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AN INVESTIGATION OF POSSIBLE
ALLELOPATHIC ACTIVITY OF Artemisia
Evidenced by SUSPECT'S Vasoyana

By

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B.S., Carroll College, 1970

Presented in partial fulfillment of the
requirements for the degree of

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1972

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Date

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CHAPTER I

INTRODUCTION

Allelopathy is chemical inhibition among plants. "Substances which can produce allelopathic effects are...universal in plants and plant communities,...and efficient" (1). Tobacco, citrus and walnut trees, sagebrush, and corn are well known examples of plants which produce allelopathy. Also it is known that a variety of plants, e.g., cucumber, barley, peach trees, and grapes respond to allelopathy (2).

Allelopathic compounds belong to several groups of organic chemicals. These groups include: terpenes, organic acids and aldehydes. Some types of compounds cause indirect allelopathic effects. For instance, indirect effects are shown by antibiotics and enzymes (3). In these cases, microorganisms in the soil are affected by the allelopath and, in turn, the organisms determine the chemical environment of the soil which can be inhibitive (4).

Several methods are used by plants to release allelopathic compounds to the surrounding environment. Root and leaf-fall leach materials from leaves and transfer the compounds to the soil. Because allelopathic are organic compounds, they can be dissolved and concentrated in organic matter, particularly in the liquid phases of seeds and seedlings in the vicinity (5). Soil adsorption also increases the concentration of allelopathic materials. It is introduced some of the allelopathic into the surrounding soil environment.

Many allelopathic substances are volatile, and are concentrated around the producing plant to form an atmospheric shield, or are washed to the ground by rainfall. Other plants produce inhibitors upon decay; decomposition releases growth-retarding chemicals to the surrounding soil (1).

Four points should be shown before compounds are established as being allelopathic: 1) the active substance should be isolated and identified, 2) the compound should have allelopathic effects when placed on or in neutral agar growth substrates, 3) allelopathic activity should also be tested in field populations, 4) a quantitative relationship between concentration of substance and level of activity should exist (1).

Some contend that the defensive chemicals are waste products and only coincidentally have inhibitory effects; such chemicals vary too much from plant to plant to be metabolites. Others believe that the compounds are produced explicitly for allelopathic effects. A general overall view suggests that the chemicals were by-products in the plant's evolutionary history but, since they had inhibitory effects on competitors, caused positive selection for the producing plant. Continuation of this process, through time and plant evolution, has resulted in actual defensive chemical production by the plant. Because the by-product protected the plant, it eventually evolved into a purposeful protectant. Specific glands for production and compartmentalization of protective chemicals in some species are considered evidence that these chemicals are used specifically for defense (1).

Concentration and age contribute to the effectiveness of the allelopathic compounds. Generally, low concentrations, around 10^{-7} or 10^{-8} molar, are sufficient to produce inhibition. These levels were established using extract application to seeds or seedlings (7). Soil seed or seedling concentrations, as well as prevailing environmental concentrations, have not been well established. Age of the plant producing the inhibitor and age of the allelopathic extract itself cause variation in toxicity. In general, produce less toxic substances and toxicity increases with age, but an extract toxicity decreases with age (8).

Although allelopathic effects are evident in all types of plant communities, the phenomena were first noticed in the arid regions. Dryness enhances the inhibitory activities of allelopathy. If all the plants are to germinate, they are in a weakened condition to compete as they mature. In an arid region seedling are too inhibited to successfully compete for water and nutrients. In some cases, fire will reduce the concentration of allelopathic substances in the soil (9). All of these factors contribute to the degree of inhibition caused by allelopathic compounds.

Allelopathy also reduces the growth rate of susceptible plants. Root, lateral root, and shoot growth are retarded; germination, reproduction, and flowering, seed production and nutrient absorption are inhibited; senescence is affected. Susceptible plants are weakened (3,7,10). Allelopathy is not only specific to particular plant groups, i.e., roots, leaves, and allelopathic way to be not a specific and also self-inhibition factor decon-

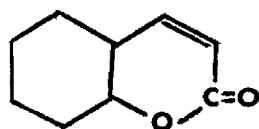
strated. Symptoms of allelopathic interference include: chlorosis, early leaf abscission, and distortion and curling of root tips or cotyledons (2).

The composition and succession patterns of plant communities are regulated, at least to a degree, by the presence of allelopathy. Allelopathic substances affect the tolerance or resistance of the plant community. The pattern of plant succession is partially determined by allelopathic production by a single dominant plant. Those plant species which are resistant to the allelopathy will become the dominant species (1). Patterns such as foliar senescence and a reduction of starch content are clear evidence of the effects of allelopathy on neighboring plants.

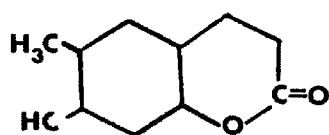
Allelopathic substances contribute to the stability of plant communities. However, if a favored species is introduced which is not affected by the inhibitors present, the invader might become dominant.

The allelopathic properties of Salvin leucodermis have been studied extensively by H. H. Miller, E. H. Miller, and associates (10,11,12,13). Stimulated by the growth pattern of S. leucodermis, which is an aquatic plant in relation to the environment, they began to study the allelopathic properties of S. leucodermis. Studies of the growth of S. leucodermis in relation to the growth of Salvin leucodermis have been reported by Miller and Miller (14), and Miller and Miller (15). The results of these studies indicate that Salvin leucodermis is a potent inhibitor of the growth of Salvin leucodermis and that the allelopathic properties of Salvin leucodermis are related to the growth of Salvin leucodermis. The results of these studies indicate that the allelopathic properties of Salvin leucodermis are related to the growth of Salvin leucodermis.

logically, the effect of soil compaction, and all its associated
consequences, in the low-altitude region is perhaps to exhib-
it the same growth pattern system as shown in Fig. 1 (). A
kind of "flare" of vigorous new vegetation, which spreads from the
center outward. A kind of an "eternity flame" is formed by the
development of beyond the bare soil, the active growth of the
the same kind of a "flare" in the center of the, "eternity flame"
and in the center of the "eternity flame" is the "eternity flame".

[illegible]

Geological is showing the last 100 years.



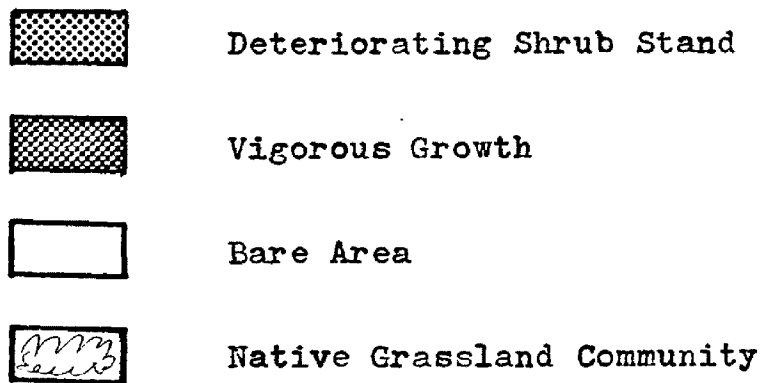
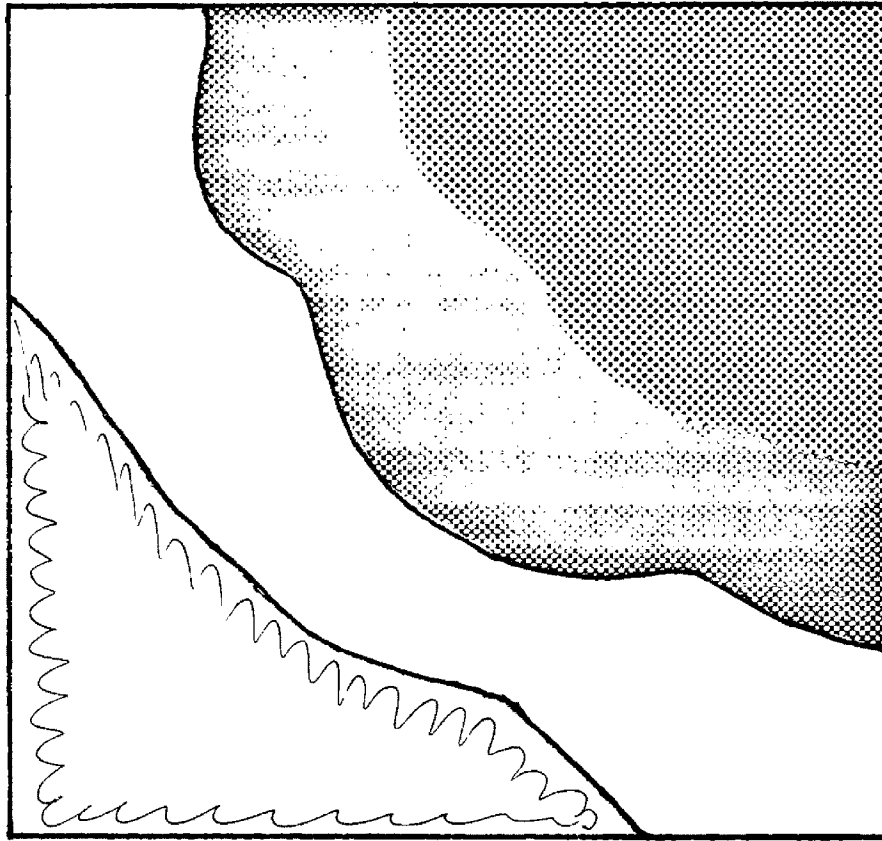
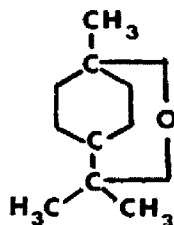


Figure 1. Growth pattern exhibited by some aromatic shrubs including chapparal and sagebrush.

Another compound found in Antennaria, coumarin, has been shown to inhibit glucose 6-phosphate dehydrogenase in tobacco (11). Coumarins fluoresce in ultra violet light and can be extracted by steam distillation. Identification and identification can be effected by several chromatographic methods (12).

Terpenes and oxygenated derivatives of terpenes make up the bulk of volatile oils of Antennaria. The most commonly occurring odors, oils from Antennaria have been used in paints, soaps, flavorings, perfumes and medicinal products. A variety of plant repellents or attractants (13). Lower terpenes have been shown to be important host specific repellents. An example is given of:



Essential oils usually contain one or two predominant terpenoids. However, oils are complex mixtures; therefore, separation of individual components is difficult. Extraction involves steam distillation, solvent extraction by saponification with alkali, and acration (14). Separation methods include: Chromatography, i.e., gas, reverse phase, thin layer, column; and derivative formation (15).

Since Antennaria exhibits some properties similar to those of S. leucophylla, the author studied essential allelochemical compounds which might be present in the plant using known biosynthetic techniques.

The initial objective of this study was to investigate the toxic effect of Antennaria tridentata var. vaughana on growth of

Cucumis sativus, Avena and three native herbs. One native herb was collected from within the A. tridentata community, a second was taken from the ecotone of an A. tridentata community, and a third was collected from an area in which A. tridentata growth was absent. Toxic effects of macerated leaf material, leaf extract, and purified growth inhibitors were quantified in seed growth chambers. The toxic or stimulatory effects on germination, radicle and hypocotyl growth, respiration and photosynthesis were determined.

However, neither Avena nor the native herb seeds could be successfully germinated; therefore, the section of the proposal concerning these species could not be developed. A study of autotoxic effects on viable A. tridentata ssp. wyomingensis seeds replaced this section. Photosynthesis studies were not carried out since seeds that were germinated and grown in dark chambers were chlorotic.

It was hoped that these tests would reveal the presence or absence of allelopathic compounds in A. tridentata ssp. vaseyana.

CHAPTER II

MATERIALS AND METHODS

Experimental Plants

Cucumis sativus, Boston pickling cucumber from the Chas H. Lilly company, was used because of its rapid and uniform germination. After soaking in aerated, distilled water for one and one-half to two and one-half hours, seeds were placed in seed chambers. These chambers were 300 ml Erlenmeyer flasks in which filter paper was placed over a layer of glass wool. Seeds were placed on the filter paper and 20 ml distilled water were added to the flasks. Flasks were sealed with parafilm and placed in the dark at approximately 36°C for four to six days. Growth inhibition was obtained by placing various quantities of macerated sagebrush in vials in the flasks or by placing various quantities of the different extracts, or purified compounds directly on the filter paper in the flasks.

Artemisia tridentata ssp. vaseyana was gathered six miles west of Missoula, Montana, between May and September of 1971. Leaves and a few buds were picked from the gathered sprigs and then macerated. Those that were picked and macerated immediately after gathering were labeled fresh and those that were first dried for several weeks after gathering, and then picked and macerated were labeled dry.

Fractionation and Purification Procedures

Extraction

The extraction procedure was as follows. First, 0.50 kg of macerated, dried sagebrush was extracted with 2.5 l of 95% ethanol. The material was soaked in the ethanol for one day and then filtered. A second portion of 1.8 l of 95% ethanol was added to the once extracted mash. The two extract filtrates were evaporated to dryness to yield 114 g of dark green, syrupy residue. Then, 100 g of this residue was partitioned between 200 ml distilled water and 750 ml chloroform. The chloroform portion was washed with 200 ml water. The water portion and wash were combined and evaporated to dryness giving approximately 30 g gold-brown syrup. Upon evaporation, the chloroform portion yielded 55 g very dark green, oily residue which was partitioned between 500 ml aqueous methanol (1 water: 2 methanol), and five 200 ml portions of petroleum ether, 30 - 110°C fraction. Each of these was evaporated; the aq. MeOH portion yielded 30 g dark green syrup and the petroleum ether portion yielded 22 g of a green, slightly viscous solution. Part of the aq. MeOH portion was used for chromatography.

Column Chromatography

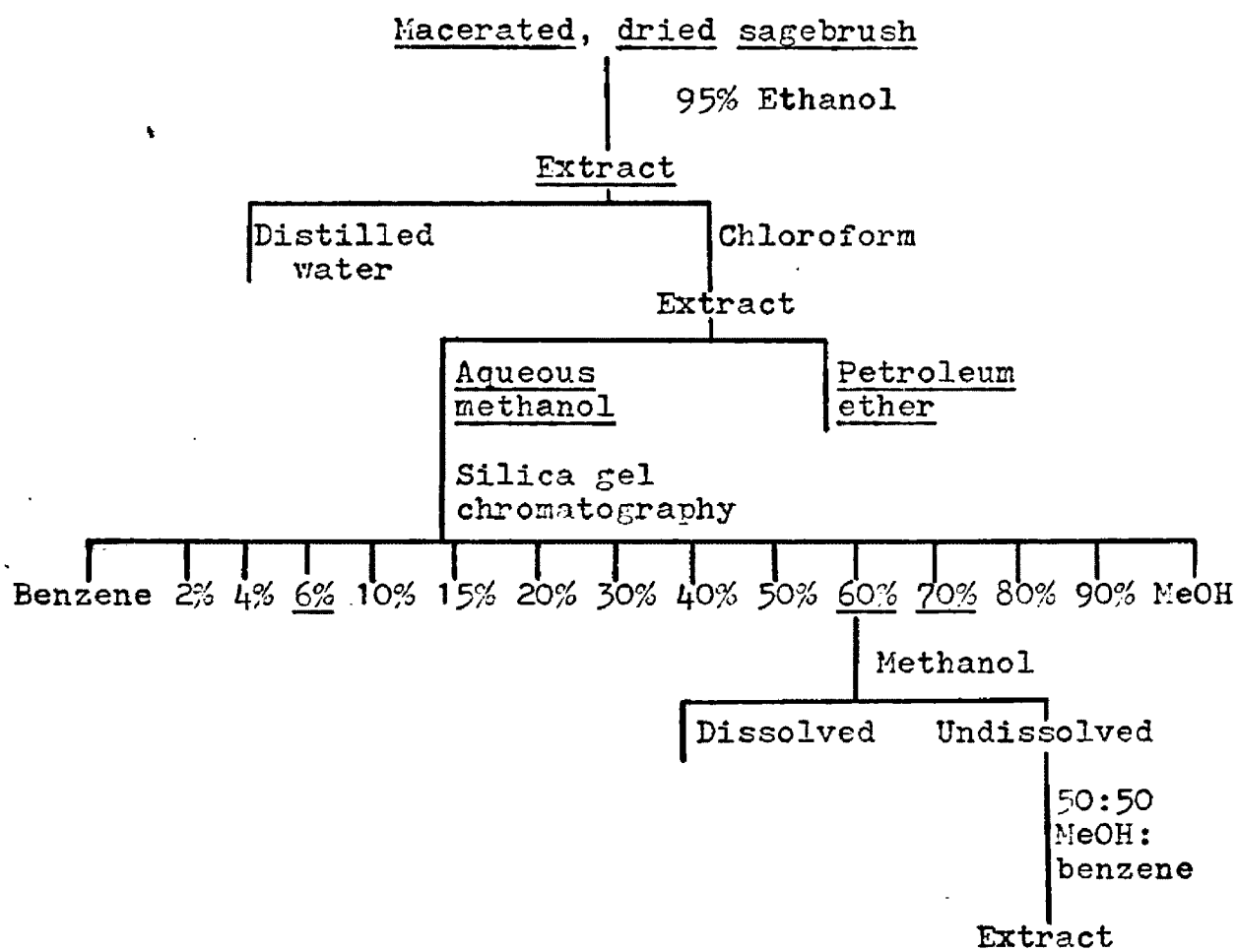
A chromatography column was prepared using 200 g powdered silica gel slurried in benzene. To this column was added 20 g of the aq. MeOH extract dissolved in 10 ml benzene. Solvents for elution were mixtures of methanol and benzene which varied in percentage by volume composition. Each solvent was passed through the column until evaporation of the eluent gave no product.

Successive elutions and evaporation of the fractions gave the following products: 17.1 l 10% benzene yielded 7.2 g dark green residue; 4.3 l 2% KOH-benzene yielded 2.1 g dark green residue; 6.2 l 4% KOH-benzene yielded 1.5 g dark green, very viscous, oily residue; 5.3 l 6% KOH-benzene yielded 1.2 g dark green, viscous residue; 7.4 l 10% KOH-benzene yielded 1.5 g dark green, crystalline and oily residue; 3.6 l 15% KOH-benzene yielded 1.5 g green-brown, crystalline and syrupy residue; 7.1 l 20% KOH-benzene yielded 1.3 g brown crystalline residue; 11.1 l 30% KOH-benzene yielded 2.1 g brown, crystalline residue and yellow-gold syrup; 6.1 l 40% KOH-benzene yielded 2.6 g dark brown, oily, crystalline material; 4.1 l 50% KOH-benzene yielded 2.2 g dark yellow, syrupy, crystalline residue; 3.2 l 60% KOH-benzene yielded 2.2 g brown crystals; 10.5 l 70% KOH-benzene yielded 2.2 g dark yellow powder; 7.3 l 80% KOH-benzene yielded 2.1 g light brown crystals; 4.2 l 90% KOH-benzene yielded 2.35 g dark yellow powder; finally, 4.4 l 100% KOH yielded 2.35 g brown powder. After activity tests, the 60% KOH-benzene fraction from this column was selected for further purification.

The 60% KOH-benzene fraction was partially dissolved in 100% KOH. The dissolved portion was entered onto a 20 g silica gel chromatography column and separated into a diffuse band. Thin layer chromatography showed that diffuse bands of the band contained one and the same compound. The undissolved portion was dissolved in 50:50 KOH:benzene, filtered and concentrated to dryness. A flow chart covering extraction, column fractionation and purification is presented in Table 1.

Table 1. Flow chart following the extraction of active inhibitors present in Artemisia tridentata ssp. vaseyana. Those fractions underlined were found to be biologically active as inhibitors. X% expresses percentage of methanol in benzene

EXTRACTION METHOD



Thin Layer Chromatography

The 75% MeOH-benzene fraction and both the dissolved and undissolved portions of the 60% MeOH-benzene fraction were compared with pure esculin using thin layer chromatography. Thin layer chromatography chambers contained 70:30 MeOH:benzene, 60:40 MeOH:benzene, and 50:50 MeOH:benzene as solvent systems. Sheets of Silica Gel II-E from Baker-flexTM were used as the chromatography matrix.

Melting point determinations were attempted using a Fisher-Johns A. P. apparatus. An attempt to obtain a nuclear magnetic resonance spectrum for the undissolved 60% MeOH-benzene fraction was made using the Varian model A-60 II, δ -chloroform solvent and TMS lock.

Cochromatography indicated that the undissolved portion of the 60% MeOH-benzene fraction was not esculin. The material fluoresced under ultra violet light and had R_f values: 0.50 in 60:40 MeOH:benzene and 0.33 in 70:30 MeOH:benzene. It did not show an inconclusive broad band at 3.2 - 3.6 μ ; i. e. determinations gave broad ranges. Amount of compound limited analytical measures. The 75% MeOH-benzene fraction did not fluoresce and its thin layer chromatography and R_f differed from that of the 60% MeOH-benzene fraction.

Germination and Growth Measurements

Germination percentage was figured on the assumption that any plant with its radicle extended from the seed pod was a germinated plant. Such plants were counted and compared to the number of

minated in a control chamber.

Growth measurements included length of radicle and hypocotyl, and number of lateral roots. Radicles were measured from "beak" to tip; hypocotyls were measured from "beak" to the point of attachment of the cotyledons.

Measurements of germination, growth and respiration recorded those effects produced by: leaves, crude extracts, the column fractions separated by chromatography and pure esculin. Macerated sagebrush leaves (fresh and dried), the aqueous-ethanol extract and pure esculin were used in varying amounts in seed chambers. One specific test measured the effects of sagebrush on seedlings of different ages. Germination and growth measurements were also carried out on the dissolved and undissolved portions of the 60% MeOH-benzene fraction. For all of the previous trials G. sativus was the assay species. A. trifidens and A. angustifolia seeds were used to test autotoxic activity of the aq. Me fraction.

Manometric Techniques

Measurement of respiration employed a Gilson respirometer. Respirometer flasks contained 0.2 g fresh weight of seedlings, 1 ml diethanolamine buffer solution, 0.4 ml in the center well and 0.6 ml in the side arm (17), which maintained a constant CO_2 level, and in some cases various amounts of fresh sagebrush. Primarily, the respiration of seedlings was measured without sagebrush present in the respirometer flask. Gas exchange was measured in microliters of five or ten minute intervals. The bath temperature was kept at 25°C in all experiments reported.

CHAPTER III

RESULTS

Macerated A. tridentata leaves were tested in order to establish whether sagebrush produces biologically active toxins. Measurements in the following tables, 2 through 10, used correct means for average lengths. Range indicated the spread of fernell clusters rather than the spread of all germinated seedlings; extremes were eliminated from calculations. "Number of sample/ number planted" recorded the number of samples which formed the cluster compared with the number of available values (number planted). Activity is based on the percentage of growth a group of plants has when compared to a control group.

Leaves were macerated in order to release volatile compounds more readily and for convenience in handling. Generally, fresh material showed inhibitory activity on growth, Table 2. Germination was not significantly affected by 0.1 g to 5.0 g of leaf material. Stimulation of growth resulted from 0.1 g material with hypocotyl growth showing the greatest increase and radicle growth showing the least. Severe inhibition occurred when 0.5 g to 5.0 g of sagebrush was present in growth chambers. Growth decreased with increasing amounts of leaf material. Hypocotyls showed most resistance to inhibition, but even in the case of hypocotyls, when 0.5 g of sagebrush was present, growth was reduced to 23% of the control. Radicle growth was cut to 3.5% of the control with 0.5 g

sagebrush. However, 1.0 g reduced growth to 1.6% of the control, while 5.0 g sagebrush reduced growth to only 3.1% of the control. This is the only deviation from a strictly proportional relationship between reduced growth and increased amounts of leaf material. No lateral roots were developed on the main root when 2.5 g to 5.0 g of sagebrush was present.

Table 2. Growth of *G. artemisiae* seedlings exposed to fresh, macerated *A. tridentata* leaves. Total germination time was 6 days.

Fresh macerated leaf material, g	0	0.1	0.5	1.0	5.0
Number of seeds germinated	20	19	8	20	19
% of Control	--	95.0	100	100	95.0
Radicule length, mm					
Average	67	67	2.7	1.0	1.0
Range	33-84	53-75	1-4	1	1-3
Number of sample/ number planted	15/20	12/19	13/20	16/20	20/20
% of Control	--	100	3.5	1.6	3.0
Hypocotyl length, mm					
Average	30	32	5.0	4.0	0.0
Range	10-70	10-33	0-11	3-7	0
Number of sample/ number planted	15/20	12/19	20/20	15/20	20/20
% of Control	--	145	23	13	0.0
Number of lateral roots					
Average	15	12	0.6	0.0	0.0
Range	0-25	10-27	0	0	0
Number of sample/ number planted	16/20	10/19	17/20	20/20	20/20
% of Control	--	120	0	0.0	0.0

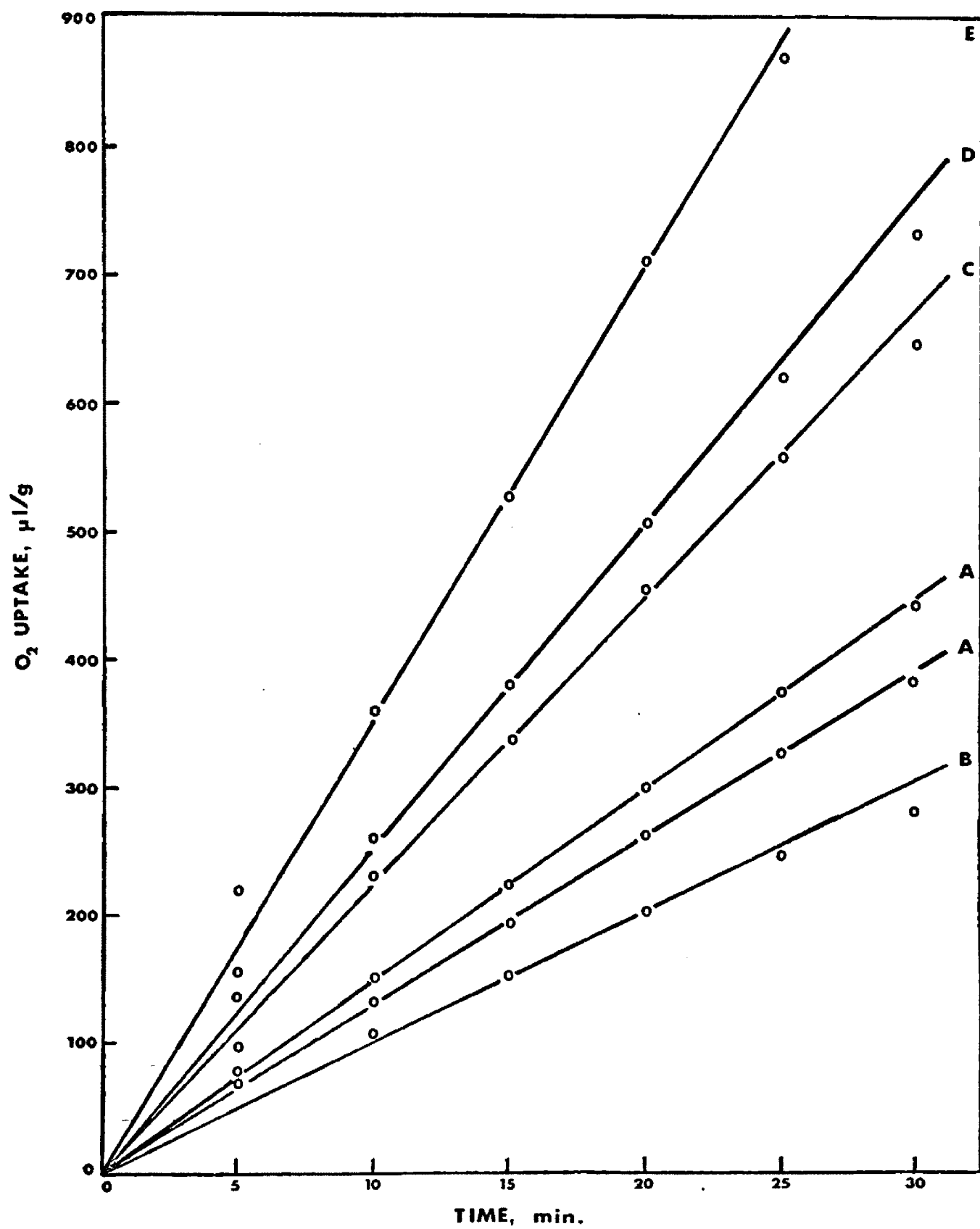
Since cineole and camphor are known inhibitors (13), and it is known that they are present in *A. tridentata*, dry sagebrush was tested to see if the macerate was still biologically active after these allelopaths had volatilized. Dry material was also more easily obtained.

Dry leaf macerate proved inhibitory, however, the degree of inhibition was less than that of fresh material, Table 3. Germination was not affected. A very slight effect on growth was caused by 0.1 g dry material. Inhibition of growth increased as the amount of inhibitory material present increased from 0.5 g to 5.0 g. Hypocotyl growth was reduced slightly, radicles were more severely affected and lateral roots were inhibited the most with no root extensions when 1.0 g and 5.0 g macerate were present. Seedlings grown in the presence of 0.5 g to 5.0 g of macerate exhibited stimulation of germination, Fig. 2. Rootination was inhibited when seedlings were grown with 0.1 g of macerate.

Table 3. Growth of *C. sativus* seedlings exposed to dry macerated *A. tridentata* leaves. Total germination time was 6 days.

Dry macerated leaf material, g	0	0.1	0.5	1.0	5.0
Number of seeds germinated	20	20	20	20	20
% of Control	--	100	100	100	100
Radicle length, mm					
Average	63	66	33	4.0	1.6
Range	33-84	54-71	12-25	2-12	1-3
Number of sample/ number planted	15/20	13/20	10/20	15/20	17/20
% of Control	--	105	30	75	2.5
Hypocotyl length, cm					
Average	22	21	2.0	7.0	8.6
Range	15-30	8-28	5-10	5-10	7-9
Number of sample/ number planted	15/20	20/20	11/20	15/20	13/20
% of Control	--	100	55	75	65
Number of lateral roots					
Average	15	16	1.1	0.1	0.0
Range	9-25	13-19	5-10	0	0
Number of sample/ number planted	16/20	15/20	15/20	11/20	20/20
% of Control	--	107	57	0.0	0.0

Figure 2. Rate of oxygen uptake by excised radicles of mauricea sativus as affected by various amounts of dried, ground Artemisia tridentata. Radicles were excised from 6-day old seedlings. A - control; F - 0.1 g; G - 0.5 g; H - 1.0 g; I - 5.0 g sagebrush.



Since dry leaf material was toxic, extracts from it were prepared. The 95% ethanol extract and subsequent water, petroleum ether and aqueous methanol extracts were tested for allelopathic activity. In Table 4, the ethanol, pet ether and ag. MeOH extracts showed inhibitive biological activity on radicle growth. The water extract was extremely stimulatory, causing radicle growth to be 137% of the control. Hypocotyl and lateral root growth were completely inhibited by all extracts. The water extract and the aqueous methanol extract affected germination, the ag. MeOH being the most inhibitory.

Table 4. Growth of *S. sativum* seedlings exposed to various extracts, solvents evaporated. Total germination time was 5 days.

Types of solvents (0.1 g extract)	Control	EtOH	H ₂ O	pet ether	ag. MeOH
Number of seeds germinated	19	19	17	20	15
% of Control	--	100	89.5	105	78.9
Radicle length, mm					
Average	6.6	1.0	12	1.0	1.0
Range	1-12	1	1-75	1	1
Number of sample/ number planted	15/20	13/20	12/19	14/20	14/1
% of Control	--	86	137	93	16
Hypocotyl length, mm					
Average	0.0	0.0	0.0	0.0	0.0
Range	0	0	0	0	0
Number of sample/ number planted	16/20	20/20	12/17	20/20	15/15
% of Control	--	--	--	--	--
Number of lateral roots					
Average	0.0	0.0	0.0	0.0	0.0
Range	0	0	0	0	0
Number of sample/ number planted	11/17	20/20	12/17	20/20	15/15
% of Control	--	--	--	--	--

Because it was the most effective extract, the aq. MeOH fraction was subjected to tests to relate amount of extract present to amount of inhibition produced. In all cases from 0.01 g to 0.1 g, except that of 0.07 g, inhibition increased as the amount of extract increased. Inhibition was marked in all cases, and reached maximum inhibition in the presence of 0.1 g aq. MeOH extract. In general, the growth of radicles was inhibited least. However, considering the case of 0.07 g material, results differed from the general pattern. This exception was regarded as experimental error rather than a deviation of significance. Germination was not significantly affected by the presence of these amounts of aqueous methanol extract, Table 5. In general, respiration was stimulated by the aq.

Table 5. Growth of *G. sativum* seedlings exposed to various amounts of aqueous methanol extract. Total germination time was 5 days.

Aq. MeOH extract, g	0	0.01	0.03	0.05	0.07	0.10
Number of seeds germinated of Control	10 --	10 100	10 100	9 90.0	9 90.0	10 100
Radicle length, cm						
Average	21	23	2.2	1.1	2.3	1.0
Range	42-116	15-32	2-4	1-2	1-5	1
Number of sample/ number planted	7/1	7/10	6/10	7/10	7/10	8/10
% of Control	--	39	5.2	2.0	3.2	1.1
Hypocotyl length, cm						
Average	55	30	3.2	0.0	4.8	0.0
Range	32-67	11-47	2-2	0	0-14	0
Number of sample/ number planted	6/10	10/10	7/10	6/10	10/10	9/10
% of Control	--	55	6.0	0.0	4.7	0.0
Number of lateral roots						
Average	26	13	0.0	0.0	1.0	0.0
Range	22-30	11-14	0	0	0-11	0
Number of sample/ number planted	5/10	6/10	6/10	6/10	1/10	10/10
% of Control	--	50	0.0	0.0	15	0.0

MeOH extract, Fig. 3. In all cases where growth was inhibited respiration was stimulated. However, amount of growth inhibition was not proportional to the amount of increase in the rate of respiration.

Tables 6 and 7 present data concerning biological activity of the chromatography column fractions of the aqueous methanol extract. This extract was chosen for chromatography because of its high biological activity. Table 6 fractions were tested to determine which showed inhibitive properties. The 1' and 12' MeOH-benzene fractions appeared most active of those presented in Table 6. Inhibitory activity decreased from the 2' fraction to the

Table 6. Growth of *S. sativus* seedlings exposed to various chromatography column fractions, 5 mg. Total germination time was 6 days.

Column fraction no. (% MeOH-benzene)	Control	2'	4'	6'	10'	15'	25'
Number of seeds germinated	12	11	8	13	10	10	9
% of Control	--	100	83.3	108	100	100	83.3
Radicle length, mm							
Average	100	101	87	62	83	82	117
Range	31-142	31-114	31-111	12-176	32-92	61-92	31-176
Number of sample/ number planted	1/10	1/10	7/10	1/10	7/10	6/10	3/10
% of Control	--	83	83	57	70	60	107
Hypocotyl length, mm							
Average	7	75	83	62	52	43	37
Range	63-19	64-21	53-19	49-21	55-61	61-111	71-11
Number of sample/ number planted	3/10	1/10	7/10	7/10	6/10	7/10	3/10
% of Control	--	100	85	83	75	117	107
Number of lateral roots							
Average	37	22	17	15	14	12	13
Range	18-55	11-33	11-17	13-22	11-17	2-13	17-26
Number of sample/ number planted	7/10	13/17	7/10	6/10	13/10	7/10	3/10
% of Control	--	110	83	75	64	57	82

Figure 3. Rate of oxygen uptake by excised radicles of Cucumis sativus as affected by various amounts of aqueous methanol extract residue in solution. Radicles were excised from 5-day old seedlings. A - control; B - 0.01 g; C - 0.03 g; D - 0.05 g; E - 0.07 g; F - 0.10 g residue.

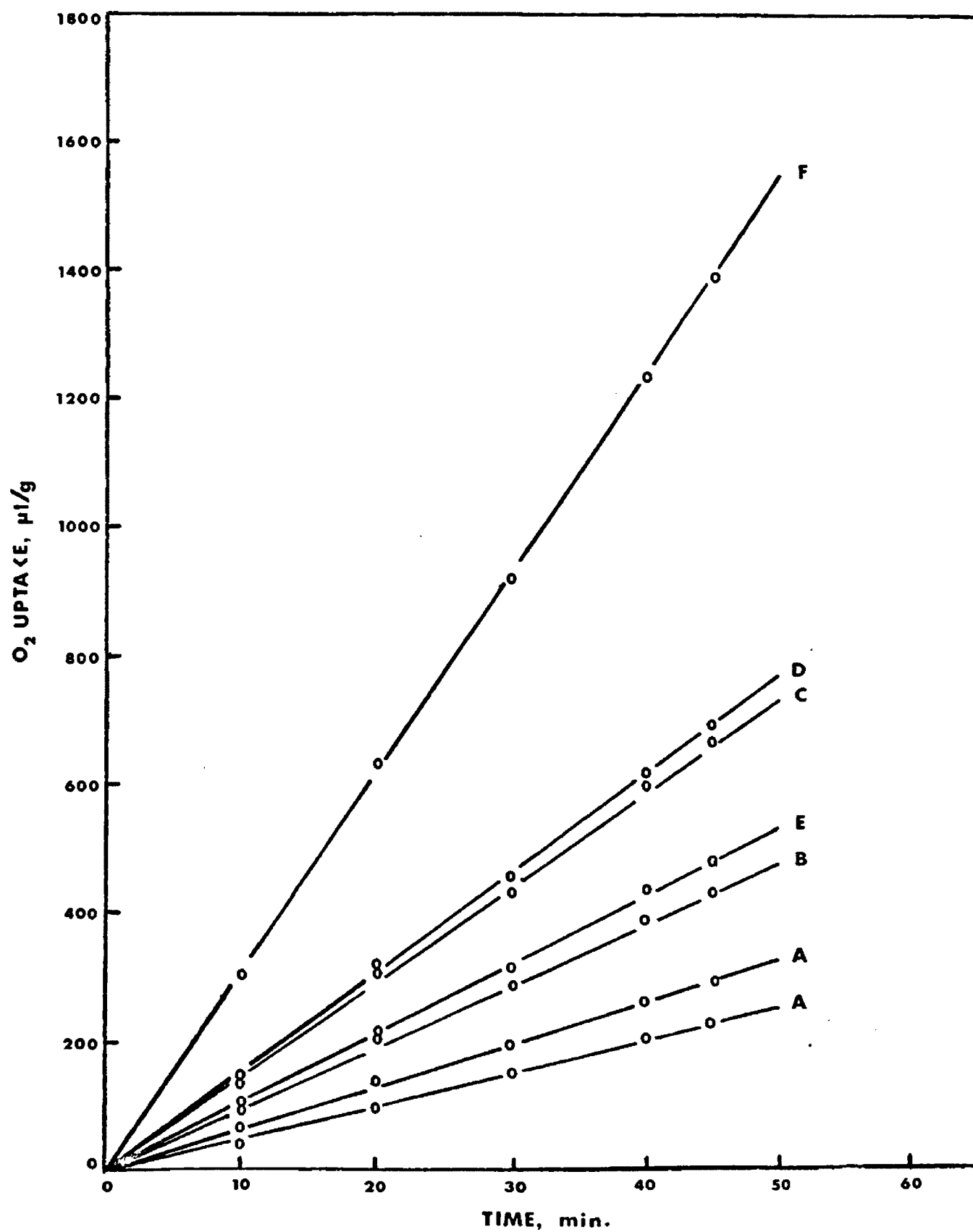


Table 7. Growth of *C. sativus* seedlings exposed to various column fractions, 10 mg. Total germination time was 4 days.

Column fractions (% MeOH-benzene)	Control	10%	15%	20%	30%	40%	50%	60%	70%	80%	90%
Number of seeds germinated	9	10	10	10	10	10	10	10	10	10	10
% of Control	--	110	110	110	110	110	110	110	110	110	110
Radicle length, mm											
Average	69	80	81	95	79	71	68	71	50	89	79
Range	51-85	73-89	75-85	85-104	62-103	57-85	52-82	61-84	37-61	83-92	71-91
Number of sample/ number planted	8/9	8/10	7/10	8/11	10/10	10/10	10/10	9/10	10/10	5/10	8/10
% of Control	--	116	117	138	114	103	98	103	72	129	114
Hypocotyl length, mm											
Average	30	42	30	39	21	39	30	19	17	33	38
Range	25-42	35-49	27-34	17-50	17-26	35-44	27-31	13-25	8-29	24-50	36-40
Number of sample/ number planted	9/9	7/10	7/10	11/11	5/10	7/10	8/10	8/10	10/10	10/10	5/10
% of Control	--	140	100	130	70	130	100	63	57	110	127
Number of lateral roots											
Average	15	18	13	15	16	17	13	15	13	17	14
Range	12-19	12-22	10-17	7-22	11-17	15-19	11-15	12-16	9-17	12-21	11-16
Number of sample/ number planted	8/9	9/10	8/10	11/11	9/10	8/10	10/10	6/10	10/10	10/10	8/10
% of Control	--	120	87	100	107	113	87	100	87	113	93

6% and 10% fractions and then decreased to the 2% fraction. There was no set pattern concerning effect on specific plant organs. In Table 7 it is shown that the 6% and 7% MeOH-benzene fractions exhibited the strongest inhibitory activity, while the 2% and 3% fractions exhibited stimulatory activity. A typical dose influence pattern exhibited generalist action concerning plant organ selectivity. Root growth was not inhibited.

As a result of the tests on column fractions, the 6% (6% and 7% MeOH-benzene fractions were assumed to contain active, inhibitory compounds. It was also reasoned that the 6% and 7% fractions might contain the same active compound. Therefore, these two fractions were subjected to further analysis including re-fractionation of the 6% fraction (not viable and not done). As shown in Tables 8 and 9, the undissolved portion of the 6% fraction had inhibitory properties, while the dissolved portion showed no kind of activity. The 7% fraction exhibited relative inhibitory activity. The undissolved portion of the 6% MeOH-benzene fraction, lateral root growth was most resistant to activity, and inhibiting stimulation. Hypocotyls were not inhibited. Root lateral growth was not affected by the 7% MeOH-benzene fraction, however, radicle growth was reduced to 55% of the control and hypocotyl growth was reduced to around 20 - 25% of the control. These results, plus the analytical procedure as described in the next section, suggested that the 6% and 7% fractions do not contain the same compound.

Isolation and analytical procedures indicated that the active

Table 3. Growth of *G. sativus* seedlings exposed to refined 60% KOH-benzene fraction and 70% KOH-benzene fraction. Total germination time was 4 days.

Treatment	Control	Undissolved portion 60% fraction, 10 mM	70% fraction 10 mM
Number of seeds germinated	10	11	10
% of Control	--	100	100
Radiicle length, mm			
Average	27	67	40
Range	64-92	46-92	31-96
Number of sample/ number planted	10/10	9/10	10/10
% of Control	--	97	75
Approx. 1st root length, mm			
Average	27	15	20
Range	11-33	11-17	16-24
Number of sample/ number planted	10/10	9/10	9/10
% of Control	--	56	76
Number of lateral roots			
Average	13	11	17
Range	11-15	13-16	11-14
Number of sample/ number planted	9/10	5/10	7/10
% of Control	--	115	100

compounds could possibly be coumarins. Therefore, pure esculin, an available coumarin, was tested for biological activity. Table 10 shows that esculin did inhibit plant growth of *G. sativus*, but did not affect germination. Degree of inhibition was not proportional to the concentration of esculin used. However, in all cases lateral root growth was the least inhibited and radiicle growth was most inhibited. This was the same pattern observed by the 70% KOH-benzene fraction, however TLC and fluorescence studies did not show a favorable comparison for the two compounds (materials and methods). In general, the rate of germination was stimulated in the presence

Table 9. Growth of L. sativus seedlings exposed to reduced 60% MeOH-benzene fraction and 70% MeOH-benzene fraction. Total germination time was 5 days.

Treatment	Control	70% fraction 15 mg	Dissolved portion from 2nd column 60% fraction, 5 mg
Number of seeds germinated	10	1	10
% of Control	--	10%	100%
Radicle length, mm			
Average	105	60	100
Range	85-130	44-72	104-111
Number of sample/ number planted	3/10	1/10	6/10
% of Control	--	33	100
Hypocotyl length, mm			
Average	45	41	45
Range	30-71	37-63	32-60
Number of sample/ number planted of Control	10/10	6/10	9/10
	--	60	90
Number of lateral roots			
Average	13	10	13
Range	13-22	11-25	13-21
Number of sample/ number planted	11/10	10/10	10/10
% of Control	--	100	100

of esculin although no direct correlation could be made between esculin concentration and stimulation of germination, Fig. 1.

It is clearly shown in Table 11 that the aqueous ethanol extract inhibits both germination and root growth of L. sativus ssp. vesiculosa.

Maximum germination for L. sativus controls occurred between 35 and 55 hours of germination. From 35 to 55 hours if any, presence of ascarabum in growth chamber caused inhibition of germination from 55 to 104 hours of age, in terms of ascarabum

Table 10. Growth of A. s. lyma seedlings exposed to gamma radiation. Total germination time was 6 days.

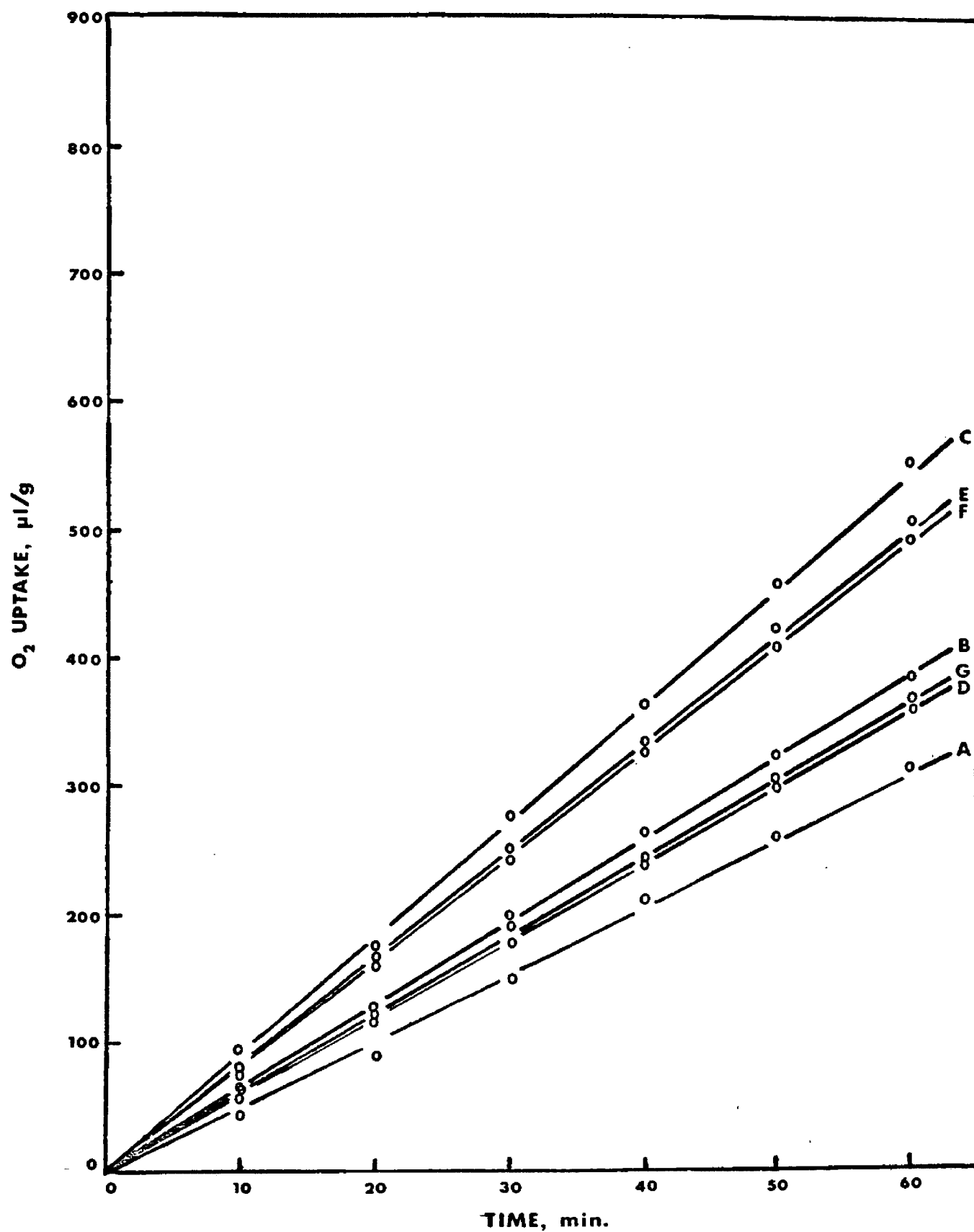
Dose rate, r/hr	0	5	10	15	20	25	30
Number of seeds germinated	10	10	10	10	10	10	10
% of Control	--	100	100	100	100	100	100
Radiicle length, mm							
Average	128	53	61	46	33	23	23
Range	111-146	46-61	57-61	36-71	17-52	17-30	14-32
Number of sample/number planted	7/10	6/10	5/10	2/10	10/10	10/10	7/11
% of Control	--	61	49	36	26	12	13
Hypocotyl length, mm							
Average	55	50	44	37	31	17	22
Range	55-53	42-56	33-46	19-39	22-50	7-23	14-31
Number of sample/number planted	6/10	3/10	6/10	10/10	10/10	10/10	10/11
% of Control	--	60	55	57	45	27	29
Length of leaf #2, mm							
Average	27	17	22	17	13	11	10
Range	15-34	12-25	20-31	15-23	11-16	7-17	8-12
Number of sample/number planted	1/10	2/10	6/10	6/10	7/10	11/11	7/11
% of Control	--	67	61	63	47	41	37

Table 11. Growth of A. tridentata var. xyomingeensis seedlings exposed to gamma radiation. Total germination time was 6 days.

Dose rate	Control	100 r/hr
Number of seeds germinated	10	10
% of Control	--	100
Radiicle length, mm ^a	5.6	5.7
% of Control	--	101
Hypocotyl length, mm ^a	4.7	5.0
% of Control	--	106

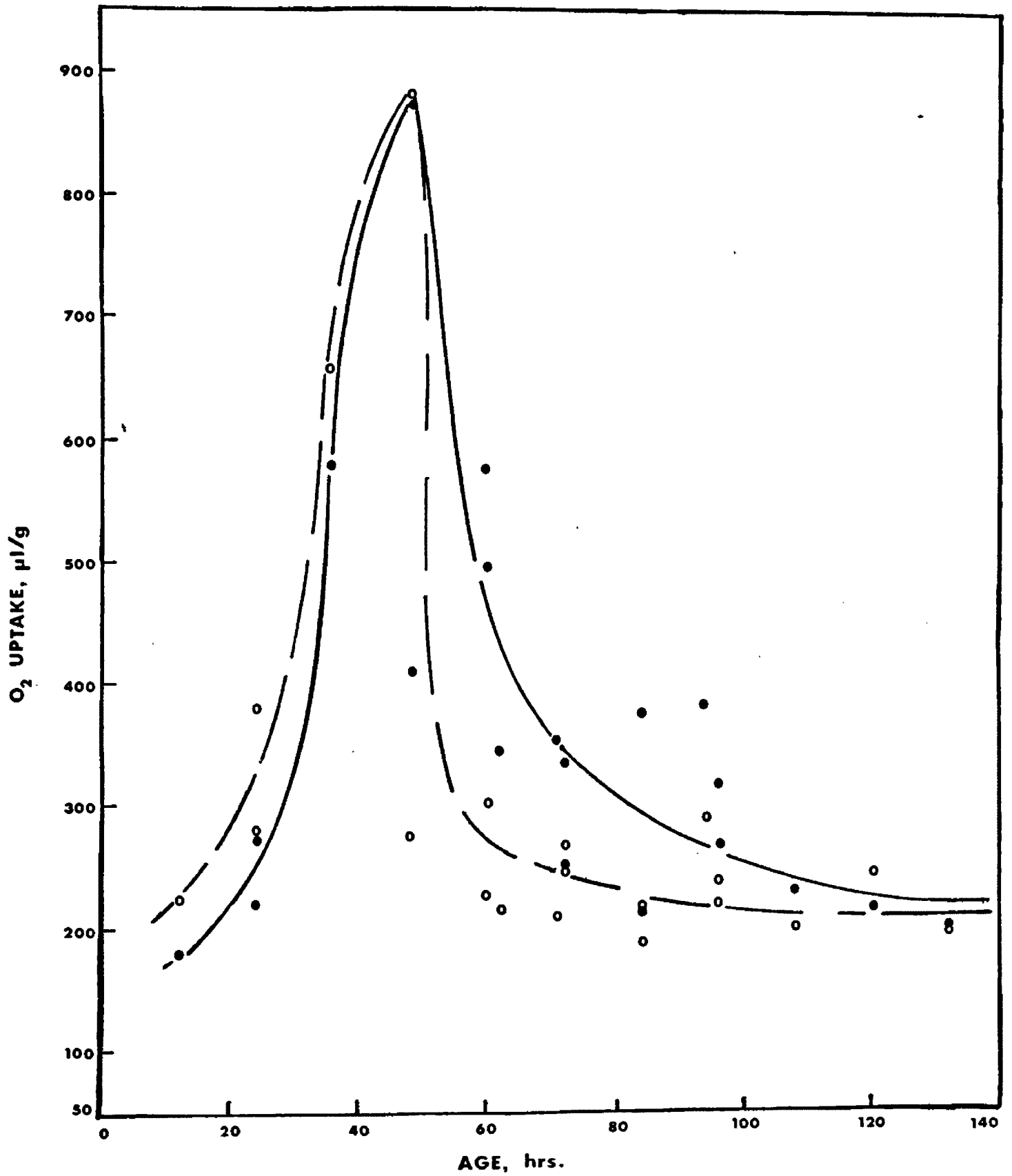
^a Average length of 10 plants/group.

Figure 4. Rate of oxygen uptake by excised radicles of Gnaphalium guttatum as affected by various amounts of esculin in solution. Radicles were excised from 6-day old seedlings. A - control; B - 3 mg; C - 5 mg; D - 10 mg; E - 15 mg; F - 20 mg; G - 25 mg esculin.



appeared to cause stimulation of germination, Fig. 3. The results were not so clear; however, such results must be considered in need of the control or testing procedure carrying out similar experiments.

Figure 5. Total oxygen uptake over a thirty
min. time period by excised radicles of Saccharis
sp.. Radicles were excised from seedlings of
various ages. Respiration flasks were paired for
each age: one containing no sagebrush, the other
containing 0.15 g dried, powdered sagebrush.
● - with sagebrush (solid line); ○ - without sage-
brush (dashed line).



CHAPTER IV

SUMMARY

Growth of C. setivus was inhibited by the presence of A. tridentata esp. varoxana as shown in Tables 2 through 9. Fresh macerated A. tridentata leaves reduced growth of seedlings to a greater extent than did dried macerate. The known inhibitors camphor and cineole (16) are present in fresh sagebrush and might account for its greater amount of inhibitory activity. However, macerated leaves exhibited inhibitory activity even after drying. This indicates that non-volatile compounds were also active as plant growth inhibitors.

The aqueous methanol extract of sagebrush leaves caused inhibition of growth in proportion to the amount present. This extract was also the one which most significantly reduced germination. Because of this activity, the aq. MeOH extract was chosen for further purification by column chromatography.

Fractions from the column showed both stimulatory and inhibitory biological activity. Fractions which stimulated growth were not studied. The 60% and 70% MeOH-benzene fractions which exhibited inhibitory activity were subjected to further analytical measures. Thin layer chromatography and fluorescence studies suggested that the 60% MeOH-benzene fraction possibly contains a coumarin. Although the 70% MeOH-benzene fraction has a polarity similar to that of the coumarins, it did not show the fluorescence typical of coumarins. Cochromatography with coumarin, a known

coumarin found in A. tridentata, showed that these fractions were not esculin. IR analysis of the 60% MeOH-benzene fraction gave only one broad band and melting points covered broad ranges of temperature. Both tests indicated that further purification is necessary.

It was found that the toxins tested inhibited growth of plant organs differently, especially esculin. With esculin as the growth inhibitor, radicle growth was reduced the most and lateral root growth was inhibited the least for all concentrations of esculin tested.

In all instances of growth reduction, stimulation of respiration was evident. However, it was also found that from 0 to 35 hours of age, G. sativus seedling respiration was inhibited by the presence of sagebrush leaf macerate and that from 35 to 144 hours of age, seedling respiration was stimulated. All instances of growth reduction reported herein were measured from seedlings of 36 to 144 hours of age which showed stimulation of respiration.

Growth inhibition found together with inhibition of respiration indicated that there is interference with the metabolic pathway of the experimental organism. Growth inhibition coupled with stimulation of respiration suggests that an energy uncoupling reaction is reducing the amount of available ATP.

The aqueous methanol extract was shown to inhibit both germination and growth of A. tridentata ssp. wyomingensis. Since A. tridentata ssp. vascovana and A. tridentata ssp. wyomingensis are very closely related, autotoxic effects can be considered a possibility.

Allelopathy is a known phenomenon and, since A. tridentata ssp. vasaryana has been shown to inhibit the growth of C. sativus and, even more severely, the growth of A. tridentata ssp. wyomingensis, it can be suggested that A. tridentata ssp. vasaryana exhibits allelopathic activity in the field. However, the study of allelopathic phenomena must include the consideration of other environmental factors. The plant must be shown to have toxic effects on species native to its habitat. Lab studies and field studies must be correlated with regard to presence, concentration and transportation mechanisms of the allelopathic compound. Other possibilities such as growth limitation by foraging animals, climatic conditions and soil type must also be considered.

In this study, not only volatile compounds, but non-volatile substances were indicated as possible allelopaths. Also, previous studies (12) found inhibition of respiration accompanying inhibition of growth, whereas this study opens the possibility of an uncoupling mechanism, since plants exhibit stimulation of respiration along with inhibition of growth. Tracing inhibitory activity through plants' extracts to find purified compounds which are active is a technique that could be explored further. Indications that biologically active compounds are specific to plant organs provide a lead to determine type and site of inhibition. These advances broaden the scope of investigation of allelopathic activity.

Many questions have been opened for discussion. Fractions which have been found to be active must be purified and indi-

vidual compounds must be identified. Continuation of the research could include several problems. One would be the determination of the type of inhibitor, i.e., enzyme inhibition or uncoupling mechanism in respiration, determination of the specific site of inhibition, and elucidation of the form the inhibitor takes within the inhibited plant. Another study, as in the follow-up work Fuller and Fuller conducted, would be to check field conditions as they exist as methods of transport of the compound in the field. A study could be done on the column fractions which appear to stimulate growth. As a practical consideration, studies could be carried out concerning the possibilities of developing "natural" herbicides.

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