INHIBITIONAL EFFECTS OF *RHUS GLABRA* AND *RHUS AROMATICA* ON THE NATIVE TALL GRASS PRAIRIE IN EAST CENTRAL KANSAS

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INTRODUCTION

It has been customary until recently to consider competition between higher plants solely a function of the relative abilities of the plants to obtain the necessary minerals, water, and light required for their growth and reproduction. Considerable evidence has accumulated indicating that the release of chemical inhibitors by higher plants is also a factor in plant competition affecting the establishment, growth, reproduction, and distribution of plants within a community.

Gray and Bonner (1948) found that leaves of Encelia farinosa produce 3-acetyl-6 methoxybenzaldehyde which causes severe retardation in the growth of other plants, but does not inhibit E. farinosa itself. Parenti (1968) showed that substances were produced by Digitaria sanguinalis, an early invader in old-field succession, which inhibited not only the germination and growth of associated species but also inhibited the growth and development of its own seedlings, thus serving as a built-in population control mechanism. He identified three of the inhibitors as chlorogenic acid, isochlorogenic acid, and sulfosalicylic acid. Muller and Hanawalt (1968) conducted a thorough investigation of the inhibition of annual plants by Arctostaphylos in southern California. The ground beneath Arctostaphylos and certain other chaparral shrubs is characteristically devoid of annual plants. Following fire, which removes the crowns as well as the litter of the shrubs, a stand of annual species occupies the area. This stand of annuals dies out after several years when the shrubs have re-established. They have attributed

this phenomenon to toxins produced by the shrubs and identified three inhibitors: arbutin, p-hydroxycinnamic acid, and a derivative of coumarin.

Indirect chemical inhibition between higher plants and microorganisms has also been reported (Rice, 1964, 1965b, 1965c). Rice, et. al. (1960) found a definite correlation between the sequence of species invading abandoned fields and their requirements for nitrogen. Thus any factor that would control the accumulation of available nitrogen in revegetating fields could play a major role in determining the duration and sequence of species during succession. Rice (1964, 1965b, 1965c) showed that several low nitrogen requiring species of the weed stage and annual grass stage are inhibitory to nitrogen-fixing and nitrifying bacteria. These low nitrogen requiring species could control the amount of nitrogen accumulated in disturbed fields confering upon them a selective advantage in competition over species of plants which have higher nitrogen requirements (Rice, 1965a, 1965b, 1965c). Rice and Parenti (1967) have isolated and identified several phenolic inhibitors from species of this weed stage including chlorogenic acid, isochlorogenic acid, ferulic acid, B-resorcylic acid, gentisic acid, a glucose ester of caffeic acid, gallic acid, and gallotannic acid. According to Blum (1968), gallic and gallotannic acid have been found in several species of Euphorbia (Rice, 1965a, 1965b) and Rhus copallina (Nierenstein, 1934). Rice (1965a) found these compounds to be inhibitory to a free-living nitrogen-fixer, Azotobacter, and to Rhizobium, a symbiotic nitrogen-fixer on legumes (Blum, 1968). It was shown that

these compounds were highly effective in the reduction of nodulation and the amount of leghemoglobin produced in bean plants when added to sand culture or soil in low concentrations and also that soils from underneath Rhus copallina and Euphorbia supina were highly effective in the reduction of nodulation (Blum, 1968).

Rhus glabra and R. aromatica normally occupy rocky slopes and ravines, ridges of outcrops, fencerows and roadside banks, and form scattered clones in rocky prairie. Field observations and preliminary productivity studies conducted on the Ross Natural History Reservation indicated that these two woody species are successfully competing with, and rapidly overtaking sizeable areas of this native tall grass prairie. Although this may be in part due to vigorous root competition for water, and/or their aggressive means of vegetative reproduction, I hypothesized that: (1) Rhus glabra and R. aromatica might release substances affecting the germination and growth of the climax grasses, (2) that these compounds may inhibit the growth of free-living nitrogen-fixing and symbiotic nitrogen-fixing bacteria, and (3) that, if present, these compounds could be isolated and extracted from fresh plant material and soil underneath the Rhus glabra and R. aromatica clones. This project was undertaken to obtain evidence concerning the ability of R. glabra and R. aromatica to inhibit certain strains of the nitrogen-fixer Azotobacter, the symbiotic nitrogen-fixer, Rhizobium, as well as certain species of grass comprising the climax vegetation of the prairie and to identify the inhibitory compounds if present.

MATERIALS

Rhus glabra and R. aromatica plants were collected from plots A 38 and A 39, respectively, on the Ross Natural History Reservation located in east central Kansas. Soil samples 0 - 6" deep were taken from the study sites in October, 1968, and April and June, 1969.

Free-living nitrogen fixers and symbiotic nitrogen-fixing bacteria used in this study were obtained from Dr. Elroy L. Rice, University of Oklahoma.

Extracts of Rhus glabra and R. aromatica were prepared by grinding 10 g of fresh sumac material in 100 ml of distilled water in a Waring Blender for 10 minutes. The mixture was allowed to stand for twenty minutes, then filtered through Whatman No. 1 filter paper in a Buchner funnel. The volume of the filtrate was brought to 100 ml with distilled water.

EXPERIMENTATION AND RESULTS

Seedling inhibition by extracts of Rhus glabra and R. aromatica.

Tomato seedlings were used to initially test the inhibitional effects of Rhus glabra and R. aromatica extracts. These were started in white quartz sand and supplied with Hoagland's complete nutrient solution (Hoagland and Arnon, 1950). After two weeks, the seedlings were transferred to plastic vials containing a 5:1 ratio of extract to nutrient solution. The controls were grown in a 5:1 solution of distilled water to nutrient solution. The vials were placed in a growth chamber (Percival, Model PT .80, No. .8336.2) which was set for an alternating 14 hour light period (85° F) and 10 hour dark period (65° F). After two weeks the seedlings were harvested, dried for 48 hours at 37° C, and the oven-dry weight determined.

The plant extracts from leaf, root, and seeds of both Rhus glabra and R. aromatica caused a significant reduction in growth of the tomato seedlings (Table I). The experiment was repeated using the four dominant species of the tall-grass prairie: Sorghastrum nutans, Panicum virgatum, Andropogon gerardi, and A. scoparius. A significant reduction in the growth of these four dominants was observed (Table II).

Effects of extracts of Rhus glabra and R. aromatica on seed germination.

To test the effect of Rhus glabra and R. aromatica leaf and root extracts on seed germination of selected plants, two hundred seeds of Plantago ovata, Panicum virgatum, Sorghastrum nutans, Andropogon gerardi,

TABLE I. Effects of fresh plant water extracts of Rhus aromatica and R. glabra on 14-day old tomato seedlings.

	Mean Oven-Dry Weight, mg with Standard Error	
Source of Extract	Control	Test[a]
Rhus aromatica seed	46.3±.004	20.3±.002
Rhus aromatica leaf	56.2±.003	7.7±.0007
Rhus aromatica root	56.2±.003	4.6±.0004
Rhus glabra seed	46.3±.004	15.4±.001
Rhus glabra leaf	56.2±.003	6.4±.0007
Rhus glabra root	56.2±.003	4.7±.0003

[a]All test weights significantly different from the control at the 0.01 level or below.

TABLE II. Effects of fresh aqueous extracts of Rhus aromatica and R. glabra on selected 14-day old plants.

Source of Extract	Mean Oven-Dry Weights, mg with Standard Error							
	Andropogon gerardi		Andropogon scoparius		Panicum virgatum		Sorghastrum nutans	
	(Control)	(Test)[a]	(Control)	(Test)[b]	(Control)	(Test)[c]	(Control)	(Test)[d]
Rhus aromatica leaf	26.9±.001	12.5±.001	11.2±.002	6.5±.001	22.4±.002	6.9±.0005	44.5±.003	12.0±.001
Rhus aromatica root					22.4±.002	5.7±.0008		
Rhus glabra leaf	26.9±.001	9.9±.001	11.2±.002	5.3±.0003[e]	22.4±.002	7.4±.0005	44.5±.003	13.9±.001
Rhus glabra root					22.4±.002	5.1±.0003		

[a,c,d,e] Dry weights significantly different from the control at the .01 level.
[b] Dry weight significantly different from the control at the .05 level.

and A. scoparius were placed on germination discs in sterile Petri dishes (100 per dish) saturated with 7 ml of a 5:1 ratio of distilled water to nutrient solution. Extracts for the test were prepared as before except that they were passed through a cellulose membrane filter holder (Metricel, GA-6 grid, 0.45 micron pore size, 1 inch diameter, Gelman Instrument Co., Scientific Products--F2932) to eliminate fungal contaminants. The Petri dishes were sealed with rubber seals and the seeds incubated at 27° C. Germination counts were made after 24 hours and subsequently every 12 hours for four days.

There was a reduction in percentage of germination in all test plants exposed to Rhus glabra root extract and R. aromatica leaf extract. Rhus aromatica root extract and Rhus glabra leaf extracts caused a reduction in final percentages of germination in Andropogon gerardi, Plantago ovata, and Sorghastrum nutans but not in A. scoparius and Panicum virgatum, respectively, although they were initially affected (Table III). All test seeds were heavily infected by fungi at the end of the 108 hour period.

Effect of root exudates of Rhus aromatica on test plants.

Procedures used to test the effect of root exudates were those of Parenti (1968). Fourteen-day old seedlings of Andropogon gerardi, A. scoparius, Panicum virgatum, and Sorghastrum nutans were transferred to plastic containers (2 seedlings per container) filled with white quartz sand and designed to maintain a continuous circulation of culture solution. The experimental group consisted of four pots of test plants

TABLE III. Effects of aqueous extracts of Rhus aromatica and R. glabra on rate and per cent germination of selected seeds.

		Number of seeds germinated/time in hours							
Plant Name	*	24	36	60	72	84	96	108	Total % Germination
Andropogon gerardi	A	128	155	166	171	172	175	175	88
	B	110	131	154	161	164	168	168	84
	C	113	143	156	164	167	169	169	85
	D	92	123	138	144	146	146	148	74
	E	98	120	136	145	150	150	151	76
Andropogon scoparius	A	22	34	50	63	73	76	79	40
	B	3	14	33	42	48	48	49	25
	C	4	21	34	46	52	56	59	30
	D	23	38	65	73	80	82	84	42
	E	12	16	32	33	41	45	47	24
Panicum virgatum	A	17	55	85	101	113	116	118	59
	B	7	32	75	95	98	98	98	49
	C	7	40	66	92	104	112	119	60
	D	9	39	81	95	104	104	106	53
	E	11	25	58	83	98	103	108	54
Plantago ovata	A	61	170	193	198	200	200	200	100
	B	22	98	137	144	147	147	150	75
	C	6	83	130	153	163	167	171	86
	D	74	142	154	157	160	160	160	80
	E	2	15	128	166	167	168	170	85
Sorghastrum nutans	A	68	73	85	91	104	106	106	53
	B	50	61	73	79	80	83	85	43
	C	40	54	68	69	69	69	69	35
	D	54	73	90	93	94	96	112	51
	E	60	72	86	96	97	100	101	51

*A: CONTROL
B: Rhus aromatica leaf extract
C: R. aromatica root extract
D: Rhus glabra leaf extract
E: R. glabra root extract

beneath two pots of sumac. The control was composed of four pots of all test plants. Hoagland's complete nutrient solution was placed in reservoirs at the bottom of the stairstep and pumped to other reservoirs at the top of the apparatus. The culture solutions dripped from the upper reservoirs into pots containing the test and control plant combinations. These solutions were then pumped back to the upper reservoirs four times daily for a period of five weeks. The oven-dry weight of the plants was determined after this time to discover whether the exudate of the sumac roots caused a reduction in growth of the test plants.

Exudate from Rhus aromatica caused a significant reduction in growth in Andropogon scoparius and Sorghastrum nutans (Table IV). Root exudates had no effect on the growth of A. gerardi but did affect the growth of Panicum virgatum, although not significantly. All test plants exhibited a chlorotic effect or bleaching of the leaves. However, the cause of this effect was not investigated further.

Effect of extracts of Rhus glabra and R. aromatica on free-living nitrogen fixers and symbiotic nitrogen fixers.

Procedures followed were those of Rice (1964). Four American Type Culture (ATC) strains of Rhizobium and two of Azotobacter were used as test bacteria: Azotobacter chroococcum Beijerinck, ATC Strain 9043, A. vinelandii Lipman, ATC Strain 9104, Rhizobium leguminosarum Frank emend. Baldwin and Fred, ATC Strain 10314, R. phaseoli Dangeard, ATC Strain 10322, 10321, and R. sp., ATC Strain 10703. All stock cultures and test organisms of Azotobacter were cultured on the mannitol medium

TABLE IV. Effects of Rhus aromatica root exudate on 5-week old plants.

	Mean Oven-Dry Weight, g with Standard Error	
Plant Name	Control	Test
Andropogon gerardi	.59±.08	.67±.06
Andropogon scoparius	.43±.06	.26±.04[a]
Panicum virgatum	3.96±.02	1.49±.28
Sorghastrum nutans	1.67±.43	.46±.03[b]

[a]Dry weight significantly different from the control at the .05 level.
[b]Dry weight significantly different from the control at the .02 level.

described in "Manual of Microbiological Methods" (Society of American Bacteriologists, 1957, p. 109). Rhizobium stock and test cultures were cultured on a yeast extract-mannitol medium (p. 113 of the above cited manual). The same media were used for liquid inocula and agar plates. Fifteen grams of bacto agar were added per liter if a solid medium was required. All stock cultures and test plates were incubated at 30° C.

Aqueous extracts from fresh leaves and roots were prepared from the sumac as before, centrifuged (Servall SS-34) in cellulose nitrate tubes (50 ml, Ivan Servall, Inc., No. 204) at a speed of 7,500 RPM for 20 minutes (Relative centrifugal force 6,780), and passed through a millipore filter. The extract was then reduced in volume in a flash evaporator, and extracted with three half volumes of ether. The water fraction was retained. The ether fraction was evaporated to near dryness and the residue dissolved in 15 ml of 95% ethanol. These fractions were tested for antibacterial activity by the diffusion technique on solid media against plates seeded with 0.5 ml of a 36-hour liquid culture of Rhizobium and Azotobacter (Rice, 1964). Sensitivity discs (ca 13 mm dia.) were sterilized and saturated with either the aqueous or ether fraction of the extract. Ten plates were used per test and the plates were examined after three days. A colony counter was used to measure the diameter of the inhibited zone in millimeters (Table V).

Extract from Rhus aromatica leaves was inhibitory to all six strains of test bacteria and in most cases, the zones were distinct. Those discs without clear-cut zones did exhibit slight to partial inhibition. Root extracts of R. aromatica inhibited Rhizobium 10703 and both strains of

TABLE V . Effect of fresh extracts of Rhus aromatica and R. glabra on free-living and symbiotic nitrogen-fixing bacteria, expressed as mean diameter of ten inhibited zones in millimeters.

Source of Extract	Test Organism											
	R 10322[1]		R 10321		R 10314		R 10703		A 9104[2]		A 9043	
	(H_2O)	(Ether)	(H_2O)	(Ether)	(H_2O)	(Ether)	(H_2O)	(Ether)	(H_2O)	(Ether)	(H_2O)	(Ether)
Rhus aromatica leaf	22.3	17.6	21.4	s	18.4	17.2	21.8	14.4par[3]	21.4	14.7par	26.6	15.1par
Rhus aromatica root	s[4]	s	0	s	s	0	s	18.3par	19.9	17.9par	22.5par	18.6par
Rhus glabra leaf	25	s	15.9	s	14.9par	13.2par	t[5]	t	16.3	0	0	16.7
Rhus glabra root	s	s	0	s	0	s	0	19.9	0	s	0	s

[1] R, Rhizobium
[2] A, Azotobacter
[3] par-partial inhibition
[4] s-slight inhibition
[5] t-total inhibition

Azotobacter. In most cases, the water extract produced greater zones of inhibition than the alcohol portion of the ether extraction. Growth of Rhizobium 10322, R 10321, and R 10314 was only slightly affected by extract of the R. aromatica root. Extract from R. glabra leaves produced zones of inhibition in all test bacteria, varying from slight to total inhibition. R. glabra root extract produced slight inhibitory zones in all test bacteria except Rhizobium 10703, where its effect was pronounced.

Identification of inhibitors.

Thin-layer chromatography (TLC) was used for the identification of inhibitors. Extracts from sumac plant material were prepared as before except that water was replaced as a solvent by 100 ml of methanol.

For initial analysis, Silica Gel G (Research Specialties Co.--8067) was spread on thin-layer plates (20 x 20 cm glass plate, 250 micron bed depth), which were then placed in a 115° C drying oven for 30 minutes. Twenty microliters of extract were applied at the origin. The plates were developed in the ascending fashion using a chloroform - ethyl acetate - formic acid (5:4:1 v/v) solvent (CEF) (Stahl, 1965). The solvent front was allowed to proceed to a height of 17.7 cm from the origin.

The spots were detected by first spraying the plates with a ferric chloride - potassium ferricyanide solution and then .1% HCl. The chromatograms were allowed to air dry between successive sprayings. Color reactions and R_f values were compared to those of known compounds (Table VI).

TABLE VI. Thin-layer chromatography of known compounds and inhibitors from Rhus glabra and R. aromatica.

Compound	R_f's on Silica Gel G[a] CEF	Reagent Color[b,c] $FeCl_3$-$K_3Fe(CN)_6$-.1% HCl
gallic acid	0.38	lt bl bordered in br
R. glabra leaf	0.37	lt bl bordered in br
R. glabra root	0.37	lt bl bordered in br
R. aromatica leaf	0.38	lt bl bordered in br
tannic acid[d]		
spot 1 (catechin)	0.08	lt bl
spot 2 (catechin)	0.22	lt bl
spot 3 (tannic)	0.24	lt bl
spot 4 (gallic)	0.38	lt bl
R. glabra leaf	0.26	lt bl
R. glabra root	0.26	lt bl
R. aromatica leaf	0.24	lt bl
R. aromatica root	0.24	lt bl

[a] R_f's are averages of three runs. Solvent system, chloroform-ethyl acetate-formic acid (5:4:1 v/v).
[b] Ferric chloride-potassium ferricyanide-.1% HCl.
[c] lt, light; bl, blue; br, brown.
[d] Tannic acid separated into four spots - gallic, tannic, and precursor catechins.

In addition to recording R_f values and color reactions of the spots, the chromatograms were recorded on photographic paper. When the paper was developed in the traditional fashion, the spots appeared white against a dark background (Fig. 1).

Isolation of inhibitors from soil.

Soil extracts for isolation of inhibitors from the soil under Rhus glabra and R. aromatica clones were prepared by mixing 75 g of dry soil in 125 ml of methanol and boiling gently for 8 hours. The extract was filtered and reduced in volume to 10 ml in vacuo. The extract was spotted on thin-layer chromatograms and developed as described under indentification of inhibitors. Although the spray indicator reacted with compounds on the plate, these compounds were not in a high enough concentration to be accurately identified. Thus, no known compounds could be isolated from the soil extracts.