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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

INHIBITION OF SYMBIOTIC NITROGEN-FIXATION BY GALLIC AND GALLOTANNIC ACIDS AND POSSIBLE ROLES IN OLD-FIELD SUCCESSION

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INHIBITION OF SYMBIOTIC NITROGEN-FIXATION BY GALLIC AND GALLOTANNIC ACIDS AND POSSIBLE ROLES IN OLD-FIELD SUCCESSION

APPROVED BY

Elroy L. Rice

James R. Estes

Lyman A. Wender

Guy J. Goodman

DISSERTATION COMMITTEE

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INHIBITION OF SYMBIOTIC NITROGEN-FIXATION BY GALLIC AND GALLOTANNIC ACIDS AND POSSIBLE ROLES IN OLD-FIELD SUCCESSION

CHAPTER I

INTRODUCTION

In central Oklahoma four prominent stages of succession occur in abandoned fields (Booth 1941). These four stages are: (1) the weed stage which lasts for 2-3 years, (2) the annual grass stage which persists for 9-13 years, (3) the perennial bunch grass stage which remains for more than 30 years, and (4) the climax prairie. The prairie climax therefore does not reoccupy such fields for considerable time after abandonment, even when the climax prairie species surround such fields (Rice, Penfound, and Rohrbaugh 1960). Finnell (1933) and Daniel and Langham (1936) demonstrated that such fields are low in nitrogen. Rice, et al. (1960) found a definite correlation between the sequence of invading species and their requirements for nitrogen. Any factor, therefore, that would control the accumulation of available nitrogen in such fields could play a major role in determining the duration and sequence

of species during succession.

Several low nitrogen requiring species of the weed stage and annual grass stage were found to be very inhibitory to nitrifying and nitrogen-fixing bacteria (Rice 1964, 1965b, 1965c). Such species as Ambrosia psilostachya,[1] Bromus japonicus, Digitaria sanguinalis, Helianthus annuus, and Euphorbia supina of the weed stage and Aristida oligantha of the annual grass stage were found to be very inhibitory to the effective nodulation of legumes (Rice 1964, 1968). By far the greatest addition of nitrogen in such fields is due to symbiotic nitrogen-fixation (Russell 1961); therefore these inhibitory species could control the amount of nitrogen accumulated in abandoned fields. Several phenolic inhibitors were isolated and identified from species of the weed stage (Rice 1965a, 1965b, 1965c, Rice and Parenti 1967). These phenolics were chlorogenic acid, isochlorogenic acid, ferulic acid, β-resorcylic acid, gentisic acid, a glucose ester of caffeic acid, gallic acid, and gallotannic acid.

Gallic and gallotannic acid were found to be produced by several species of Euphorbia (Rice 1965a, 1965b) and Rhus copallina (Nierenstein 1934). Rice (1965a) found these compounds to be very inhibitory to a free-living nitrogen-fixer, Azotobacter, and to Rhizobium, the

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[1]Nomenclature of vascular plants follows Waterfall (1966) unless authority is given.

symbiotic nitrogen-fixer on legumes. I hypothesized, therefore, that: (1) these two compounds would inhibit effective nodulation of legumes, (2) that these very water soluble compounds could be extracted from soil under plants which produce them, and (3) that resistant strains of Rhizobium could be selected which would cause effective nodulation in the presence of the inhibitors. Appropriate experiments were designed to test these hypotheses.

CHAPTER II

MATERIALS

Red kidney beans (Burpee) were used in all nodulation experiments because they grow rapidly, they nodulate well, and specific strains of Rhizobium are available for nodulation.

Specific strains of Rhizobium phaseoli Dangeard (10321, 10322) were obtained from the American Type Culture Collection (ATCC). These strains were used in all experiments involving the selection of strains resistant to gallotannic acid; they were grown on a yeast and soil extract-mannitol medium (Soc. Am. Bacteriol. 1957).

Reagent gallic and gallotannic acid were obtained from the Fisher Scientific Company.

CHAPTER III

EXPERIMENTATION AND RESULTS

Effects of different concentrations of gallic and gallotannic acid on nodulation of beans

Red kidney bean seeds were heavily inoculated with the appropriate Nitragin inoculum and five seeds were planted in each of 55 pots containing quartz sand. The bean plants were thinned to two uniform plants per pot and a reservoir containing Hoagland's solution with 1/10 the usual amount of nitrogen (Hoagland and Arnon 1950) was attached by a rubber tube to each pot. Ten pots were used as controls, and the rest were divided into nine test series with five pots in each series. Sufficient amounts of gallic or gallotannic acid were added to the pots of each series to make the following concentrations: 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}, and 10^{-2}M. A 10^{-10}M concentration of gallic acid was not included, however. The pH was adjusted initially to 5.8 in all reservoirs. Distilled water was added daily to compensate for transpiration and evaporation and twice a week nutrient solution was added to insure sufficient minerals for growth. The quartz sand in each pot was kept drained by placing these pots

above the liquid level of the reservoirs and the plants were watered daily by raising the reservoirs above the sand level. Fresh weights and nodule numbers were determined four weeks after the beans were planted.

Plants grown in 10^{-2}M gallotannic acid were killed, but no statistically significant reduction of fresh weight occurred with plants grown in the remaining concentrations of either gallic or gallotannic acid (Table 1), however, these concentrations except 10^{-2}M gallic acid significantly reduced the mean nodule number (Fig. 1). A 10^{-8}M concentration of gallic and of gallotannic acid caused the greatest reduction of nodulation. The experiment was repeated with similar results.

Effects of different concentrations of gallic acid on leghemoglobin content of nodules

A sand culture experiment as previously described was run with 10^{-2}M and 10^{-6}M gallic acid. Fresh plant weight, nodule number, and leghemoglobin content per nodule were determined. Leghemoglobin was extracted from the nodules by a pyridine-sodium hydrosulfite extraction, and the amount of heme per nodule and per plant was determined according to the method of Virtanen, Erkama, and Linkola (1947).

The 10^{-2}M concentration significantly increased the nodule number per plant, whereas the 10^{-6}M concentration

Table 1. Effects of different concentrations of gallic and gallotannic acid on weight of bean plants.

Concentrations, M	Mean Fresh Weight, g[a]			
	Gallic acid		Gallotannic acid	
	Roots	Tops	Roots	Tops
Control	8.46±1.47	13.10±0.69	8.46±1.47	13.10±0.69
10^{-10}	---	---	6.81±0.81	10.45±1.12
10^{-8}	7.69±0.58	13.01±1.12	6.83±0.67	13.04±1.26
10^{-6}	7.27±0.54	13.79±1.60	7.93±0.38	14.82±1.24
10^{-4}	6.85±0.61	12.99±0.91	7.95±0.59	13.47±1.58
10^{-2}	8.25±0.53	14.69±1.43	killed	killed

[a]Each figure represents 20 plants for controls and 10 plants in each test series.

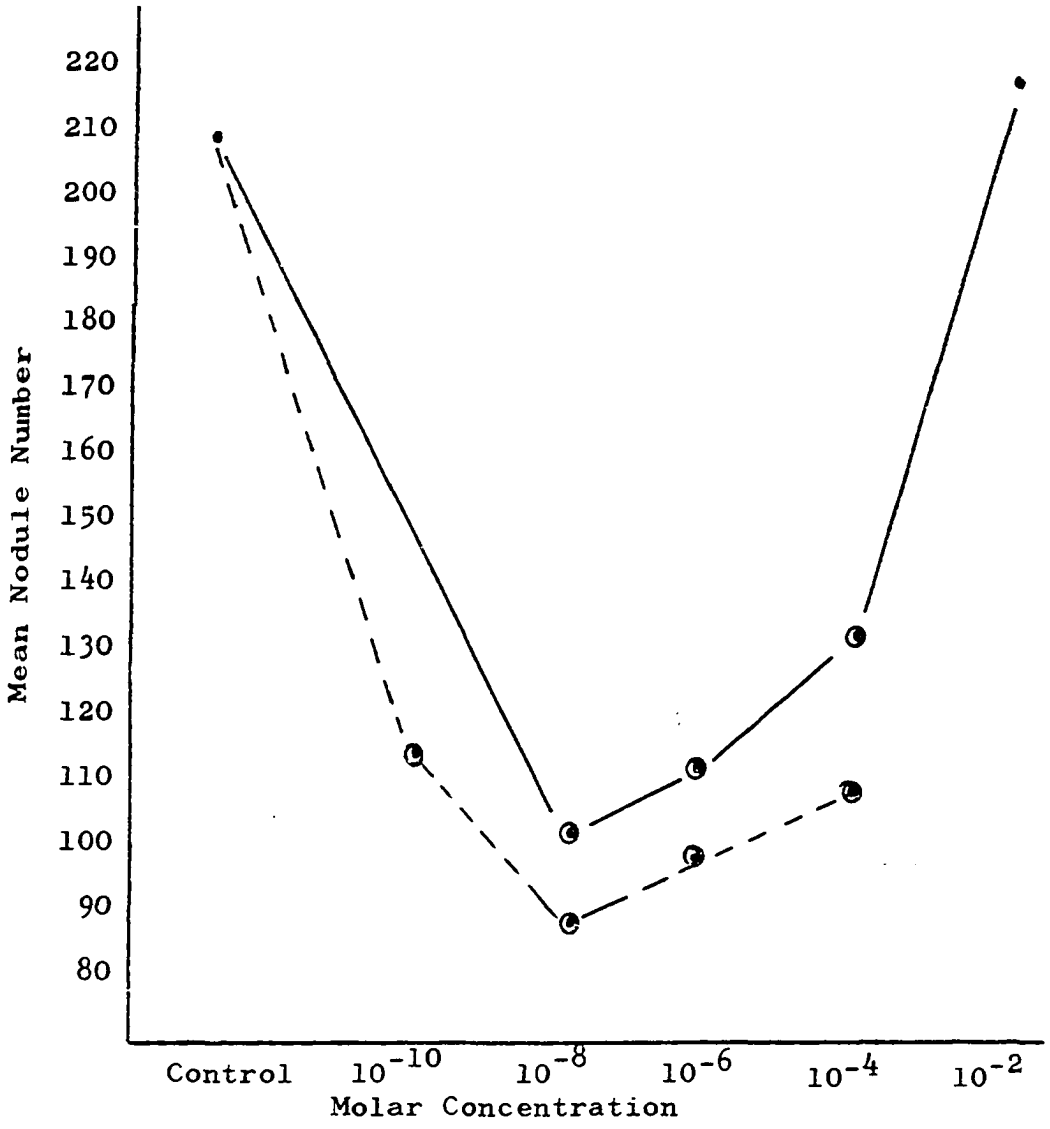


Figure 1. Effects of different concentrations of gallic and gallotannic acid on nodulation. Broken line, gallotannic acid; solid line, gallic acid. ○ indicates difference from control is statistically significant.

significantly reduced the number (Table 2). There was no statistically significant effect on plant weight. The 10^{-2}M concentration significantly reduced the mean amount of leghemoglobin per nodule and the 10^{-4}M concentration significantly reduced the mean amount per plant.

Effects of different concentrations of gallotannic acid on nodulation in soil

A mixture of three parts soil and two parts sand was placed into twenty 4 inch pots. Known concentrations of gallotannic acid were added to the upper 300 g of soil in half of the pots. Five highly inoculated bean seeds were planted in each pot and the plants were thinned to two uniform ones per pot. These were harvested 4 weeks after the initiation of the experiment and fresh weight, nodule number, and total leghemoglobin per plant were determined. Concentrations of 33.2, 100, 200, 300 and 400 parts per million of gallotannic acid reduced the mean nodule number, and all reductions were statistically significant except that due to 33.2 ppm (Fig. 2). No statistically significant reduction in mean plant weight or leghemoglobin occurred, although the amount of leghemoglobin was reduced in each case (Table 3).

Comparison of trends in nodule number per plant in soil and sand culture experiments

In sand culture the nodule number increased with increasing concentrations of gallotannic acid starting

Table 2. Effects of gallic acid on leghemoglobin content of nodules.

Concentration M	Mean[b] Nodule Number	Mean Plant Weight g	Leghemoglobin			
			ug per Nodule	% Reduction	ug per Plant	% Reduction
Control	109.50±10.04	18.50±1.28	1.09±0.09		124.25±10.8	
10^{-6}	81.83± 8.2[a]	22.32±2.42	0.90±0.61	16.92	79.00±14.5[a]	36.42
10^{-2}	182.80± 9.5[a]	17.70±0.81	0.73±0.49[a]	39.16	116.25±24.7	6.44

[a]Difference from control significant at the 0.05 level or below.

[b]Each figure represents mean of 20 plants.

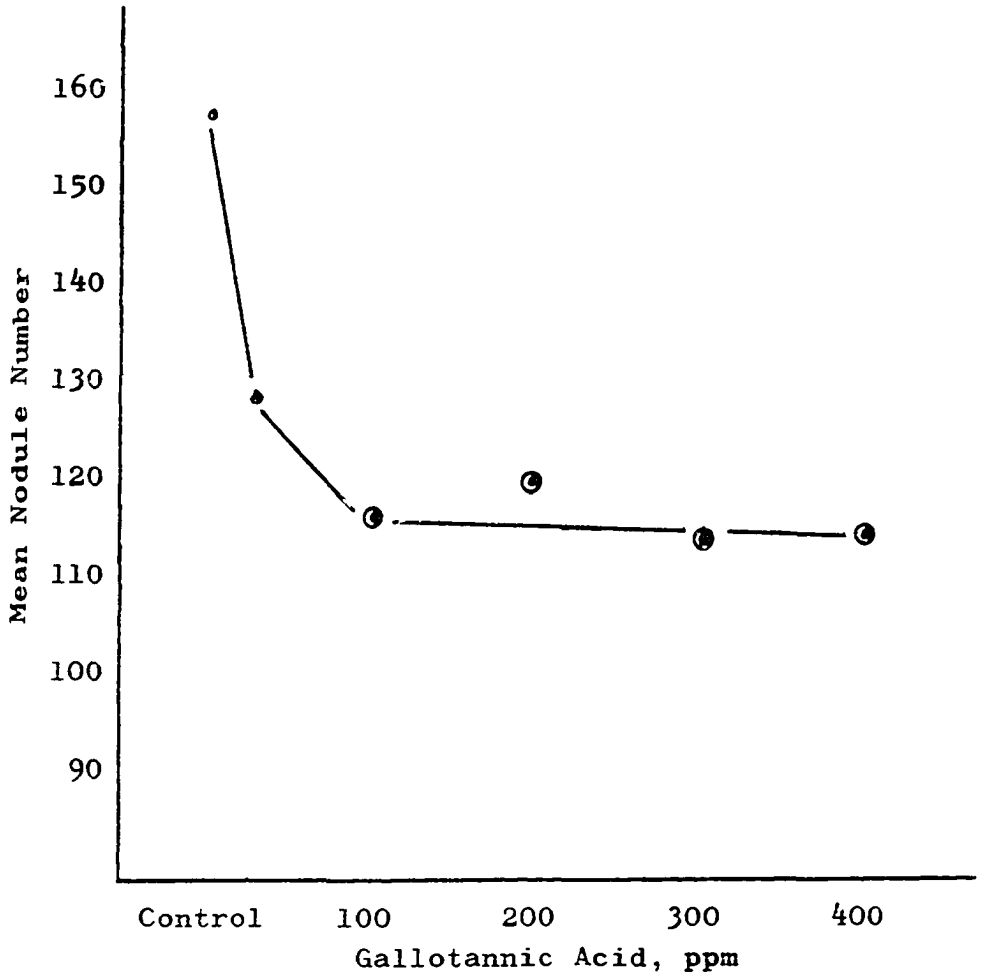


Figure 2. Effects of different concentrations of gallotannic acid on nodulation in soil. O indicates difference from control is statistically significant.

Table 3. Effects of gallotannic acid on total leghemoglobin per plant.

Concentration, ppm	Mean Plant[a] Weight g	Leghemoglobin	
		Per Plant[b] ug	% Reduction
Control	13.9±0.55	87.20±10.24	
33.2	12.3±0.62	47.75± 8.71	45.3
100	14.0±0.67	68.25±11.87	21.8
200	13.4±0.56	74.25± 8.3	14.9
300	12.9±0.56	58.50± 9.74	33.0
400	12.4±0.35	70.50± 7.34	18.9

[a]Each figure represents mean of 20 plants.

[b]Each figure represents mean of 10 plants.

with 10^{-8}M solution and continuing until the solution became lethal. In the soil experiment, however, the nodule number decreased as the concentration increased (Fig. 1, 2), but the decrease was slight with increasing concentrations above 100 ppm.

Effects on nodulation and leghemoglobin content of bean plants of soil from underneath Euphorbia supina and Rhus copallina

Soil from underneath Euphorbia supina was brought into the laboratory on October 28, 1966 for nodulation experiments. The plant and plant remains were removed from the soil, and the soil was then obtained with a post hole digger to disturb the vertical stratification as little as possible. Control soil was gathered in the same manner in an adjacent area without E. supina. The soil was placed directly in pots, and heavily inoculated bean seeds were planted in ten control and ten test pots. The plants were thinned to two per pot and were harvested 4 weeks after the seeds were planted. Soil gathered from underneath E. supina significantly reduced the mean nodule number of bean plants without appreciably affecting their weight (Table 4).

Soil was gathered from the 0-5 cm level underneath Rhus copallina after the plant residues had been removed from the surface. The soil was mixed with sand in a ratio of 3 parts soil to 2 parts sand. Approximately 300 grams of this mixture were placed on top of a similar mixture of

Table 4. Effect of field soil from underneath *Euphorbia supina* on nodulation of bean plants.

Soil	Mean Nodule Number[b]	Mean Plant Weight, g
Control	139.4±12.1	9.77±0.54
Euphorbia soil	93.7± 5.56[a]	9.20±0.34

[a]Difference from control significant at the 0.05 level or below.

[b]Each figure represents mean of 20 plants.

greenhouse soil. Control soil was gathered from an adjacent area without R. copallina and treated in the same manner. Highly inoculated bean seeds were planted and the plants were thinned and harvested as before. Soil samples were gathered at intervals between September 1966 and October 1967 and similar experiments were run. All soil samples gathered under R. copallina significantly reduced the mean nodule number on bean plants (Table 5). The leghemoglobin was extracted from nodules of plants grown in the soil collected in June, 1967 and October, 1967. A statistically significant reduction in the mean amount of leghemoglobin per plant occurred during both sampling periods (Table 6).

Duff and soil extractions

Soils from underneath Rhus copallina and Euphorbia supina were extracted by a 24 hour acetone soxhlet extraction. The acetone was reduced in volume in a flash evaporator. It was made up to a specific volume and chromatographed in two dimensions on Whatman 3 MM paper in benzene: butanol:acetic acid:water (5:4:3:1 v/v) followed by 6% aqueous acetic acid. The gallotannic acid spot was marked under UV, cut out, and eluted with water. Absorption was measured with a Beckman DB-G Spectrophotometer at 277 mu, and amounts were determined from a standard curve of gallotannic acid. The standard curve was determined by spotting known concentrations of gallotannic acid on

Table 5. Effects of field soil from underneath Rhus copallina on nodulation of bean plants.

Date of Soil Sample	Control Soil[b]		Test Soil	
	Mean Nodule Number	Mean Plant Weight g	Mean Nodule Number	Mean Plant Weight g
Sept. 23, 1966	139.2± 7.5	12.37±0.48	106.6±6.9[a]	11.6 ±0.68
April 26, 1967	163.4±10.7	10.43±0.98	125.5±6.7[a]	12.4 ±0.44
June 26, 1967	130.9± 8.0	15.70±0.63	107.1±8.2[a]	12.59±0.67
Oct. 6, 1967	130.9± 8.0	15.70±0.63	102.3±7.6[a]	13.25±0.66

[a]Difference from control significant at 0.05 level or below.

[b]Each figure represents mean of 20 plants.

Table 6. Effects of soil from underneath Rhus copallina on leghemoglobin content of nodules.

Date of Soil Sample	Leghemoglobin, ug			
	Mean Total[b] per Plant	% Reduction	Mean per Nodule	% Reduction
Control	90.9±7.68		0.6415±0.062	
June 26, 1967	57.6±9.48[a]	36.7	0.4798±0.073	25.21
Oct. 6, 1967	45.7±8.1[a]	49.8	0.4864±0.095	14.18

[a]Difference from control significant at 0.05 level or below.

[b]Each figure represents mean of 15 plants.

Whatman 3 MM paper and following the same procedure as with the soil extracts.

When known concentrations of gallotannic acid were added to soil which was obtained from areas adjacent to the Rhus copallina stand it was found that a minimum of 400 ppm had to be added before any could be recovered from the soil. To compensate for this loss, known concentrations were run during all extractions to permit calculation of the recovery percentage. Soil was gathered from underneath Rhus copallina on November 11, 1966 and June 26, 1967 after all visible plant remains were removed from the surface. The soil was air-dried, and 50 g were extracted. The November soil contained approximately 787 ppm and the June soil 600 ppm of gallotannic acid (Table 7).

Two grams of duff from underneath Rhus copallina were ground in a Waring Blendor with acetone for 10 minutes and then extracted in a soxhlet apparatus as previously described. The acetone extract was treated as before. Duff collected in November 1966 contained 45,720 ppm of gallotannic acid while duff collected the following June contained 6,375 ppm. Small quantities of gallic acid were found in similar extractions of soil from underneath Euphorbia supina, but no gallotannic acid.

Soil cores were taken with an auger on May 11, 1967 underneath Rhus copallina from the following depths: 0-15, 15-25, . . . 75-85 cm. Fifty grams from each level were

Table 7. Amounts of gallotannic acid in soil and duff underneath Rhus copallina.

Date of sample	Gallotannic acid, ppm	
	Duff	Soil (0-5 cm)
November 11, 1966	45,720	787
June 26, 1967	6,375	600

extracted as previously described. All samples were made to equal volume and aliquots of these were spotted on Whatman 3MM paper and developed as before. The paper was allowed to dry and dipped in $FeCl_3$-$K_3Fe(CN)_6$. The relative quantity of gallotannic acid was determined by the amount of blue color that developed. Gallotannic acid was found to a depth of 75 cm with a definite zone of accumulation at the 45-55 cm level (Table 8).

Selection of gallotannic acid resistant strains of Rhizobium phaseoli and their effectiveness in nodulation

Gallotannic acid resistant strains were obtained by growing Rhizobium phaseoli (ATCC 10321) in yeast and soil extract-mannitol broth with 10^{-7}M concentration by gallotannic acid for a two week period at which time they were transferred to a 10^{-6}M concentration and subsequently at two week intervals to 10^{-5} and 10^{-4}M concentration. Resistant and non-resistant types were incubated at 31°C for 10 days in yeast and soil extract-mannitol broth to use in inoculation of bean seeds. Forty pots containing a mixture of three parts soil and two parts sand were divided into two equal sets and 33 ppm of gallotannic acid were added to the upper 300 grams of soil in each pot of one set. The other set was kept for controls. Bean seeds inoculated with the resistant strain were planted in 10 control pots and 10 pots containing gallotannic acid. Other bean seeds inoculated with the non-resistant strain were planted in the remainder of the pots thus making four series of plants. Fresh plant weight, nodule number, and

Table 8. Relative amounts of gallotannic acid at various depths under Rhus copallina.

Levels of Soil Samples (cm)	Relative Intensity of Blue Color with $FeCl_3$-$K_3Fe(CN)_6$
0-15	+++
15-25	+
25-35	++
35-45	++++
45-55	+++++
55-65	++
65-75	+?
75-85	

quantity of leghemoglobin were determined 4 weeks from the time of planting. The experiment was repeated using 400 ppm of gallotannic acid. The resistant strain was not as effective in nodulation as the non-resistant strain, but it was as effective in the presence of gallotannic acid as without it (Tables 9, 10). The resistant strain was considerably less effective, also, in initiating the production of leghemoglobin in the nodules (Table 10).

Table 9. Effects of gallotannic acid resistant strains on nodulation (33 ppm gallotannic acid).

Series	Mean[b] Nodule Number	Plant Weight g
Non-resistant strain, control soil	58 ± 2.60	15.3 ± 1.4
Non-resistant strain, soil with gallotannic acid	27 ± 4.89[a]	24.8 ± 9
Resistant strain, control soil	32 ± 7.81[a]	13.8 ± 0.93
Resistant strain, soil with gallotannic acid	30 ± 6.7[a]	16.0 ± 1.85

[a]Difference from non-resistant strain in control soil significant at 0.05 level or below.

[b]Each figure represents mean of 8 plants.

Table 10. Effects of gallotannic acid resistant strains on nodulation and leghemoglobin content (400 ppm gallotannic acid).

Series	Mean[b] Nodule Number	Plant Weight g	Leghemoglobin, ug	
			per Plant	% Reduction
Nonresistant strain, control soil	66.3±4.4	12.61±0.57	90.00± 8.4	
Nonresistant strain, soil with gallotannic acid	59.2±3.7[a]	11.75±0.45	79.30± 6.7	11
Resistant strain, control soil	51.2±5.3[a]	12.28±0.53	71.87±10.5	20
Resistant strain, soil with gallotannic acid	45.5±4.5[a]	12.65±0.56	66.37± 8.1	25

[a]Difference from non-resistant strain in control soil significant at 0.05 level or below.

[b]Each figure represents mean of 20 plants.

CHAPTER IV

DISCUSSION

Gallic and gallotannic acid when added in low concentrations were found to be highly effective in the reduction of nodulation and the amount of leghemoglobin produced in bean plants grown in either sand culture or soil. By what mechanisms could such reduction be induced? The following summary of nodule formation suggests some possible answers. Multiplication of Rhizobium is stimulated by the root exudates of the plant host (Nutman 1965), and a specificity exists between host and bacterium (Wilson 1930). Kefford, Brockwell, and Zwar (1960) suggested that Rhizobium may utilize tryptophan from root exudates of the host plant to produce IAA, and Brown (1963) reported that the IAA may be important in root hair curling by promoting local extension growth. According to Brown, IAA may interfere with the establishment of covalent linkages between polysaccharide chains. Fåhraeus (1963) felt that some unknown factor is also involved in this root hair phenomenon. Rhizobium may stimulate roots to produce polygalacturonase (PG) (Fåhreaus and Ljunggren 1959) through the production of bacterial polysaccharides (Nutman 1965).

PG production seems to be specific for it has been shown that an avirulent variant of clover bacteria could not induce its formation. After treatment with virulent DNA this variant could induce PG production (Ljunggren 1961). Lim (1963) demonstrated that the number of root hair infections is proportional to the density of the bacteria, and the number of infections greatly exceeds the number of nodules. Only a few surplus infections occur in Trifolium pratense, and more bacteria are necessary for each succeeding nodule formation (Purchase and Nutman 1957). An infection thread is produced by the host plant after infection has occurred (McCoy 1932, Schaede 1940), and the thread grows from the root hair into the cortex and distributes the bacteria in the tissue of the host (Nutman 1965). The nodules are subsequently formed, and leghemoglobin has to be synthesized before the nodules can function in nitrogen-fixation (Virtanen et al. 1947, Stewart 1966).

There are, therefore, numerous points where gallic or gallotannic acid might reduce effective nodulation. Rice (1965a) demonstrated that both compounds are very inhibitory to Rhizobium, so these compounds may keep the population of Rhizobium low around legume roots. The action of tannins could depend on their ability to combine with extracellular enzymes precipitating them and thus rendering them inactive (White 1957). Rhizobium is known

also to change commonly to bacteroidal forms upon artificial media containing glucosides (Breed, Murray, and Smith 1957) and this might possibly occur in soil. Tannins may combine with proteins and produce complexes that are very resistant to breakdown by microorganisms. Such precipitates are also known to coat other substances such as cellulose which are then protected from microbial attack (Handley 1966), and this could reduce the available nutrients in the soil.

Gallic and gallotannic acid no doubt have numerous subtle effects on the host plant. Bendall and Gregory (1963) found that phenol oxidases which are important in the formation of lignin-like polymers from coniferyl alcohol (Freudenberg 1959) and oxidation products of tannins such as quinones react with each other to produce a complex that may make phenol oxidase catalytically inactive or may produce an active protein of modified properties. Hulme and Jones (1963), and Jones and Hulme (1961) found that tannins caused considerable reduction of Kreb's cycle succin oxidase and malic enzyme activity. Tannic acid and gallic acid were shown also to inhibit IAA induced growth (Zimsmeister and Hollmuller 1964), and Hall (1966) and Williams (1963) found that gallotannic acid of relatively low concentration was very inhibitory to pectolytic enzymes.

The inhibitory activities of gallic and gallotannic

acid, whether direct or indirect to the bacteria or plant host, are very effective in the reduction of nodulation and the amount of leghemoglobin produced. These reductions would definitely decrease the amount of nitrogen fixed (Virtanen et al. 1947, Stewart 1966).

The original hypothesis that resistant strains of Rhizobium could be selected which could cause effective nodulation in the presence of the inhibitors was not substantiated. Strains were obtained which were able to induce nodulation as well in the presence of gallotannic acid as without it, but they were much less effective in nodulation than the original strains from which they were isolated. Moreover the nodules produced by the resistant strains had less leghemoglobin and were thus less effective in nitrogen-fixation. Schwinghamer (1964, 1967) found that strains of Rhizobium resistant to antibiotics were less effective in inducing nodulation than the original strains, and he suggested that such reduction in effectiveness was due in part to morphological changes of the bacterial walls. Therefore, even though strains of Rhizobium evolve which are resistant to various inhibitors, their failure to be effective in nodulation and nitrogen-fixation would certainly explain how inhibitor species could continue to decrease the amount of nitrogen fixed in old-fields for prolonged periods, thus slowing succession.

Soil from underneath Euphorbia supina, an important

species in abandoned fields (Drew 1942), and Rhus copallina, a species important only in localized areas of such fields but containing large quantities of gallotannic acid, were found to be highly effective in the reduction of nodulation. Large quantities of gallic and gallotannic acid were found in these soils and in the duff underneath Rhus copallina. A considerable reduction in the amount of gallotannic acid occurred in the duff, but not in the top 5 cm of the soil, from November, 1966 to June, 1967. Tannin and protein complexes are very resistant to microbial attack (Handley 1966), and the majority of fungi which occur in the prairie are very sensitive to tannins (Cowley and Whittingham 1961). These facts suggested that such large losses of gallotannic acid in the duff were not due to breakdown. Gallotannic acid was found to a depth of 75 cm in soil extractions from different levels underneath Rhus copallina, with a definite zone at the 45-55 cm level. Gallotannic acid was obviously stable enough that it was leached from the duff into the soil where it accumulated in the B-horizon. I suspect that only the excess gallotannic acid which was not adsorbed by the soil was leached to the deeper levels, for when known concentrations of gallotannic acid were added to soil it was found that amounts below 400 ppm could not be recovered. The accumulation in the B-horizon probably resulted from greater adsorption due to more colloidal clay and to a

decreased percolation of water below that horizon. Although concentrations of 33-300 ppm of gallotannic acid could not be recovered from soil, they were still effective in reducing nitrogen-fixation. It is possible that many other phenolic inhibitors of nitrogen-fixing organisms are stable in soil, and that they may be effective also in reducing nitrogen-fixation at concentrations that cannot be recovered from the soil. The reduction in nitrogen-fixation by gallic and gallotannic acid, or any other inhibitory compounds in the soil of old-fields, is without doubt very important in decreasing the rate of accumulation of nitrogen in the soil of such fields.

Many weeds of the first successional stage in old-fields in central Oklahoma and Aristida oligantha of Stage 2 have been found to be inhibitory to nitrogen-fixation (Rice 1968). Moreover, there is a definite correlation between the sequence of invading species and their increasing requirements for nitrogen from the Aristida stage to the climax prairie (Rice et al. 1960). Unquestionably, plants which produce large amounts of gallic acid or gallotannic acid would slow the rate of succession, and this appears to be true also of plants that produce other kinds of inhibitory compounds (Rice 1965a, 1968). The decreased effectiveness in nitrogen-fixation of bacterial strains resistant to inhibitors would certainly enhance this effect.

CHAPTER V

CONCLUSIONS

Gallic and gallotannic acid were found to be highly effective in the reduction of nodulation and the amount of leghemoglobin produced in bean plants when added in low concentrations in sand culture or soil. The greatest reduction of nodulation occurred with 10^{-8}M concentrations of gallic and gallotannic acid in sand culture, and reductions in nodule number occurred in soil with concentrations of 33-400 ppm of gallotannic acid. In sand the nodule number increased with an increase or decrease in gallotannic acid from the 10^{-8}M concentration. The nodule number decreased in the soil, however, as the concentration increased up to 400 ppm, which was the greatest concentration tested.

Soils from underneath Euphorbia supina, an important species in abandoned fields, and Rhus copallina, important only in localized areas of such fields, were found to be highly effective in the reduction of nodulation. Large quantities of gallic and gallotannic acid were found in these soils and in the duff underneath Rhus copallina. A considerable reduction in the amount of gallotannic acid

occurred in the duff (45,720 ppm to 6,375 ppm), but not in the top 5 cm of soil (787 ppm to 600 ppm) from November 1966 to October 1967. This decrease in the duff was due primarily to leaching, because a definite zone of accumulation was found at the 45-55 cm level in the soil. Thus, gallotannic acid is stable enough in soil to undergo leaching and remain in the soil for prolonged periods of time. Amounts of gallotannic acid below 400 ppm could not be recovered from the soil with the best extraction procedure devised, but in spite of this, concentrations of 33-300 ppm were still effective in reducing symbiotic nitrogen-fixation. It is possible that many other phenolic inhibitors of nitrogen-fixing organisms are stable in the soil, and may be effective also in reducing nitrogen-fixation at concentrations that cannot be recovered from the soil. The reduction in nitrogen-fixation by gallic and gallotannic acid, or any other inhibitory compound in the soil of old-fields, is without doubt important in decreasing the rate of accumulation of nitrogen in the soil of such fields.

My original hypothesis that resistant strains of Rhizobium could be selected which could cause effective nodulation in the presence of the inhibitors was not substantiated. Strains were obtained which were able to induce nodulation as well in the presence of gallotannic acid as without it, but they were much less effective in inducing nodulation and synthesis of leghemoglobin than

the non-resistant strains from which they were isolated.

Unquestionably, plants which produce large amounts of gallic acid or gallotannic acid would decrease the rate of addition of nitrogen to old-fields and thus slow the rate of succession. This appears to be true also of plants that produce other kinds of inhibitory compounds. The decreased effectiveness in nitrogen-fixation of strains resistant to inhibitors would certainly enhance this effect.

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