AN ABSTRACT OF THE THESIS OF

JOY RAE JAEGER (Name of student) for the MASTER OF SCIENCE (Degree)

in Plant Pathology (Major) presented on February 25, 1974 (Date)

Title: FACTORS AFFECTING A BIOASSAY FOR AGROBACTERIUM TUMEFACIENS IN NATURAL SOIL

Abstract approved: Redacted for privacy

Larry W. Moore

Tomato seedlings were used to assay the population of A. tumefaciens in artificially contaminated soil. Aqueous soil suspensions were applied directly to the wounded stem. The bioassay was used because not all pathologically important physiological strains of A. tumefaciens would grow on any of the selective media available. This method also eliminated the need to assay each Agrobacterium-like isolate obtained on selective media forpathogenicity.

Factors affecting the bioassay such as minimum number of bacteria needed for gall initiation, age of seedling at time of inoculation, treatment of seedlings after inoculation, and effect of soil on bacterial suspension used for inoculation were tested. The infectivity of both the 3-ketoglycoside-negative and positive strains were compared, and both produced similar gall initiation in all tests.

A concentration of approximately 10^3 bacterial cells per wound

was necessary to infect 70% of the plants inoculated. Incubating seedlings at 100% relative humidity for 24 hours after inoculation resulted in a significantly ($p = 0.05$) higher percentage of plants infected over those left at 45-55% relative humidity immediately after inoculation. Mixing the bacterial suspension with either sterile or natural soil immediately prior to inoculation did not affect the number of cells needed for 70% infection.

The sensitivity of this assay is not adequate to detect the numbers of A. tumefaciens in field soil which has been reported in the literature (316/g) (41). In this work a population of 10^3 cells/wound was required to infect 7 out of 10 tomato seedlings. However, because only 0.01 ml was applied to each wound, it would require an actual concentration of 10^5 pathogens/ml of initial soil suspension to achieve 70% infection.

Factors Affecting a Bioassay for *Agrobacterium tumefaciens* in Natural Soil

by

Joy Rae Jaeger

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1974

APPROVED:

Redacted for privacy

Assistant Professor of Plant Pathology
in charge of major

Redacted for privacy

Head of Department of Plant Pathology

Redacted for privacy

Dean of Graduate School

Date thesis is presented Feb. 25, 1974

Typed by Ilene Anderton for Joy Rae Jaeger

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

FACTORS AFFECTING A BIOASSAY FOR *AGROBACTERIUM TUMEFACIENS* IN NATURAL SOIL

INTRODUCTION AND LITERATURE REVIEW

Crown gall is a widespread disease caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn occuring on plants of many families. The galls are most commonly found on the root or crown and may vary greatly in size. Crown gall is a serious problem for nurserymen in the Northwest because nursery trees showing gall formation cannot be sold. In some years thousands of nursery trees must be culled and destroyed because of crown gall infection.

A way to avoid the problem would be to develop nurseries on land free of the pathogenic bacteria. This may not be possible since the disease has been reported on nursery stock planted to previously uncultivated land (1). However, this land could have become infested by planting nursery stock with incipient infection (43), or the bacteria may have been introduced into the soil with contaminated irrigation water (38). Hence, it may be difficult to find and maintain pathogen-free soil, especially with the rapid decline of new cultivatable land in areas currently supporting nursery operations.

The necessity for repeated planting in an area raises questions about population levels of *A. tumefaciens* in the soil relative to the

incidence of disease. High populations one season, followed by low populations the following season, might explain why most nurserymen have experienced fluctuations in crown gall severity from year to year. To explain the cause of these fluctuations it would be helpful to know what and how different factors affect the population of A. tumefaciens in the soil. An accurate method of assaying population levels in field soil is a prerequisite to such a study. However, such methods are not currently available.

Patel (34) was the first of numerous workers who sought to develop a selective medium for the isolation and enumeration of A. tumefaciens from soil. Crystal violet and sodium taurocholate (bile salts) were utilized to increase selectivity. However, fast-growing bacterial saprophytes from the soil and many fungi grow in the medium, concealing Agrobacterium colonies. Addition of actidione (12) enhanced selectivity. More recently selective media have also been reported by Schroth (37), Kado (19), Clark (9) and New (33).

Variation in the physiological strains of Agrobacterium has also contributed to the complexity of developing a selective medium. Until 1969, the main form of A. tumefaciens implicated as the causal agent of crown gall was thought to be one which utilized nitrate (8) and produced 3-ketolactose from lactose (5). In 1969, Kerr (21) reported that a ketoglycoside-negative isolate (unable to produce 3-ketolactose) was the principal cause of crown gall losses in

Australian nurseries. This form would not grow on Schroth's medium (37), even without the addition of antibiotics, or on Kado's medium (19); and Kerr found it necessary to return to Patel's medium for isolation. This was due to the inability of the ketoglycoside-negative strains to utilize nitrate unless biotin, and sometimes L-glutamic acid,(20) were supplied. Although different physiological strains have been reported (5, 11, 16, and 42), including vitamin-requiring strains (7), they had not previously been regarded as important pathological strains because of the low number which had been identified. Recent work in Australia (20, 21), and our work in Oregon, indicates the presence of greater numbers of these pathologically important ketoglycoside-negative strains exist than was previously recognized.

An additional problem in developing a soil assay using a selective medium is the difficulty of distinguishing colonies of A. tumefaciens from those of a closely related bacterium, Agrobacterium radiobacter (Beijerinck and van Delden) Conn (21). A. radiobacter is a soil saprophyte reported to exist in the soil in a numerical ratio of 100-500 colony-forming units to one such unit of A. tumefaciens (41). Biochemical and serological tests and other refined techniques, such as DNA homology and G/C ratio, have not differentiated between A. tumefaciens and A. radiobacter (11, 16). The only distinguishing characteristic is the inability of A. radiobacter

to incite galls. The similarity of these two species and the abundance of A. radiobacter in field soils have created a major obstacle to the development of a selective medium to assay populations of pathogenic A. tumefaciens because of the laborious process of purifying each isolate and assaying its pathogenicity.

Because pathogenicity, as determined by inoculation of a susceptible host, is the only diagnostic test for pathogenic A. tumefaciens, several attempts have been made in the past to find a highly sensitive host to monitor proposed planting sites. Stapp (39) recommended that carefully inspected pear wildlings (seedlings) be set out in the intended planting area and dug up some time later and examined for galls. This same procedure was also recommended by Cochran (10), using stone fruit seedlings. Field bioassays of this type involve at least a season's growth before plants can be read.

In 1953, Ark and Schroth (2) proposed the use of carrot discs to bioassay soil for A. tumefaciens in preference to other fleshy roots. Previous workers inoculated carrot discs with A. tumefaciens (3, 6, 23, 24, 25) in conjunction with soil assay but reported it as unsatisfactory. There are a number of problems associated with the carrot disc assay which seriously limits its effectiveness as an assay tool. A major difficulty is that other substances can produce tumorous proliferations on the surface of carrot discs identical in appearance to crown gall (3, 6). Blumenthal and Meyer (6) noted

that a solution of 1% lactic acid had the same effect as the bacterium in producing gall proliferations. Auler (3) also reported induction of gall tissue, histologically identical to those produced by bacteria on the carrot surface, using formic acid, formamide and acetamide. Another serious problem with use of carrot discs to assay natural soil was the initiation of decay by other organisms before gall proliferation (2). Susceptibility of carrot discs to formation of tumors also varied with side of disc, distance from stem end, and from carrot to carrot (25).

In search of a better host, Datura was used in studies by Beaud et al., (4) and Manigault and Beaud (30). They found, however, that nonpathogenic mutants and sterile cell extracts also caused short-lived proliferations resembling the early stages of gall formation caused by A. tumefaciens. Hildebrand, Thompson, and Schroth (18) reported that Datura had a low threshold for gall induction and wounds inoculated with other bacteria or foreign agents will produce short-lived galls. Other solanaceous hosts have also been used for crown gall bioassays. An attempt was made by Levine (26) to measure quantitatively inoculum density to disease ratios on tobacco, but only concentrated bacterial suspensions were used.

Tomato plant stem tissue has been used to test for the presence of crown gall bacteria in soil but not estimate populations (31, 32, 35, 36). Soil artificially contaminated with bacterial suspension was

either placed on stems, or stems with wounds were buried below the soil line.

A greenhouse bioassay, based on techniques similar to those used for mechanical inoculation of plant viruses, was developed by Lippincott and Heberlein (28, 29) using the primary leaves of pinto bean. This procedure was used to study gall initiation and the infection process by El Khalifa and Lippincott (14, 15). Their method required aqueous suspensions of bacteria in the range of 10^5 and 10^6 bacteria per leaf or per ml of inoculum to achieve a mean of one tumor per leaf. The test was rapid and quantitative and employed plants that could be easily cultured. However, it required larger numbers of A. tumefaciens than have been reported in naturally infested soil (41).

In 1970, El Khalifa and El Nur (13) developed a bioassay similar to Lippincott and Heberlein's (28, 29) using the primary leaves of castor beans instead of pinto beans. The assay was more sensitive than Lippincott's by a factor of 100 but still required a larger number of A. tumefaciens cells than have been reported in naturally infested soil.

The objective of the present study was the development of a reliable method of assaying the amount of A. tumefaciens inoculum in natural soil, so nurserymen could have a measure of possible contamination before planting a susceptible crop. The occurrence

of pathologically important physiological forms of A. tumefaciens, which did not grow on any of the then available selective media, and the ultimate need to assay each soil isolate for pathogenicity, led to the attempt to develop a bioassay system using tomato seedlings. The assay was tested under laboratory conditions, using known concentrations of inoculum, to establish the practical limits of the method for assaying nursery soil.

MATERIALS AND METHODS

Isolation of *Agrobacterium* Species From Soil

The influence of different agronomic plants on the natural population of *Agrobacterium* spp. in soils from several Oregon locations was assayed using selective media. Samples were collected from soils supporting growth of the following agricultural crops: bentgrass, wheat, apple, thornless evergreen blackberry, black raspberry, red raspberry, hops, beans, and garlic. Five gram soil samples were collected at a depth of one-half to three inches below the surface and placed in 50 ml of distilled water. Aliquots of this were diluted to 1:100 and 1:500. Two agar plates were spread using 1/10 ml per plate according to the following schedule of dilutions: Schroth's medium (37) 1:10; Kado's medium (19) 1:10 and 1:100; and Patel's medium (34) 1:100 and 1:500.

Well-separated colonies resembling *A. tumefaciens* were chosen from each medium and streaked onto Kings' medium B (22) so that colonies of fluorescent Pseudomonads could be separated and discarded. After 24 hours growth, single colonies were streaked on potato dextrose agar (PDA) containing 0.5% calcium carbonate. Forty-eight hours later, single colonies were transferred to slants of PDA plus 0.5% calcium carbonate and stored at room temperature.

To determine whether cultures were A. tumefaciens, A. radiobacter, or some other bacterium, isolates were tested for ability to produce 3-ketolactose using the method of Bernaerts and DeLey (5) and for pathogenicity by inoculation of tomato plants in the greenhouse. In some cases isolates were also tested for pathogenicity on Datura stramonium, sunflower, and black raspberry.

Parameters Involved in Direct Assay of Soil Containing Agrobacterium tumefaciens on Tomato Seedlings

Assay Host and Growing Conditions; Including Wounding, Method of Inoculation, and Treatment of Seedlings After Inoculation

Young tomato seedlings (Bonny Best var.) were used in the bioassay because of their established use as an assay host, ease of culture under laboratory conditions, and rapid initiation of galls. Seeds were germinated at room temperature between moist paper toweling in Petri dishes and planted in plastic ice cube trays filled with moist sand. The sand was kept wet until the seedlings emerged. The seed coats frequently had to be removed from the emerging seedlings to obtain uniform plants. The seed coat was softened with a drop of water then readily removed by hand.

Age of Host. Seedlings were inoculated 2 to 27 days after transplanting to determine the susceptibility of different aged tissues

to form galls. Seedlings were routinely inoculated 8-17 days after transplanting, although plants as young as two days old (if handled with extra care) could be successfully inoculated. A 25-gauge hypodermic syringe needle was used to make a shallow slit four to six mm long, beginning six to twelve mm below the cotyledons. A shallow slit was found to be less damaging than a puncture at this stage. A 0.01 ml drop of suspension was placed in each slit from the hypodermic needle. Ten plants were inoculated for each dilution and each experiment replicated three or four times.

Humidity and Other Growing Conditions. To study the effect of relative humidity upon gall initiation, twenty plants were inoculated with each dilution. Ten of these plants were immediately placed in vegetable crispers lined with wet paper towels (100% R.H.). The other ten plants were left at room humidity (45-55% R.H.). After 24 hours the plants under 100% R.H. were removed from the crispers and placed at room humidity with the other plants until galls developed. Relative humidity was measured with a Durotherm-Hygrometer.

Seedlings were grown in the laboratory at room temperature (24-29^{o}C) using artificial lighting. Light intensities of five hundred or more foot candles were necessary to produce short, strong-stemmed seedlings that were easily inoculated.

Nutrient supplementation was begun when the seedlings were two weeks old. The plants were watered one to two times weekly

with a one gram per liter solution of Ra-pid-gro (Ra-pid-gro Corp., Dansville, New York).

Initial gall symptoms were observed in seven to ten days, with maximum numbers of plants becoming infected in eleven to fourteen days. Symptoms of plants inoculated with bacteria were compared to control plants inoculated with sterile water.

Agrobacterium Isolates, Their Sources and Dilution Plate Counts

The inability to differentiate between pathogenic and nonpathogenic species of Agrobacterium using only selective media led to the selection of tomato seedlings to bioassay soil for gall-inducing bacteria. The two physiological types of A. tumefaciens known to infect nursery stock (21), were tested (Table 1) as well as an isolate of Agrobacterium rhizogenes (Riker, et al.) Conn.

The minimum number of A. tumefaciens cells required to give a consistent percentage of infected seedlings was determined by correlating dilution plate counts with infectivity. Bacteria used for inoculations were grown for 24 hours on slants of PDA containing 0.5% calcium carbonate and then suspended in ten ml of sterile distilled water. Counts of the viable bacteria in the suspension were made from serial dilutions of the suspension by spreading 0.1 ml aliquots from 10^{-5} and 10^{-6} dilutions to agar plates (2.3% Difco

Table 1. Origin and ketoglycoside reaction of Agrobacterium isolates used in this study.

Isolate identity	Ketoglycoside reaction	Source
A. tumefaciens		
B6	positive	Dr. R. Baker Colorado State University Fort Collins, Colorado
Kerr 27	negative	Dr. A. Kerr Waite Research Institute Glen Osmond, South Australia
V-15	positive	isolated from thornless evergreen blackberry gall
Q_{51}	negative	isolated from cherry gall
A. rhizogenes		
A-4	negative	Dr. D. Huisingh North Carolina State University Raleigh, North Carolina

nutrient agar, 0.1% Difco yeast extract, and 0.5% sucrose) (27). Six plates from each dilution were spread and incubated at room temperature for 48-72 hours. Plates having 50-600 colonies per plate were counted.

Preparation of Soil Suspension for Inoculating Seedlings

To stimulate natural field conditions, an aqueous suspension of bacterial cells was added to sterile or natural silty loam soil before inoculating tomato plants.

Sterile soil samples were prepared by autoclaving tubes containing five gram samples of a field soil "Willamette silty loam" for one-half hour on two successive days. They were tested for sterility by adding one tube of soil to 50 ml of nutrient agar at 45°C, swirling and pouring plates. None of the plates showed bacterial growth after three weeks incubation at room temperature.

Soil suspensions were prepared in duplicate by diluting a concentrated bacterial suspension and adding 9 ml of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions to three sets of two culture tubes containing five grams of either sterile or natural silty loam soil. The tubes were shaken for 30 seconds and then aliquots of the supernatent were immediately removed with a syringe and the seedlings inoculated. For certain experiments, soil suspension tubes were retained up to 24 hours, periodically shaken, and aliquots removed to inoculate plants.

RESULTS

Isolations of *Agrobacterium* Species From Soil

Agrobacterium was isolated from 22 of 34 different samples of field soil with no relationship to locality or crop, indicating a wide distribution in Oregon soils. Some samples yielded as many as 10,000 *Agrobacterium*-like colonies/gram of soil. Of 216 isolates selected from Schroth's medium, 215 were ketoglycoside-positive. This was due to the inability of the ketoglycoside-negative forms of *A. tumefaciens* to grow on this medium (37), which had not been reported at the time the soil isolations were being undertaken. Fifteen selections were made from Patel's medium and all were ketoglycoside-negative. However, it is doubtful that these keto-negative isolates were *Agrobacterium* because their colony morphology was atypical when streaked on PDA with 0.5% $CaCO_3$. Twenty-one isolates were taken from plates of Kado's medium and six were ketoglycoside-positive. Representative colonies were chosen from each sample and assayed for pathogenicity. One hundred and twenty-five of the ketoglycoside-positive and twenty of the ketoglycoside-negative isolates were tested for virulence. All pathogenicity tests were negative.

Direct Assay of Agrobacterium tumefaciens on Tomato Seedlings

Assay Host and Growing Conditions; Including Wounding, Inoculation, and Treatment of Seedlings After Inoculation

Age of Host. The hypocotyl and 1st internode of seedlings of different ages were inoculated to determine which area was most susceptible and would give consistent gall initiation.

Young tissue was the most susceptible to infection. Tomato seedlings varying in age from 8-15 days were equally susceptible to infection. The hypocotyls of more mature seedlings (25-27 days old) were less susceptible ($p = 0.05$) than the hypocotyls of younger seedlings (Table 2), however, the internodes above the cotyledons of more mature seedlings (25-27 days old) were found to be equally as susceptible as the hypocotyls of younger seedlings (Table 2). Similar results were obtained with isolates of both physiological strains (Table 3).

Humidity and Other Growing Conditions. The sensitivity of the bioassay, relative to numbers of bacteria required to induce galls, may be influenced by environmental conditions favoring a survival and establishment of infection by pathogenic A. tumefaciens bacteria. Webb (40) has reported that a relative humidity greater than 90% was essential for bacterial survival, hence an experiment was

Table 2. The effect of tomato seedling age and inoculation site on gall formation.

Isolate	Plant age in days and place of inoculation	Cells/ml of initial cell suspension (X 10^8)	Percent plants infected at each dilution[a]		
			10^{-3}	10^{-4}	10^{-5}
Kerr 27	6-9 (hypocotyl)	4	80	55	40
		3	80	40	10
	11-13 (hypocotyl)	4	70	10	10
		3	70	30	20
	25-27 (hypocotyl)[b]	4	50	20	10
		3	60	20	0
	25-27 (1st internode)[c]	4	80	40	20
		3	60	50	10

[a] A total of 12 plants was used for each dilution; 0.01 ml of each dilution was used to inoculate each plant.

[b] Significantly less galls ($p = 0.05$) when compared to hypocotyls of seedlings aged 6-9 days and 11-13 days.

[c] The first internode was the top one (except where a new internode was just forming).

Table 3. The influence of plant age on susceptibility of tomato seedlings inoculated with different concentrations of A. tumefaciens.

Isolate	Plant age	Cells/ml of initial cell suspension (X 10^8)	Percent plants infected at each dilution[a]				
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
B6	8- 9	7	100	83	75	17	25
		9	83	100	67	17	18
	14-15[b]	5	91	75	58	17	8
		8	91	83	58	8	17
Kerr 27	8- 9	3	100	67	64	42	25
		4	100	91	25	17	25
	14-15[b]	2	91	67	33	25	25
		7	91	91	42	0	8

[a] A total of 12 plants was used for each dilution, 0.01 ml of each dilution was used to inoculate each plant.

[b] The difference between ages 14-15 days and 8-9 days are not significant at $p = 0.05$.

conducted to determine the influence of relative humidity on gall initiation. Seedlings placed under 100% relative humidity immediately after inoculation had a higher percentage of gall initiation ($p = 0.05$) than those left at room humidity (45-55% R.H.) immediately after inoculation (Figures 1 and 2).

Comparison of Infectivity of Agrobacterium Isolates

It was shown that the tomato seedling assay could be used with the isolates tested and that physiologically different strains produced similar inoculum density/disease severity curves (Table 4, Figure 3). Five different isolates were tested in the bioassay to determine if the number of bacteria needed to induce gall formation differed significantly for any physiological strain. Regression lines for the inoculum density/disease severity curves of each isolate were calculated and found not to differ significantly ($p = 0.05$) from a common regression line with a correlation coefficient of 0.851. The four A. tumefaciens isolates were also found not to differ significantly ($p = 0.05$) from the A. rhizogenes isolate (Table 4, Figure 3).

Response of Tomato Seedlings Inoculated With A Soil Suspension Containing A. tumefaciens

Because A. tumefaciens is considered a soil-inhabiting microorganism, tests were conducted to determine the influence of soil

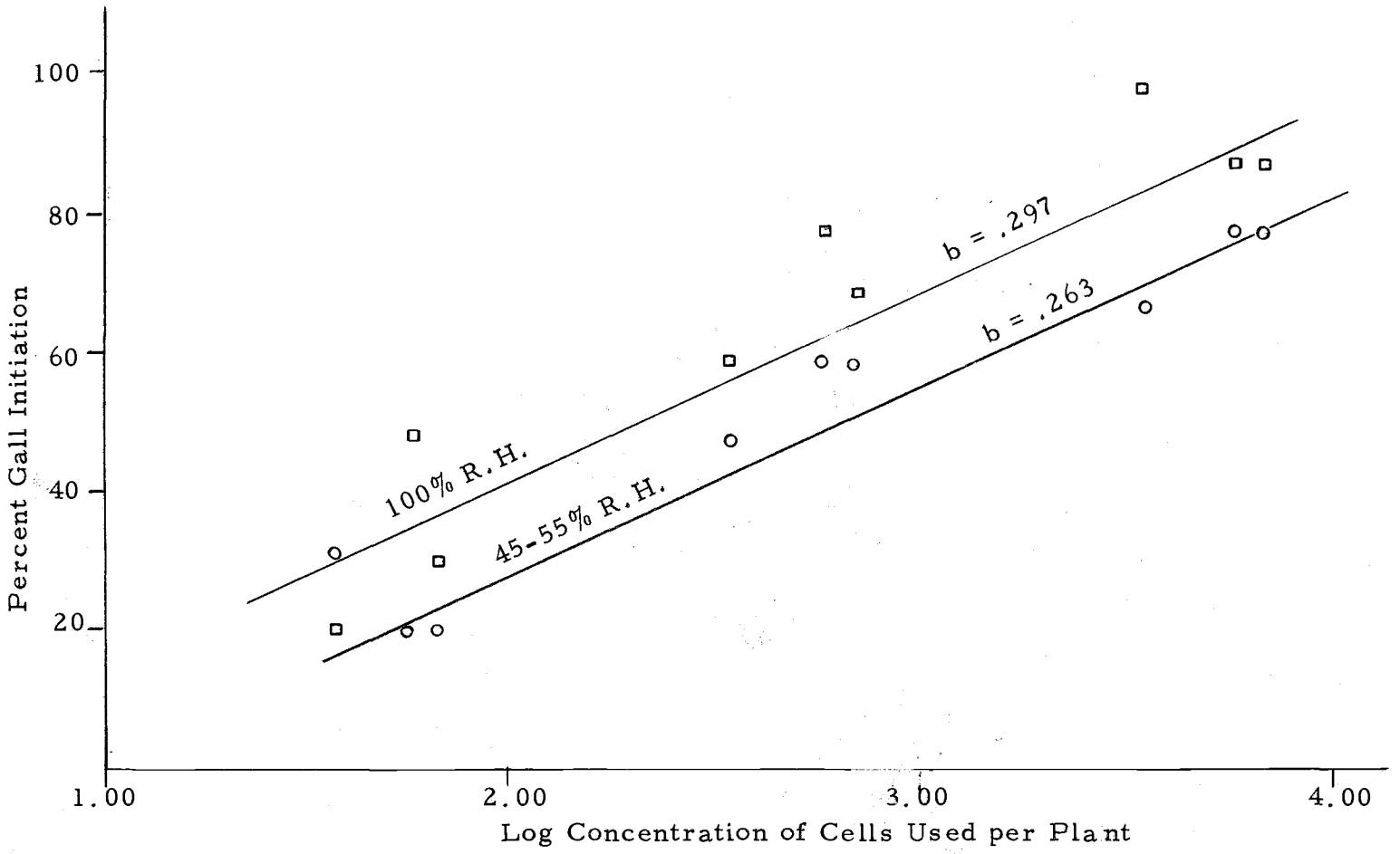

Figure 1. Influence of relative humidity on susceptibility of tomato seedlings immediately following inoculation with A. tumefaciens (isolate 27 from Kerr). Analysis of variance was significant ($p = 0.05$).

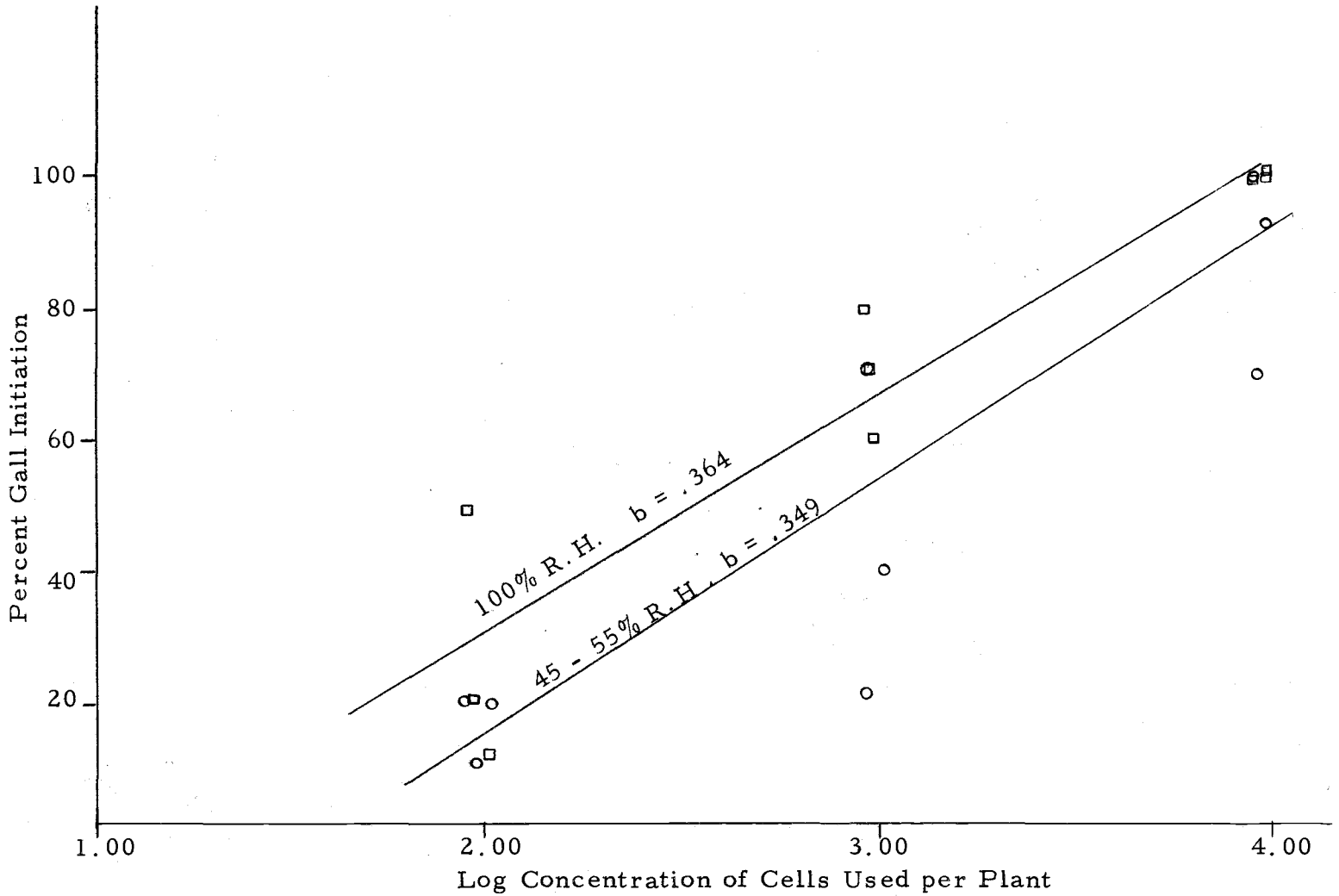

Figure 2. Influence of relative humidity on susceptibility of tomato seedlings immediately following inoculation with A. tumefaciens (isolate B6). Analysis of variance was significant ($p = 0.05$).

Table 4. Comparison of average percent gall formation from inoculation with five *Agrobacterium* isolates.

Isolate	Correlation coefficient	Cells/ml of initial cell suspension (X 10^8)	Average percent plants infected at each dilution[a]		
			10^{-3}	10^{-4}	10^{-5}
A-4	0.823	8	75	50	21.5
V-15	0.890	14	90	64.3	34.3
Kerr 27	0.862	4	76.6	42.5	21.5
Q_{51}	0.823	7	88.7	61.7	29.0
B6	0.874	7	88.5	54.4	19.0

[a] The range of variation for the different dilutions is as follows: 10^{-3}, 66% to 100%; 10^{-4}, 10% to 80%; and 10^{-5}, 0% to 50%.

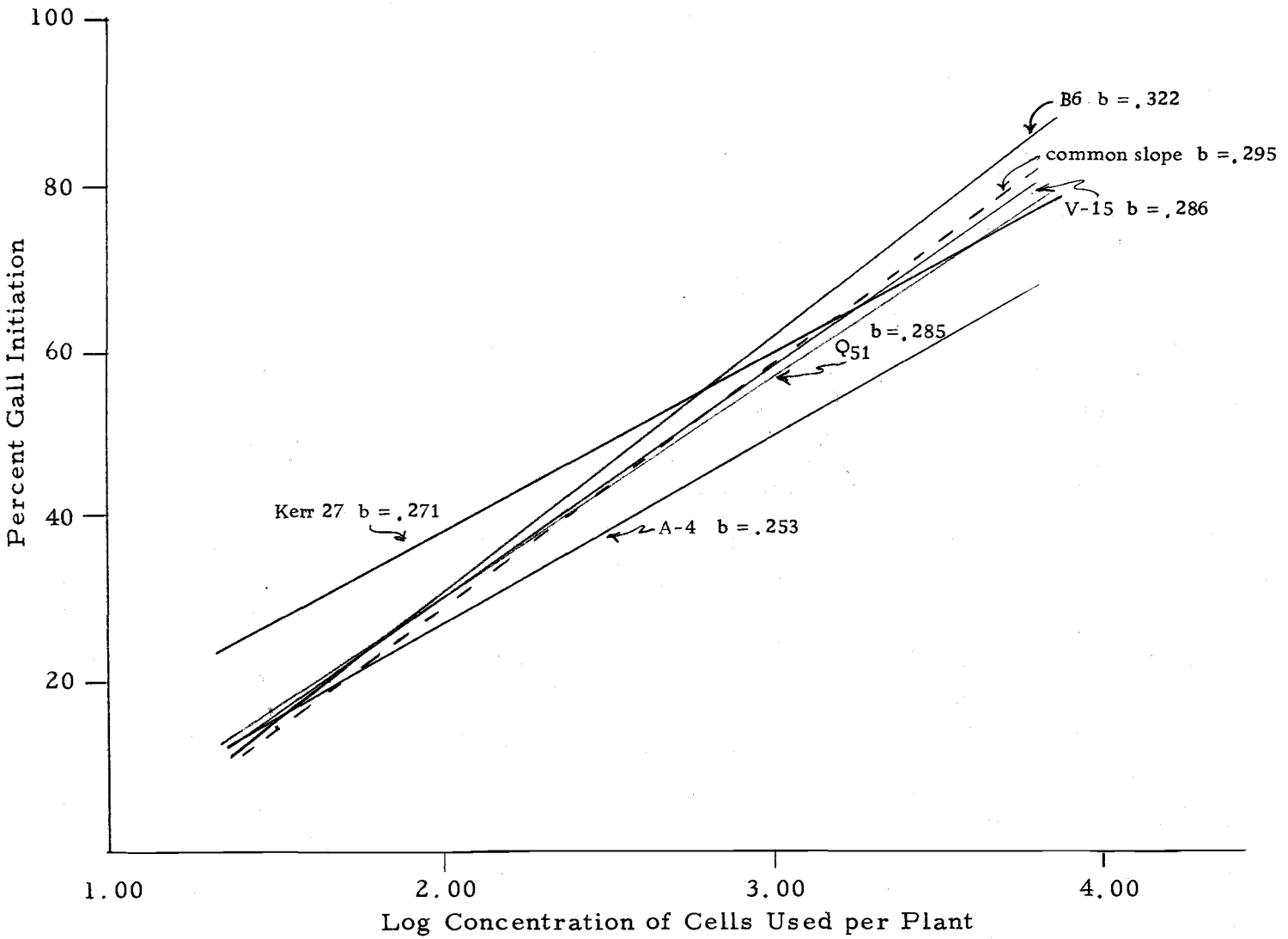

Figure 3. Relationship between inoculum density and gall initiation for five isolates of Agrobacterium and their common regression line.

on the bioassay. There was no difference in gall initiation ($p = 0.05$) when the Kerr 27 isolate of A. tumefaciens was added to either natural or sterile soil and plants inoculated immediately or two hours later (Table 5). There did appear to be an increase in gall initiation when bacteria were incubated for 24 hours in an aqueous suspension of sterile soil. However, bacterial growth could be expected since the bacteria would not only have access to nutrients but would be free of microbial competition in the sterile soil.

Table 5. The effect of inoculation of tomato seedlings with sterile and natural soil suspensions containing A. tumefaciens (Kerr's isolate 27).

Cells/ml in initial cell suspension ($X 10^8$)	Dilution	Percent gall initiation[a/]					
		Time zero		2 hours		24 hours	
		sterile	natural	sterile	natural	sterile	natural
4.5	10^{-2}	70	100	90	100	100	70
	10^{-3}	50	90	80	50	90	80
	10^{-4}	20	10	20	30	100	40
6.0	10^{-2}	90	80				
	10^{-3}	80	70				
	10^{-4}	30	30				
9.6	10^{-2}	100	100	90	100		
	10^{-3}	80	100	90	70		
	10^{-4}	30	40	30	30		
9.5	10^{-2}	100	80	100	80		
	10^{-3}	80	80	90	80		
	10^{-4}	60	40	50	30		

[a/] Ten plants per treatment were used at 45-55% relative humidity and 24-29°C.

DISCUSSION

This work reports the results of experiments to develop a bioassay for the estimation of A. tumefaciens populations in naturally infested soil. Tomato seedlings were effective for detection of high populations in experimentally infested soil but were not sensitive enough to detect naturally occurring populations of A. tumefaciens in field soil (41).

The sensitivity of the bioassay was enhanced by putting the seedlings under 100% relative humidity immediately after inoculation. It is possible that the high humidity provided a more favorable infection court because of the delayed drying of the wound as well as favoring survival of the bacteria.

Methods could be developed which would possibly increase the sensitivity of the bioassay even further, such as increasing the concentration of bacteria by desorption from soil particles and centrifugation. Another method could be the addition of selective antibiotics or other growth inhibitors to the soil samples and then an enrichment factor which would favor the growth of Agrobacterium, although this may also increase the population of A. radiobacter present. Kerr has shown that high numbers of an A. radiobacter (Biotype 2 radiobacter) present in the infection court inhibits crown gall infection. The bioassay might also be made more sensitive by the addition

of compounds such as Mitomycin C to the soil suspension which is reported to increase the virulence (or amount of infection) of A. tumefaciens (17).

The tomato assay is an improvement over the use of selective media which support growth of the ketoglycoside-positive strains, as both strains respond similarly on tomato. If the bioassay were sensitive enough to detect the A. tumefaciens present in natural soil, then both ketoglycoside strains would be found. Recent research (20, 21) has indicated the presence of the ketoglycoside-negative strain, which has heretofore been ignored, in agriculturally important areas. At the present time an improved bioassay technique seems to be the only way to estimate populations of pathogenic A. tumefaciens in field soil so that the potential danger from this source of inoculum can be determined.

BIBLIOGRAPHY

1. Anonymous. 1923. Crown gall with nursery stock. South African Fruit Grower 10:242-243.

2. Ark, P. A. and M. N. Schroth. 1958. Use of slices of carrot and other fleshy roots to detect crown gall bacteria in soil. Plant Disease Reporter 42:1279-1281.

3. Auler, H. 1925. Uber chemische und anaerobe Tumorbildung bei Pflanzen. (Chemical and anaerobic tumor formation in plants). Zeitschrift Fuer Krebsforschung 22:393-403. (Abstracted in Botanical Abstracts Volume 15 No. 8870. 1926)

4. Beaud, G., P. Manigault, and C. Stoll. 1963. Observations sur des tumeurs végétales incomplétes. Phytopathologische Zeitschrift 47:25-37.

5. Bernaerts, M. J. and J. DeLey. 1963. A biochemical test for crown gall bacteria. Nature 197:406-407.

6. Blumenthal, F. and P. Meyer. 1924. Uberdurch Acidum lacticum erzeugte Tumoren auf Mohrrubenscheiben. (Tumors produced by lactic acid on carrot discs). Zeitschrift Fuer Krebsforschung 21:250-252. (Abstracted in Botanical Abstracts Volume 15 No. 8875. 1926)

7. Braun, A. C. 1950. Tumor-inducing bacterium isolated from Euonymous galls. Phytopathology 40:1058-1060.

8. Breed, R. S., E. G. D. Murray, and N. R. Smith (eds.). 1957. Bergey's Manual of Determinative Bacteriology. 7th edition. Baltimore. The Williams and Wilkins Co. 1094 p.

9. Clark, A. G. 1969. A selective media for the isolation of Agrobacterium species. Journal of Applied Bacteriology 32:348-351.

10. Cochran, L. C. 1941. A case of crown gall organism persistent in grain land. Plant Disease Reporter 25:73.

11. DeLey, J. et al. 1966. Approach to an improved taxonomy of the genus Agrobacterium. Journal of General Microbiology 43(1):7-17.

12. Dickey, R. S. 1961. Relation of some edaphic factors to Agrobacterium tumefaciens. Phytopathology 51:607-614.

13. El Khalifa, M. D. and E. E. El Nur. 1970. Crown gall on castor bean leaves. 1. Crown gall bioassay on primary castor bean leaves. Angewandte Botanik 44(1-2):29-37.

14. El Khalifa, M. D. and J. A. Lippincott. 1968a. The influence of plant growth factors on the initiation of crown-gall tumours on primary pinto bean leaves. Journal of Experimental Botany 19(61):749-759.

15. El Khalifa, M. D. and J. A. Lippincott. 1968b. Promotion of crown gall tumor initiation on primary pinto bean leaves by certain inorganic salts. Plant and Cell Physiology 9:217-225.

16. Heberlein, G. T., J. DeLey, and R. Tijtgat. 1967. Deoxyribonucleic acid homology and taxonomy of Agrobacterium, Rhizobium, and Chromobacterium. Journal of Bacteriology 94(1):116-124.

17. Heberlein, G. T. and J. A. Lippincott. 1967. Enhancement of Agrobacterium tumefaciens infectivity by mitomycin C. Journal of Bacteriology 94:1470-74.

18. Hildebrand, D. C., J. P. Thompson, and M. N. Schroth. 1966. Bacterial enhancement of self-limiting outgrowth formation of Datura. Phytopathology 56:365-366.

19. Kado, C. I. and M. C. Heskett. 1970. Selective media for isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, and Xanthomonas. Phytopathology 60(6): 969-976.

20. Keane, P. J., A. Kerr, and P. B. New. 1970. Crown gall of stone fruit. 2. Identification and nomenclature of Agrobacterium isolates. Australian Journal of Biological Sciences 23:585-595.

21. Kerr, A. 1969. Crown gall of stone fruit. 1. Isolation of Agrobacterium tunefaciens and related species. Australian Journal of Biological Sciences 22:111-116.

22. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine 44:301.

23. Klein, R. M. 1955. Resistance and susceptibility of carrot roots to crown gall tumor formation. Proceedings of the National Academy of Science 41(5):271-274.

24. Klein, R. M. and J. L. Knupp, Jr. 1957. Sterile induction of crown gall tumors on carrot tissues in vitro. Proceedings of the National Academy of Sciences 43:199-203.

25. Klein, R. M. and I. L. Tenenbaum. 1955. A quantitative bioassay for crown gall formation. American Journal of Botany 42:709-712.

26. Levine, M. 1923. Studies of plant cancers. 4. The effects of inoculating quantities of different dilutions of Bacterium tumefaciens into the tobacco plant. Bulletin of the Torrey Botanical Club 50:231-243.

27. Lippincott, B. B. and J. A. Lippincott. 1966. Characteristics of Agrobacterium tumefaciens auxotrophic mutant infectivity. Journal of Bacteriology 92(4):937-945.

28. Lippincott, J. A. and G. T. Heberlein. 1965a. The induction of leaf tumors by Agrobacterium tumefaciens. American Journal of Botany 52(4):396-403.

29. Lippincott, J. A. and G. T. Heberlein. 1965b. The quantitative determination of the infectivity of Agrobacterium tumefaciens. American Journal of Botany 52:856-863.

30. Manigault, P. and G. Beaud. 1967. Expression de l'efficacité de la bacterie Agrobacterium tumefaciens (Smith and Townsend) Conn dans l'induction tumorale (Datura stramonium L.). Annales de L'Institut Pasteur 112:445-457.

31. Muncie, J. H. 1926a. The effect of crown gall on apple and peach trees and longevity of Bacterium tumefaciens in the soil. Phytopathology 16:79.

32. Muncie, J. H. 1926b. A study of crown gall caused by Pseudomonas tumefaciens on Rosaceous hosts. Iowa State College Journal of Science 1:67-117.

33. New, P. B. and A. Kerr. 1971. A selective medium for Agrobacterium radiobacter biotype 2. Journal of Applied Bacteriology 34(1):233-236.

34. Patel, M. K. 1926. An improved method of isolating Pseudomonas tumefaciens (Smith and Town.) Phytopathology 16:577.

35. Patel, M. K. 1928. A study of pathogenic and nonpathogenic strains of Pseudomonas tumefaciens (Smith and Town.) Phytopathology 18:331-343.

36. Riker, A. J. 1926. Studies on the influence of some environmental factors on the development of crown gall. Journal of Agricultural Research 32(1):83-96.

37. Schroth, M. N., J. P. Thompson, and D. C. Hildebrand. 1965. Isolation of Agrobacterium tumefaciens-A. radiobacter group from soil. Phytopathology 55(6):645-647.

38. Smith, C. O. and L. C. Cochran. 1944. Crown gall and irrigation water. Plant Disease Reporter 28(4/5):160-162.

39. Stapp, C. 1961. Bacterial Plant Pathogens. (Translated by A. Schoenfeld) London, Oxford University Press. 292 p.

40. Webb, S. J. 1965. Bound water in biological integrity. Charles C. Thomas, Publisher, Springfield, Illinois, 187 p.

41. Weinhold, A. R. in Root Diseases and Soil-Borne Pathogens. T. A. Toussoun, R. V. Bega, and P. E. Nelson (eds.) University of California Press. Berkeley, Los Angeles, London. 1970. 252 p.

42. Wormald, J. 1945. Physiologic races of the crown gall organism in Britain. Transactions of the British Mycological Society 28:134-146.

43. Young, R. A. 1954. Dissemination of plant pathogens on nursery and ornamental plants. Plant Disease Reporter 38(6):417-420.