the soil, directly or as by-products, as the plant material decays. The observed high concentration of chlorogenic acid in mature sunflower plants and the ensuing rapid build-up of inhibitors in the soil surrounding sunflower clones would contribute to the rather short duration of the weed stage of succession.

Inhibitory effect on bioassay seed germination

Helianthus annuus extract reduced germination in all Psyllium tests. However, the degree of inhibition had no direct correlation with the concentration of chlorogenic acid in the extract. Lowest inhibition of germination occurred with extract from 12-week H. annuus plants; highest germination inhibition occurred with extract from 10-week old plants. Concentration of chlorogenic acid was approximately equal in 10 and 12-week plants (Table III and Figure 3).

The lack of correlation between chlorogenic acid concentration and allelopathic effect may indicate that the total phytotoxic effect of sunflower extract is due to a phytotoxin other than chlorogenic acid or the combined effects of several phytotoxins, including chlorogenic acid.

The greatest degree of suppression of germination was observed during the first 24 hours of each test. This effect is probably caused by a degeneration of phytotoxins after the initial hours of incubation (Figure 4).

As a conclusion to this paper, I would like to mention two major suggestions for improvement in investigations of this type: 1.) Because of environmental variables to which field samples are subject, error in quantitative results is certain. Environmental conditions influence the concentration of chlorogenic acid and might obscure the effects of plant age alone. Samples for this type of investigation should be grown in growth chambers, if possible. 2.) Whole-plant extracts are an inaccurate basis for tests on a lapsed-time schedule due to root-stem-leaf proportion differences between seedlings and mature plants. Limiting the material for extraction to one plant organ would be advisable.

TABLE III. Inhibitory effects of Helianthus annuus extract on Psyllium ovata seed germination.

Extract: Age of plant (in weeks)	Percentage of seeds germinated/ time in hours			
	24	36	48	Total % germination
2	0%	33%	69%	69%
4	28	77	79	79
6	2	55	72	72
8	1	47	73	73
10	1	35	63	63
12	9	80	88	88
14	6	58	73	73
16	3	55	68	68
Control (average)	36	84	90	90

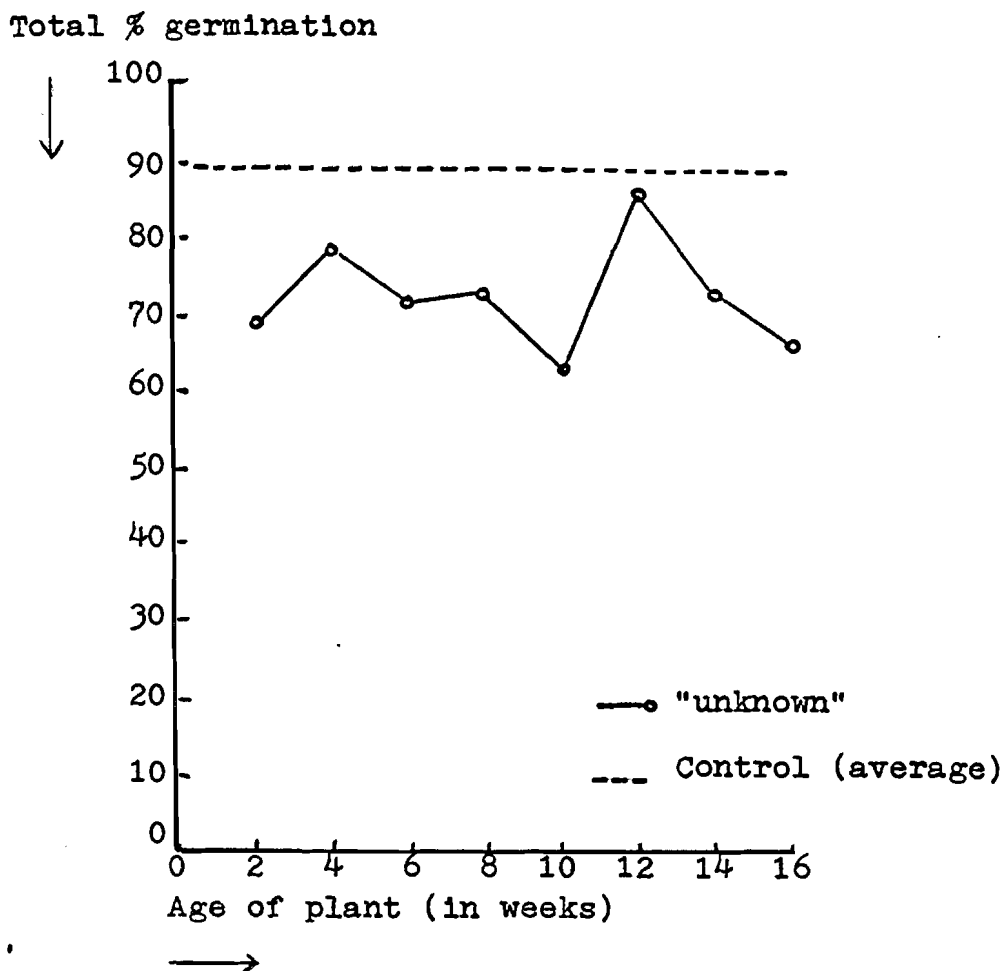


Figure 3. Inhibitory effects of Helianthus annuus extract on Psyllium ovata seed germination: total % germination.

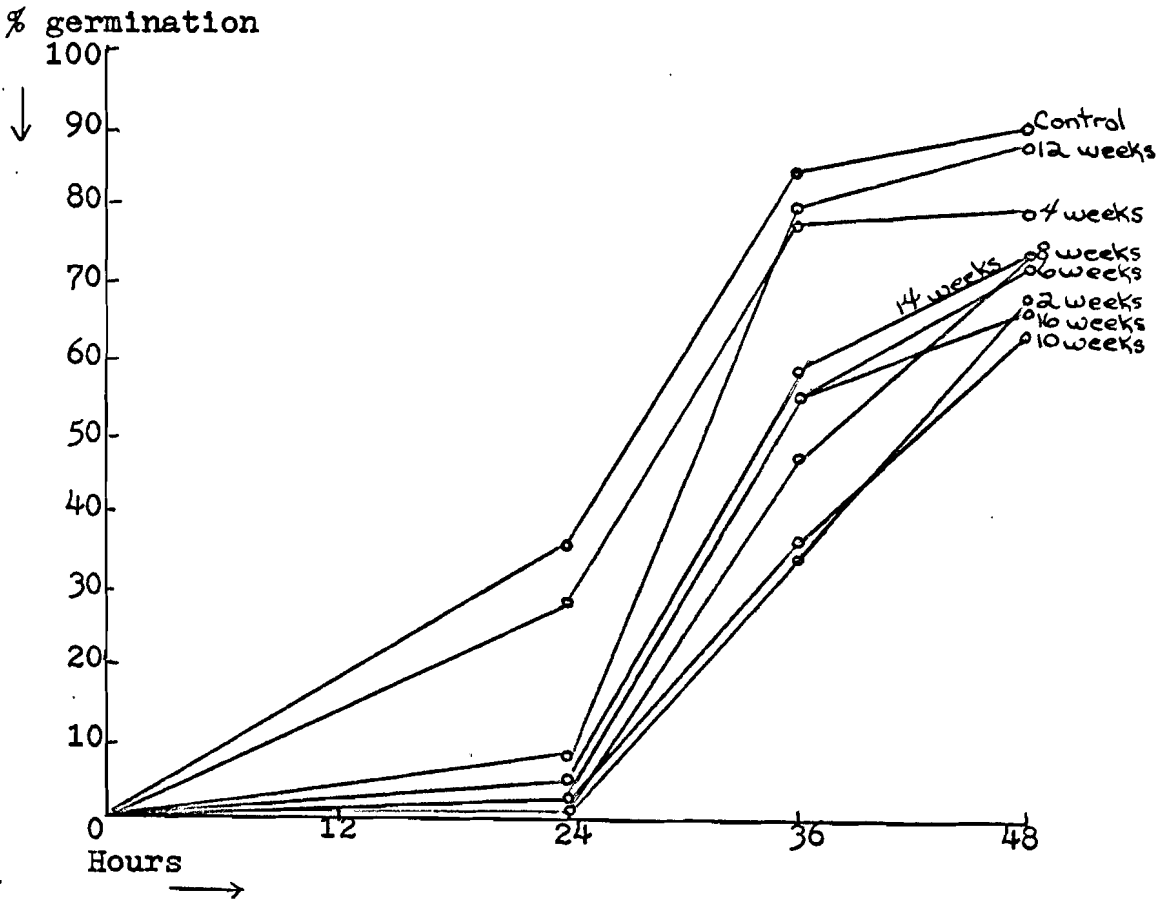


Figure 4. Inhibitory effects of Helianthus annuus extract on Psyllium ovata seed germination.

SUMMARY

A prominent pioneer species of the weed stage of succession in southeastern Kansas, Helianthus annuus (sunflower), has been found to play an allelopathic role in the self-elimination of the weed stage and its subsequent transition to the annual grass stage. The phytotoxic effects of this species have been attributed to high concentrations of chlorogenic acid, scopolin, and isochlorogenic acid present in all organs of the plant. It has been postulated that the concentration of chlorogenic acid per gram of plant material varies due to changes in plant age. Quantitative analyses of chlorogenic acid were made on H. annuus extracts from plants 2 to 16 weeks of age. Concentration was highest in 2-week plants, then decreased until the eighth week. An increase occurred between the eighth and tenth weeks; a plateau was maintained from the tenth to the fourteenth week. Between the fourteenth and sixteenth weeks, the concentration again increased to a level slightly below that observed in 2-week plants. H. annuus whole-plant extract reduced germination in Psyllium seed tests. However, the degree of inhibition showed no direct correlation with the concentration of chlorogenic acid in the extract; this may indicate that the major germination inhibitor in H. annuus is not chlorogenic acid.

LITERATURE CITED

LITERATURE CITED

- Booth, W.E. 1941. Revegetation of abandoned fields in Kansas and Oklahoma. *Amer. J. Bot.* 28: 415-422.
- Koeppel, D.E. and L.M. Rohrbaugh. 1968. The effect of environmental stress conditions on the concentration of chlorogenic acid and scopolin in tobacco and sunflower. International Biology Program, Santa Barbara, California.
- Lane, F.E. 1965. Dormancy and germination in fruits of the sunflower. Ph.D. Dissertation, University of Oklahoma, Norman.
- Parenti, R.L. and E.L. Rice. 1969. Inhibitional effects of Digitaria sanguinalis and possible role in old-field succession. *Bull. Torr. Bot. Club.* 96(1): 70-78.
- Rabin, R.S. and R.H. Klein. 1957. Chlorogenic acid as a competitive inhibitor of IAA oxidase. *Arch. Biochem. Biophys.* 70: 11-15.
- Rice, E.L. 1965. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. II. Characterization and identification of inhibitors. *Physiol. Plant.* 18: 225-268.
- Sondheimer, E. 1960. The chlorogenic acids and related compounds. In Plant phenolics and their industrial significance (V.C. Runeckles, ed.), p. 15-37. Symp. Plant Phenolics Group of North America. Imperial Tobacco Co. of Canada, Montreal, Quebec.
- _____ 1964. Chlorogenic acids and related depsides. *Bot. Rev.* 30: 667-712.
- Wilson, R.E. and E.L. Rice. 1968. Allelopathy as expressed by Helianthus annuus and its role in old-field succession. *Bull. Torr. Bot. Club.* 95(5): 432-448.
- Zane, A. 1963. Isolation, identification, synthesis and pyrolysis of plant phenolic compounds. Ph.D. Dissertation, University of Oklahoma, Norman.