T H E S I S

METHODS FOR DETERMINING *AZOTOBACTER VINELANDII* IN SOILS

Submitted by

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In partial fulfillment of the requirements

for the Degree of Master of Science

Colorado State College

of

Agriculture and Mechanic Arts

Fort Collins, Colorado

May 1941

COLORADO STATE COLLEGE

OF

AGRICULTURE AND MECHANIC ARTS

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **Walter N. Gonick**

ENTITLED

Methods for Determining Azotobacter Vinelandii in Soils

BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF **Science**

MAJORING IN **Soil Microbiology**

CREDITS **17½**

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TABLE OF CONTENTS

TABLE OF CONTENTS (Cont'd)

ACKNOWLEDGMENTS

The writer wishes to express his gratitude to Dr. H. W. Reuszer under whose direction this work was begun and to Dr. D. P. Glick whose assistance and inspiration enabled the author to complete this undertaking.

The author is also indebted to Dr. L. A. Brown for his assistance in sampling and classifying many of the soils from eastern Colorado, to J. F. Brandon for his kind permission and assistance in selecting soil samples at the Akron Field Station, to the field staff of the Agronomy Station of the Experiment Station at Fort Collins for their kind permission and assistance in selecting soil samples at the Fort Collins Station.

The writer is also grateful to Mr. M. J. Russell for his assistance in editing the manuscript.

LIST OF TABLES

LIST OF CONTENTS (Cont'd)

LIST OF TABLES (Cont'd)

LIST OF FIGURES

LIST OF FIGURES (Cont'd)

LIST OF FIGURES (Cont'd)

LIST OF FIGURES (Cont'd)

METHODS FOR DETERMINING AZOTOBACTER VINELANDII IN SOILS

By Walter N. Gonick

INTRODUCTION

The study of Azotobacter as a non-symbiotic, aerobic, nitrogen-fixing organism has aroused considerable interest both from academic and practical points of view. The mechanism of nitrogen fixation possessed by this organism has been a subject of intense research since the organism was first isolated by Beijerinck (1 & 2), yet the apparent ease with which this organism assimilates free nitrogen into an organic form still remains to be solved. Azotobacter is just as much an enigma in the realm of practical value as it is in the academic field. How much nitrogen Azotobacter is able to fix under natural conditions still is not known. However, depending on the individual viewpoint, answers may range from none or very little to 10 to 40 pounds of nitrogen per acre per year (28). Waksman (43) claims that

> . . . there is one point not yet settled, namely, whether these organisms (Azotobacter) really fix

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(1 & 2) Reference by number (in parenthesis) is to "Bibliography."

> nitrogen in the soil under field conditions. This has been emphasized by the fact that available energy is not supplied in the soil in the form of mannitol or dextrose universally used in the study of pure cultures . . . but rather as cellulose and other complex carbohydrates, which these organisms are quite unable to use as a source of energy.

Winogradsky (43) is of the same opinion, for he states that " . . . all research work from Beijerinck's time to 1930 has failed to make the role of Azotobacter in the soil any better understood."

Compared with the foregoing complexities much progress has been made in the study of distribution of Azotobacter, particularly the species chroococcum. This is not at all surprising for Az. chroococcum is the most common species of the genus, and its appearance in nitrogen-free mannite or glucose media, or on the agar or silica plates of the same composition, is easily recognized. However, studies upon the distribution of the species vinelandii are relatively unknown in scientific literature. This can be explained, perhaps, by the fact that Az. vinelandii is not as ubiquitous as Az. chroococcum, although its nitrogen-fixing ability in the laboratory is much greater than that of the latter (23, 24). Those who have isolated Az. vinelandii from soils or waters have done so while investigating Az. chroococcum or Az. agile.

It would seem, therefore, that because of the apparent scarcity of Az. vinelandii in nature a method would have to be developed which would facilitate the study of ecological distribution of this organism. A method that would greatly stimulate the development of this species but not that of others would be most desirable. An approach to this problem has been made by Reuszer (37) who found that in soils Az. vinelandii is " . . . better able to develop in the presence of large amounts of benzoic acid compounds than is Az. chroococcum." This observation was confirmed by the fact that Az. vinelandii is able to withstand higher concentrations of sodium benzoate in artificial media than Az. chroococcum (36). It therefore seems evident that if benzoates can be made to stimulate the development of Az. vinelandii in soils to such a degree as to make possible its isolation, even if it was originally present in very small quantities, then the ecological distribution of this species in nature may be readily studied.

To work out and test a method or methods based on the principle of stimulating the growth of Az. vinelandii while inhibiting the growth of other species, particularly Az. chroococcum, is the purpose of the present undertaking. In this connection it is proposed to investigate:

1) The value of various non-nitrogenous organic compounds, including benzoic acid

and some of its salts, for demonstrating the presence of Az. vinelandii in soils; and

2) the distribution of Az. vinelandii in some Colorado soils and waters.

REVIEW OF LITERATURE

In 1901 Beijerniok (1,2) succeeded in isolating a group of large aerobic, nitrogen-fixing bacilli to which he gave the generic name of Azotobacter. To the organism isolated from garden soil he gave the species name chroococcum and to the organism isolated from canal water he applied the species name agilis. He separated the two species on the basis of morphology, ecology, and physiology. Since then numerous organisms have been identified (23,25,29,40) for which the authors have claimed the generic name of Azotobacter and a species name of their own choosing. However, many of these species have been discarded because of their rareness or perhaps because of their poor description. The position of the remaining species has been hotly contested by the workers in the field of soil microbiology.

Az. vinelandii is one of the strains whose qualifications to the title of a separate species have been widely questioned. Isolated by Lipman (24) in 1903 from a soil in the locality of Vineland, New Jersey, Az.

vinelandii answers to the following description: Four-days-old colonies on mannite agar about 4mm. in diameter, round, raised, semitransparent, concentric in structure, with denser whitish centers; mannite culture forms range from large rods with rounded ends to spherical organisms (2.0 to 3.0 by 3.0 to 6.0 microns); in most cases progressive or rotary motility is evident; yellow to yellowish-red pigment occurs on mannite agar plates or in solution and in old cultures diffuses throughout the medium.

There appears to be among some workers a prevailing opinion that Az. vinelandii is very closely related to Az. agilis and should be classified as a variety of the latter. Löhnis and Smith (27) after a study with 30 strains of Azotobacter concluded that two species of this organism may be recognized: Az. chroococcum and Az. agilis. They consider Az. vinelandii a synonym for Az. agilis.

Prazmowski (34) is of the opinion that the various species described are merely races of one great variable species Azotobacter. He reached this conclusion by observing changes of the dark brown Az. Chroococcum into a colorless race similar to Az. vinelandii and Az. agilis and a yellow race similar to Az. beijerinckii. His work was upheld by Omeliansky (33) who found that the pigment formation of Azotobacter is dependent entirely on the composition of the medium.

Smith (39) in his studies concerning the utilization of carbon by various Azotobacter species found that Az. vinelandii and Az. agilis, obtained from various laboratories, utilized dextrose, sucrose, mannitol, and glycerine but could not use lactose, starch, and dextrin. He concludes that " . . . this lack of variation in their carbon requirements probably means that these cultures had common origin."

Green (20) analyzed bacterial growth of Az. vinelandii and Az. agilis which were grown on nitrogen-free mannitol agar for four days and found them to be very similar not only in composition but also in nitrogene-fixing ability. Az. chroococcum, however, under the same conditions gave different results.

Waksman (42), however, commenting upon the work of Omeliansky, states that there is no sufficient reason to deny altogether the existence of the various species since the genus itself is extremely variable.

Winogradsky (49) recognizes Az. vinelandii as a separate species from Az. agilis. Working with a number of specimens from each species, he found differences not only in motility, size, and form but also in the cycle of development. In case of Az. vinelandii he found a definite stage of cyst formation which was not present in the specimens of Az. agilis examined. The young forms of Az. vinelandii were small, short, mobile rods, and the

adult forms were round and diminutive. The adult stage gives rise to cyst formation which eventually is followed by coccoid forms and therefore the repetition of the cycle. In case of Az. agilis there was no succession of generation, the old cells being distinguished from the young by the presence of oil droplets and by an increase in size.

From his observations Winogradsky concludes that the most important difference between the two species is the presence of a resting stage in Az. vinelandii and its absence in Az. agilis. Since the resting stage is a characteristic common to all three of the best known species of Azotobacter (chroococcum, beijerinckii, and vinelandii), then cyst formation must be considered as a characteristic of the genus Azotobacter. Hence, in order to avoid confusion, Winogradsky proposes that Az. agilis be given a new genus name Azomonas.

Energy Sources

Azotobacter depends upon complex organic sompounds as sources of energy and carbon. Löhnis and Pillai (26) list the following compounds in the order of their preference by Azotobacter: Mannitol, xyclose, lactose, laevulose, dextrose, starch, sodium tartrate, glycerine, sodium succinate, calcium lactate, sodium citrate, sodium propionate, potassium oxalate, calcium butyrate, and humus. Winogradsky (47,49), in addition to some of

the foregoing compounds, adds the following: Sodium acetate, sodium benzoate, ethanol, and butanol.

There are numerous compounds that have been tested and found toxic to Azotobacter even in small quantities. The following compounds are listed (17,35) as unsuited for Azotobacter: Caffein, alloxan, betaine, trimethylamine, legumin, cinnamic acid, aspartic acid, asparagine, hippuric acid, creatin, creatinine, xanthine, and hypoxanthine. Esculin, vanillin, daphnetine, cumarin, heliotropin, arbutin, resorcinol, pyrogallol, phlorogluoine, hydroquinone, salicylic aldehyde, and oxalic acid do not stimulate in any concentration and are not toxic until large quantities have been added. Urea, glycerol, and formamide likewise produce depressing effects on Azotobacter (35). Difficulty is also experienced with sodium formate, sodium oleate, methyl alcohol, phenol, and sodium salicylate (47).

Various strains of Azotobacter may react differently to the same source of carbon. Gainey (13) found that some cultures are rather limited in their ability to use fatty acids while others can assimilate a wide variety of these compounds. He concludes that the cation with which the acid is combined and the molecular weight of the acid are important in determining the ability of the organism to use the acid. Smith (39) found that not all strains of Azotobacter chroococcum

isolated by him were able to use mannitol, although all were able to utilize dextrose and sucrose.

Lipman (24) in studying the value of various organic compounds as sources of energy for Az. vinelandii found that mannite was more favorable than glycerine, sodium propionate, sodium succinate, or ethyl alcohol. Sodium citrate was not used and sodium succinate only slightly. He sums up the result by stating that

> . . . A. vinelandii like A. chroococcum can make use of a great variety of organic compounds for its development. These include not only higher alcohols and mono saccharides but also the salts of the simpler organic acids of the paraffin series and of the lower alcohols.

Results of Smith (39) show that Az. vinelandii is able to use dextrose, sucrose, mannitol, and glycerine but not lactose, starch, and dextrin. Winogradsky (49) states that ethanol, butanol, calcium acetate, calcium butyrate, and calcium benzoate cause no deviation from the regular cycle possessed by this bacterium; while the normal evolution is deranged by glucose and mannite. Az. agilis, however, favors ethanol but not benzoate and mannite

The use of benzoate compounds as a means of isolating Azotobacter from soil has been strongly recommended by Winogradsky (48). The silica-gel plate method with calcium or sodium benzoate as a source of carbon has been found more efficient in producing pure cultures

of the organism than has the same method with glucose or mannite. Reuszer (37) found that additions of benzoate compounds to soil may stimulate the development of Az. vinelandii to such an extent as to inhibit entirely the growth of Az. chroococcum. The addition of 1.0, 2.5, and and 5.0 percent of calcium benzoate, and benzoic acid to soil which was not suspected of containing Az. vinelandii, yielded only Az. chroococcum at first. After five weeks, however, Az. chroococcum was replaced entirely by a green-pigmented form (Az. vinelandii) in treatments containing 2.5 and 5.0 percent calcium benzoate and benzoic acid.

Katznelson (22), studying the survival of Azotobacter in soils, found that additions of one percent of ethyl alcohol, butyl alcohol, and calcium benzoate to soil suppressed Azotobacter growth. The same compounds in quantities of one-half percent acted favorably. Large increases in numbers occured only in the presence of readily available sources of energy such as mannite or glucose.

Although numerous organic compounds cannot serve as sources of carbon for Azotobacter in pure cultures, it is possible that in soil they may be converted into compounds readily available to Azotobacter. That the soil contains organisms capable of acting upon benzin ring compounds has been established by Söhngen (38)

and by Gray and Thornton (15). Organisms were isolated from the soil which were capable of utilizing phenol, meta-cresol, naphthalene, ortho-cresol, para-cresol, phloroglucinol, resorcinol, and toluol.

Distribution

The distribution of Az. chroococcum in soils has been studied successfully by many investigators. This has been facilitated by the fact that the organism grows readily in nitrogen-free media containing readily available sources of energy such as glucose, sucrose, or mannite. Lipman and Burgess (23) in examining soils from all parts of the world found that about one-third of them contained Azotobacter. Other workers (2,4,12,21,50), both in this country and abroad testify to the common occurrence of this organism in soils.

Studies upon the distribution of Az. Vinelandii, however, have been discouraged by the fact that this bacterium does not appear to be as commonly distributed in soils as is Az. chroococcum. Lipman (25), who first isolated the organism, states that Az. Vinelandii " . . . is widely distributed in the arable soils of the State (New Jersey), especially in the red shale area of Middlesex County." Burgess (4), studying the distribution of Azotobacter in Hawaiian soils, isolated and identified Az. vinelandii and several strains which appeared similar to it. Lipman and Burgess (23) found

Az. vinelandii in a soil from Smyrna.

Yamagata and Itano (50) examined 300 soils from Japan and found that out of forty-three belonging on an alkali group, three contained Az. vinelandii.

Winogradsky (49) isolated Az. vinelandii from the soil of Brie but contends that this organism is rare in soils and rather common in water, its natural habitat. He recommends examination of clear waters for Az. vinelandii and muddy water and soils for Az. chroococcum.

Some Environmental Factors

Of the many factors capable of influencing Azotobacter development, soil reaction has received considerable attention. This appears to be justifiable since it has been definitely established that Azotobacter does not develop in soils whose reaction is below that of pH 6. Gainey (14) from his numerous observations concluded that the presence or absence of Azotobacter in soils is very closely associated if not dependent upon the absolute reaction of the soil solution. However, Starkey and De (40) have recently isolated an organism, Az. indicum, which will grow under more acid condition than any of the species of Azotobacter recognized at present.

Investigation has revealed that in solution cultures the limiting pH appears to be between 5.9 and

6.1 (11). Yamagata and Itano (50) found that the optimum pH for Az. chroococcum was between 7.45 and 7.60, while the limiting pH was 5.80. For Az. vinelandii, however, the optimum pH was between 7.50 and 7.70 and the limiting pH was 5.90.

It was originally believed that Azotobacter was common in soils which were well supplied with calcium carbonate. Because of this it was suggested that the organism be used as an index of lime requirement of the soil. Martin and Brown (30) in their studies with Iowa soils found that the addition of lime was essential for the prolonged growth of Azotobacter and that other treatments, organic and inorganic, with the exception of oat straw were not essential. Gainey (14) wrote that the addition of calcium carbonate or magnesium carbonate sufficient to raise the pH of an acid soil to 6.0 rendered it fit for Azotobacter. He was not able to isolate Azotobacter from soil known to contain the organism before its reaction was lowered below pH 6.0.

It appears that the presence of carbonates in large quantities does not necessarily mean the presence of Azotobacter. The value of carbonates is found in their ability to neutralize acids and raise the pH to 6.0, thus making the soil a favorable medium for nitrogen-fixers.

It seems that not all investigators agree on the factors influencing the development of Azotobacter in soil. Wilson (46) stated that he was able to induce the growth of microscopic colonies of Azotobacter upon the surface of soil plaques containing proper salts and carbohydrates regardless of acidity.

Nicklas et al. (32) attribute the failure to find Azotobacter in some soils with favorable reaction and high lime content to lack of phosphoric acid. Martin and Walker (31) are of the opinion that other factors than pH and phosphorus content limited the growth of Azotobacter in some of the soils examined by them.

A complete disappearance of Azotobacter from soils because of some unknown cause has been observed by several workers. Vandecavey (41) observed that Berkfeld filtrates from soils which had lost their Azotobacter population had an inhibiting effect when added to Azotobacter solution cultures. Christensen (7) wrote that the disappearance of Azotobacter from soil is usually associated with the absence of basic substances and not with the presence of toxic ones.

The optimum temperature for the activities of Azotobacter lies at 28° C. and the extreme limits are between 9° C. and 33° C. (29). Recent work of Greene (19), however, places the optimum temperature at 32.5° C.

The resistance of Azotobacter to drying has been attested by Lipman and Burgess (23). They found that many soils containing Azotobacter showed a vigorous nitrogen-fixing power even after being allowed to remain in museum jars from five to twenty years. However, the activities of Azotobacter in the soil appear to be optimum at 20 percent moisture (44). Greaves (16) states that the moisture content for maximum nitrogen fixation lies between 15 and 22 percent.

The topography of the land from which the soil samples are taken seems to have some influence on the development of Azotobacter. In comparing samples taken from depressed areas with those taken from elevated areas, it was found that a much greater percentage of the former contained Azotobacter (9,31).

METHODS AND PROCEDURE FOR PART 1

Experiments described in Part 1 were carried out with the intention of determining the value of various organic compounds in stimulating the growth of Az. vinelandii in soil. Compounds used here fall into two general groups: Those that present a readily available source of carbon without undergoing any further change, and those that do not appear, according to the literature, to be beneficial to Az. vinelandii but which may undergo changes in the soil and eventually present an available source of carbon for this organism.

The soil came from the Colorado Agricultural Experiment Station farm at Fort Collins. In the laboratory the soil was partly air dried, passed through a ten-mesh sieve, and then thoroughly mixed. Its moisture content was determined just before use. Two hundred-gram samples were then placed in a 40 by 100 mm. Petri dish and treated in the following manner:

Series 1--methyl, ethyl, n-propyl, amyl, and hexyl alcohols and mannite and glycerol in quantities of 1.0, 3.0, and 5.0 percent.

Series 2--glucose, galactose, mannose, fructose, raffinose, sucrose, maltose, lactose, inulin,

starch, and dextrin in quantities of 1.0 and 2.5 percent.

Series 3--phenol, thymol, resorcinol, quinol, pyrogallol, phloroglucinol, benzene, and toluene in quantities of 1.0 and 2.5 percent.

Series 4--salicylic acid, gallic acid, tannic acid, potassium hydrogen phthalate, and cinniamic acid in quantities of 1.0 and 2.5 percent; sodium benzoate, calcium benzoate, and benzoic acid in quantities of 2.0, 3.0, and 5.0 percent.

Series 5--calcium and potassium citrate, calcium and potassium lactate, calcium and potassium tartrate, potassium oxalate, and potassium malate in quantities of 1.0 and 2.5 percent.

Series 6--calcium and potassium propionate, calcium and potassium palmitate, calcium and potassium butyrate, and potassium acetate in quantities of 1.0 and 2.5 percent.

Series 7--cinnamic acid, 0.5 percent and glucose, 1.5 percent.

All treatments were duplicated and each set of treated soils was accompanied by untreated samples in duplicate, designated as "check".

The treated and untreated soils were then inoculated with one milliliter of water solution containing Az. vinelandii which was grown on sucrose agar for

48 hours. The culture of Az. vinelandii was previously isolated in this laboratory. The moisture content of all treatments was kept at 20 percent, with slight variation in either direction, by weekly check on the weight of the individual container.

The treatments were incubated at 28° C. in a constant-temperature room.

On the first day and at the end of the fourth day each treatment was thoroughly mixed to insure a homogeneous distribution of chemical and Az. vinelandii. At the end of 1, 2, 4, 6, 8, and in some cases 10 weeks the soil was examined for Azotobacter, other bacteria and Actinomyces, molds, and soil reaction. Ten-gram samples were taken for the determination of microorganisms and one-gram samples for pH determination.

Counts of the organisms were made by the agar-plate method. Azotobacter counts were made on modified Wenzl's medium:

K_2HPO_4	0.75	gr.
$MgSO_4 \cdot 7H_2O$	0.30	gr.
NaCl	0.20	gr.
$Al_2(SO_4)_3$	0.20	gr.
$Na_2MoO_4 \cdot 2H_2O$	0.00025	gr.
Sucrose	20.0	gr.
$CaCO_3$	5.0	gr.
Agar	12.0	gr.
Water (tap)	1,000	ml.

Agar plates were poured 24 to 48 hours before being used, to permit some evaporation. They were then inoculated by pouring one milliliter of the desired dilution and distributing it over the surface of the plate. The plates were then allowed to stand in the upright position for about one hour, after which time they were inverted and incubated at 28° C. Preliminary work showed that this method was more satisfactory than the one previously used in this laboratory where the plates were uncovered and the excess moisture allowed to evaporate. Possibility of Azotobacter contamination was avoided by the improved method.

Azotobacter counts were made after five to seven days. Az. vinelandii was distinguished from Az. chroococcum by its brilliant greenish-yellow to yellowish-green pigment. Az. chroococcum produces a growth which is at first milky in appearance and which with age becomes brown to dark-brown.

Bacteria and Actinomyces were grown on sodium albuminate agar, and molds were grown on peptone-glucose acid agar.

Just before plating, the soil reaction was determined with the aid of a Beckman pH meter, using one part of soil to two parts of distilled water.

Because of the biological factors involved and because of the insufficient number of determinations, it

was found impractical to analyze the results statistically.

METHODS AND PROCEDURE FOR PART 2

The second part of this work was devoted to the study of the distribution of Az. vinelandii in some Colorado soils and waters and to the identification of the strains isolated from these sources.

The methods used in studying the distribution of Az. vinelandii were worked out and tested in the preliminary investigations and are as follows: The plate-culture method, the enrichment-culture method, and the soil-enrichment method. The plate method is based on the fact that Az. vinelandii develops readily on agar in presence of an available source of carbon. In this connection sodium benzoate in place of sucrose was commonly used in modified Wenzl's medium, already given. The method is rapid but it is useful only if Az. vinelandii organisms are present in large enough quantities to enable their isolation from 0.1 gram of soil or 1 milliliter of water. The plates were prepared in the manner previously described, incubated at 28° C., and examined after five to seven days for greenish-yellow colonies.

The enrichment culture method is based on the supposition that at least one or more Az. vinelandii cells are present in a definite amount of soil used, which was from 5 to 10 grams. The soil is added to a flask containing 40 to 80 milliliters of sterile modified Wenzl's liquid medium and incubated at 28° C. from five to seven days. As a source of energy calcium benzoate or sodium benzoate was used in place of sucrose, and the pH was adjusted to approximately 7.6 before sterilization. If sodium benzoate is used, the treated soil sample should not be incubated for more than five days because of the highly alkaline condition that develops after that time. After the incubation period, agar plates are prepared in the usual manner and finally examined for the greenish-yellow colonies. By this treatment Az. vinelandii, if present in the soil, is stimulated in its development, thus making its isolation possible.

If the number of Az. vinelandii cells in the soil is less than one organism per ten grams of soil, then a larger quantity of soil must be used, and for that purpose the soil enrichment method is employed. The method, in principle, is the same as described in Part 1 for stimulating the development of Az. vinelandii in the soil by the addition of various chemicals. In this case, however, soil of unknown Az. vinelandii content

was used and as a source of carbon one or more of the following compounds was added: Glucose, calcium butyrate, cinnamic acid, and benzoate compounds. The treatments were prepared in duplicate, incubated for a period of six weeks or more, and examined periodically for Az. vinelandii in the usual manner by the agar-plate method.

All soil samples were collected in sterile cans. The first several inches of the surface soil were removed with a trowel and the next four to eight inches were sampled. Where possible, several samples were taken from the same field. In the laboratory the soils were mixed in the can with a sterile spatula and the pH and Azotobacter population were determined in the usual manner. After that the soils were subjected to one, two, or all three of the afore-mentioned methods to determine the presence of Az. vinelandii.

The water samples were collected in sterile flasks from lakes, irrigation ditches, and rivers. In the laboratory the pH, and in some cases the Azotobacter population were determined, the latter being ascertained by the agar-plate method.

Following the preliminary determinations, all water samples were treated in duplicate by the addition of the following media:

a. Wenzl's modified with 2 percent sucrose as a source of carbon.

b. Wenzl's modified with 0.05 percent sodium benzoate as source of carbon.

c. Winogradsky's medium with 0.5 percent ethyl alcohol as a source of carbon.

One hundred millileters of the water sample were added to 250 ml. flasks, each flask containing one of the afore-mentioned media in concentrated form. In this manner culture media were obtained whose final dilution was approximately the same as prescribed by formula. Ethyl alcohol, however, was sterilized separately and was added to the medium after the water sample. The treatments were then incubated for from five to ten days and examined for Az. vinelandii by the agar-plate method, using the same source of carbon as was used in the original treatment.

The identification of the isolated strains was performed with the aid of the method recommended by Winogradsky (49), which is based on the fact that Az. vinelandii forms cysts while Az. agilis does not. Az. vinelandii was distinguished from Az. chroococcum on the basis of pigmentation, the former developing a greenish-yellow pigment and the latter a yellowish-brown to dark-brown pigment. No attempt was made to distinguish Az. chroococcum from Az. beijernickii.

To establish the identity of the organisms suspected of being Az. vinelandii, the cultures were grown in Winogradsky's liquid medium in tubes and on agar

slants of the same composition. Periodic examinations for cyst formation were made over a period of eighteen days. Six different media were prepared using the following compounds as sources of energy: 0.5 percent ethyl alcohol, 0.3 n. butyl alcohol, 0.3 percent calcium butyrate, 0.3 percent calcium benzoate, 0.3 percent glucose, and 0.3 percent mannite.

PART 1

Series 1

The purpose of experiments in series 1 was to determine the effect of methyl alcohol, ethyl alcohol, n-propyl alcohol, amyl alcohol, hexyl alcohol, glycerol, and mannite on the distribution of *Az. vinelandii* in the soil. The effect of these compounds on *Az. chroococcum*, other bacteria and Actinomyces, molds, and soil reaction was also recorded. It was felt that although many of the compounds added were not suitable as sources of energy for *Az. vinelandii* in pure culture, in the soil the decomposition of these compounds by autochthonous soil flora might result in the production of substances that would have the desired stimulating effect on *Az. vinelandii*.

The experiments relating to the compounds just mentioned were set up and carried as outlined under Methods and Procedure.

From the figures in Tables 1 and 2 and the summary in Table 14, the effect of the afore-mentioned alcohols on the distribution of Azotobacter is clearly evident. Methyl, ethyl, hexyl, amyl, and n-propyl alcohols were not only non-stimulating to the development of *Az. vinelandii* but actually appeared injurious. In all cases

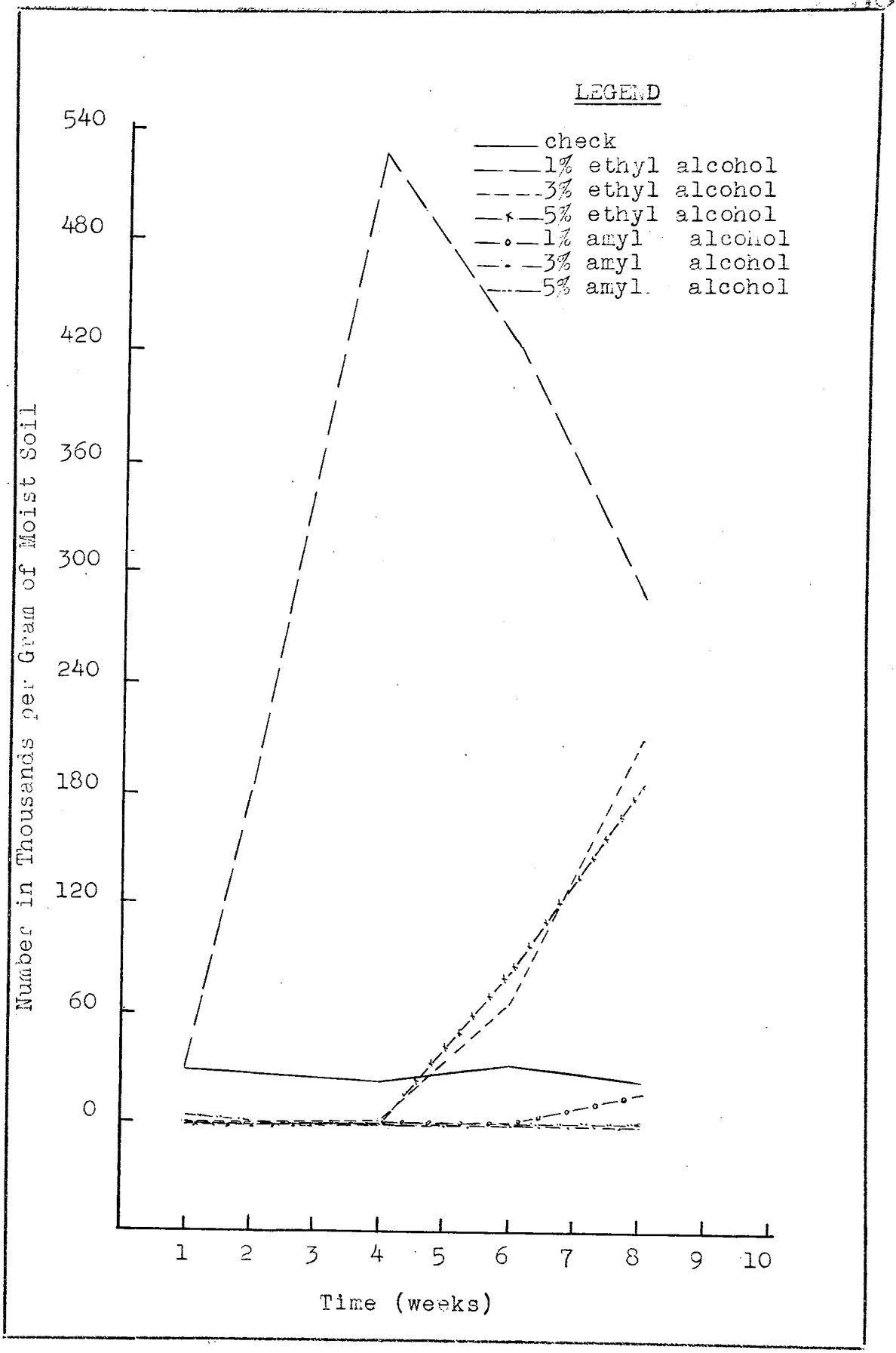

Fig. 1 Variation in Numbers of Molds in Treated Soil Series I

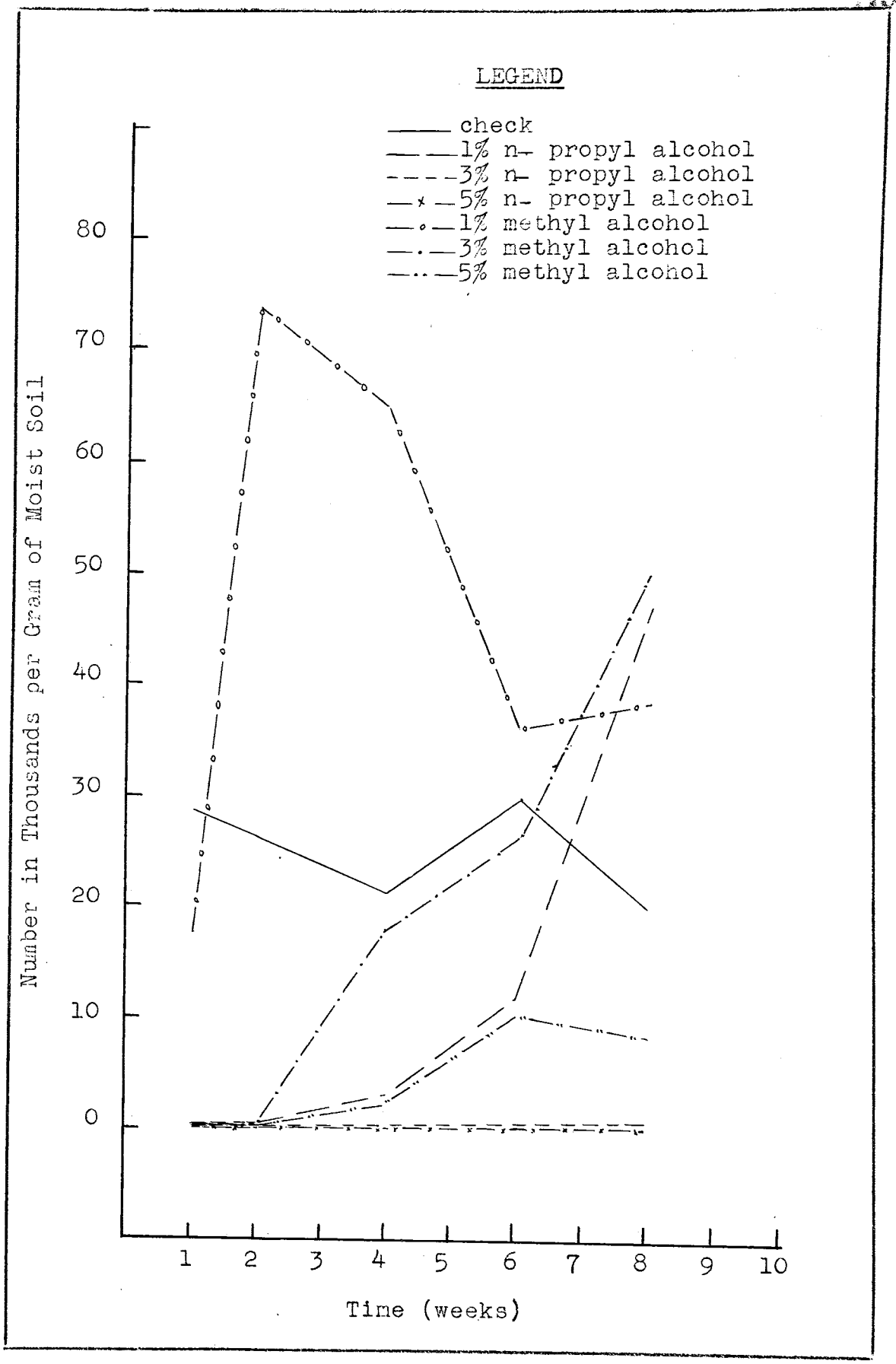

Fig. 2 Variation in Numbers of Molds in Treated Soil Series 1

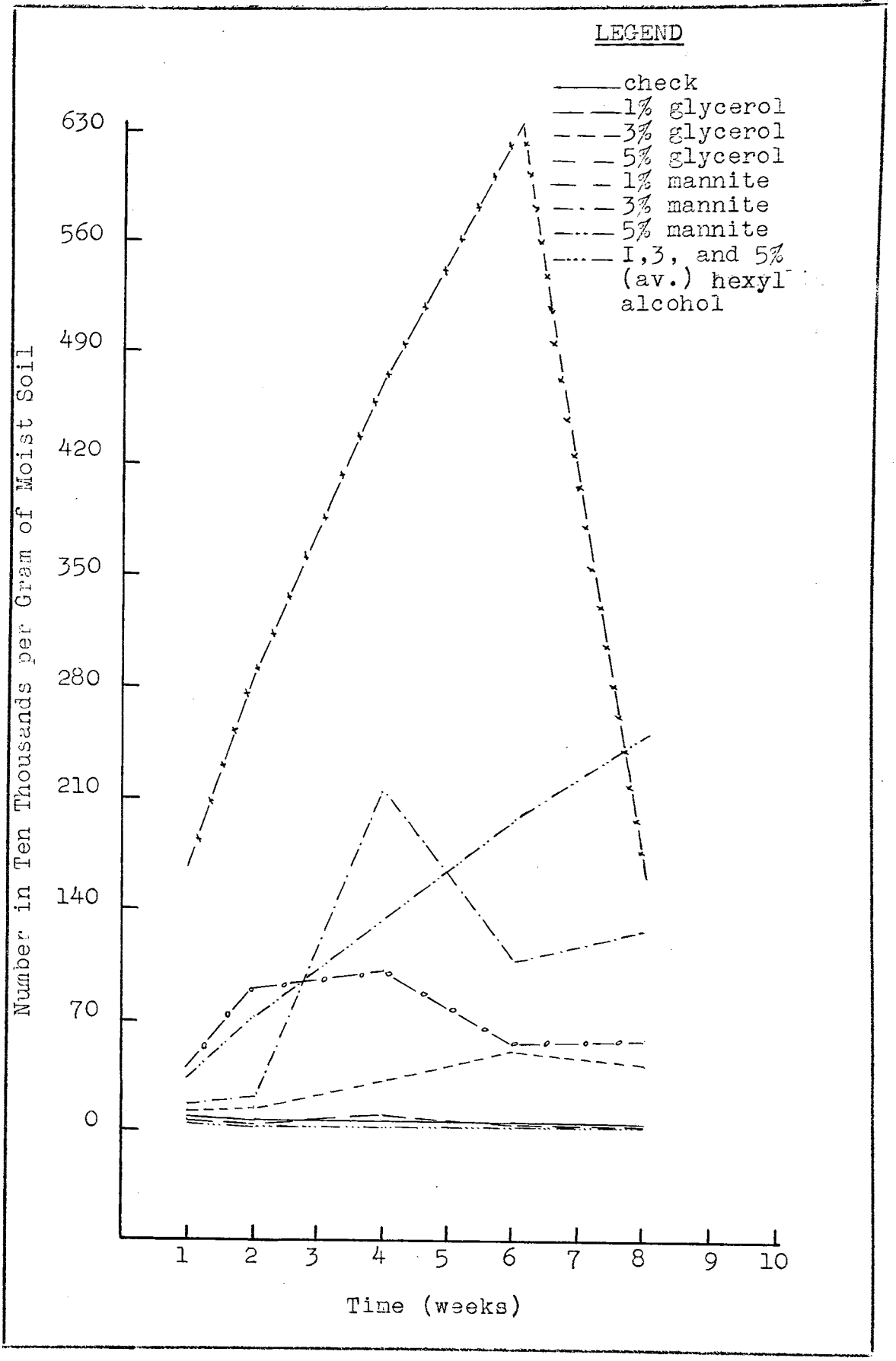

Fig. 3 Variation in Numbers of Molds in Treated Soil. Series 1

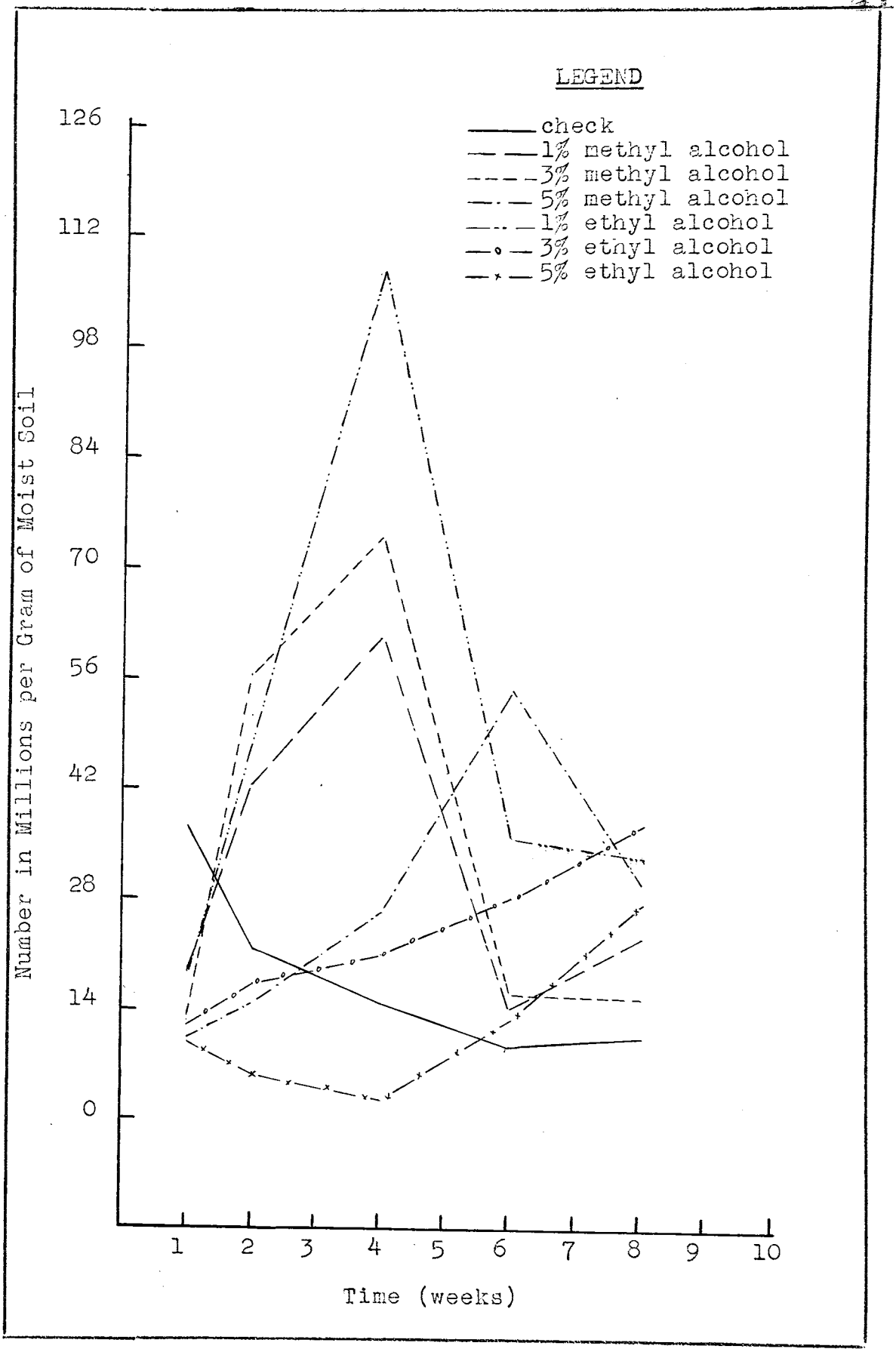

Fig. 4 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 1

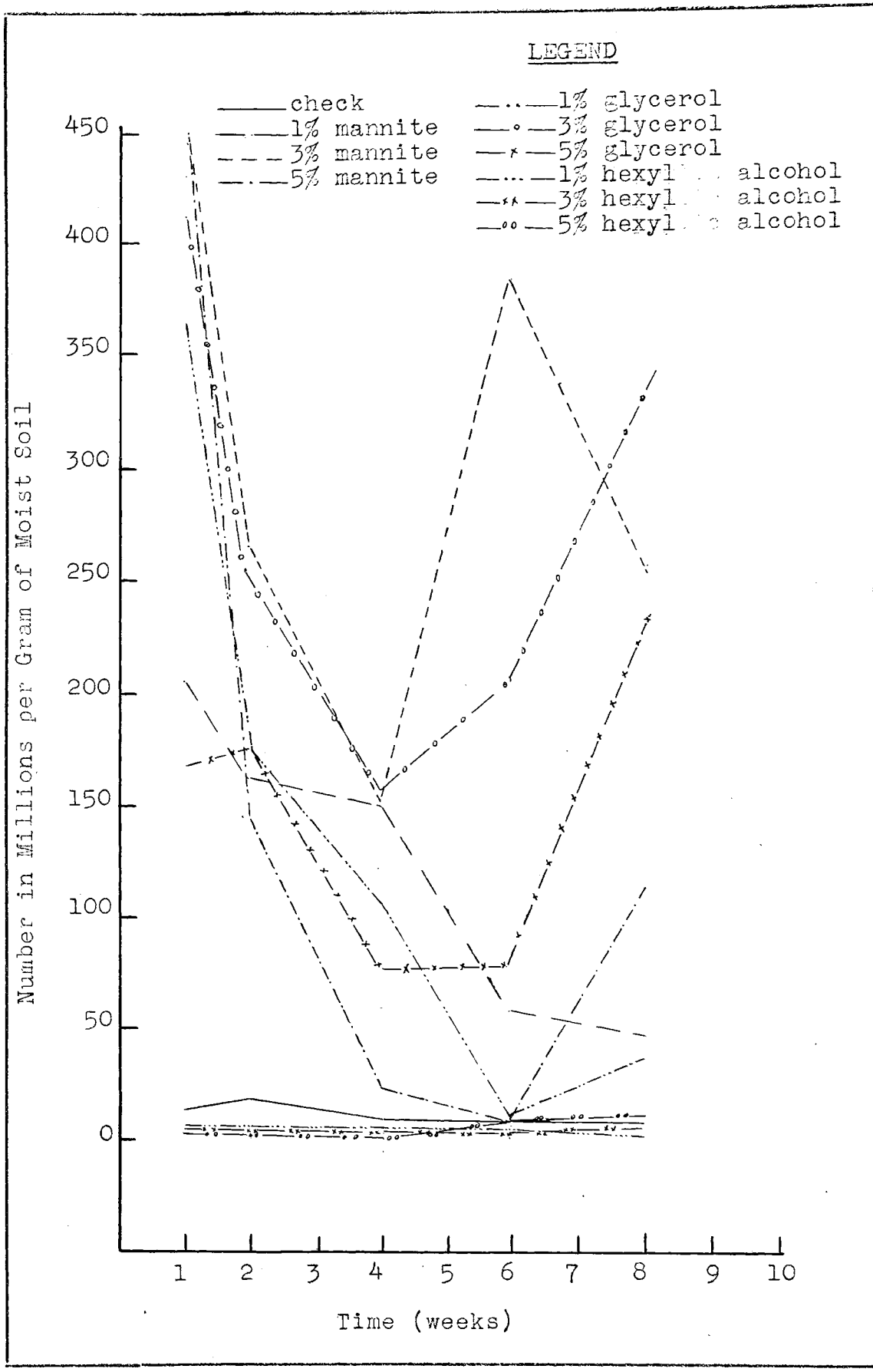

Fig. 5 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 1

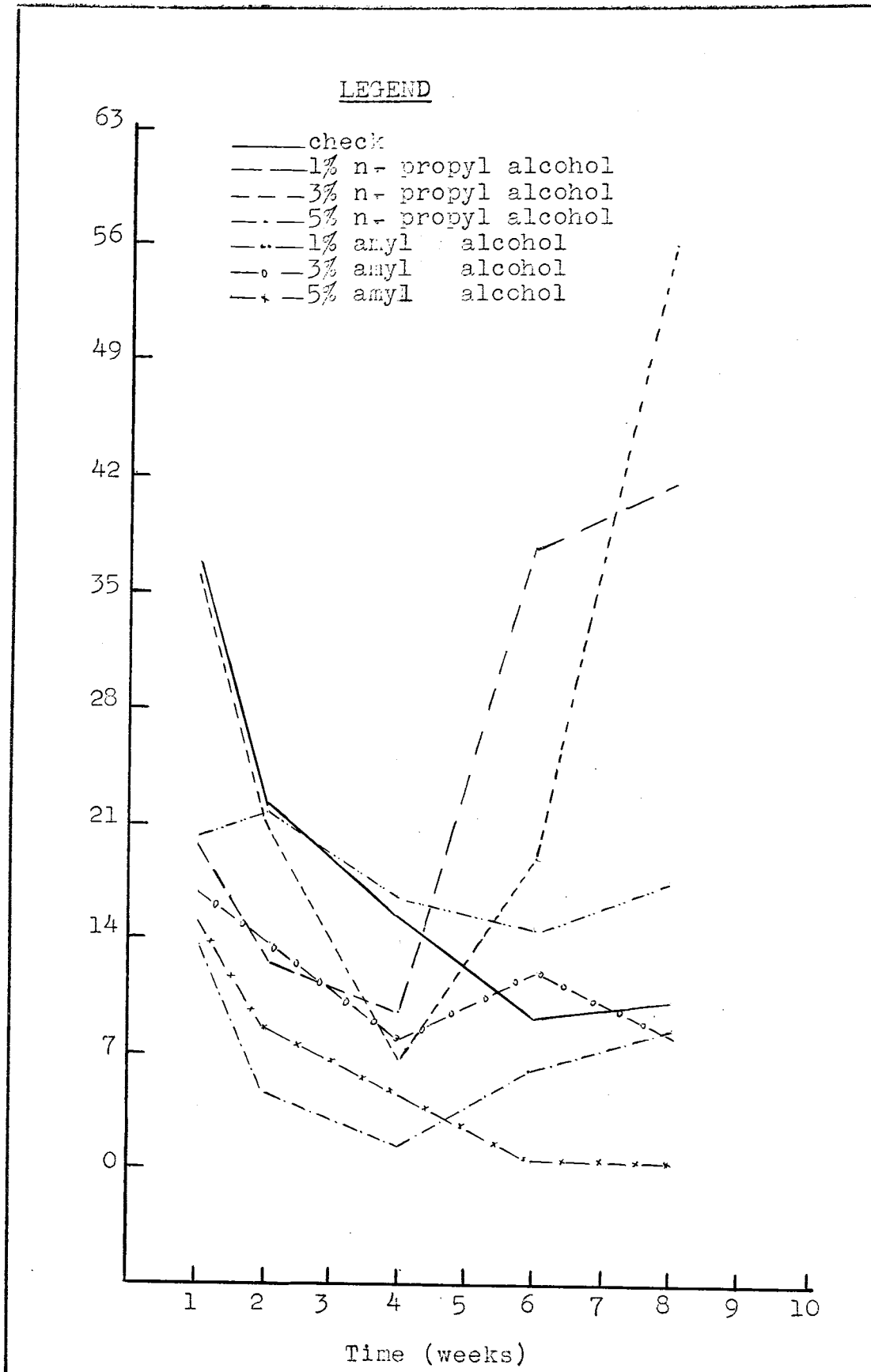

Fig. 6 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 1

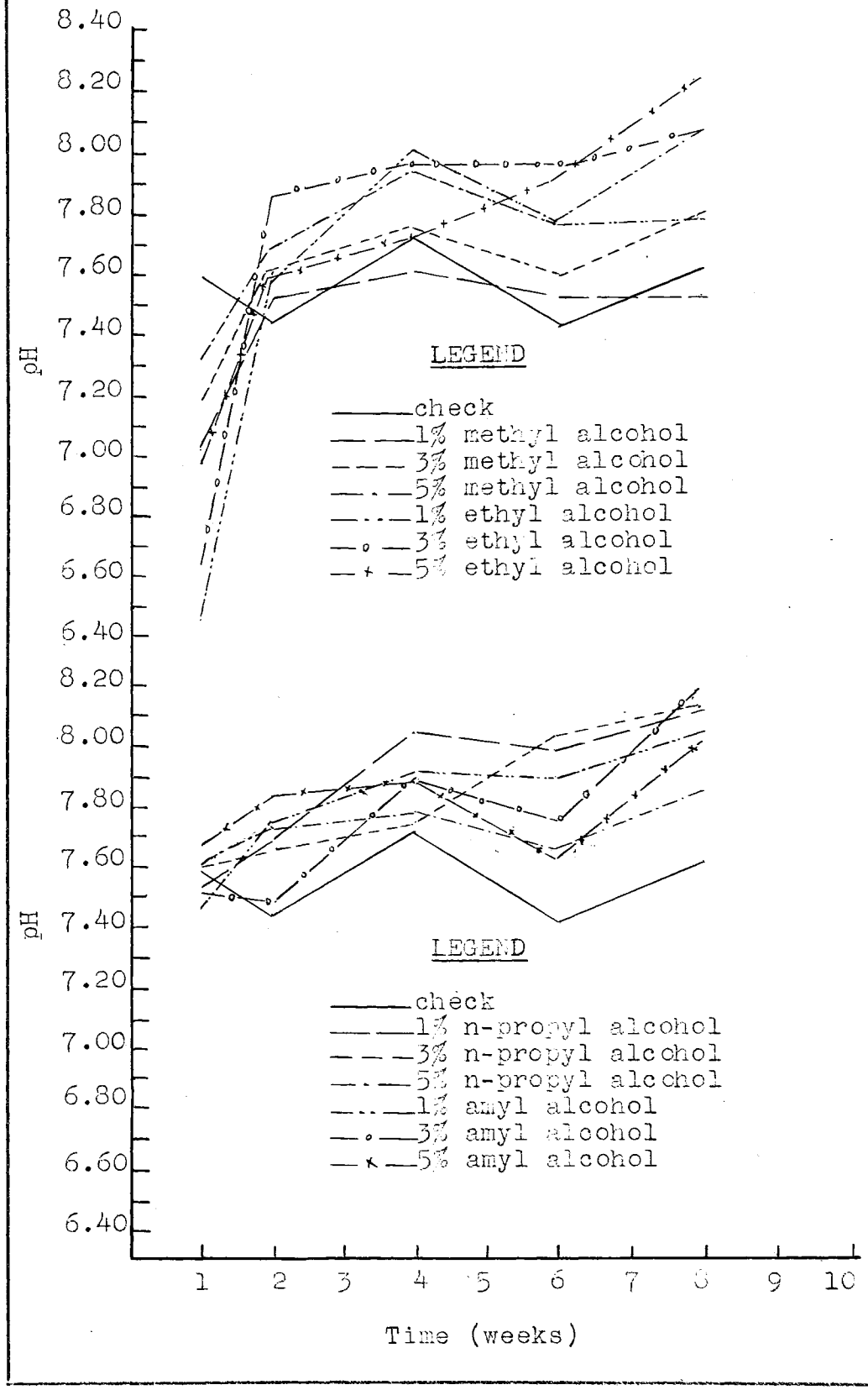

Fig. 7 Variation in pH values in Treated Soils. Series 1

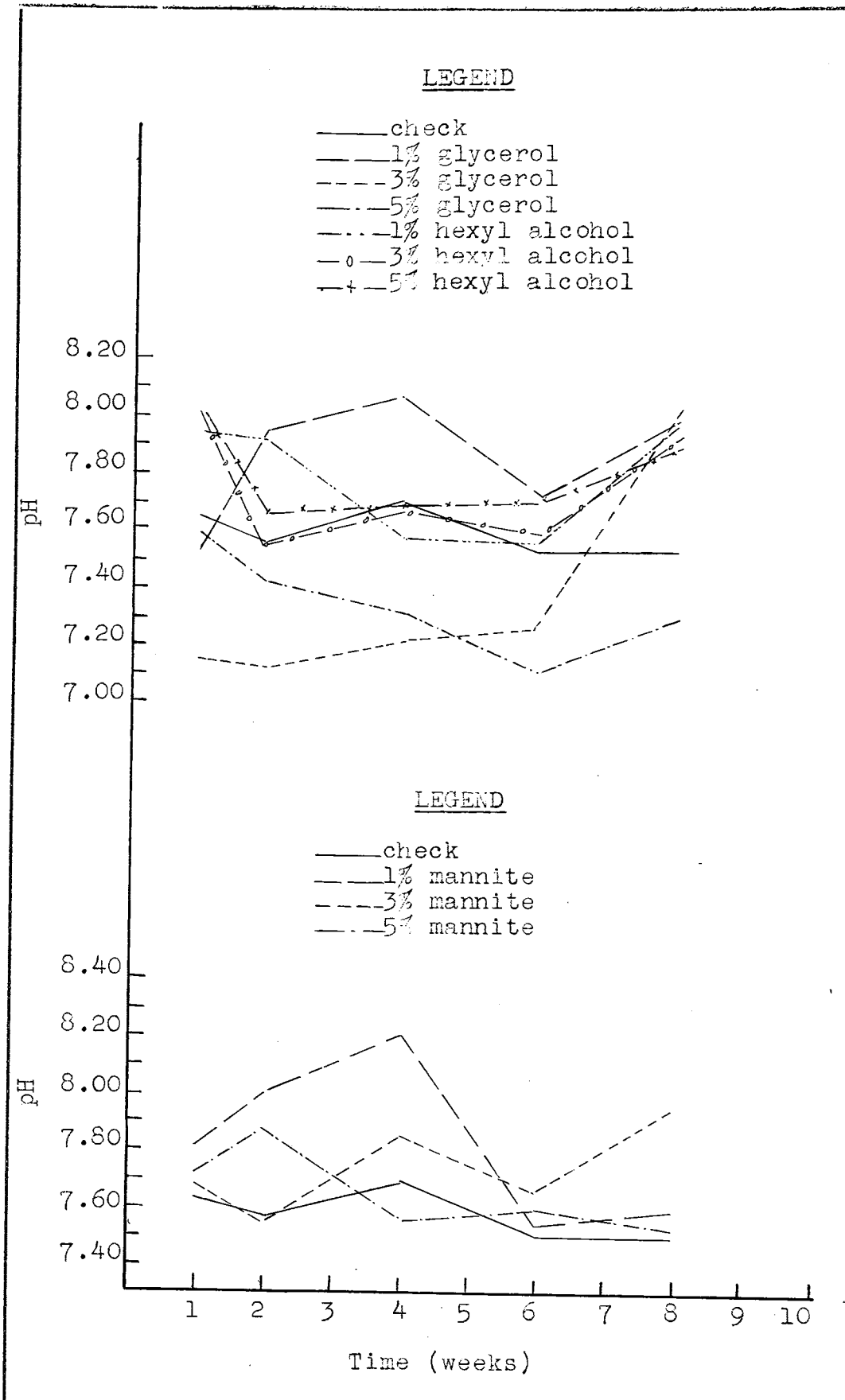

Fig. 8 Variation in pH values in Treated Soils. Series 1

TABLE 1--AZOTOBACTER IN TREATED SOIL. SERIES 1
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8
1.0% mannite	vinelandii	2.0*	4.2*	15.0*	13.0*	1.2*
	chroococcum	21.0*	63.0*	130.0*	116.0*	72.0*
3.0% mannite	vinelandii	4.5*	25.7*	1,700.0*	1,300.0*	2,120.0*
	chroococcum	17.0 *	30.0*	1,900.0*	1,860.0*	1,100.0*
5.0% mannite	vinelandii	13.2*	55.0*	290.0*	100.0*	5,850.0*
	chroococcum	9.0*	22.0*	190.0*	350.0*	1,600.0*
1.0% glycerol	vinelandii	2.0*	3.7*	100.0*	3,100.0*	350.0*
	chroococcum	1.2*	1.3*	120.0*	900.0*	600.0*
3.0% glycerol	vinelandii	100	370	60.0*	2,450.0*	14,000.0*
	chroococcum	100	470	20.0*	1,050.0*	3,500.0*
5.0% glycerol	vinelandii	10	10	10	10	0
	chroococcum	10	10	10	0	0
1.0% methyl alcohol	vinelandii	0	0	0	0	0
	chroococcum	650	2.0*	1.0*	300	350
3.0% methyl alcohol	vinelandii	0	0	0	0	0
	chroococcum	10	10	0	0	0
5.0% methyl alcohol	vinelandii	0	0	0	0	0
	chroococcum	0	0	0	0	0
Check	vinelandii	150	120	200	100	140
	chroococcum	100	520	450	190	170

*Numbers in thousands

TABLE 2--AZOTOBACTER IN TREATED SOIL. SERIES 1
(Average Counts per Gram of Moist Soil)

Treatment		Azotobacter	Time (weeks) 1	2	4	6	8
1.0%	ethyl alcohol	vinelandii	0	0	0	0	0
		chroococcum	420	560	100	100	110
3.0%	ethyl alcohol	vinelandii	0	0	00	0	0
		chroococcum	20	10	0	0	0
5.0%	ethyl alcohol	vinelandii	0	0	0	0	0
		chroococcum	0	0	0	0	0
1.0%	hexyl alcohol	vinelandii	0	0	0	0	0
		chroococcum	0	0	0	0	0
3.0%	hexyl alcohol	vinelandi	0	0	0	0	0
		chroococcum	0	0	0	0	0
5.0%	hexyl alcohol	vinelandii	0	0	0	0	0
		chroococcum	0	0	0	0	0
1.0%	n-propyl alcohol	vinelandii	0	0	0	0	0
		chroococcum	0	0	0	0	0
3.0%	n-propyl alcohol	vinelandii	0	0	0	0	0
		chroococcum	0	0	0	0	0
5.0%	n-propyl alcohol	vinelandii	0	0	0	0	0
		chroococcum	0	0	0	0	0
check		vinelandii	150	120	200	100	140
		chroococcum	100	520	450	190	170

the pH was favorable for Azotobacter activity, as shown in Figures 7 and 8. In none of the mentioned treatments was Az. vinelandii recovered from the soil at any time during the incubation period, while in the untreated soil its presence was clearly evident. Az. chroococcum was present, however, in 1.0 and 3.0 percent methyl and ethyl alcohol treated soils.

Favorable results were obtained with mannite and glycerol treatments. High Az. vinelandii counts were recorded in 3.0 and 5.0 percent mannite treatments and still higher counts with 3.0 percent glycerol. The 5.0 percent glycerol treated soil proved unfavorable for the development of Azotobacter.

Mold counts are presented graphically in Figures 1, 2, and 3. One percent of ethyl alcohol was suitable for mold development, but the 2.0 and 5.0 percent concentrations did not exceed the check until the sixth week (Fig. 1). Amyl and hexyl alcohols showed depressing effect in all concentrations used. Methyl alcohol (1.0 percent), however, was utilized effectively (Fig. 2), while n-propyl alcohol did not exceed the check until the eighth week. High concentrations of these two alcohols depressed the mold growth. Unlike glycerol, which appeared to be a poor source of carbon for molds, mannite greatly stimulated mold growth.

Figures 4, 5, and 6 illustrate the distribution of bacteria and Actinomyces in treated soils. It is

evident from Figure 4 that with methyl alcohol the 3.0 percent treatment presented the most favorable source of carbon for bacteria and Actinomyces, while with ethyl alcohol the 1.0 percent treatment proved most effective. Hexyl alcohol was depressing in all concentrations used, and amyl alcohol appeared to be of little importance.

Figures 7 and 8 show that in all cases the soil reaction was above pH 6.0, the limiting pH value for Azotobacter development.

Series 2

The experiments in this series were carried out in order to determine the effect of certain carbohydrates on the distribution of Azotobacter in soil. Counts of other bacteria and Actinomyces, and molds, as well as soil reaction, were also determined. The carbohydrates used were: Glucose, galactose, mannose, fructose, raffinose, lactose, inulin, sucrose, starch, dextrin, and maltose. The experiments were set up and carried out as outlined under Methods and Procedure.

Azotobacter counts are presented in Tables 3, 4, and 5 and are summarized in Tables 14 and 15. The results indicate that for Az. vinelandii, in the majority of cases, carbohydrates in quantities of 2.4 percent were more efficient than in quantities of 1.0 percent. Glucose, galactose, and raffinose gave the best results and are stated in the order of decreasing Az. vinelandii

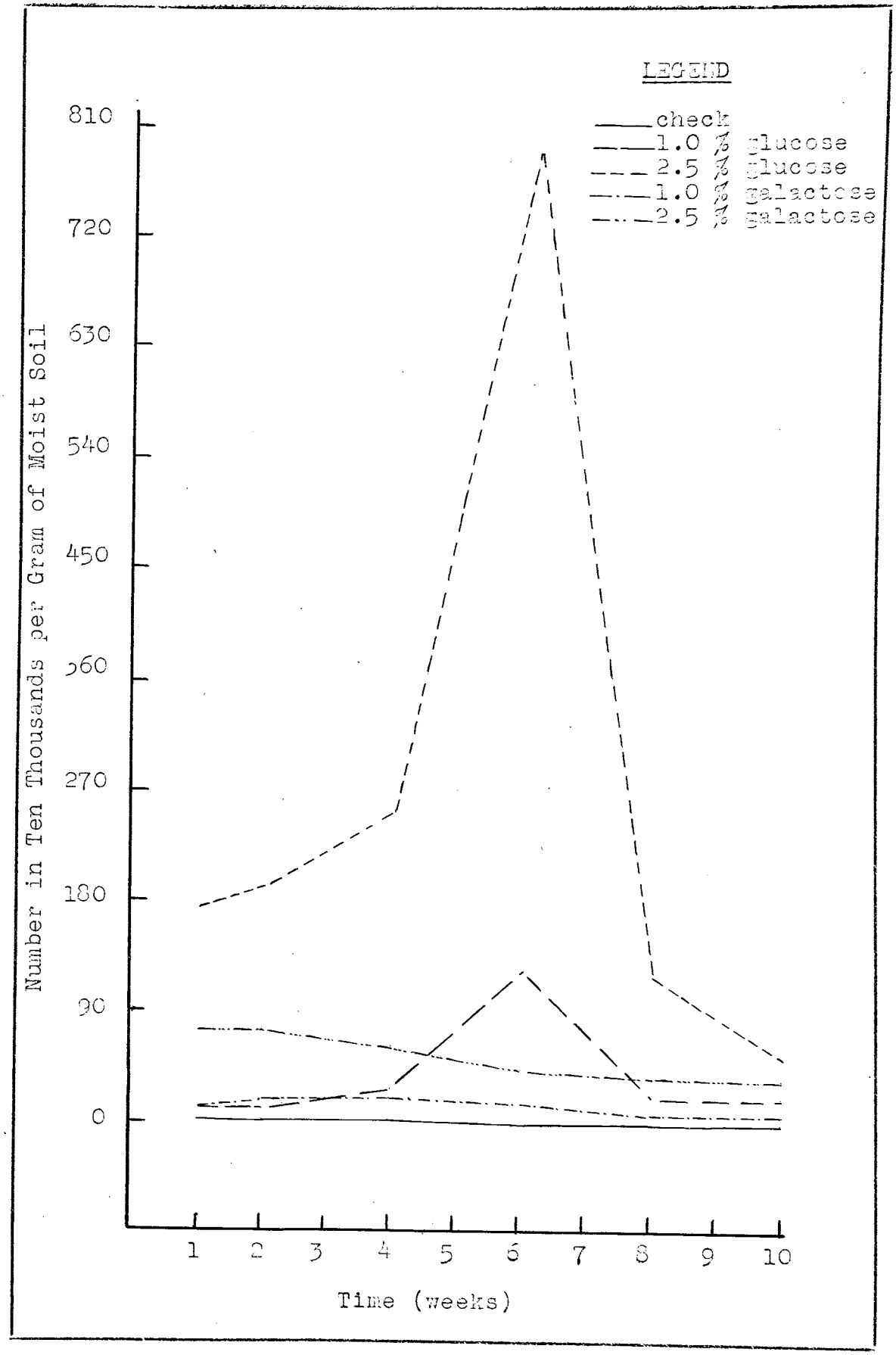

Fig. 9 Variation in Numbers of Molds in Treated Soil. Series 2

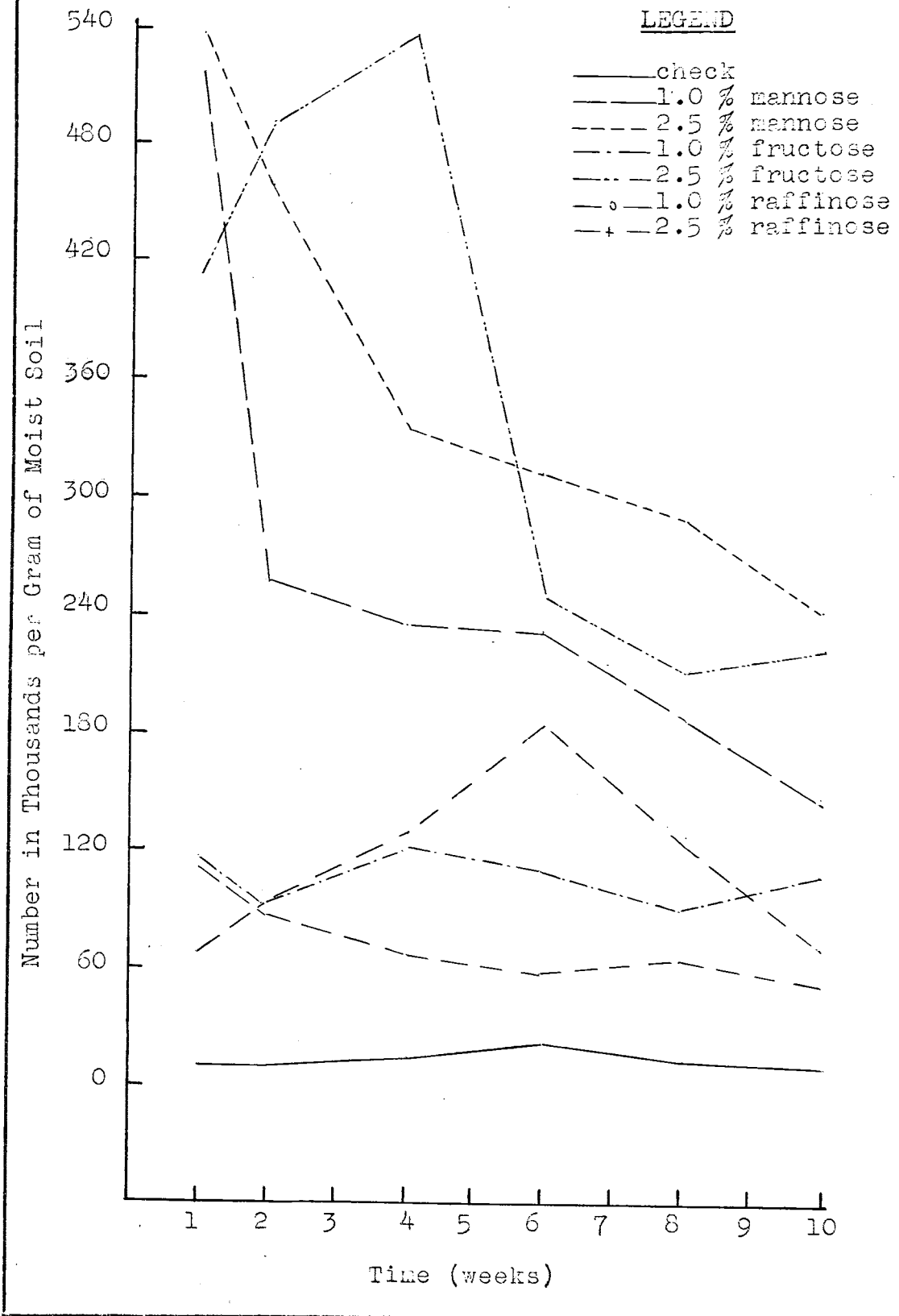

Fig. 10 Variation in Numbers of Molds in Treated Soil Series 2

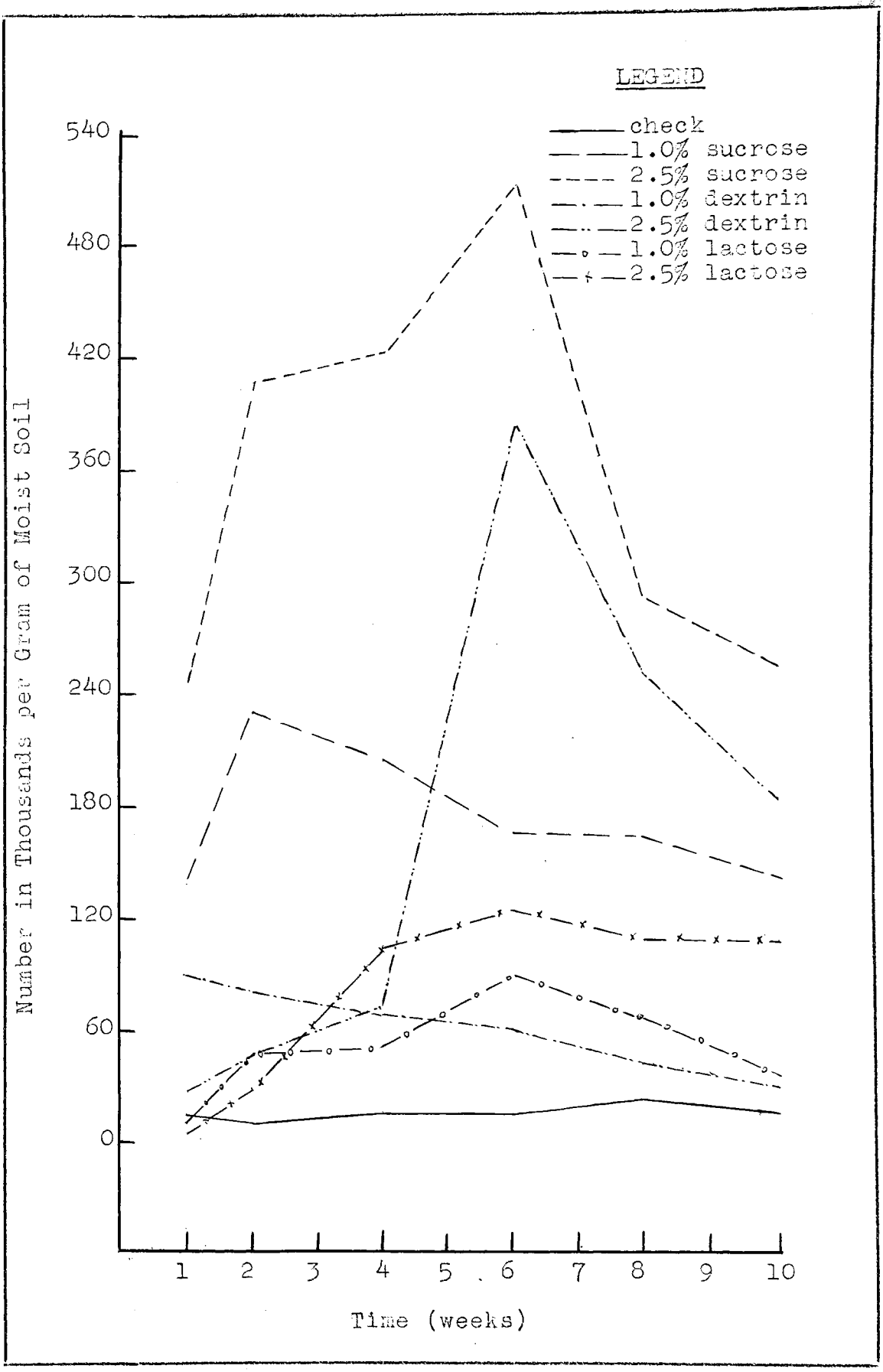

Fig. 11 Variation in Numbers of Molds in Treated Soil. Series 2

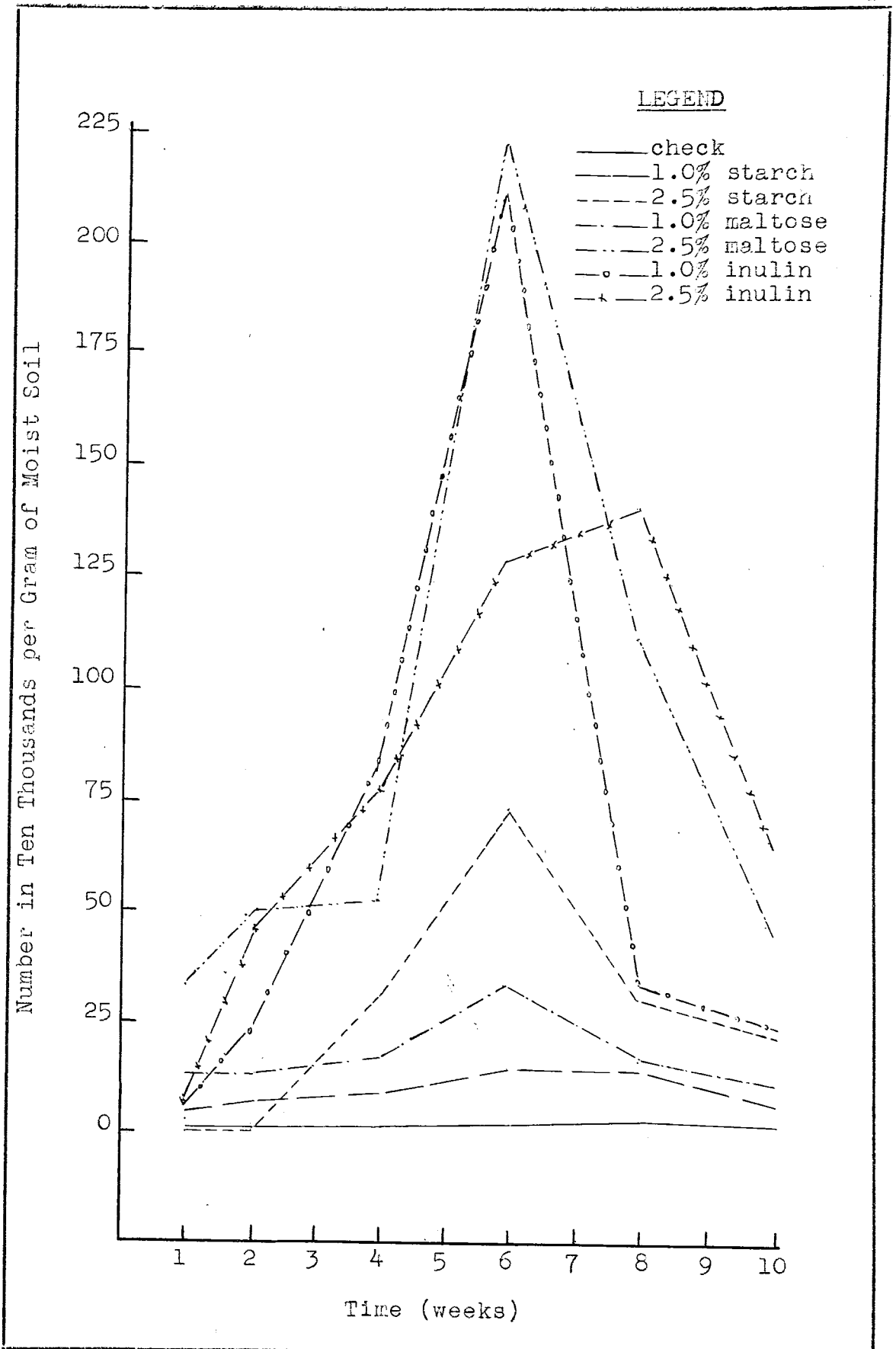

Fig. 12 Variation in Numbers of Molds in Treated Soil. Series 2

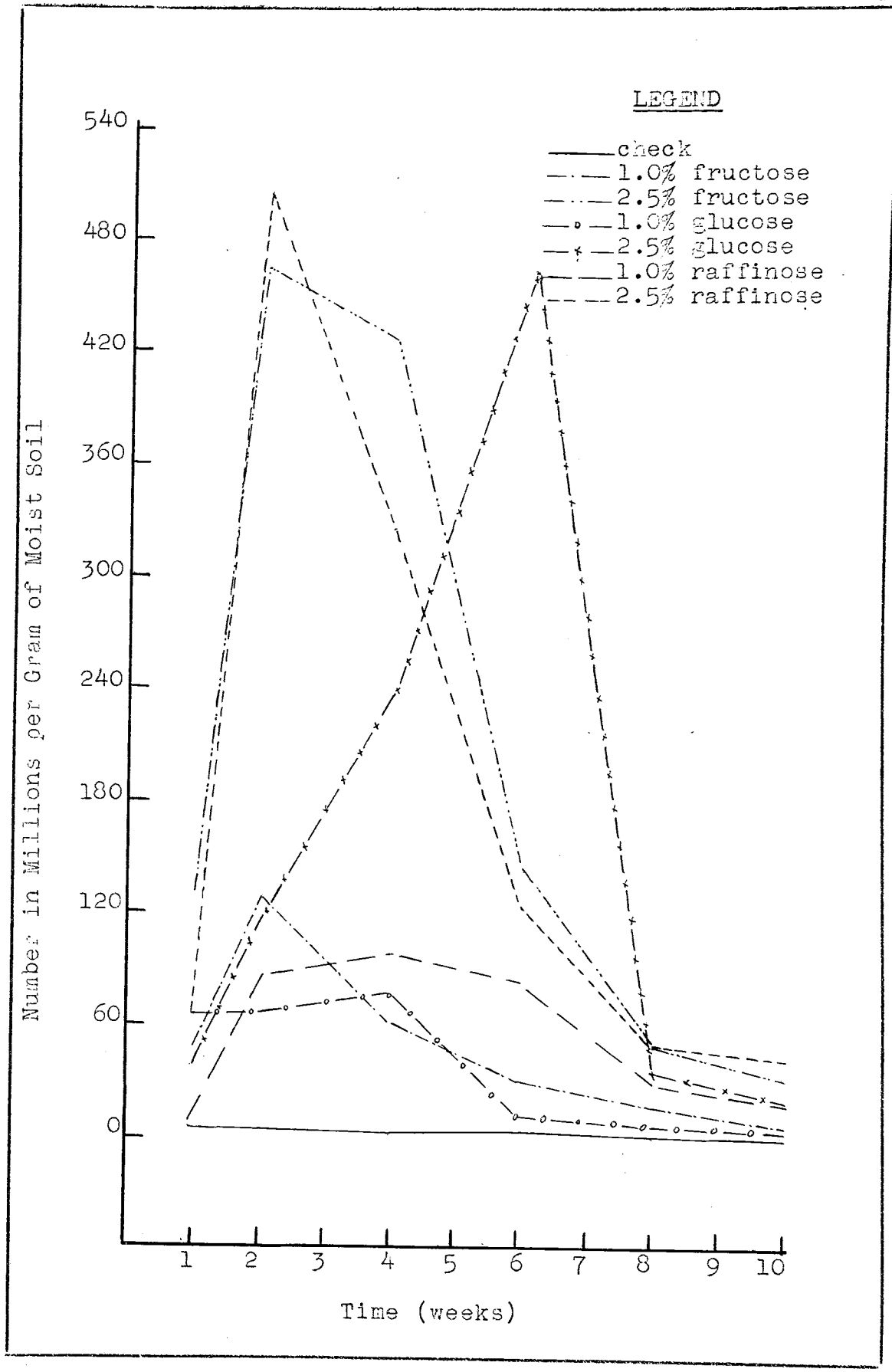

Fig. 13. Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 2

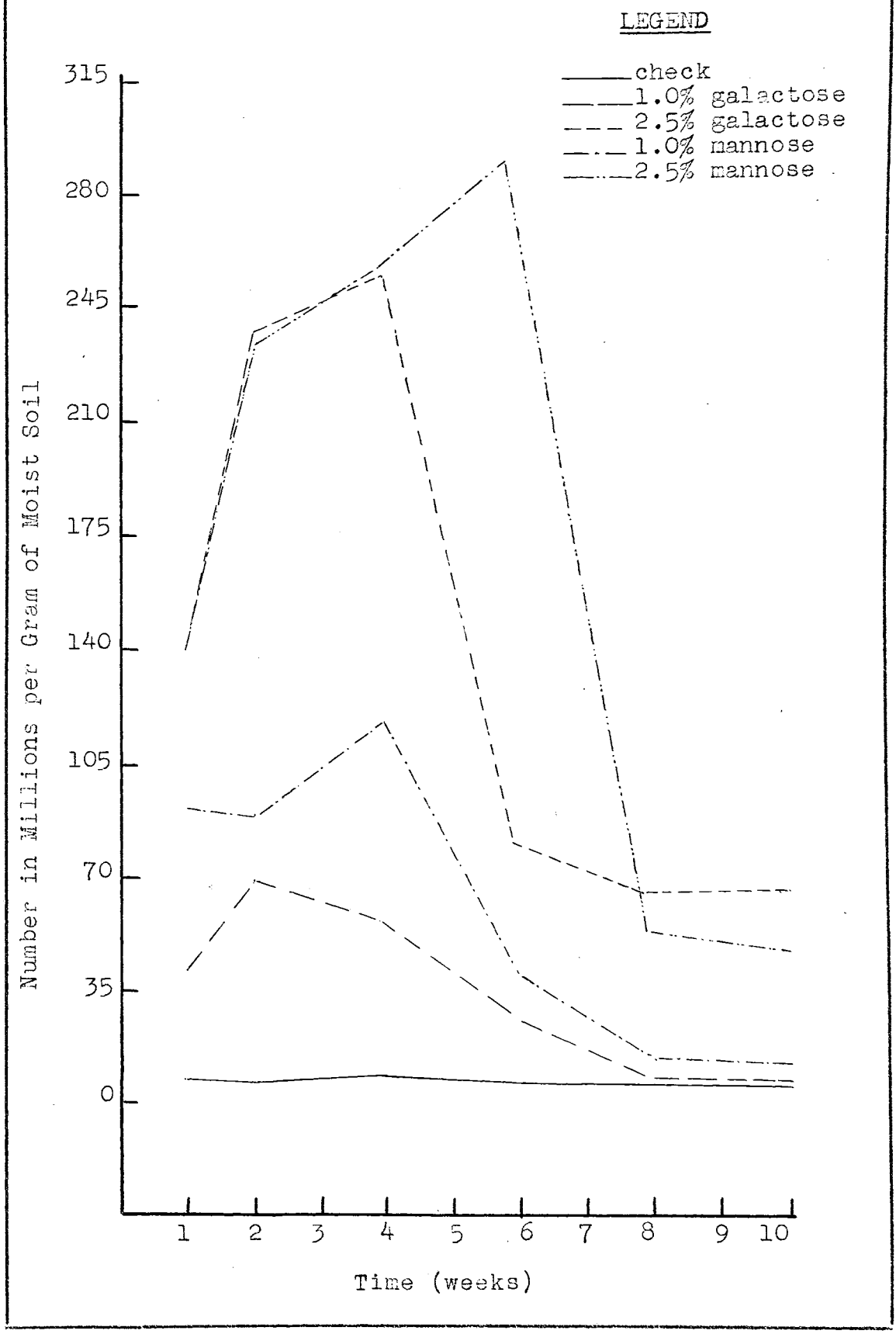

Fig. 14 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 2

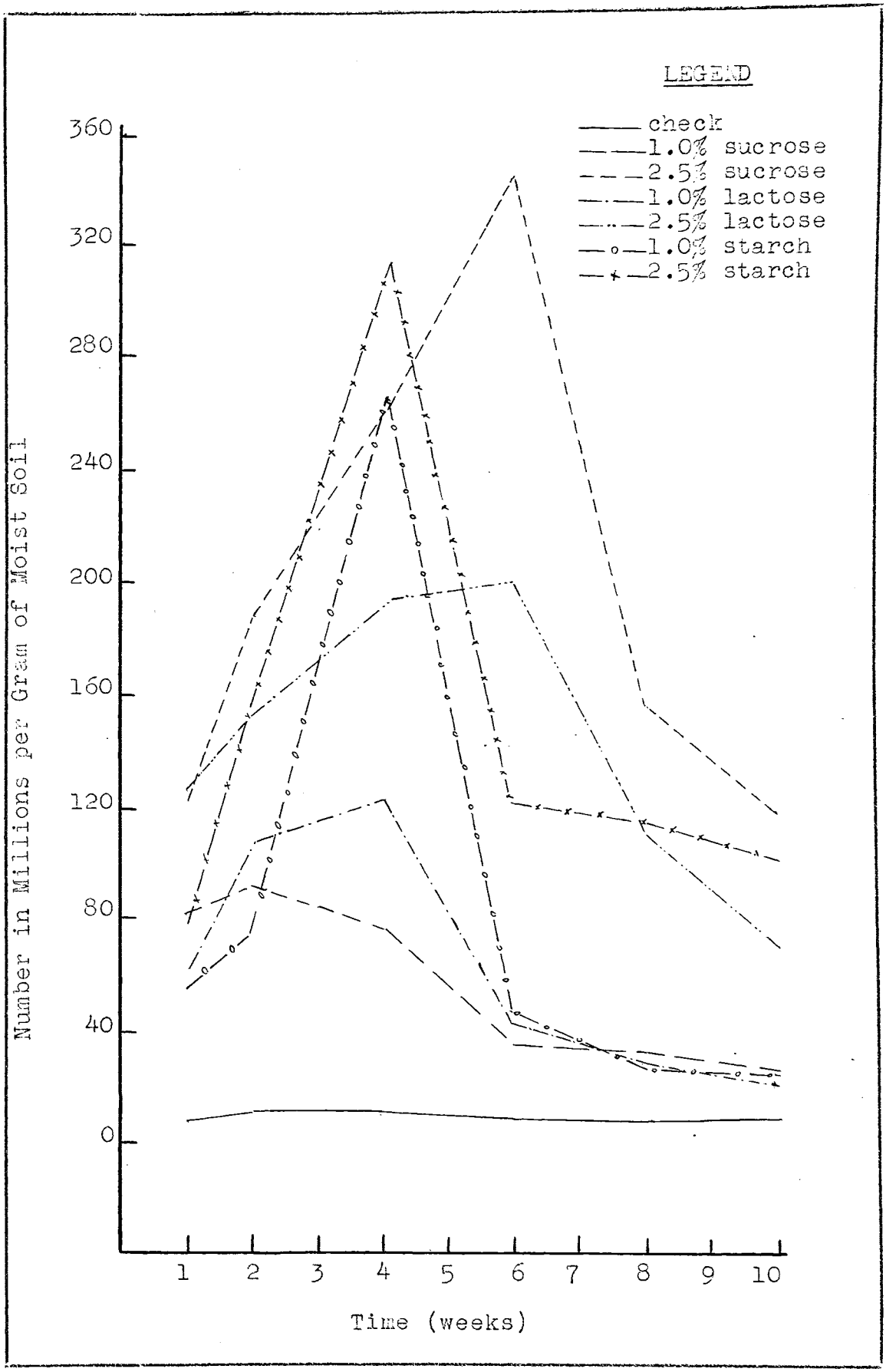

Fig. 15 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 2

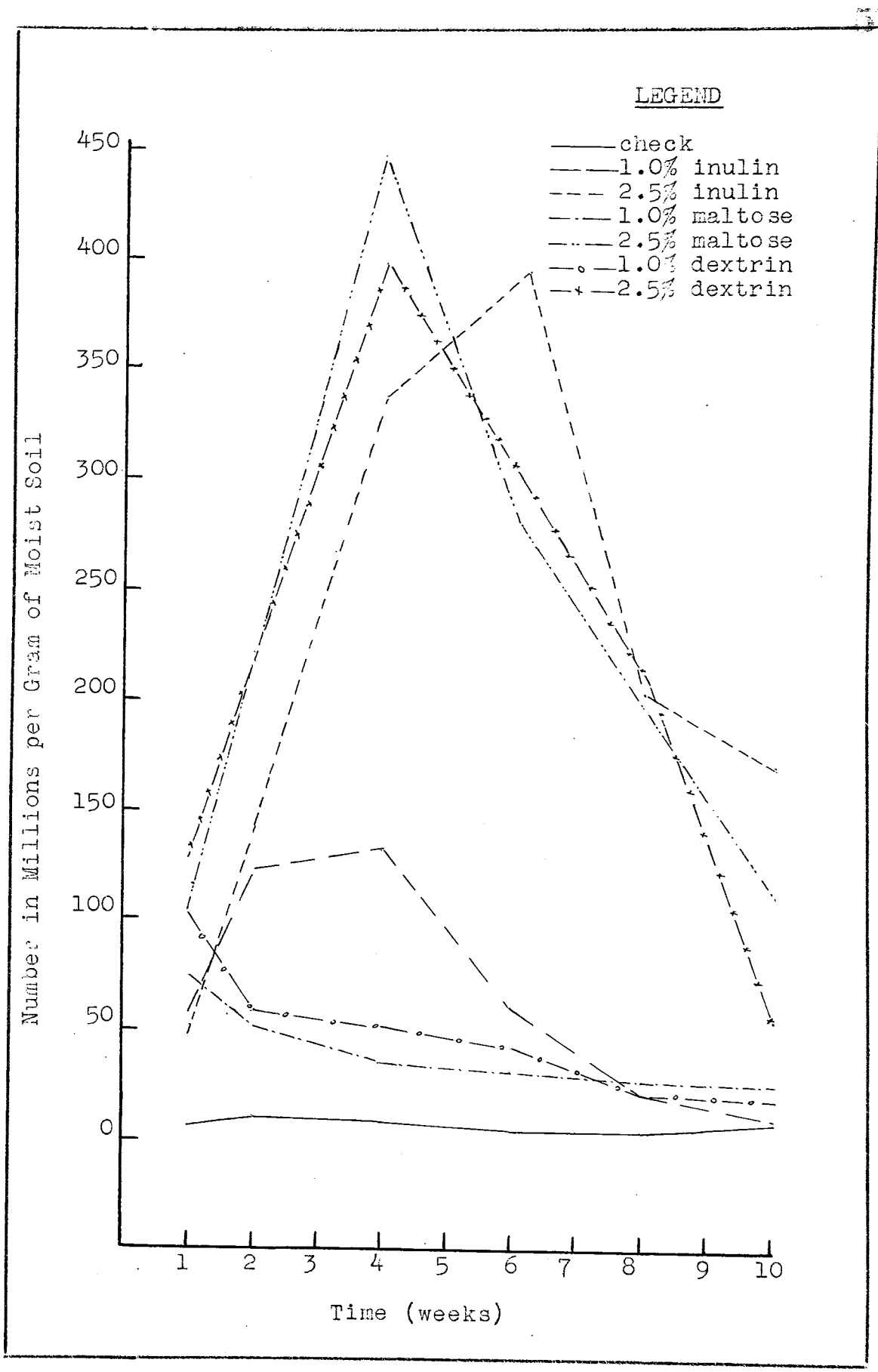

Fig. 16 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 2

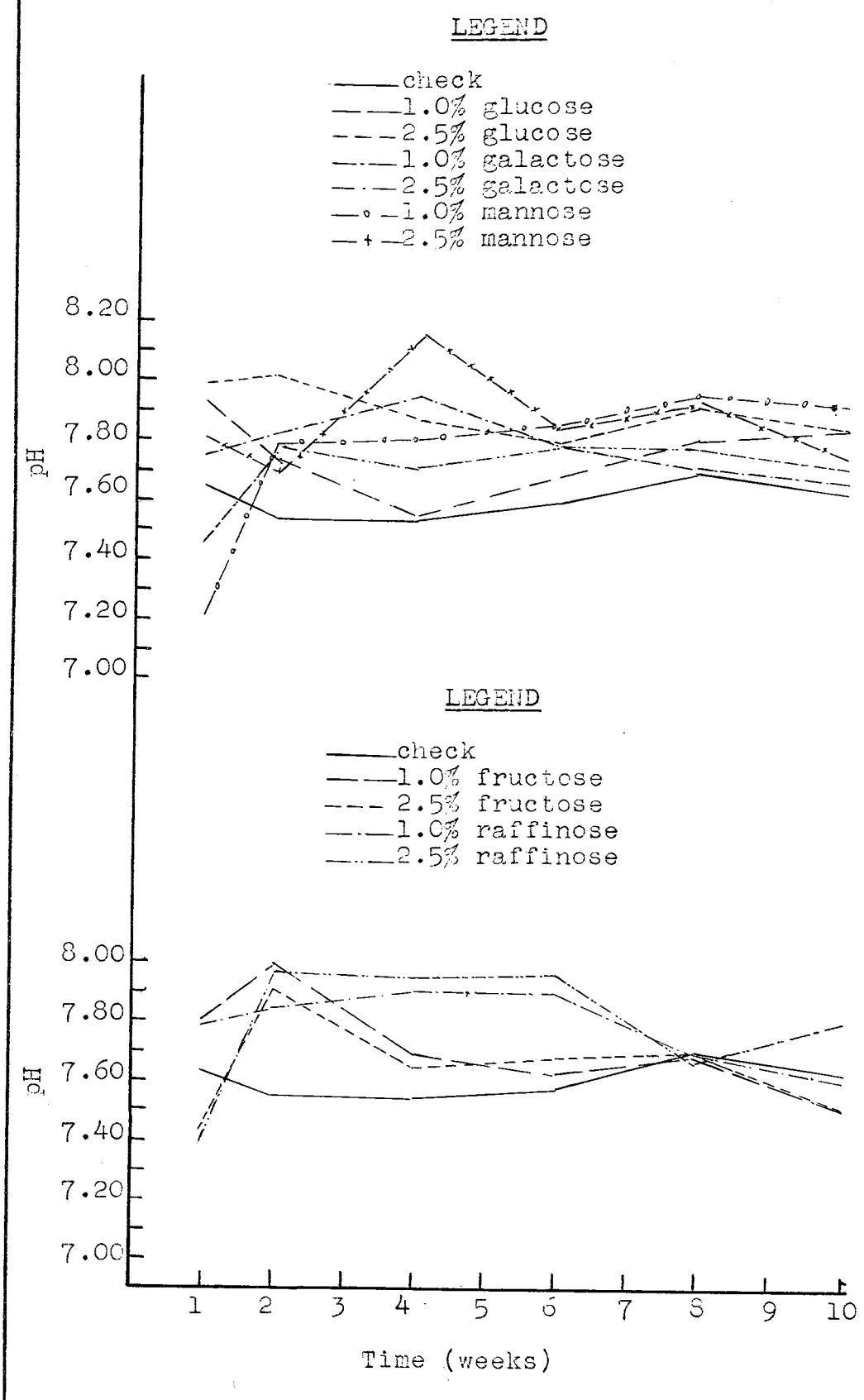

Fig. 17 Variation in pH values in Treated Soils. Series 2

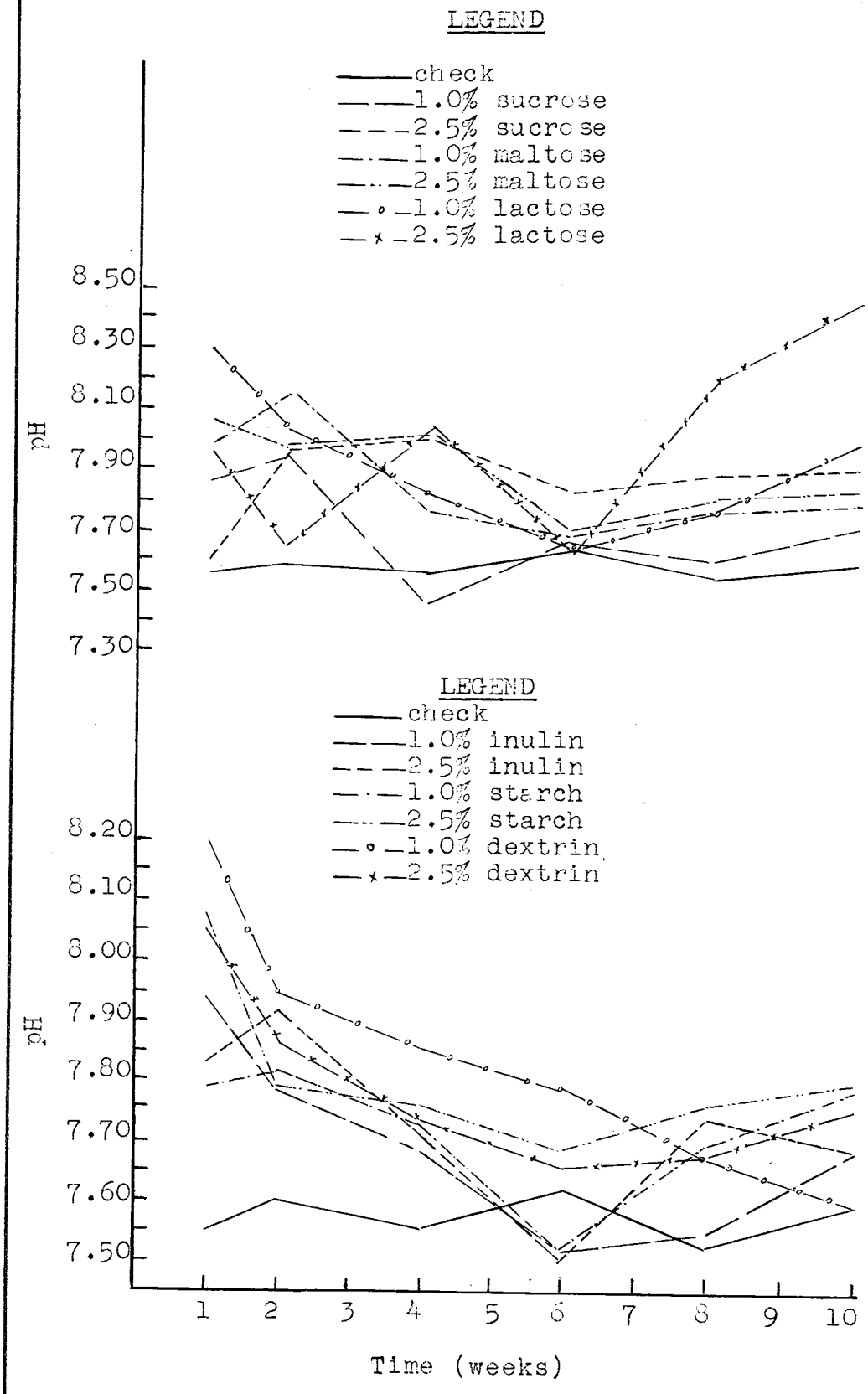

Fig. 18 Variation in pH values in Treated Soils. Series 2

TABLE 3--AZOTOBACTER IN TREATED SOIL. SERIES 2
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% glucose	vinelandii	10.0*	32.0*	73.0*	160.0*	100.0*	100.0*
	chroococcum	720.0*	908.0*	3,650.0*	2,800.0*	2,500.0*	1,500.0*
2.5% glucose	vinelandii	16.3*	1,200.0*	1,800.0*	15,700.0*	1,300.0*	1,400.0*
	chroococcum	870.0*	3,500.0*	6,500.0*	55,000.0*	2,500.0*	2,200.0*
1.0% galactose	vinelandii	10.0*	210.0*	100.0*	160.0*	100.0*	100.0*
	chroococcum	430.0*	620.0*	1,300.0*	1,700.0*	3,700.0*	1,100.0*
2.5% galactose	vinelandii	30.0*	860.0*	9,900.0*	1,200.0*	2,100.0*	2,200.0*
	chroococcum	3,200.0*	4,200.0*	6,900.0*	7,300.0*	6,100.0*	4,300.0*
1.0% mannose	vinelandii	52.0*	31.0*	2.8*	1.1*	100	100
	chroococcum	20.6*	27.1*	8.3*	5.0*	3.9*	2.3*
2.5% mannose	vinelandii	82.0*	280.0*	76.0*	42.0*	27.0*	8.3*
	chroococcum	92.0*	250.0*	680.0*	35.0*	30.0*	27.0*
1.0% fructose	vinelandii	10.0*	1,600.0*	935.0*	620.0*	100.0*	100.0*
	chroococcum	620.0*	1,100.0*	1,900.0*	2,100.0*	3,200.0*	1,200.0*
2.5% fructose	vinelandii	42.0*	68.0*	280.0*	790.0*	1,500.0*	650.0*
" "	chroococcum	920.0*	1,300.0*	2,600.0*	3,100.0*	3,200.0*	3,800.0*
1.0% raffinose	vinelandii	22.0*	86.0*	110.0*	130.0*	10.0*	10.0*
	chroococcum	820.0*	860.0*	1,100.0*	1,400.0*	1,300.0*	1,100.0*
2.5% raffinose	vinelandii	310.0*	2,100.0*	2,950.0*	1,070.0*	1,200.0*	980.0*
	chroococcum	1,200.0*	5,700.0*	6,100.0*	1,300.0*	1,800.0*	1,200.0*
check	vinelandii	1,000	800	100	100	100	100
	chroococcum	2,000	2,200	5,000	4,800	3,700	4,100

* Numbers in thousands

TABLE 4--AZOTOBACTER IN TREATED SOIL SERIES 2
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% lactose	vinelandii	0	0	0	0	0	0
	chroococcum	180	220	60	70	40	50
2.5% lactose	vinelandii	100	100	430	2.0*	870	100
	chroococcum	1.6*	4.3*	8.0*	40.0*	31.0*	26.0*
1.0% inulin	vinelandii	100	100	110	320	0	0
	chroococcum	100	100	800	1.1*	230	260
2.5% inulin	vinelandii	6.2*	14.0*	25.0*	1.8*	100	100
	chroococcum	33.0*	65.0*	180.0*	62.0*	36.0*	32.0*
1.0% sucrose	vinelandii	12.0*	1.0*	1.0*	1.0*	100	100
	chroococcum	8.2*	12.0*	17.7*	20.0*	5.0*	4.0*
2.5% sucrose	vinelandii	52.0*	81.0*	151.0*	340.0*	208.0*	180.0*
	chroococcum	140.0*	760.0*	820.0*	260.0*	160.0*	142.0*
1.0% maltose	vinelandii	7.3*	1.1*	670	820	730	500
	chroococcum	24.0*	13.0*	8.0*	6.2*	3.4*	3.0*
2.5% maltose	vinelandii	185.0*	220.0*	290.0*	420.0*	260.0*	180.0*
	chroococcum	110.0*	520.0*	730.0*	310.0*	240.0*	160.0*
1.0% dextrin	vinelandii	1.0*	1.0*	1.0*	100	100	100
	chroococcum	20.0*	32.0*	22.0*	5.0*	2.8*	3.0*
2.5% dextrin	vinelandii	10.0*	60.0*	21.0*	6.7*	2.9*	1.5*
	chroococcum	10.0*	71.0*	27.0*	18.0*	12.2*	16.0*
check	vinelandii	820	530	730	360	100	100
	chroococcum	1.6*	2.0*	980	1.6*	1.2*	1.9*

* Numbers in thousands

TABLE 5--AZOTOBACTER IN TREATED SOIL SERIES 1
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8
1.0% amyl alcohol	vinelandii	0	0	0	0	0
	chroococcum	0	0	0	0	0
3.0% amyl alcohol	vinelandii	0	0	0	0	0
	chroococcum	0	0	0	0	0
5.0% amyl alcohol	vinelandii	0	0	0	0	0
check	vinelandii	150	120	200	100	140
	chroococcum	100	520	450	190	170

AZOTOBACTER IN TREATED SOIL SERIES 2
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% starch	vinelandii	1.0*	380	150	100	10	10
	chroococcum	2.0*	450	300	200	130	200
2.5% starch	vinelandii	10	10	10	0	0	0
	chroococcum	1.8*	2.3*	3.6*	7.0*	10.0*	18.0*
check	vinelandii	820	530	730	360	100	100
	chroococcum	1.6*	2.0*	980	1.6*	1.2*	1.9*

* Numbers in thousands

counts. Dextrose and starch were poor stimulants, while the remaining carbohydrates fell in the intermediate class. However, in almost every case, Az. chroococcum counts were higher than those of Az. vinelandii. This indicates that carbohydrates like glucose, galactose, and raffinose were just as good, if not better, sources of carbon for Az. chroococcum as for Az. vinelandii.

Mold counts are presented graphically in Figures 9, 10, 11, and 12. Of all the carbohydrates used, glucose and galactose gave the greatest increase in mold counts. From Figure 9 it is evident that the 1.0 and 2.5 percent curves of the two sugars are very similar. Only in a few instances did the mold counts in untreated soils exceed those of treated soils. As a rule, greater increase in molds was noted in 2.5 percent treatments than in 1.0 percent treatments. In case of inulin, however, a slightly greater increase was noted in 1.0 percent than in 2.5 percent treatments.

The effect of the carbohydrates studied on the distribution of bacteria and Actinomyces is shown graphically in Figures 13, 14, 15, and 16. It is evident from these graphs that 2.5 percent treatments were more favorable for the development of these organisms than 1.0 percent treatments. Also the treated soils gave larger counts than the untreated soils.

Changes in soil reaction during the entire incubation period are given in Figures 17 and 18. It is seen that all pH fluctuations were within the limits permitting favorable Azotobacter development.

Series 3

In this series the effect of quinol, benzene, toluene, phenol, resorcinol, pyrogallol, phloglucinol, and thymol upon the distribution of Azotobacter in the soil was investigated. Routine determinations for other bacteria and Actinomyces, molds, and soil reaction were likewise made. The experiments were set up and carried out as outlined previously.

Tables 6, 7, and 15 illustrate the distribution of Azotobacter in treated and untreated soils. It is evident that the chemicals, in the concentrations used, were not favorable for the development of Az. vinelandii and Az. chroococcum. That this inhibiting effect was due entirely to the chemicals themselves and not to the soil reaction brought about by these chemicals is seen from Figures 24 and 25. The pH values in all cases but one, 2.5 pyrogallol, were above 6.0 and therefore were favorable for Azotobacter development. The isolation of Azotobacter from untreated soils met with no difficulty, as is shown in Tables 6 and 7.

Phenol, thymol, benzene, and quinol depressed mold development during the entire incubation period

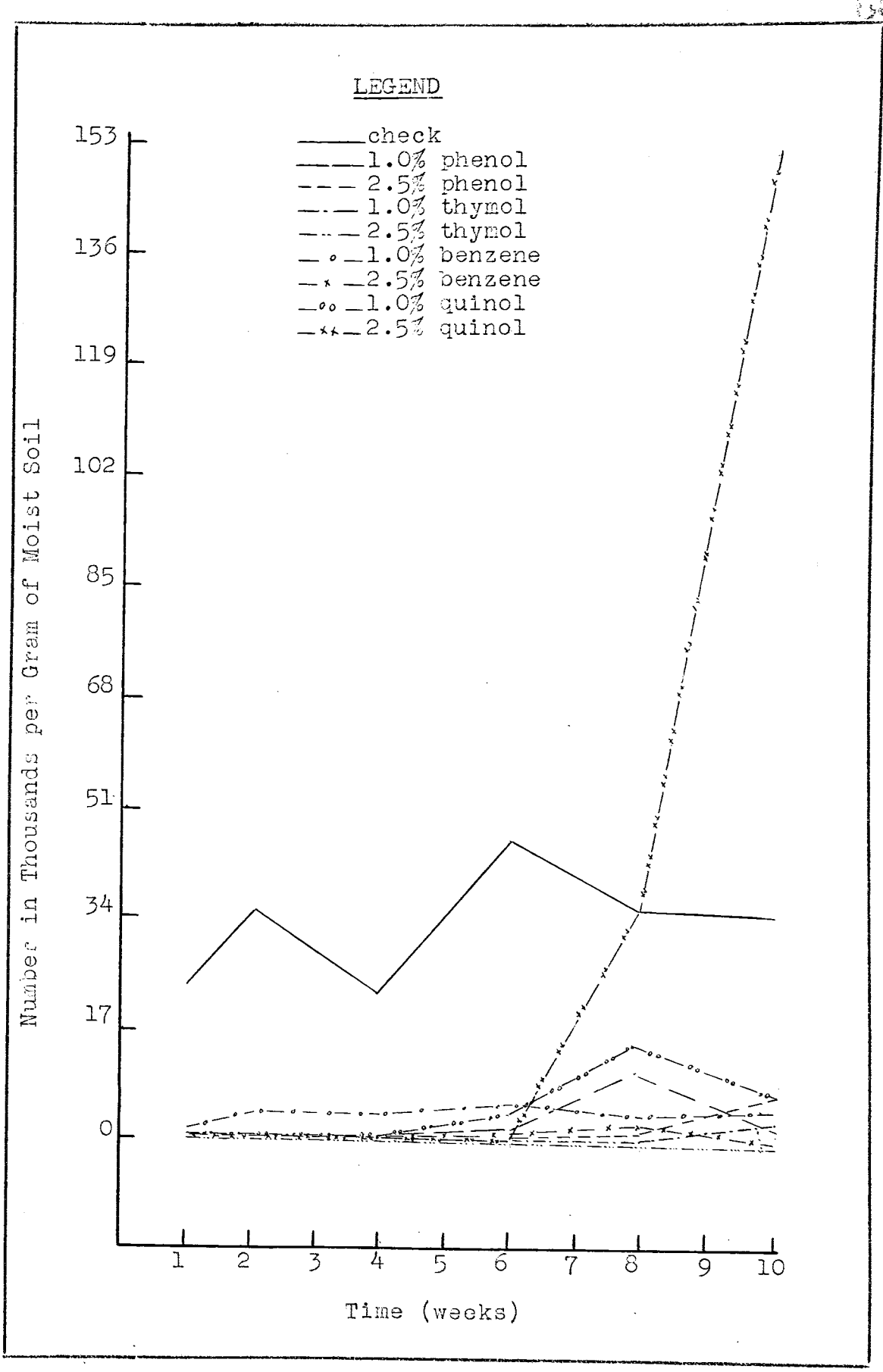

Fig. 19 Variation in Numbers of Molds in Treated Soil. Series 3

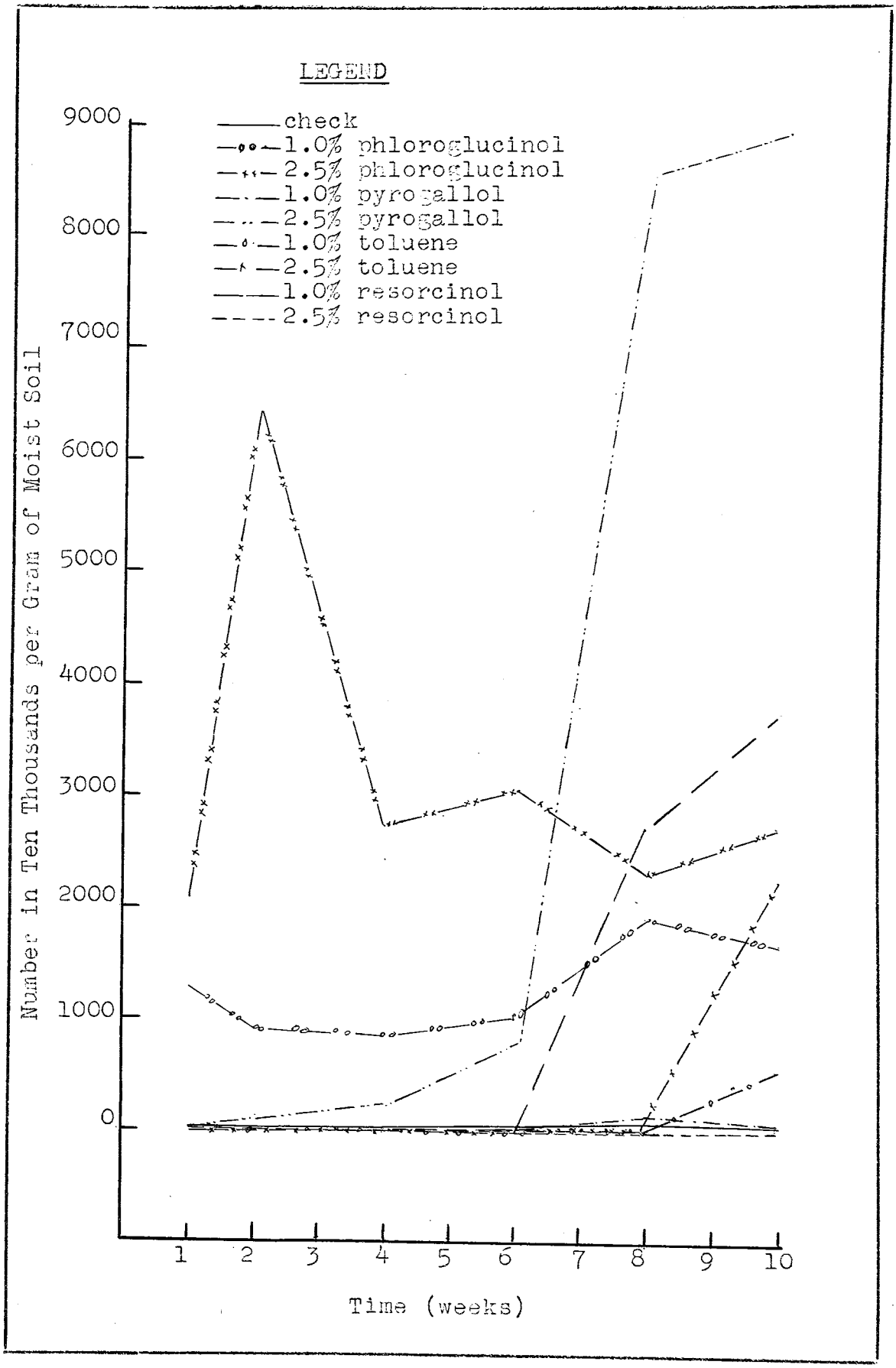

Fig. 20 Variation in Numbers of Molds in Treated Soil Series 3

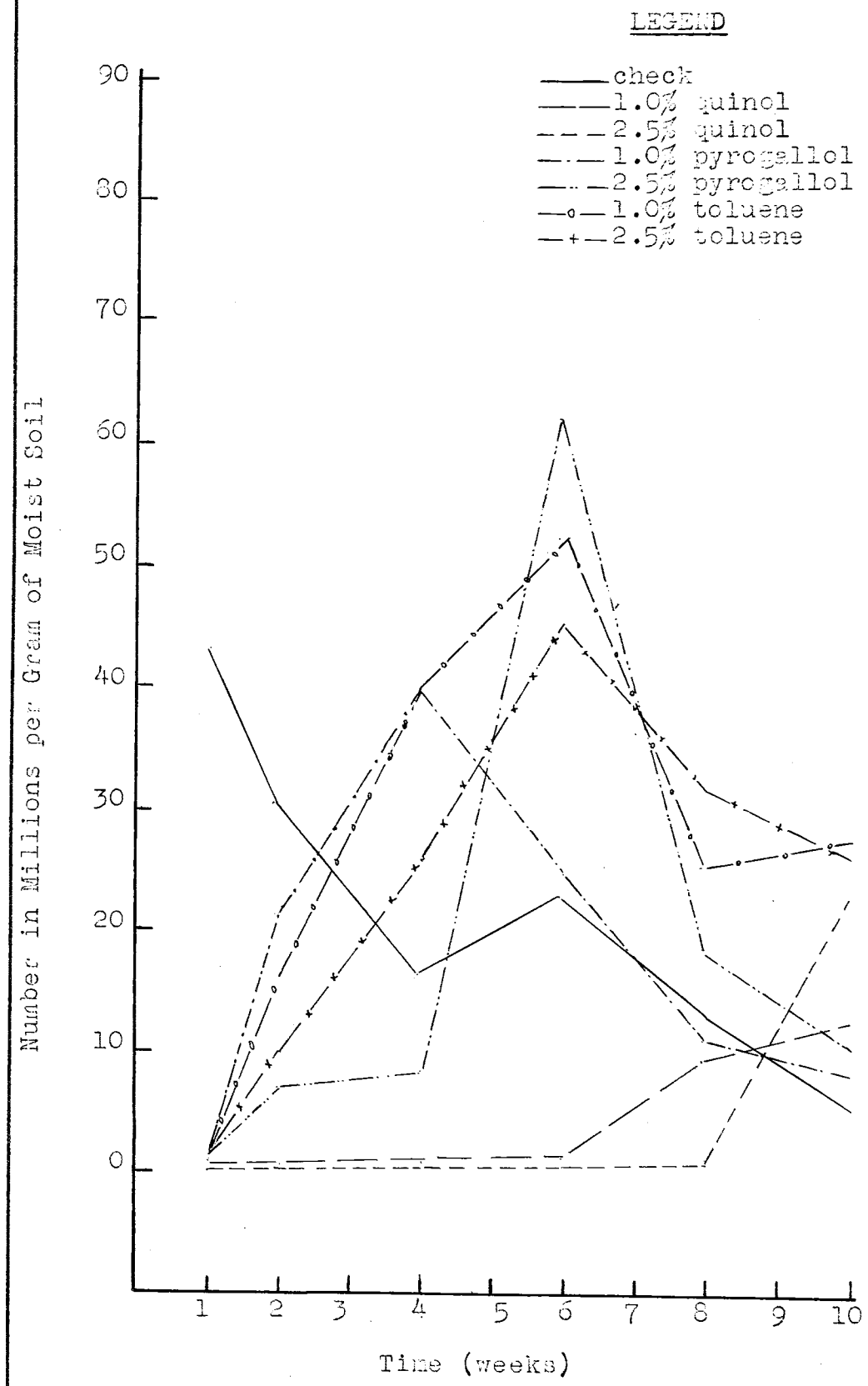

Fig. 21 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 3

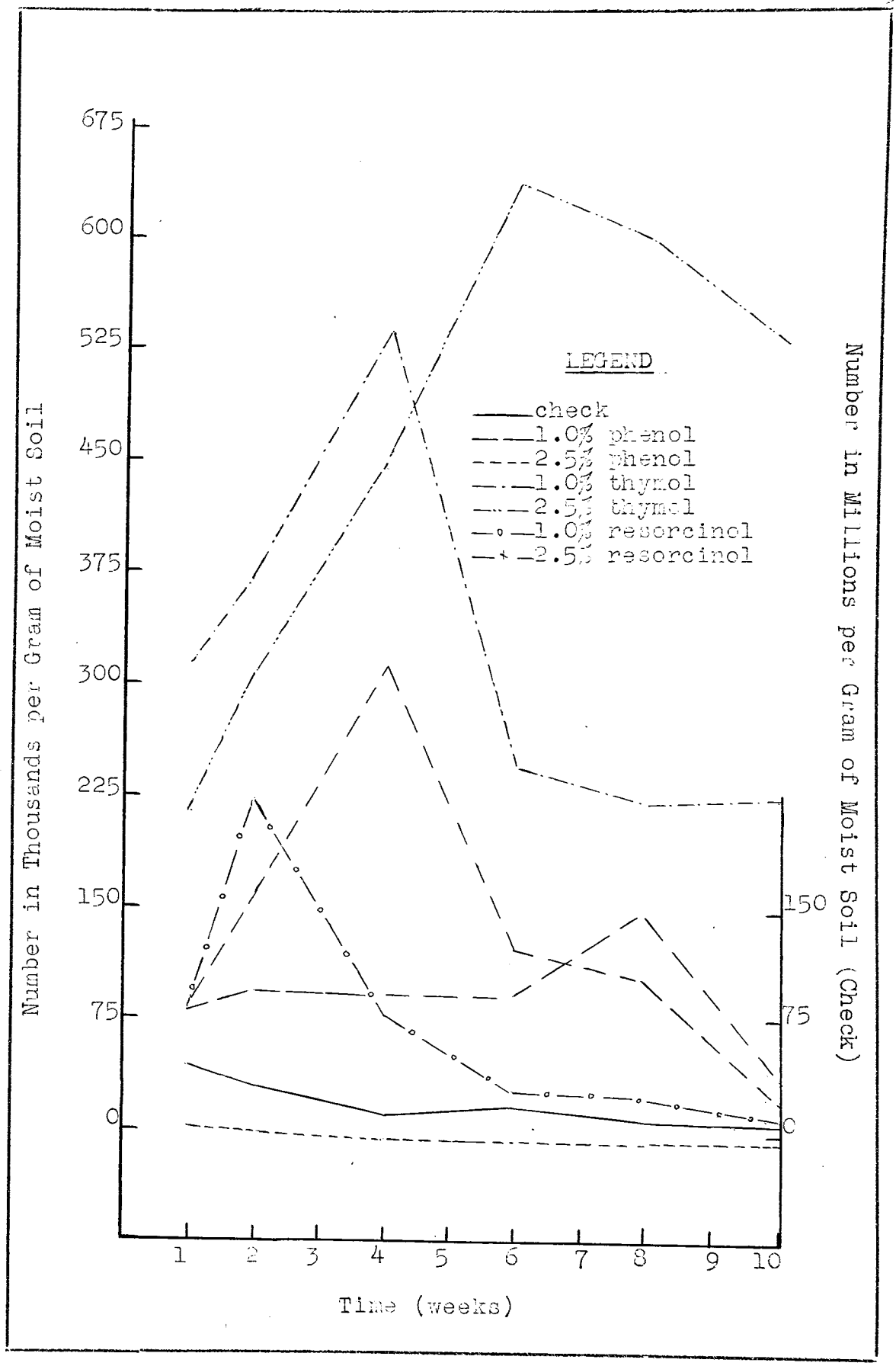

Fig. 22 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 3

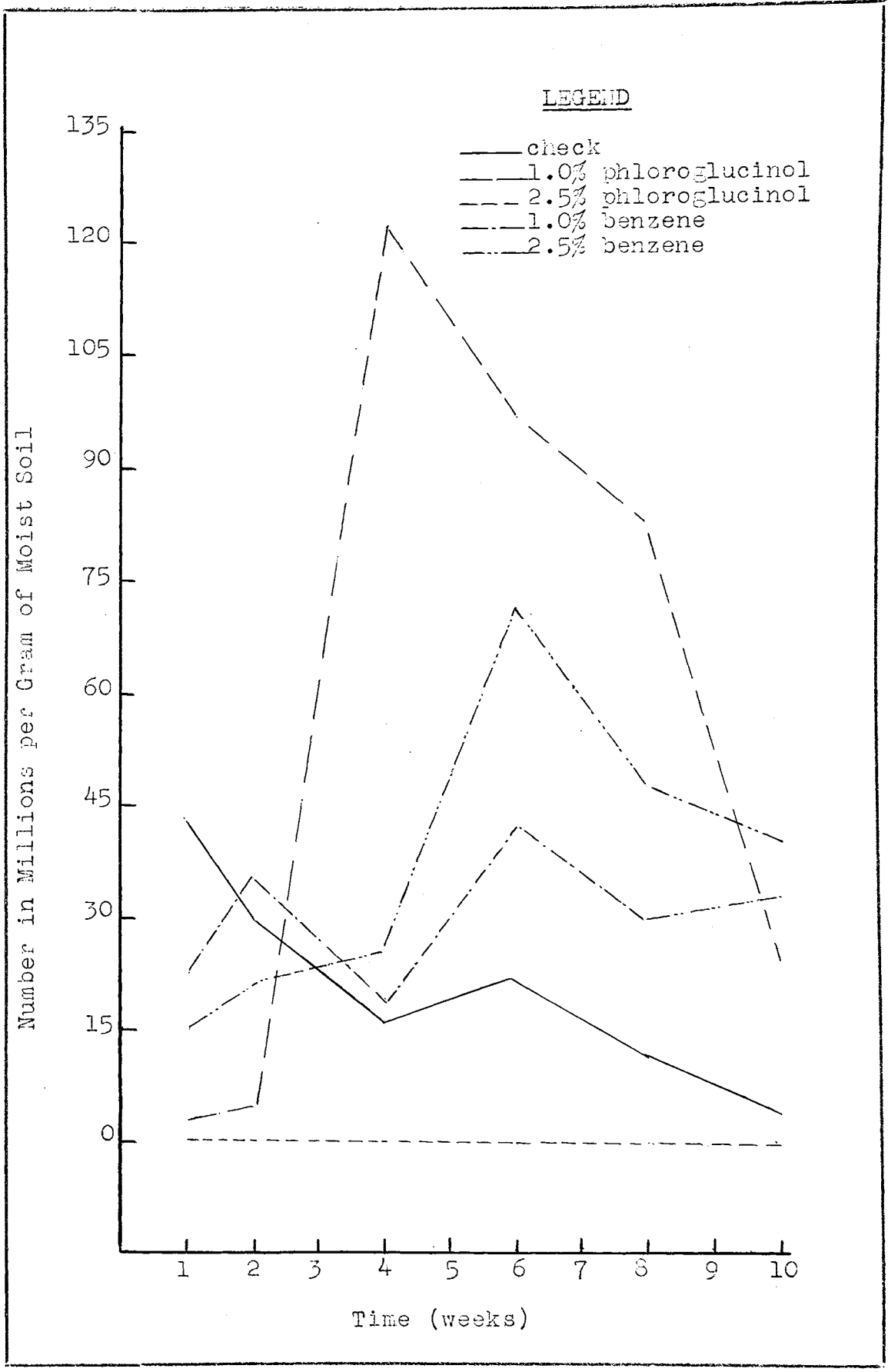

Fig. 23 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 3

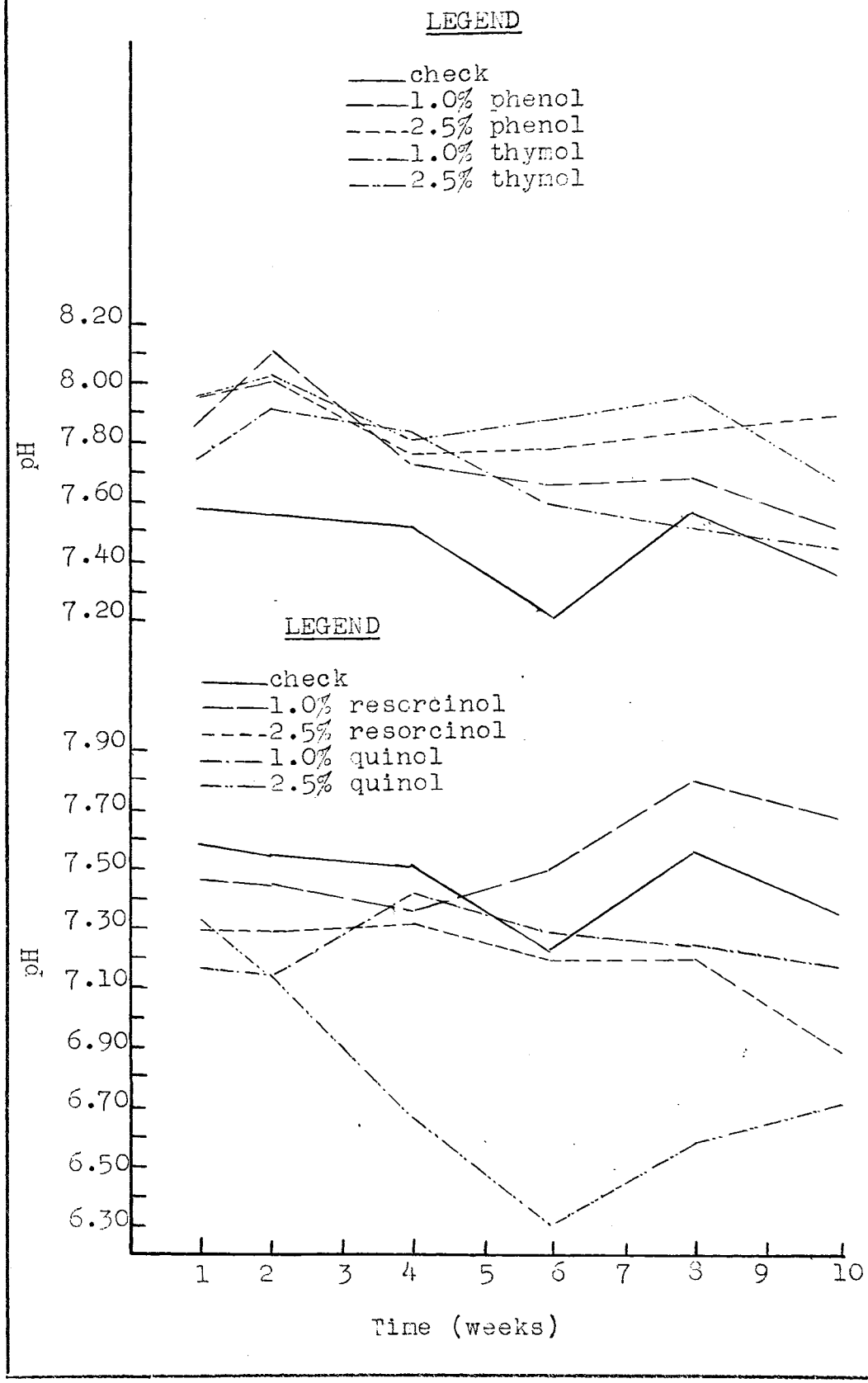

Fig. 24 Variation in pH values in Treated Soils. Series 3

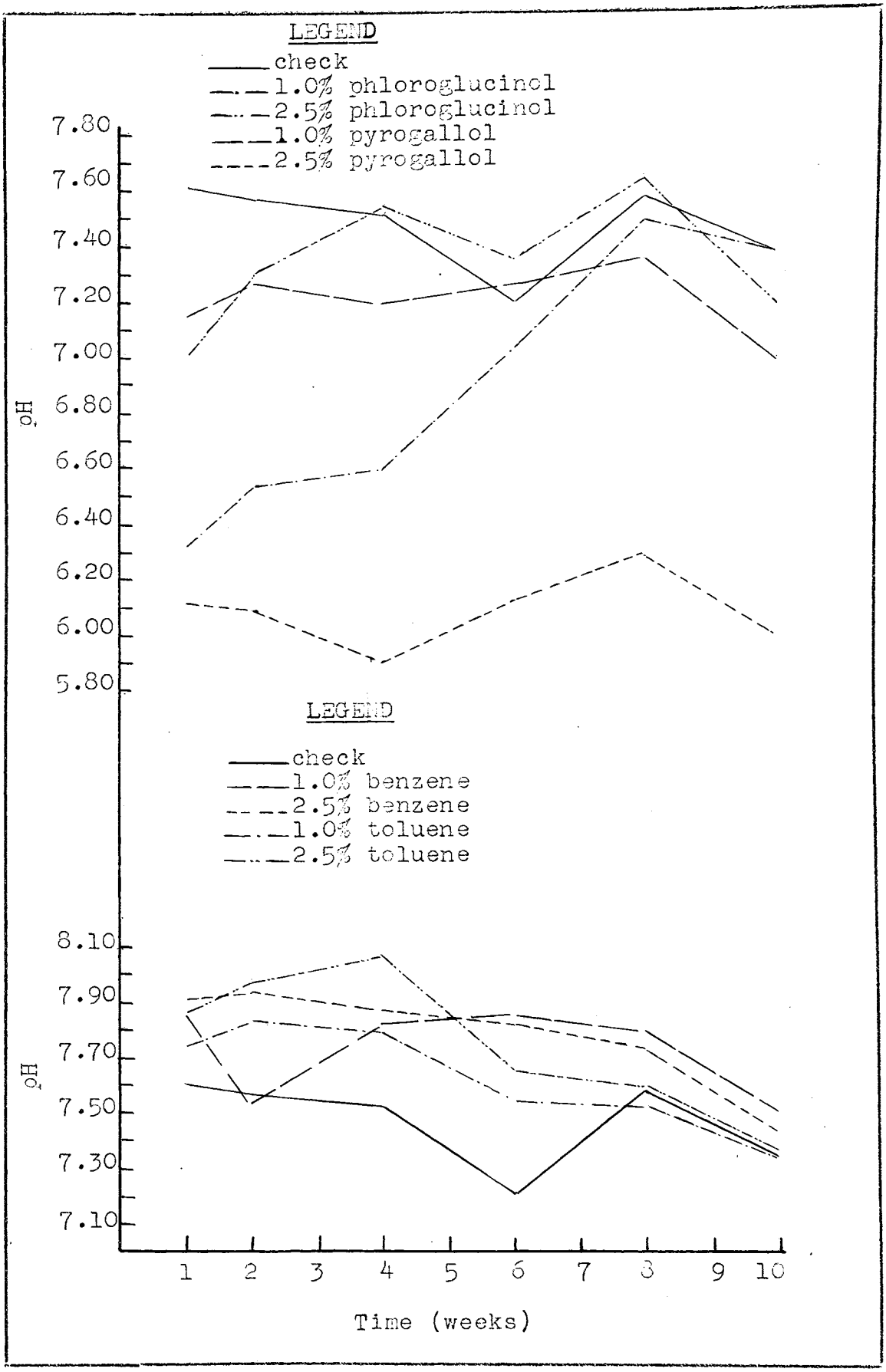

Fig. 25 Variation in pH values in Treated Soils. Series 3

TABLE 6--AZOTOBACTER IN TREATED SOIL SERIES 3
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% quinol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% quinol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
1.0% benzene	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% benzene	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
1.0% toluene	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% toluene	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
1.0% phenol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% phenol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
check	vinelandii	1.0*	4.2*	3.0*	2.6*	3.5*	1.5*
	chroococcum	2.6*	6.3*	6.1*	5.6*	6.5*	3.0*

* Numbers in thousands

TABLE 7--AZOTOBACTER IN TREATED SOIL SERIES 3
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% resorcinol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% resorcinol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
1.0% pyrogallol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% pyrogallol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
1.0% phloroglucinol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% phloroglucinol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
1.0% thymol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.5% thymol	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
check	vinelandii	1.0*	4.2*	3.0*	2.6*	3.5*	1.5*
	chroococcum	2.6*	6.3*	6.1*	5.6*	6.5*	3.0*

* Numbers in thousands

(Fig. 19). The 2.5 percent quinol treatment, however, proved stimulating after eight weeks. Phloroglucinol and 2.5 percent pyrogallol were very effective in increasing the mold counts (Fig. 20). Toluene showed beneficial results after eight weeks and 1.0 percent resorcinol after six weeks. The 2.5 percent resorcinol treatment, however, proved depressing throughout the entire incubation period.

The distribution of bacteria and Actinomyces is given graphically in Figures 21, 22, and 23. Quinol, pyrogallol, and toluene treatments (Fig. 21) depressed the development of bacteria and Actinomyces at first but increased their numbers over the check later in the incubation period. Phenol, thymol, and resorcinol were injurious to the development of these organisms (Fig.22). The phloroglucinol treatment was more effective at 1.0 percent than at 2.5 percent. The benzene treatment showed some increase over the untreated soil after several weeks of incubation (Fig. 23).

Series 4

In series 4 soils were treated with salicylic acid, gallic acid, tannic acid potassium hydrogen phthalate, cinnamic acid, sodium benzoate, calcium benzoate, and benzoic acid. The experiments were carried out as outlined under Methods and Procedure.

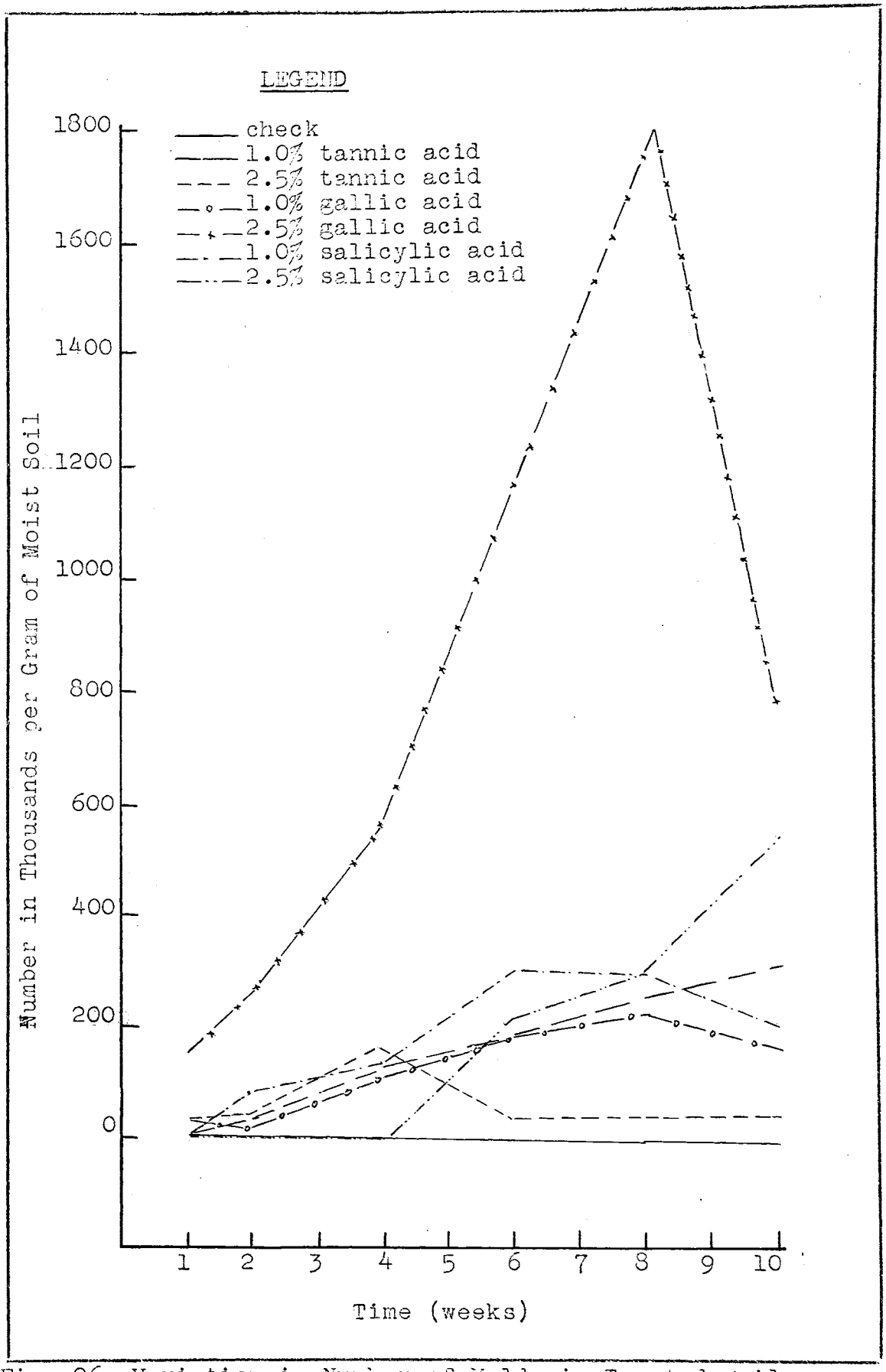

Fig. 26 Variation in Number of Molds in Treated Soil. Series 4

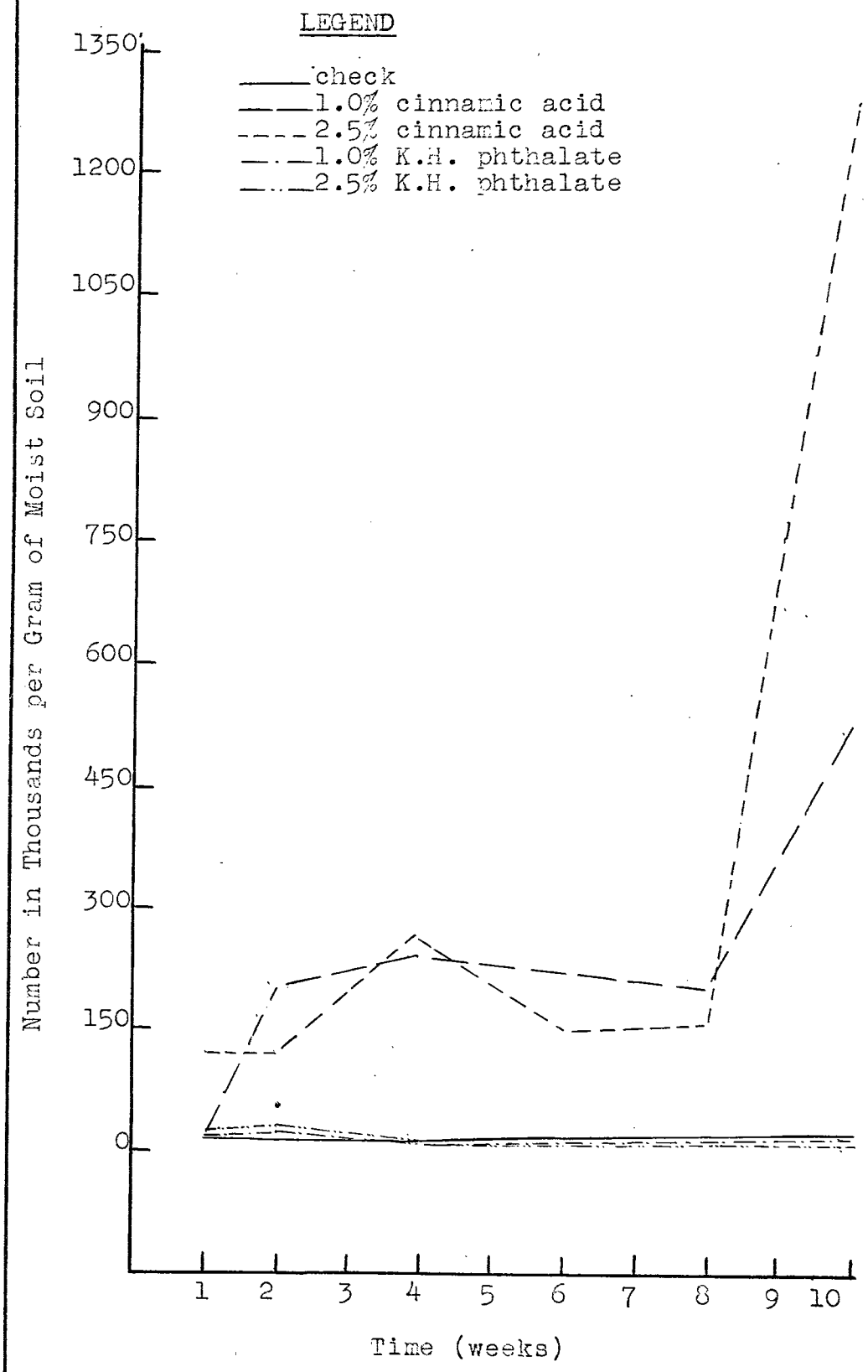

Fig. 27 Variation in Numbers of Molds in Treated Soil. Series 4

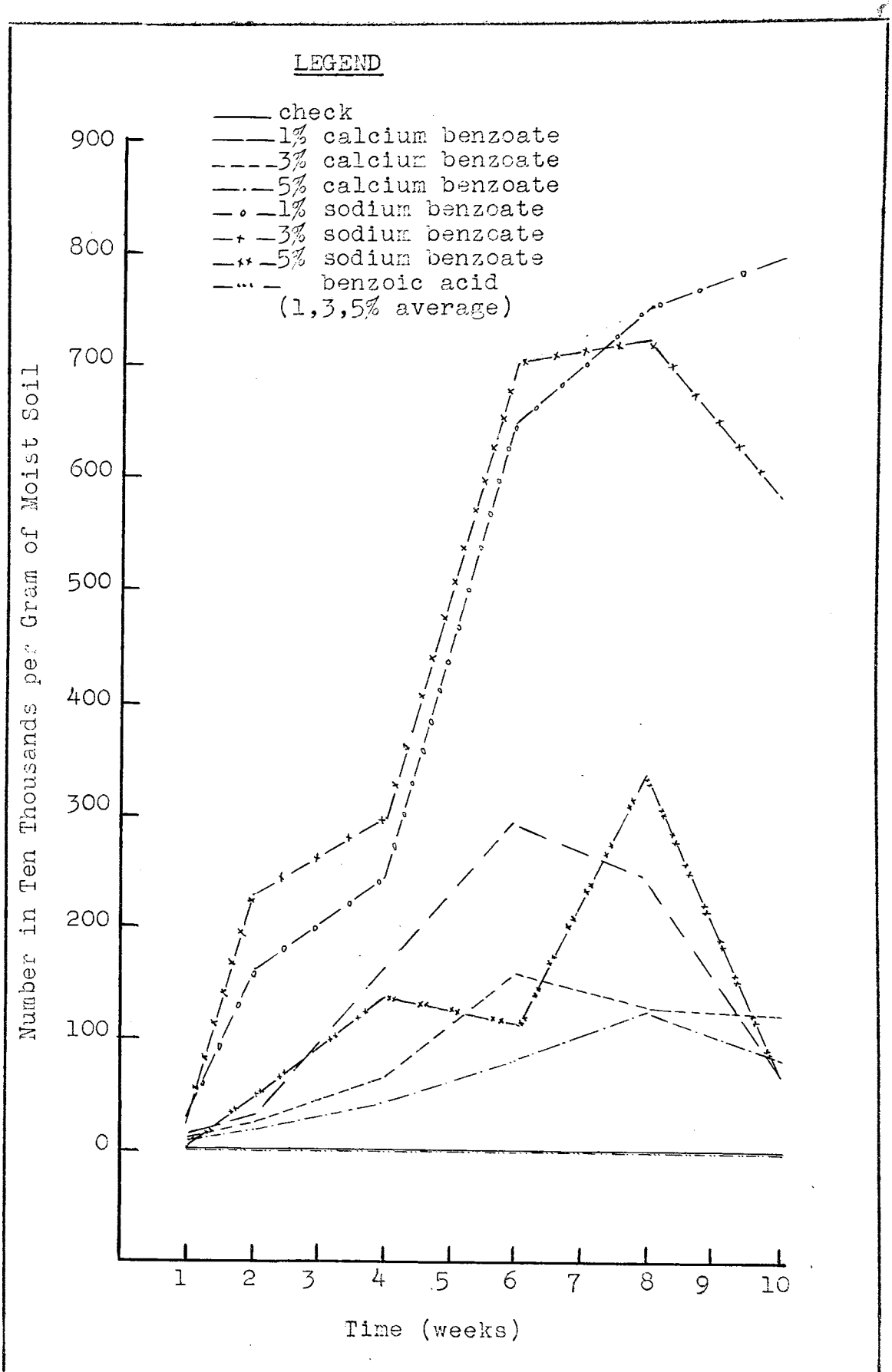

Fig. 28 Variation in Numbers of Molds in Treated Soil. Series 4

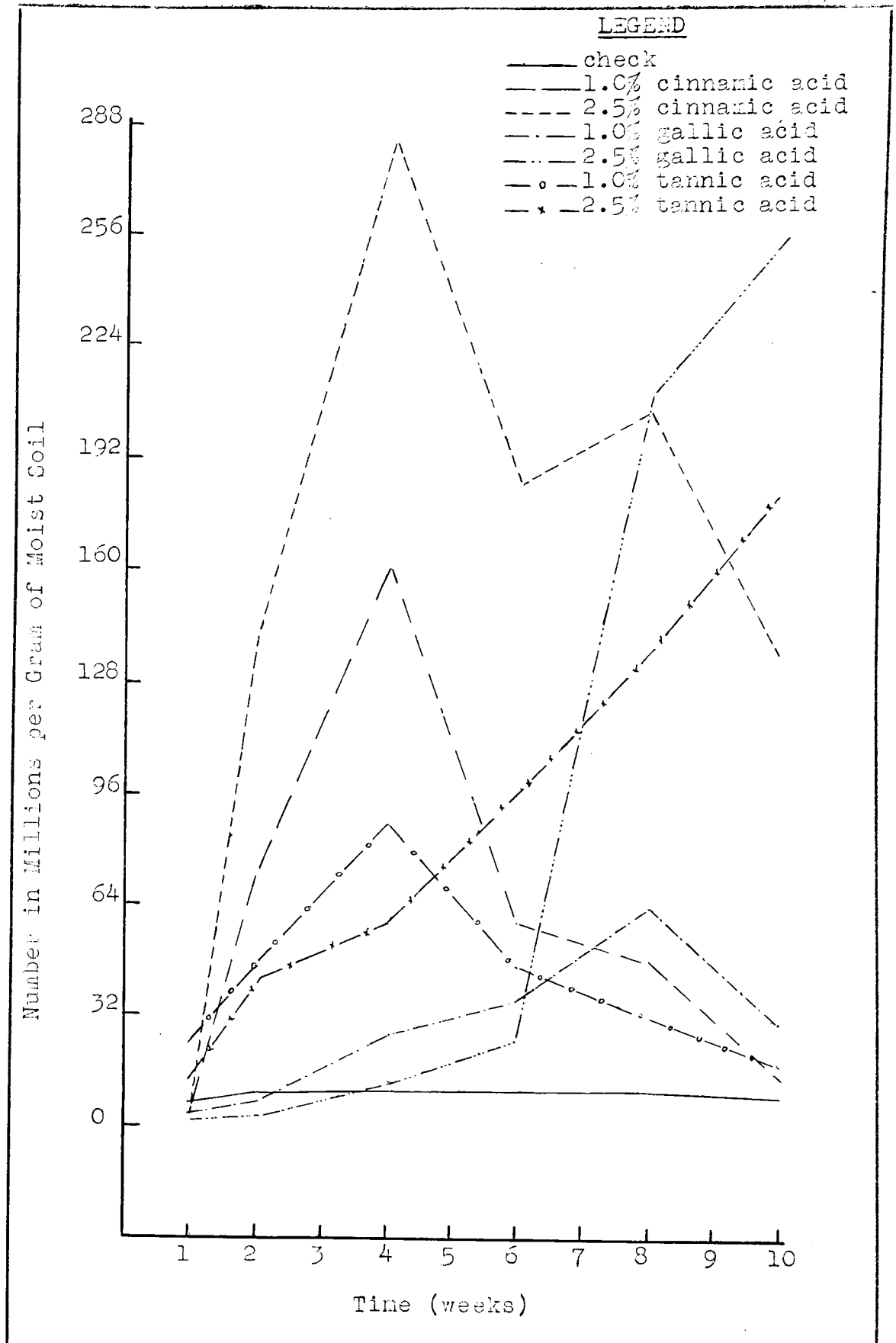

Fig. 29 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 4

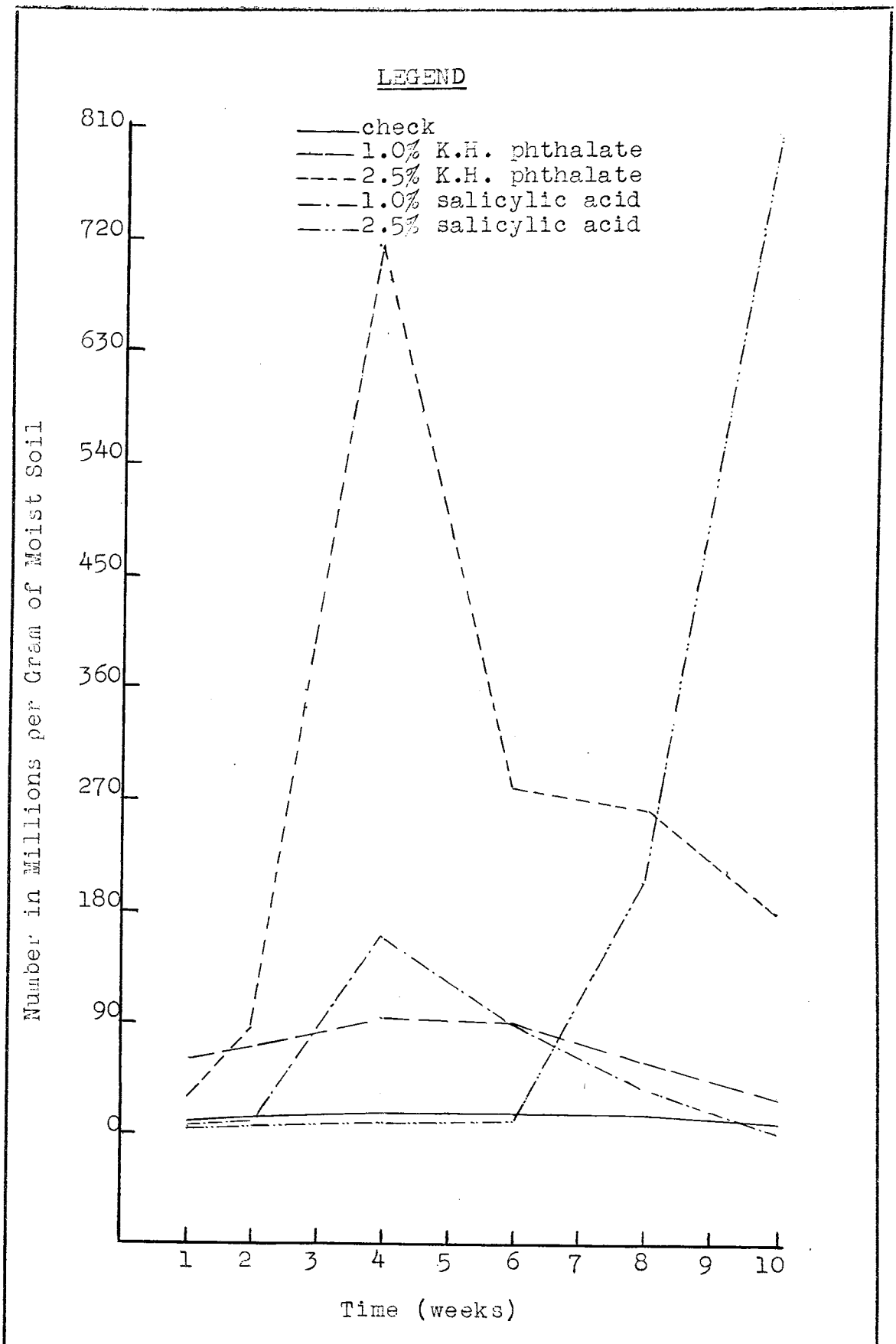

Fig. 30 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 4

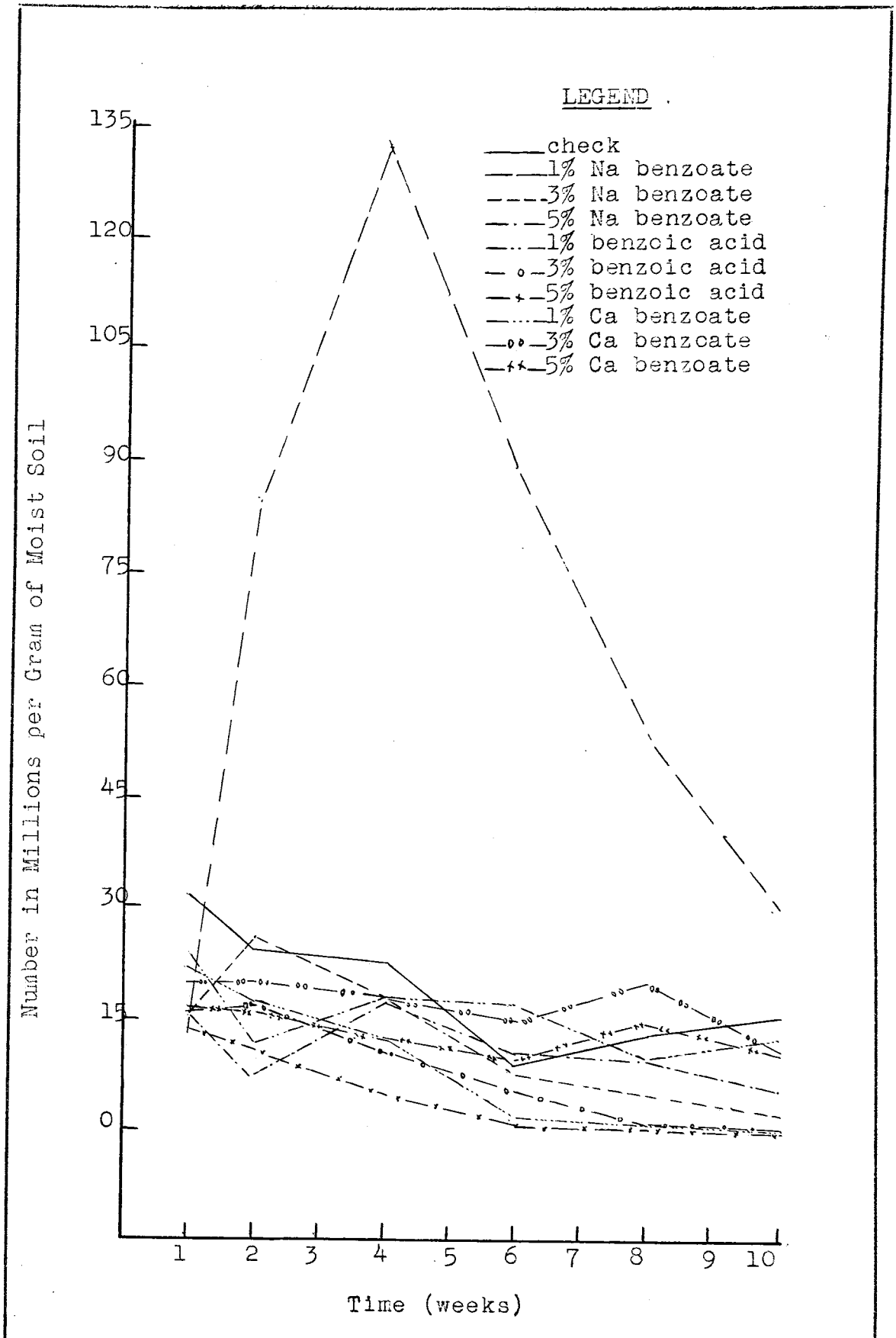

Fig. 31 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 4

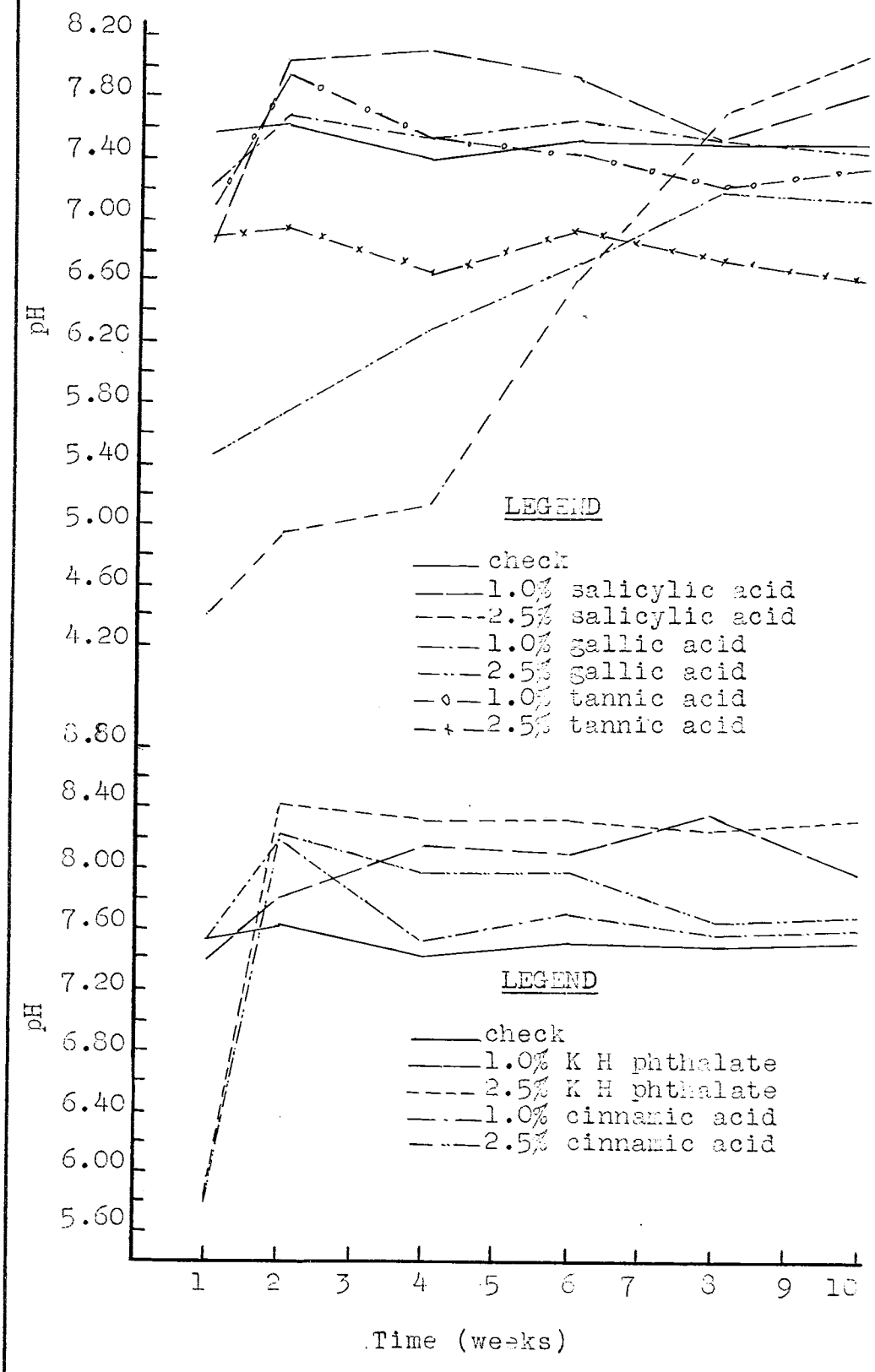

Fig. 32 Variation in pH values in Treated Soils. Series 4

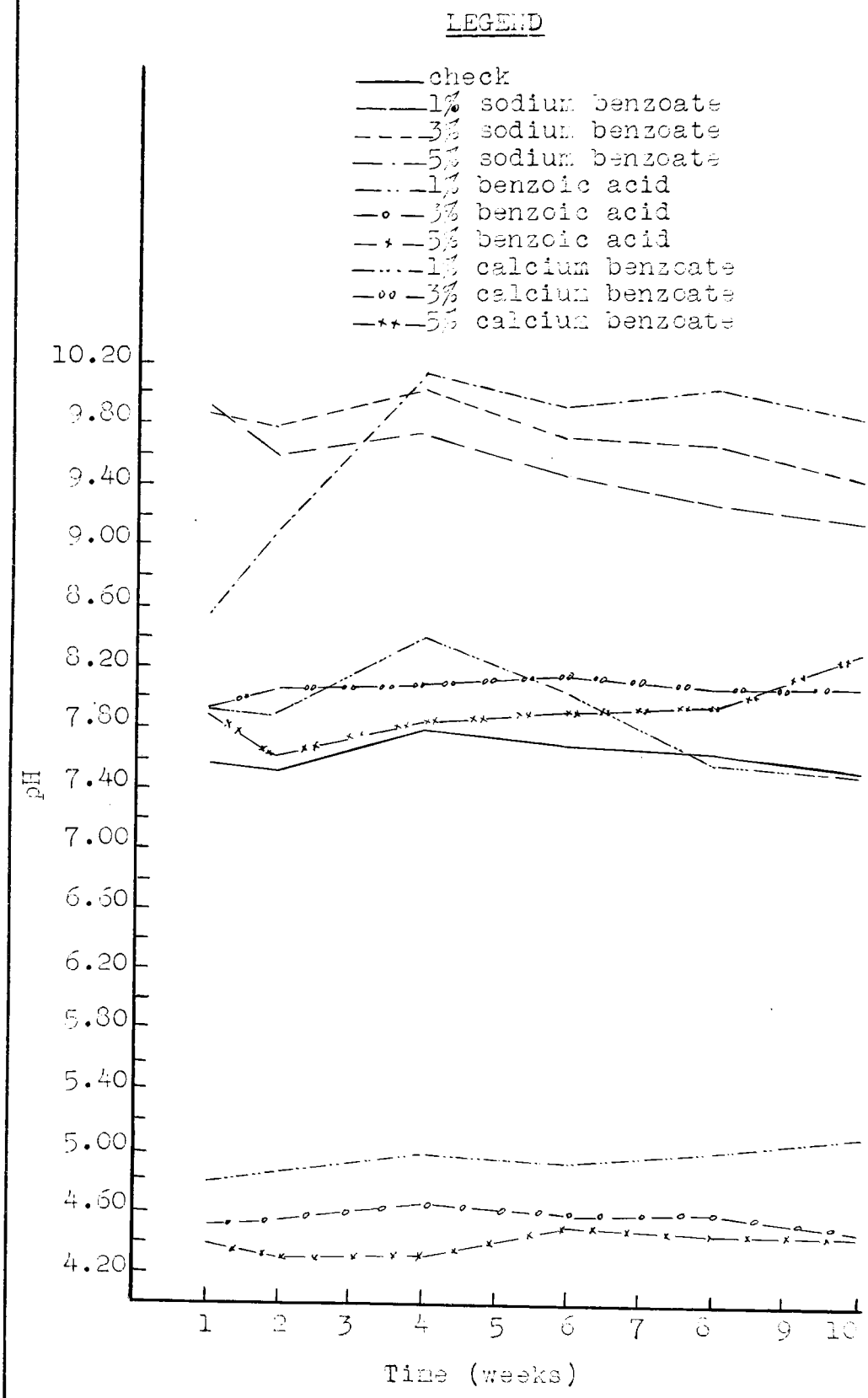

Fig. 33 Variation in pH values in Treated Soils. Series 4

TABLE 8--AZOTOBACTER IN TREATED SOIL SERIES 4
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% tannic acid	vinelandii	60	610	600	630	870	200
	chroococcum	320	2.2*	2.7*	4.0*	1.7*	500
2.5% tannic acid	vinelandii	1.1*	26.7*	210.0*	1,200.0*	170.0*	240.0*
	chroococcum	8.0*	108.0*	460.0*	12.9**	1.3**	520.0*
1.0% K H phthalate	vinelandii	30	3.0*	4.1*	7.3*	9.7*	8.2*
	chroococcum	160	1.8*	5.0*	6.4*	2.4*	7.1*
2.5% K H phthalate	vinelandii	80	600	700	12.3*	1.6*	100
	chroococcum	170	1.2*	2.6*	18.4*	3.9*	5.1*
1.0% salicylic acid	vinelandii	0	0	0	10	10	20
	chroococcum	0	40	130	160	140	60
2.5% salicylic acid	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	10	850
1.0% gallic acid	vinelandii	0	0	0	0	0	0
	chroococcum	10	0	10	10	10	20
2.5% gallic acid	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
1.0% cinnamic acid	vinelandii	320.0*	2.4**	8.1**	210.0**	4.6**	1.8**
	chroococcum	0	0	0	0	0	0
2.5% cinnamic acid	vinelandii	890.0*	6.1**	45.1**	460.0**	8.2**	1.5**
	chroococcum	0	0	0	0	0	0
check	vinelandii	500	3.0*	2.2*	6.5*	2.3*	2.6*
	chroococcum	700	5.0*	2.0*	3.1*	4.0*	5.5*

* Numbers in thousands
** Numbers in millions

TABLE 9--AZOTOBACTER IN TREATED SOIL SERIES 4
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
2.0% calcium benzoate	vinelandii	10.0*	43.0*	1.1**	730.0*	250.0*	205.0*
	chroococcum	10.0*	30.0*	980.0*	230.0*	120.0*	90.0*
3.0% calcium benzoate	vinelandii	1.0*	4.0*	670.0*	140.0*	14.0*	5.9*
	chroococcum	1.0*	13.0*	790.0*	208.0*	9.2*	2.1*
5.0% calcium benzoate	vinelandii	1.0*	1.0*	1.0*	22.6*	480.0*	1.4**
	chroococcum	1.0*	1.0*	1.0*	0	0	0
2.0% sodium benzoate	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
3.0% sodium benzoate	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
5.0% sodium benzoate	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
2.0% benzoic acid	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
3.0% benzoic acid	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
5.0% benzoic acid	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
check	vinelandii	670	580	460	870	450	430
	chroococcum	520	650	700	900	800	330

* Numbers in thousands
** Numbers in millions

The distribution of Azotobacter is presented in Tables 8 and 9 and is summarized in Tables 15 and 16. The changes in soil reaction are given graphically in Figures 32 and 33. It is evident from Table 15 that 2.5 percent of tannic acid produced a more favorable effect on the Azotobacter development than 1.0 percent. The pH values in both cases were above 6.0. the 1.0 percent potassium hydrogen phthalate treatment proved to be a better stimulant for Az. vinelandii than for Az. chroococcum. However, the 1.0 percent treatment gave just the opposite results. In both cases the counts were low (Table 15). Salicylic acid was even a poorer stimulant for Azotobacter than was potassium hydrogen phthalate. The pH for the 2.5 percent treatment was well below 6.0 during the first four weeks, yet Az. chroococcum appeared in the soil after eight and ten weeks (Table 8 and Fig. 32). This was possibly due to the survival of the organisms until the conditions were more favorable for its development or perhaps due to reinoculation by contamination. Az. vinelandii was not found in this treatment.

Treatments containing 2.5 percent of gallic acid gave a reaction below pH of 6.0 after the first week but remained above 6.0 for the remainder of the incubation period (Fig. 32). Both the 1.0 and 2.5 percent additions of this acid were injurious to Azotobacter (Table 8). A remarkable increase in Az. vinelandii num-

bers was noted in cinnamic acid treated soils (Table 15). In this case Az. chroococcum was entirely absent and the soil reaction remained favorable for the growth of Azotobacter throughout the experiment (Fig. 32). The greatest increase in Az. vinelandii numbers took place after the sixth week.

Sodium benzoate and benzoic acid were injurious to Azotobacter in the concentrations used (Table 9). At no time during the entire incubation period was it possible to isolate Az. chroococcum or Az. vinelandii from the treated soils. The influence of soil reaction was undoubtedly important here. Figure 33 shows that in the case of benzoic acid the pH values were below 6.0 in all the treatments during the entire incubation period. In case of sodium benzoate, however, the pH values were high, ranging from 8.15 to 10.18. For the calcium benzoate treatments the pH readings were within the limits for the maximum development of Azotobacter. In this case both Az. vinelandii and Az. chroococcum increased substantially over the untreated soil.

Variations in mold counts are shown in Figures 26, 27, and 28. Mold counts for tannic, gallic, and salicylic acid are above those for the untreated soil, with greatest increase in the 2.5 percent gallic acid treatment (Fig. 26). The greatest increase for soils receiving cinnamic acid takes place after the

eighth week, the 2.5 percent treatment resulting in higher counts than the 1.0 percent treatment (Fig. 27).

Additions of benzoic acid to the soil resulted in lower counts than in the case of untreated soils (Fig. 28). The additions of sodium and calcium benzoate to the soil enhanced greatly the development of molds, especially the 3.0 and 5.0 percent treatments.

Variations in bacteria and Actinomyces are presented graphically in Figures 29, 30, and 31. A rapid development of bacteria and Actinomyces was evident during the first four weeks in case of cinnamic acid treatments (Fig.29). For the same acid, however, the mold counts began to increase rapidly only after the first eight weeks (Fig.27) and it was previously noted that Az. vinelandii reached the maximum development at the end of the sixth week (Table 8). It therefore appears that bacteria and Actinomyces were more instrumental in the decomposing cinnamic acid into a compound or compounds highly suitable for Az. vinelandii than for molds.

A rapid development of bacteria and Actinomyces was apparent in the 2.5 percent potassium hydrogen phthalate and 2.5 percent salicylic acid treatments after the first six weeks (Fig.30). With the benzoate compounds the greatest increase in the number of these organisms was noted in the case of the 2.5 percent sod-

ium benzoate treatment. The greatest depressing effect was found in benzoic acid treated soils (Fig.31).

Series 5

In series 5 the soils were treated with calcium and potassium citrate, calcium and potassium lactate, calcium and potassium tartrate, potassium oxalate, and potassium malate. The experiments were performed in the manner outlined under Methods and Procedure, and the usual determinations were made.

The distribution of Azotobacter in treated and untreated soils is shown in Tables 10, 11, and 16, and the variation in pH values in Figures 40 and 41. In two cases did Az. vinelandii counts exceed a million: In the 2.5 percent calcium lactate treated soil and in the 2.5 percent calcium tartrate treated soil. On the basis of counts obtained, calcium tartrate was more suitable for Azotobacter development than potassium tartrate. A concentration of 2.5 percent of the last-named salt was more detrimental than a 1.0 percent concentration. This was probably due to the high pH values obtained (Fig.41). Possibly for the same reason the 1.0 percent potassium malate treatment is somewhat better than the 2.5 percent treatment (Fig.40). The 2.5 percent potassium oxalate treatment also showed a higher pH value than the 1.0 percent treatment, but in this case even the lower amount of this chemical was not a

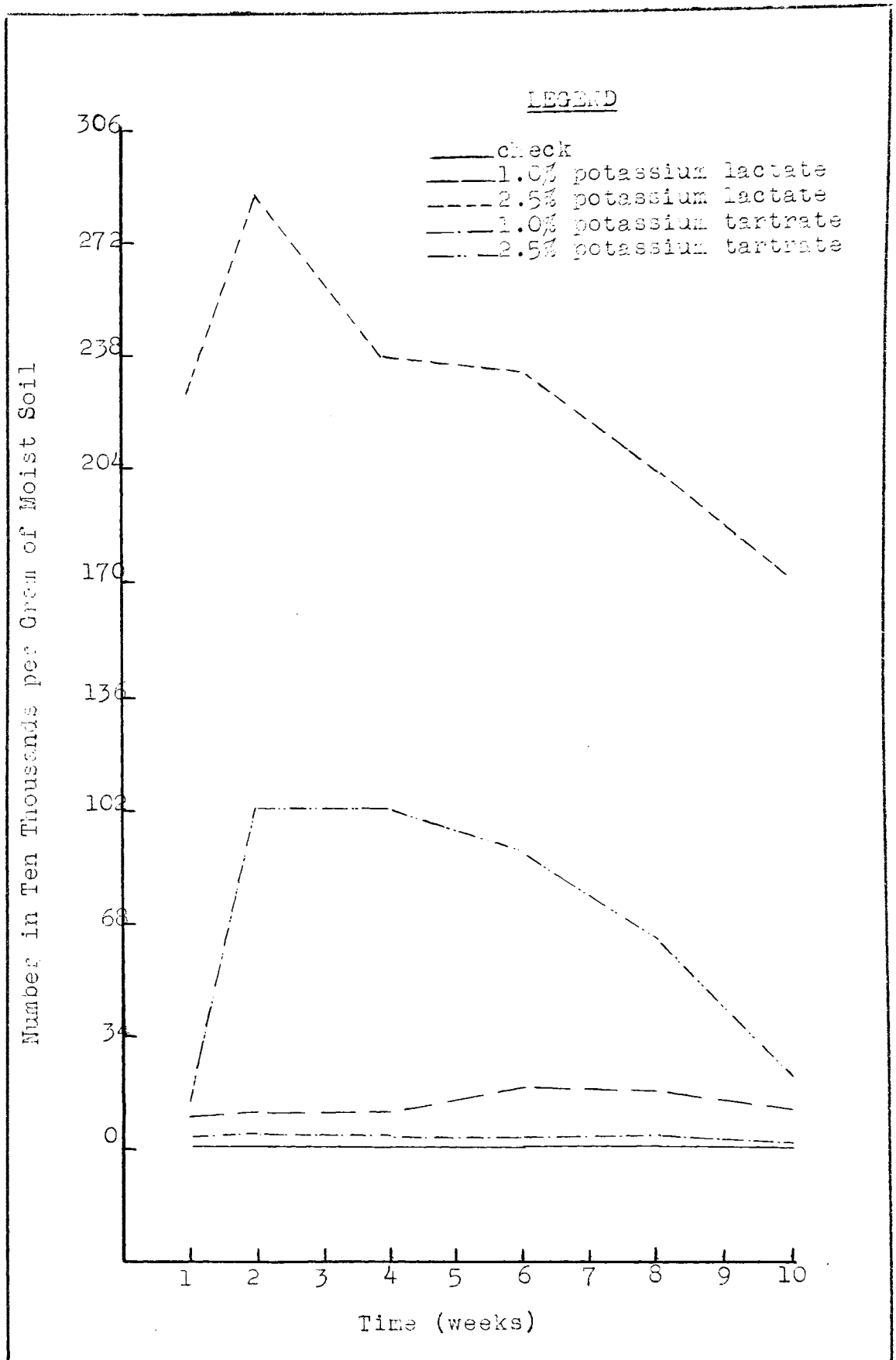

Fig. 34 Variation in Numbers of Molds in Treated Soil. Series 5

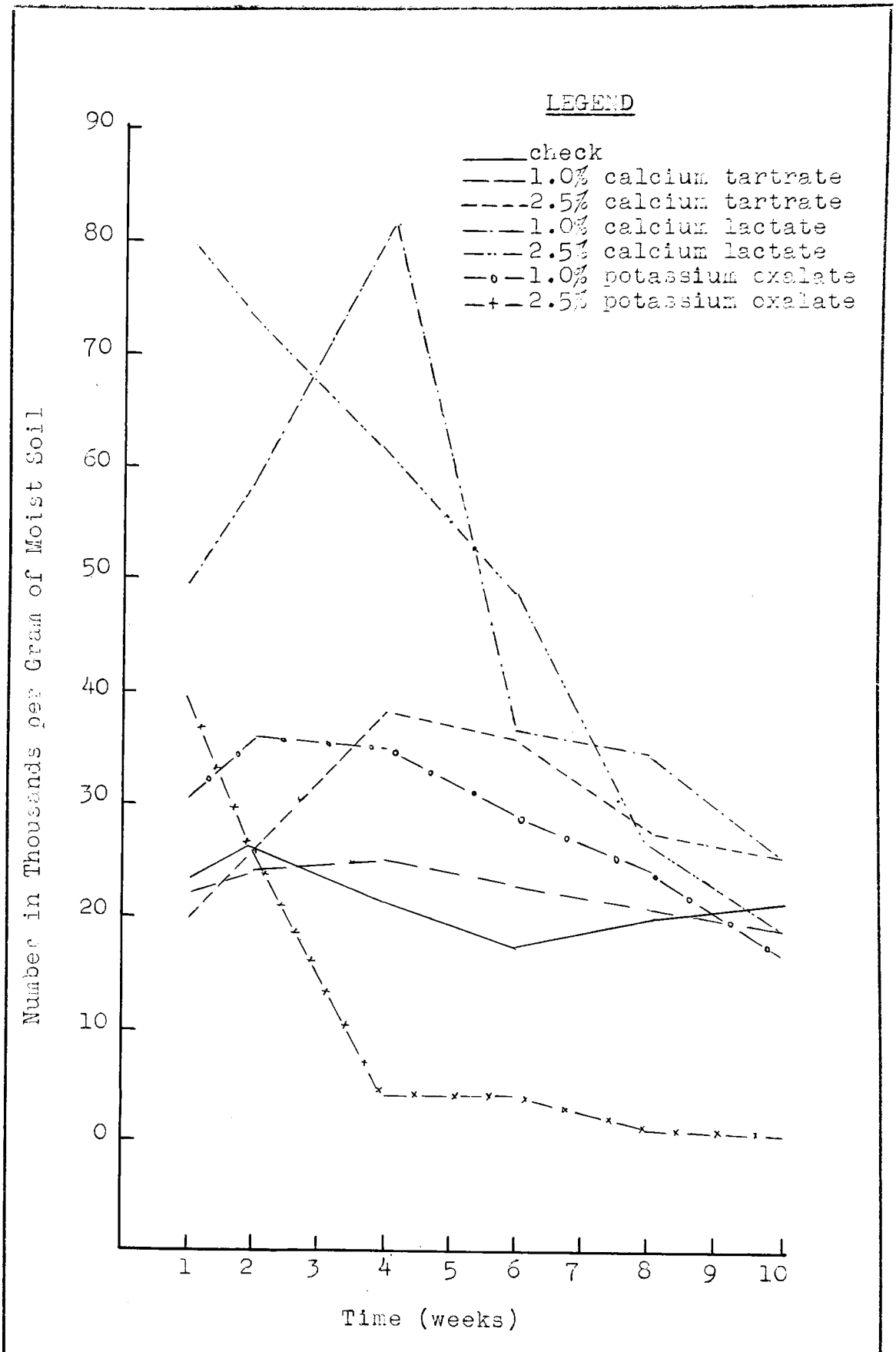

Fig. 35 Variation in Numbers of Molds in Treated Soil. Series 5

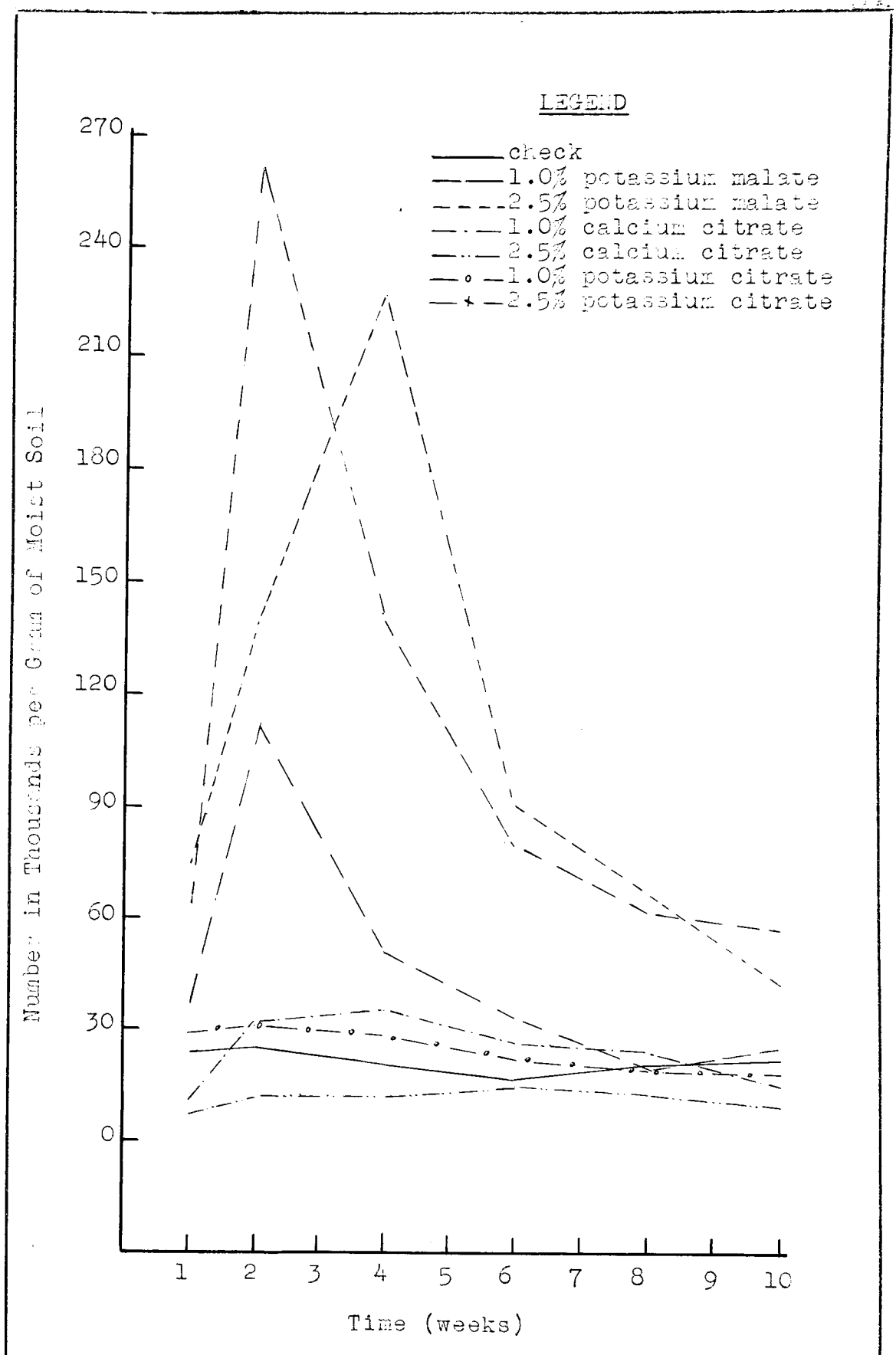

Fig. 36 Variation in Numbers of Molds in Treated Soil. Series 5

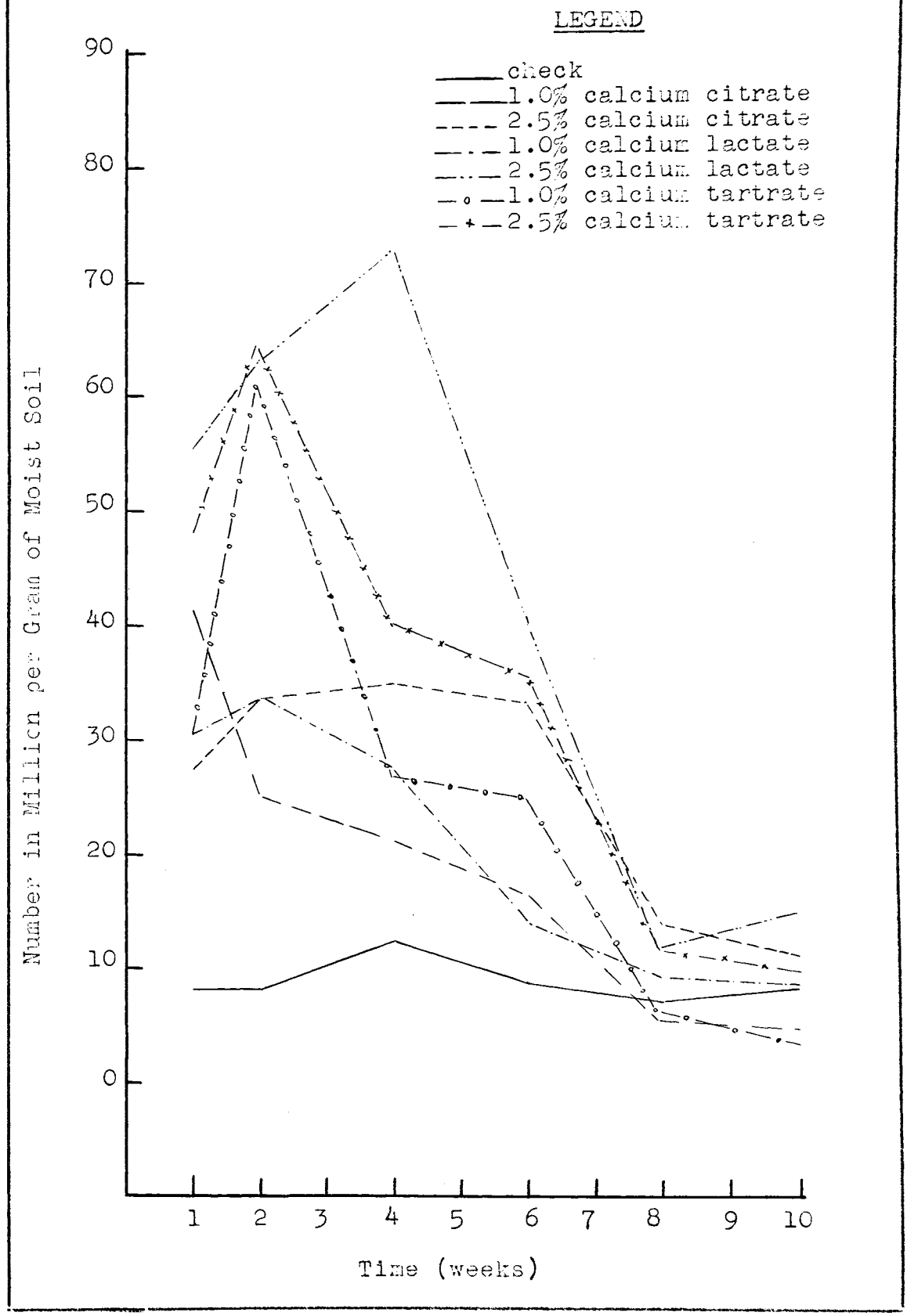

Fig. 37 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 5

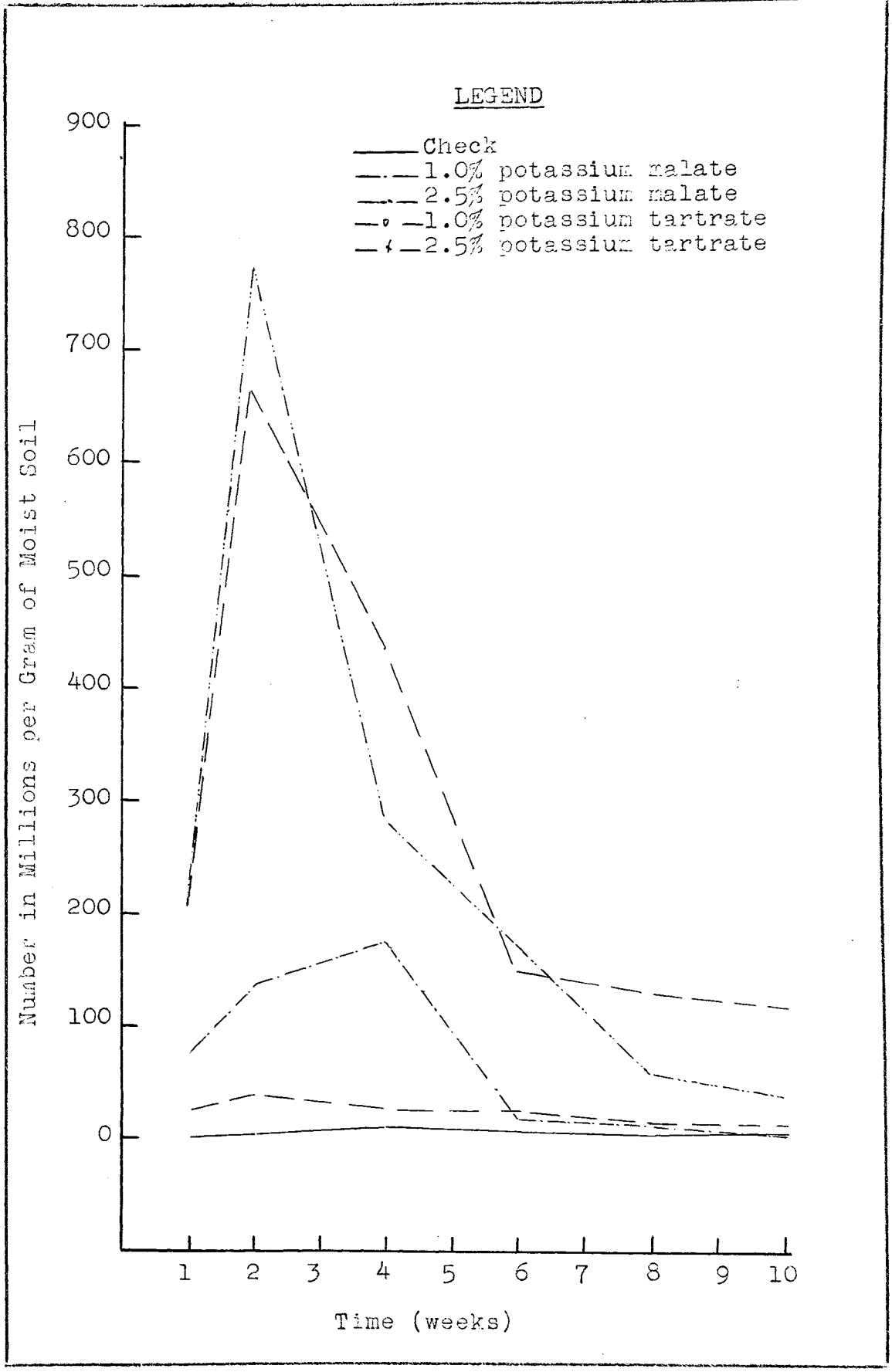

Fig. 38 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 5

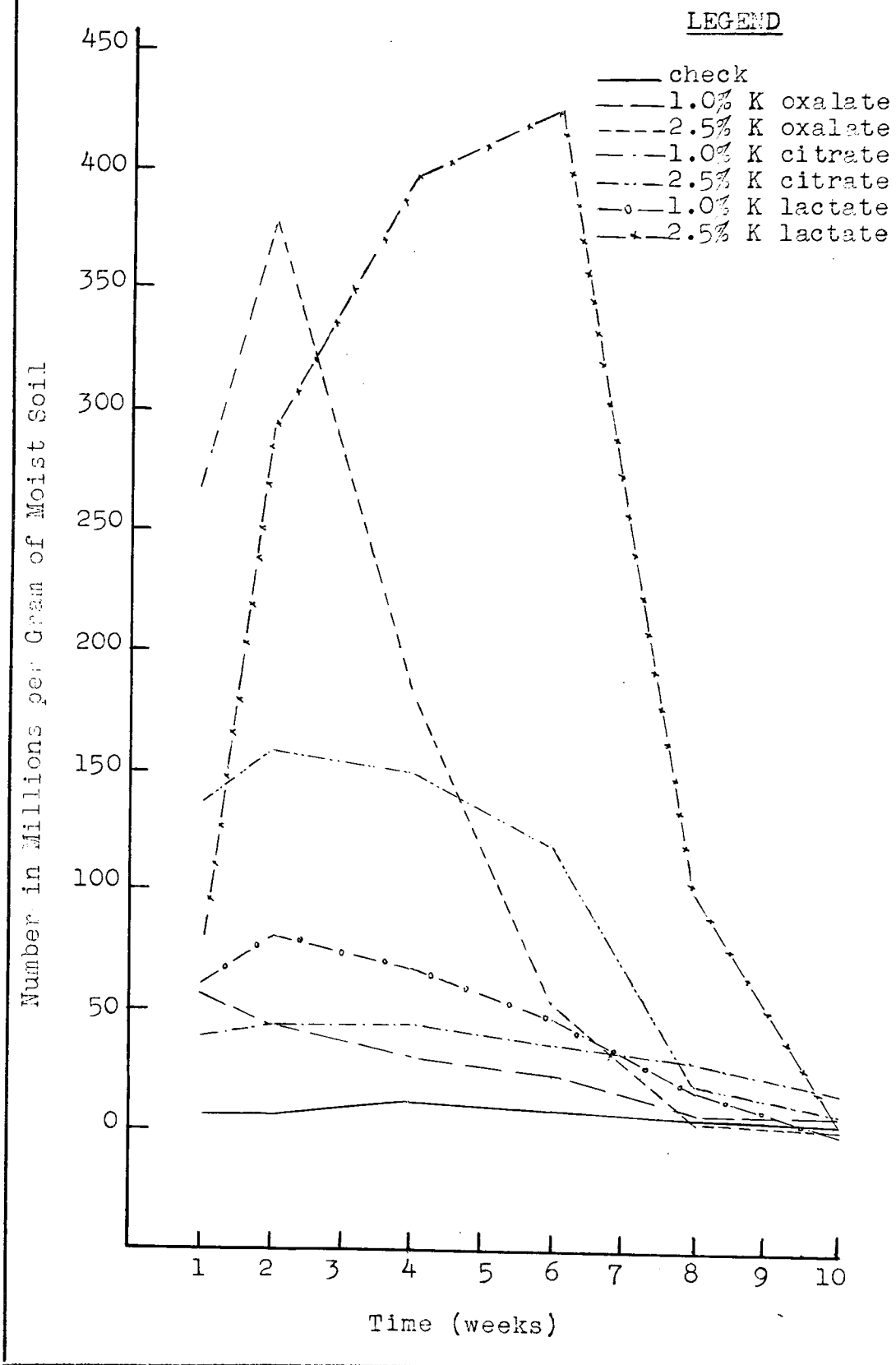

Fig. 39 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 5

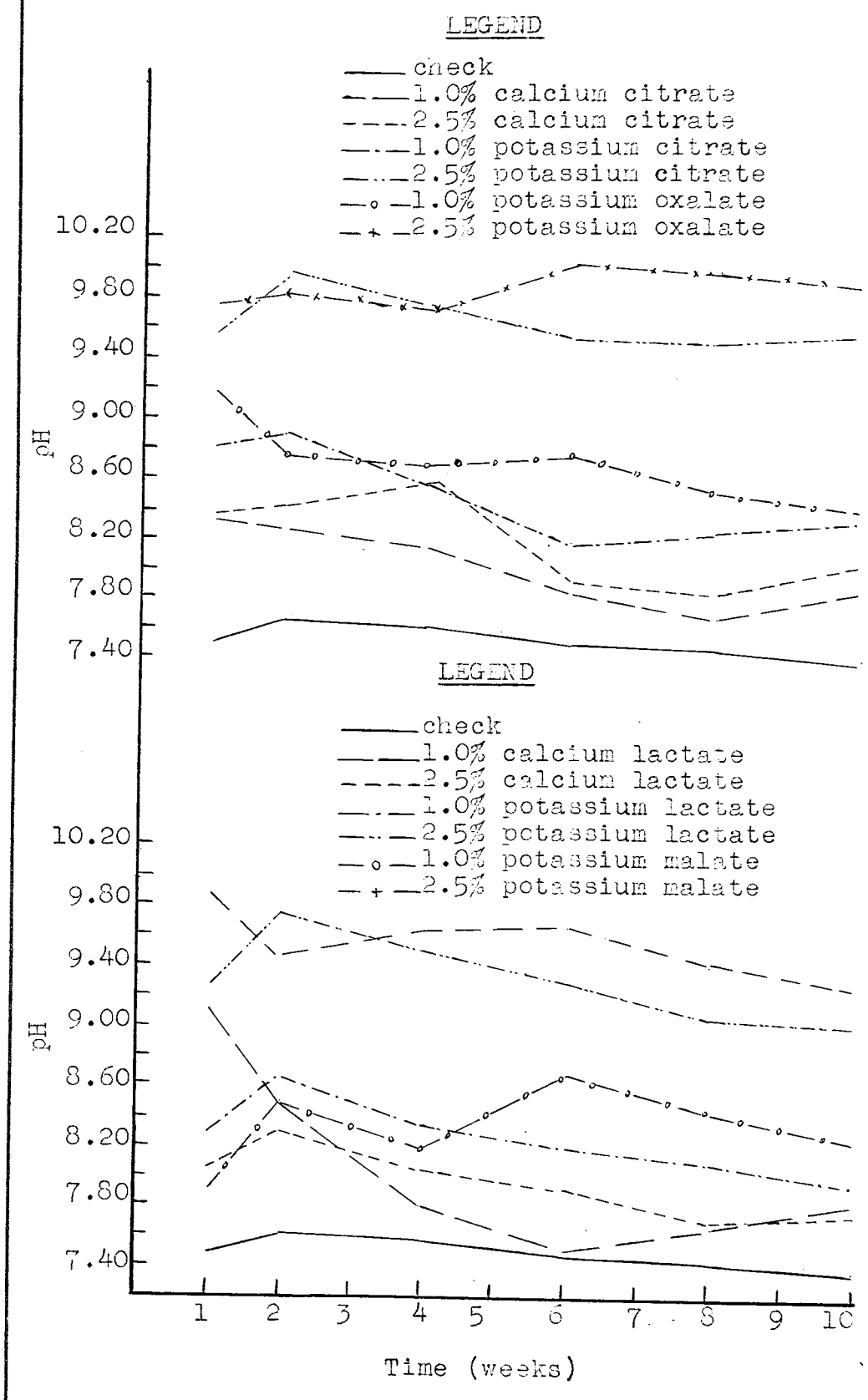

Fig. 40 Variation in pH values in Treated Soils. Series 5

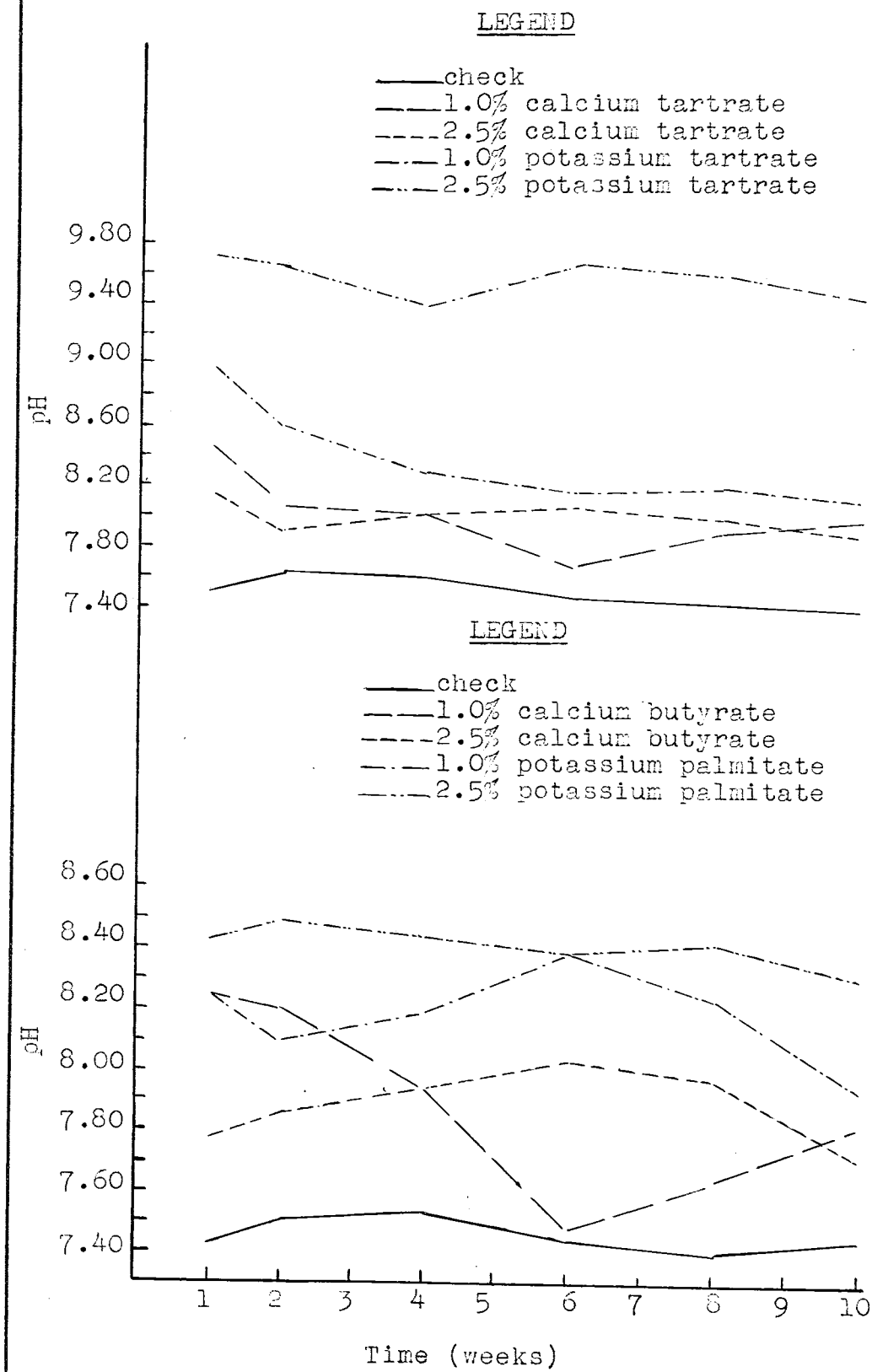

Fig. 41 Variation in pH values in Treated Soils.
Series 5 (top) Series 6 (bottom)

TABLE 10--AZOTOBACTER IN TREATED SOIL SERIES 5
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% calcium lactate	vinelandii	0	0	0	0	0	0
	chroococcum	1.5*	550.0*	130.0*	2.5**	5.1**	1.4**
2.5% calcium lactate	vinelandii	0	0	0	3.8**	3.1**	2.5**
	chroococcum	210.0*	2.2**	6.8**	8.5**	6.5**	5.1**
1.0% potassium lactate	vinelandii	0	0	720.0*	410.0*	108.0*	200.0*
	chroococcum	36.1*	1.8**	2.9**	6.6**	3.2**	2.0**
2.5% potassium lactate	vinelandii	0	0	36.0*	22.0*	16.0*	10.0*
	chroococcum	72.0*	2.6**	1.2**	680.0*	595.0*	510.0*
1.0% calcium tartrate	vinelandii	0	11.0*	30.0*	58.0*	100.0*	100.0*
	chroococcum	6.3*	62.0*	810.0*	1.7**	5.1**	2.3**
2.5% calcium tartrate	vinelandi	0	67.0*	230.0*	1.1**	1.9**	3.1**
	chroococcum	12.0*	208.0*	1.1**	4.9**	2.9**	2.2**
1.0% potassium tartrate	vinelandii	1.0*	5.6*	51.0*	160.0*	206.0*	810.0*
	chroococcum	12.3*	135.0*	260.0*	420.0*	680.0*	1.5**
2.5% potassium tartrate	vinelandii	780	2.1*	2.6*	3.2*	1.0*	100
	chroococcum	3.2*	8.0*	35.2*	45.0*	19.0*	8.0*
1.0% potassium malate	vinelandii	6.7*	42.0*	125.0*	470.0*	210.0*	10.0*
	chroococcum	31.0*	520.0*	810.0*	1.2**	950.0*	420.0*
2.5% potassium malate	vinelandii	400	1.2*	1.0*	1.0*	10	10
	chroococcum	2.8*	6.1*	22.0*	1.0*	10	10
check	vinelandii	200	150	100	100	130	100
	chroococcum	1.1*	2.3*	2.1*	1.6*	700	800

*Numbers in thousands **Numbers in millions

TABLE 11--AZOTOBACTER IN TREATED SOIL SERIES 5
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% calcium citrate	vinelandii	0	0	2.8*	36.0*	42.0*	26.0*
	chroococcum	22.0*	350.0*	410.0*	850.0*	3.2**	250.0*
2.5% calcium citrate	vinelandii	2.1*	10.0*	10.0*	110.0*	100.0*	200.0*
	chroococcum	68.1*	530.0*	620.0*	3.1**	4.5**	2.3**
1.0% potassium citrate	vinelandii	1.4*	6.7*	18.3*	195.0*	120.0*	110.0*
	chroococcum	16.3*	450.0*	800.0*	920.0*	740.0*	630.0*
2.5% potassium citrate	vinelandii	0	0	0	0	0	0
	chroococcum	780	10	10	20	10	10
1.0% potassium oxalate	vinelandii	60	100	0	0	0	0
	chroococcum	100	120	130	100	200	400
2.5% potassium oxalate	vinelandii	0	0	0	0	0	0
	chroococcum	0	0	0	0	0	0
Check	vinelandii	200	150	100	100	130	100
	chroococcum	1.1*	2.3*	2.1*	1.6*	700	800

*Numbers in thousands
**Numbers in millions

suitable source of energy for Azotobacter (Fig.40 and Table 16).

Of the citrates used, the calcium salt is a better source of carbon for Azotobacter than is that of potassium. Az. chroococcum, however, develops more favorably than Az. vinelandii in the presence of these salts (Table 16).

Mold counts are presented graphically in Figures 34, 35, and 36. In this series the highest mold counts were obtained with 2.5 percent potassium lactate (Fig.34). Potassium malate and potassium citrate proved to be more stimulating in the 2.5 percent concentration than in 1.0 percent concentration (Fig.36) while potassium oxalate showed depressing effects in the 2.5 percent concentration (Fig.35).

Variations in bacteria and Actinomyces are shown in Figures 37, 38, and 39. In calcium citrate, calcium lactate, and calcium tartrate treatments the counts increased in the first two to four weeks and fall off rather rapidly after that (Fig.37). In case of calcium citrate a decrease in numbers was noted after the first week. The potassium salts of oxalic acid, citric acid, and lactic acid, in quantities of 2.5 percent, greatly stimulated the development of bacteria and Actinomyces during the first four weeks (Fig.39). At the end of ten weeks, however, these counts approached those

of the untreated soil indicating the exhaustion of energy supply or accumulation of toxic substances. Similar results were noted in this series as well as in some of the previous series.

Series 6

In series 6 the effects of calcium and potassium salts of various fatty acids on the distribution of Azotobacter in soil were studied. The salts included in this series were calcium and potassium propionate, calcium and potassium palmitate, calcium and potassium butyrate, and potassium acetate. The usual determinations were made according to directions given under Methods and Procedure.

The distribution of Azotobacter in treated and untreated soils is shown in Tables 12, 13, and 16. Variations in soil reactions are given graphically in Figures 41 and 48. Of the salts tested calcium butyrate appeared to be most favorable for the development of Az. vinelandii and Az. chroococcum. One percent of calcium butyrate gave somewhat higher Az. vinelandii counts than 1.0 percent of cinnamic acid. However, the 2.5 percent cinnamic acid treatment gave the highest count obtained in all six series (Table 15). Potassium butyrate was less effective than the calcium salt of the same acid.

Calcium and potassium propionate appeared to be next best in this series in stimulating Az. vine-

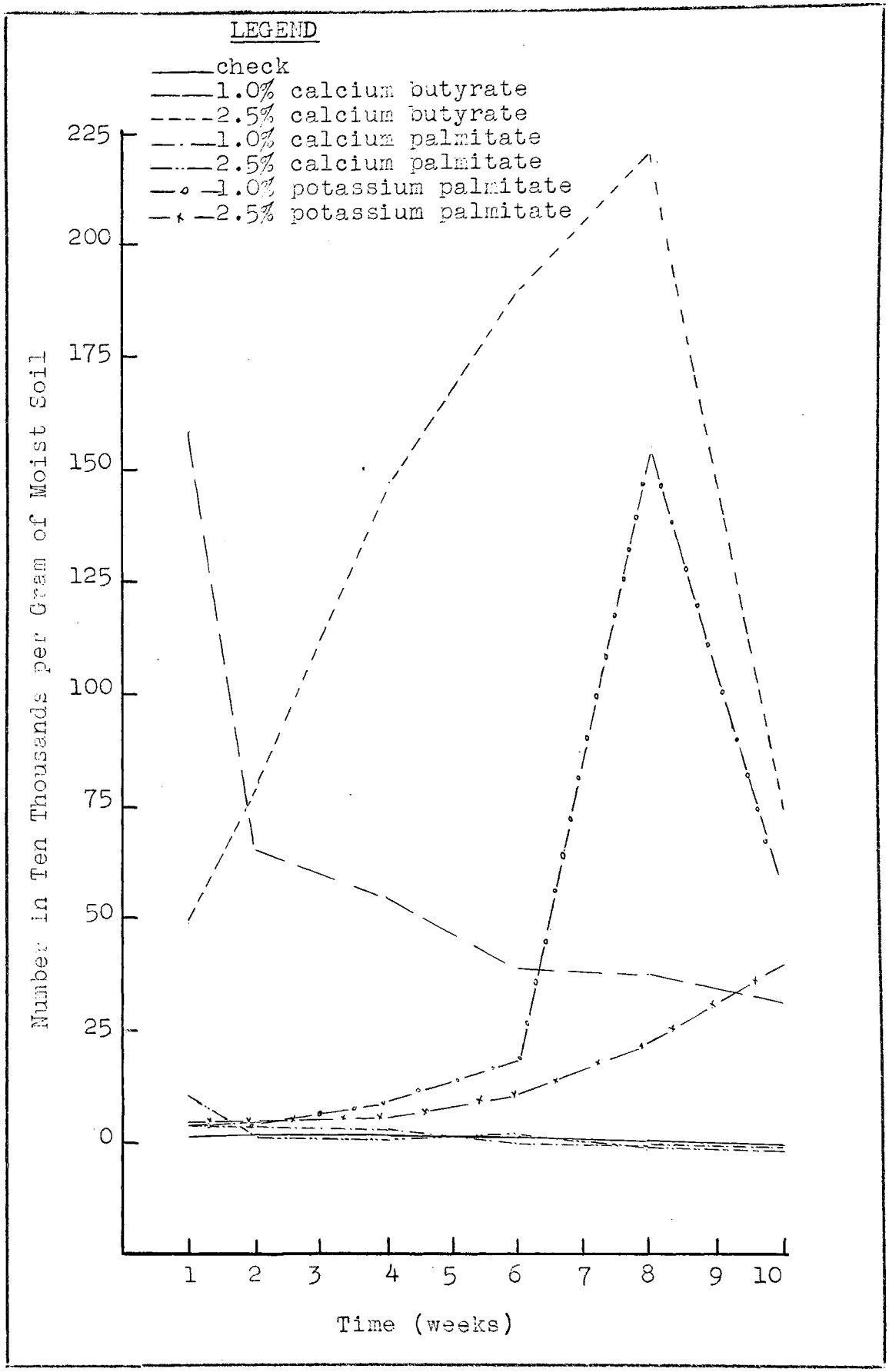

Fig. 42 Variation in Numbers of Molds in Treated Soil. Series 6

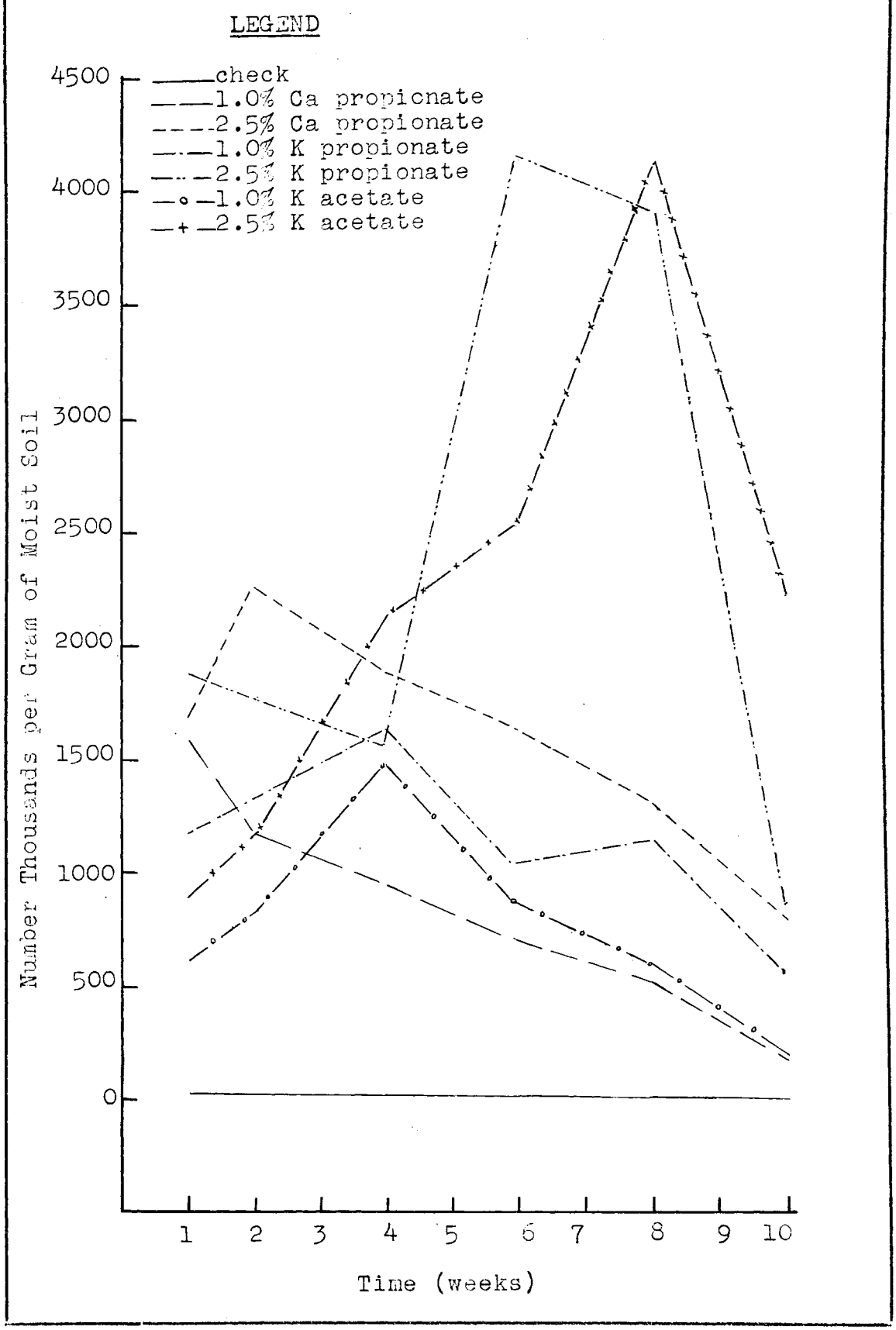

Fig. 43 Variation in Numbers of Molds in Treated Soil. Series 6

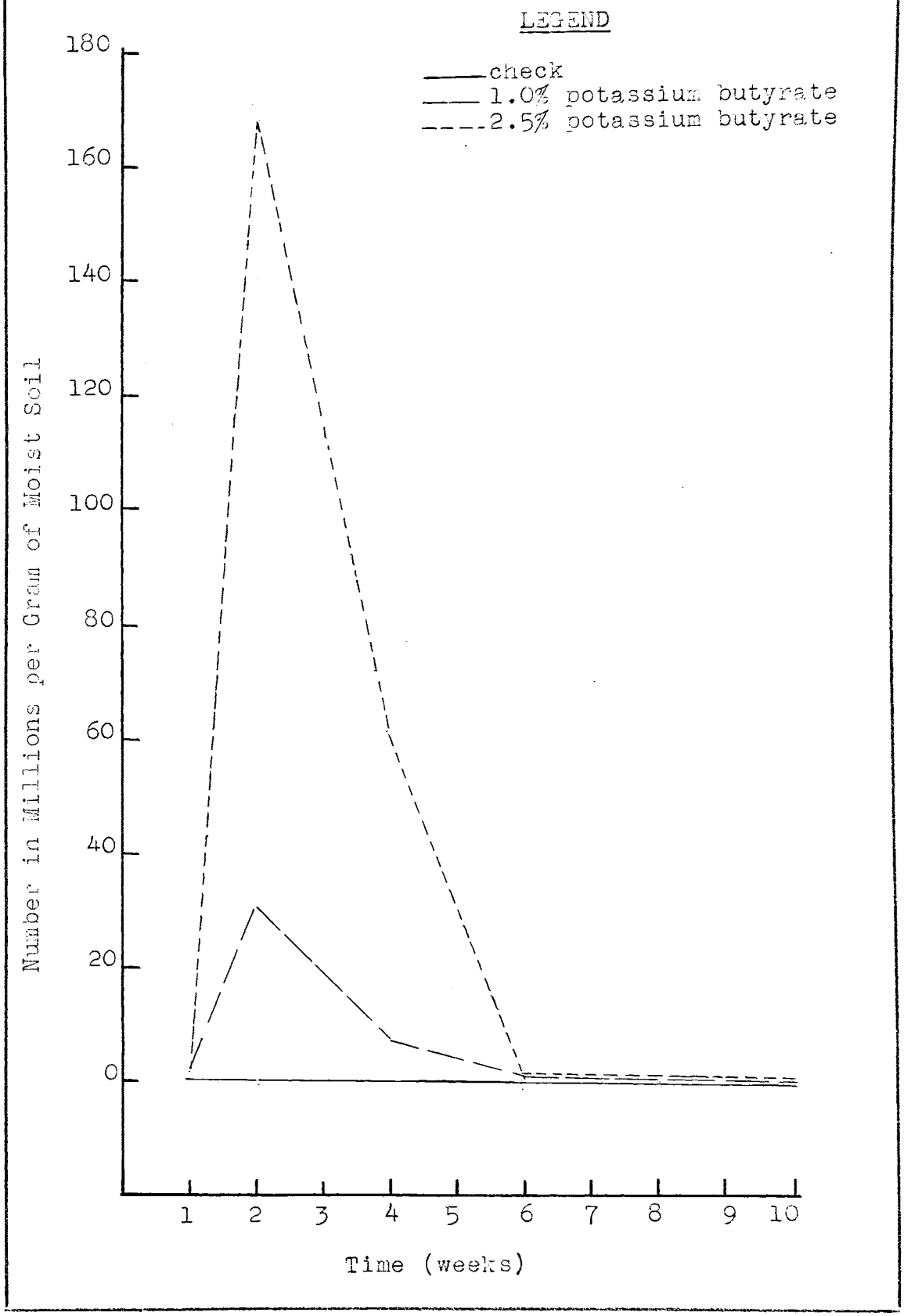

Fig. 44 Variation in Numbers of Molds in Treated Soil. Series 6

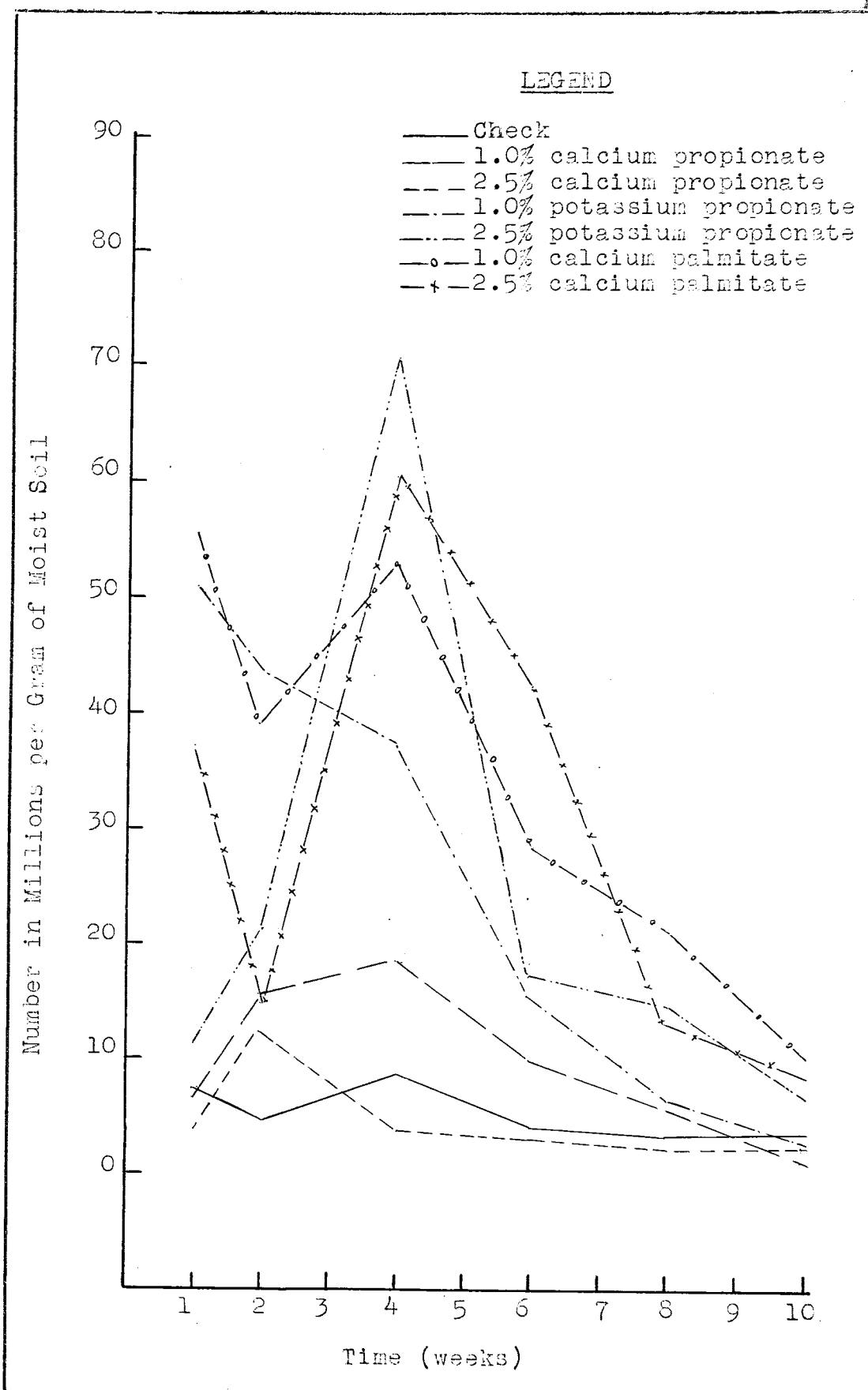

Fig. 45 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 6

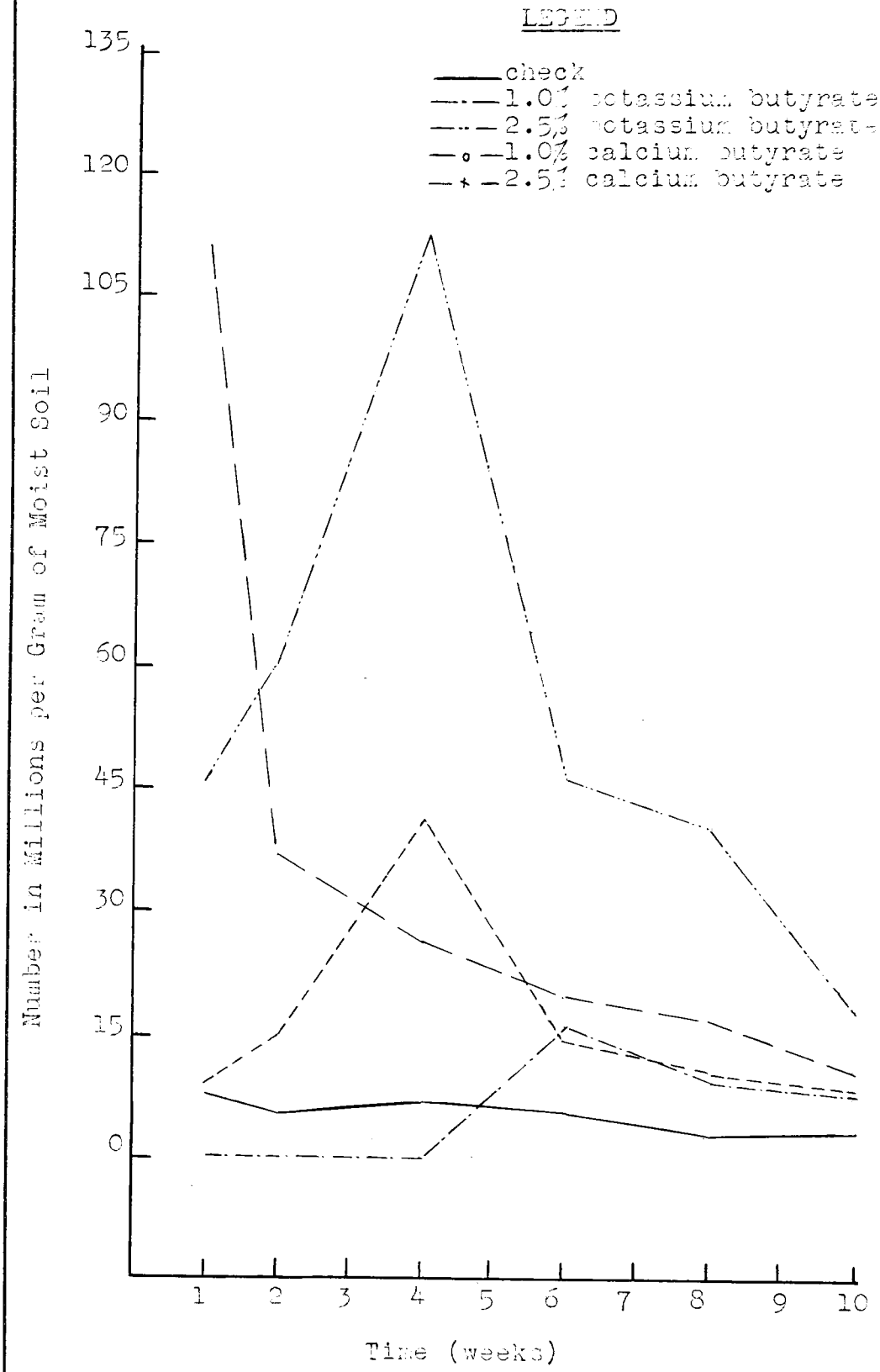

Fig. 46 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 6

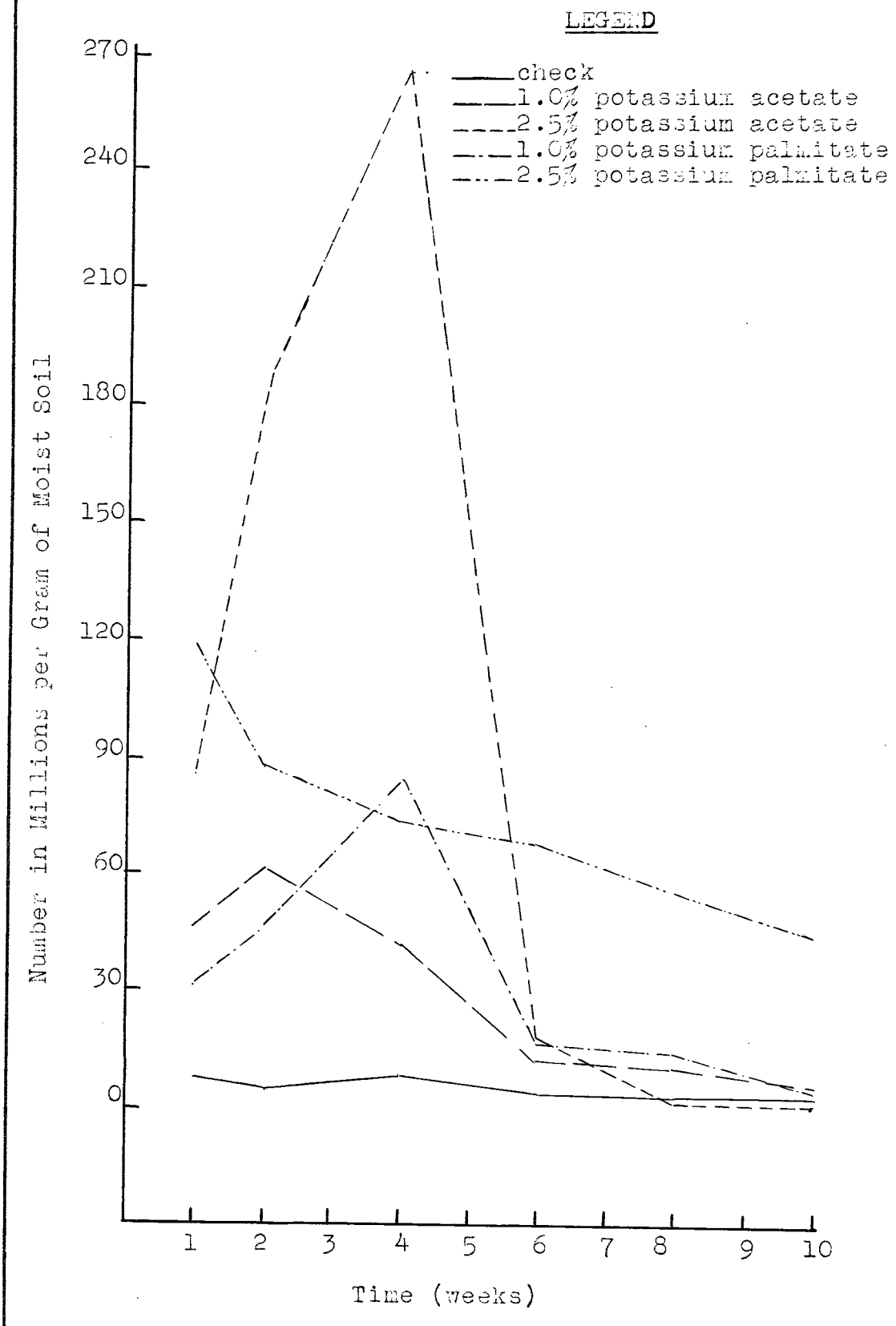

Fig. 47 Variation in Numbers of Bacteria and Actinomycetes in Treated Soil. Series 6

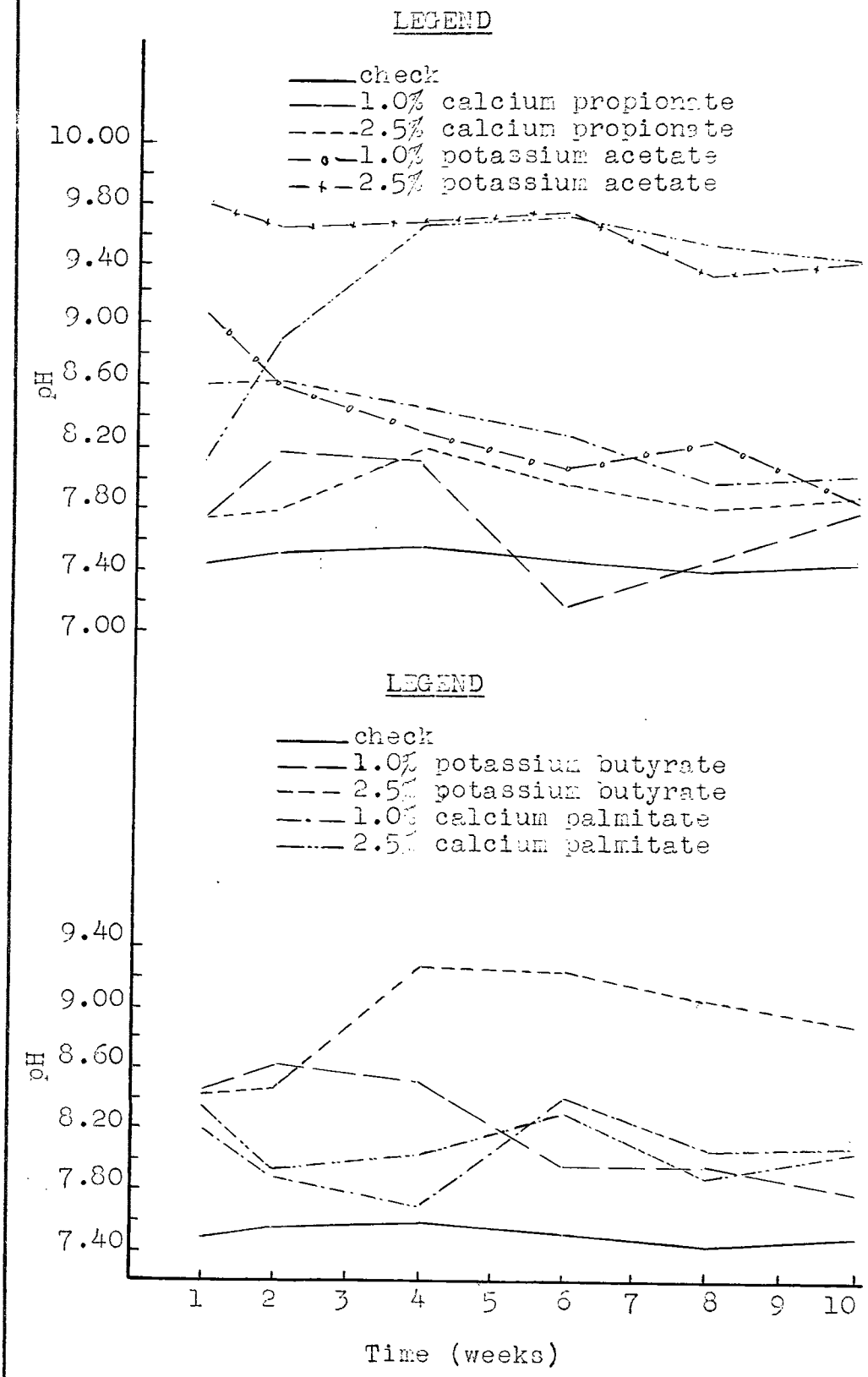

Fig. 48 Variation in pH values in Treated Soils. Series 6

TABLE 12--AZOTOBACTER IN TREATED SOIL SERIES 6
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% calcium palmitate	vinelandii	700	1.3*	1.2*	1.8*	650	300
	chroococcum	1.0*	2.3*	5.0*	20.6*	2.8*	750
2.5% calcium palmitate	vinelandii	400	15.1*	2.3*	1.9*	150	870
	chroococcum	120	36.0*	57.5*	12.1*	1.2*	6.0*
1.0% potassium palmitate	vinelandii	2.0*	4.5*	1.1*	200	300	250
	chroococcum	2.2*	5.0*	2.6*	1.9*	1.8*	1.2*
2.5% potassium palmitate	vinelandii	400	2.3*	150	110	160	120
	chroococcum	3.1*	2.5*	1.6*	810	300	600
check	vinelandii	1.8*	2.4*	780	200	320	600
	chroococcum	2.2*	2.1*	4.3*	5.1*	4.0*	2.0*

*Numbers in thousands

TABLE 13--AZOTOBACTER IN TREATED SOIL SERIES 6
(Average Counts per Gram of Moist Soil)

Treatment	Azotobacter	Time (weeks) 1	2	4	6	8	10
1.0% calcium propionate	vinelandii	26.1*	180.0*	1.2**	5.9**	2.3**	1.4**
	chroococcum	76.0*	680.0*	1.1**	960.0*	831.0*	620.0*
2.5% calcium propionate	vinelandii	0	0	22.1*	870.0*	16.3**	320.0*
	chroococcum	30	1.2*	145.0*	420.0*	4.3**	720.0*
1.0% potassium propionate	vinelandii	29.1*	250.0*	450.0*	4.2**	22.0**	2.3**
	chroococcum	18.0*	67.0*	2.1**	2.8**	3.6**	380.0*
2.5% potassium propionate	vinelandii	200	4.3*	310	0	0	0
	chroococcum	1.8*	12.1*	1.6*	200	40	0
1.0% calcium butyrate	vinelandii	620.0*	1.9**	2.1**	230.0**	13.5**	18.2**
	chroococcum	960.0*	3.5**	6.1**	4.7**	5.2**	800.0*
2.5% calcium butyrate	vinelandii	32.0*	1.1**	4.2**	25.1**	6.8**	2.5**
	chroococcum	270.0*	890.0*	1.5**	3.1**	2.3**	1.2**
1.0% potassium butyrate	vinelandii	12.0*	83.0*	1.8**	5.3**	3.8**	1.8**
	chroococcum	86.1*	130.0*	750.0*	4.1**	2.9**	450.0*
2.5% potassium butyrate	vinelandii	500	37.0*	680.0*	2.6**	6.9**	620.0*
	chroococcum	1.2*	108.0*	990.0*	670.0*	520.0*	490.0*
1.0% potassium acetate	vinelandii	30	1.0*	1.0*	1.0*	300.0*	120.0*
	chroococcum	4.6*	91.0*	560.0*	2.5**	1.4**	1.2**
2.5% potassium acetate	vinelandii	0	1.0*	0	0	0	0
	chroococcum	1.6*	2300*	180.0*	0	0	0
check	vinelandii	1.8*	2.4*	780	200	320	600
	chroococcum	2.2*	2.1*	4.3*	5.1*	4.0*	2.0*

* Numbers in thousands
** Numbers in millions

landii (Table 16). Potassium propionate in 1.0 percent concentration was more desirable than calcium propionate in 2.5 percent concentration. However, the 2.5 percent potassium propionate treatment brought about a soil reaction detrimental to Az. vinelandii (Fig.48). It is evident from Table 13 and Fig. 48 that as the soil reaction became more akaline, the development of Azotobacter retarded more and more until finally Azotobacter disappeared entirely from the soil. At the end of six weeks when the pH was 9.78 there were still 200 Az. chroococcum cells per gram of soil but no Az. vinelandii cells.

The distribution of molds is illustrated graphically in Figures 42, 43, and 44. In this series potassium butyrate was most effective in increasing the mold counts in the soil (Fig.44). Two and five-tenths percent of this salt was more effective than 1.0 percent, but the opposite was true in case of potassium palmitate (Fig.42). Molds appeared to be able to withstand high alkaline reaction more than Azotobacter since they developed readily in the potassium propionate treated soil (Fig.43).

Figures 45, 46, and 47 show that bacteria and Actinomyces were stimulated to develop far above the numbers present in the untreated soil in the presence of the majority of chemicals used in this series. There was

TABLE 14--AVERAGES OF AZTOBACTER COUNTS PER GRAM OF MOIST SOIL

Treatment	Gain or Loss During Incubation Az. vinelandii	Az. chroococcum
1.0% mannite	6,983	80,114
3.0% mannite	1,029,858	981,114
5.0% mannite	1,261,498	43,114
1.0% glycerol	710,998	342,214
3.0% glycerol	3,301,952	913,828
5.0% glycerol	-136	-280
1.0% methyl alcohol	0	574
3.0% methyl alcohol	0	-282
5.0% methyl alcohol	0	0
1.0% ethyl alcohol	0	-28
3.0% ethyl alcohol	0	-280
5.0% ethyl alcohol	0	0
1.0% hexyl alcohol	0	0
3.0% hexyl alcohol	0	0
5.0% hexyl alcohol	0	0
1.0% n-propyl alcohol	0	0
3.0% n-propyl alcohol	0	0
5.0% n-propyl alcohol	0	0
1.0% amyl alcohol	0	0
3.0% amyl alcohol	0	0
5.0% amyl alcohol	0	00
1.0% glucose	78,793	2,009,350
2.5% glucose	3,569,016	11,758,017
1.0% galactose	-225	1,471,350
2.5% galactose	2,715,000	5,329,683
1.0% mannose	14,150	6,650
2.5% mannose	85,516	182,017
1.0% fructose	577,133	1,683,017
2.5% fructose	554,000	2,483,017
1.0% raffinose	60,966	1,093,017
2.5% raffinose	1,434,633	2,879,683
1.0% lactose	0	-1,444
2.5% lactose	600	16,936
1.0% inulin	-335	-380
2.5% inulin	7,427	666,453
1.0% sucrose	2,093	9,603
2.5% sucrose	168,227	378,786

* Check for Az. vinelandii and Az. chroococcum
mannite to amyl alcohol - 142, 286,
glucose to raffinose - 367, 3,650,
lactose to sucrose - 440, 1,547

TABLE 15--AVERAGES OF AZTOBACTER COUNTS PER GRAM OF MOIST SOIL

Treatment*	Gain of Loss During Incubation	
	Az. vinelandii	Az. chroococcum
1.0% maltose	1,413	8,053
2.5% maltose	258,727	343,453
1.0% dextrin	120	12,586
2.5% dextrin	15,572	24,153
1.0% starch	-165	-1,000
2.5% starch	-433	5,570
1.0% quinol	0	0
2.5% quinol	0	0
1.0% benzene	0	0
2.5% benzene	0	0
1.0% toluene	0	0
2.5% toluene	0	0
1.0% phenol	0	0
2.5% phenol	0	0
1.0% resorcinol	0	0
2.5% resorcinol	0	0
1.0% pyrogallol	0	0
2.5% pyrogallol	0	0
1.0% phloroglucinol	0	0
2.5% phloroglucinol	0	0
1.0% thymol	0	0
2.5% thymol	0	0
1.0% tannic acid	-2,355	-1,480
2.5% tannic acid	321,783	2,529,284
1.0% KH phthalate	2,538	427
2.5% KH phthalate	-287	1,845
1.0% salicylic acid	-21843	-3,295
2.5% salicylic acid	0	-3,223
1.0% gallic acid	0	-3,375
2.5% gallic acid	0	0
1.0% cinnamic acid	37,867,150	0
2.5% cinnamic acid	86,828,650	0

*Check for Az. vinelandii and Az. chroococcum;
maltose to starch- 440, 1,547,
quinol to thymol- 2,633, 5,017,
tannic acid to cinnamic acid- 2,850, 3,383.

TABLE 16--AVERAGES OF AZTOBACTER COUNTS PER GRAM OF MOIST SOIL

Treatment	Gain or Loss During Incubation	
	Az. vinelandii	Az. chroococcum
2.0% sodium benzoate	0	0
3.0% sodium benzoate	0	0
5.0% sodium benzoate	0	0
2.0% benzoic acid	0	0
3.0% benzoic acid	0	0
5.0% benzoic acid	0	0
2.0% calcium benzoate	389,956	242,683
3.0% calcium benzoate	136,472	169,900
5.0% calcium benzoate	245,023	4,350
1.0% calcium lactate	0	1,445,483
2.5% calcium lactate	1,566,547	4,883,567
1.0% potassium lactate	239,547	2,773,834
2.5% potassium lactate	13,870	941,400
1.0% calcium tartrate	49,703	1,661,617
2.5% calcium tartrate	1,066,037	1,885,234
1.0% potassium tartrate	203,803	499,950
2.5% potassium tartrate	1,500	18,300
1.0% potassium malate	142,320	653,734
2.5% potassium malate	473	3,887
1.0% calcium citrate	17,670	845,567
2.5% calcium citrate	71,886	1,850,534
1.0% potassium citrate	75,103	592,950
2.5% potassium citrate	0	-1,310
1.0% potassium oxalate	-103	-1,258
2.5% potassium oxalate	0	0
1.0% calcium palmitate	-25	2,125
2.5% calcium palmitate	2,436	15,537
1.0% potassium palmitate	376	833
2.5% potassium palmitate	-477	-1,798
1.0% calcium propionate	1,833,333	709,550
2.5% calcium propionate	2,917,666	927,755
1.0% potassium propionate	4,870,500	1,490,884
2.5% potassium propionate	-215	-660
1.0% calcium butyrate	44,385,650	3,540,050
2.5% calcium butyrate	6,620,983	1,540,050
1.0% potassium butyrate	2,130,983	1,399,400
2.5% potassium butyrate	1,805,233	459,917
1.0% potassium acetate	69,488	955,984
2.5% potassium acetate	-850	30,817

*Check for Az. vinelandii and Az. chroococcum;
benzoates- 577, 650,
Ca lactate to K oxalate- 130, 1,433,
Ca palmitate to K acetate- 1,017, 3,283.

however, after a period of stimulation a decline in bacterial numbers. In some cases the bacterial counts dropped even below those of the check, as in the case of calcium and potassium propionate (1.0 percent), Figure 45, and potassium acetate (2.5 percent), Figure 47.

Series 7

The experiments in series 7 were performed with the intention of determining the number of Az. vinelandii cells that must be present in untreated and treated soil in order that they may be isolated by means of the agar-plate method.

As a source of carbon 1.0 percent sucrose and 0.5 percent cinnamic acid were employed. A prepared dilution of a 48-hour agar-slant culture of Az. vinelandii, containing 318 organisms per milliliter as determined by the agar-plate method, was added in quantities of one milliliter to soils of the following weights: 50, 100, 300, 600, 1,000, 2,000, and 4,000 grams. In this manner soils containing different numbers of Az. vinelandii per gram of soil were prepared. All treatments were duplicated and arranged in the following manner:

Table 17 - treatment 1. 1.5% sucrose.

- treatment 2. 1.5% sucrose and Az. vinelandii

TABLE 17--AZOTOBACTER IN TREATED SOIL SERIES 7
(Average Counts per Gram of Moist Soil)

Grams of Soil Treated	Azotobacter	Time (weeks) 1	2	3
Treatment 1. 1.5% sucrose				
50	vinelandii	0	0	0
	chroococcum	1.5**	12.4**	8.6**
100	vinelandii	0	0	0
	chroococcum	1.6**	8.8**	9.7**
300	vinelandii	0	0	0
	chroococcum	1.7**	6.8**	1.1**
600	vinelandii	0	0	0
	chroococcum	1.8**	6.0**	5.7**
1,000	vinelandii	0	0	0
	chroococcum	1.6**	8.9**	5.2**
2,000	vinelandii	0	0	0
	chroococcum	810.0*	7.1**	8.8**
4,000	vinelandii	0	0	0
	chroococcum	980.0*	7.5**	8.1**
Treatment 2. 1.5% sucrose and Az. vinelandii				
50	vinelandii	110.0*	1.5**	1.2**
	chroococcum	900.0*	9.1**	14.6**
100	vinelandii	470.0*	3.0**	1.5**
	chroococcum	1.3**	12.4**	18.3**
300	vinelandii	190.0*	1.2**	1.8**
	chroococcum	980.0*	2.9**	5.7**
600	vinelandii	800.0*	1.5**	1.2**
	chroococcum	1.2**	3.6**	12.4**
1,000	vinelandii	700.0*	400.0*	1.0**
	chroococcum	1.9**	1.8**	11.9**
2,000	vinelandii	600.0*	1.9**	1.0**
	chroococcum	1.7**	2200**	31.6**
4,000	vinelandii	480.0*	1.1**	1.0**
	chroococcum	1.1**	4.5**	16.9**
Treatment 3. Az. vinelandii				
50	vinelandii	0	0	0
	chroococcum	1.2*	360	830
100	vinelandii	0	0	0
	chroococcum	600	290	700
300	vinelandii	0	0	0
	chroococcum	740	220	640
600	vinelandii	30	0	0
	chroococcum	660	440	550
1,000	vinelandii	20	0	0
	chroococcum	980	370	490
2,000	vinelandii	0	0	0
	chroococcum	480	480	450
4,000	vinelandii	0	0	0
	chroococcum	450	350	400

*Numbers in thousands
**Numbers in millions

TABLE 18--AZOTOBACTER IN TREATED SOIL SERIES 7
(Average Counts per Gram of Moist Soil)

Grams of Soil Treated	Azotobacter	Time (weeks) 1	2	3
Treatment 1. 0.5% Cinnamic Acid				
50	vinelandii	0	0	0
	chroococcum	3.1**	4.9**	5.8**
100	vinelandii	0	0	0
	chroococcum	3.6**	7.2**	7.1**
300	vinelandii	0	0	0
	chroococcum	2.2**	5.9**	5.2**
600	vinelandii	0	0	0
	chroococcum	2.8**	4.1**	4.9**
1,000	vinelandii	0	0	0
	chroococcum	2.5**	3.6**	5.5**
2,000	vinelandii	0	0	0
	chroococcum	3.7**	6.2**	8.3**
4,000	vinelandii	0	0	0
	chroococcum	2.9**	5.5**	4.0**
Treatment 2. 0.5% Cinnamic Acid and Az. vinelandii				
50	vinelandii	3.8**	2.2**	200.0*
	chroococcum	3.3**	4.0**	2.8**
100	vinelandii	4.0**	3.1**	1.3**
	chroococcum	2.6**	3.1**	7.2**
300	vinelandii	2.9**	3.6**	1.5**
	chroococcum	2.2**	4.2**	7.1**
600	vinelandii	2.6**	2.7**	780.0*
	chroococcum	3.7**	7.0**	5.3**
1,000	vinelandii	3.4**	4.7**	900.0*
	chroococcum	2.4**	1.0**	4.7**
2,000	vinelandii	1.2**	1.1**	200.0*
	chroococcum	2.6**	3.7**	2.9**
4,000	vinelandii	450.0*	2.5**	240.0*
	chroococcum	8.6**	1.4**	2.6**
Treatment 3. Az. vinelandii				
50	vinelandii	0	0	0
	chroococcum	1.2*	360	830
100	vinelandii	0	0	0
	chroococcum	600	290	700
300	vinelandii	0	0	0
	chroococcum	740	220	640
600	vinelandii	30	0	0
	chroococcum	660	440	550
1,000	vinelandii	20	0	0
	chroococcum	980	370	490
2,000	vinelandii	0	0	0
	chroococcum	480	480	450
4,000	vinelandii	0	0	0
	chroococcum	450	350	400

*Numbers in thousands
**Numbers in millions

Table 18 - treatment 1. 0.5% cinnamic acid

- treatment 2. 0.5% cinnamic acid and *Az. vinelandii*

- treatment 3. *Az. vinelandii*

The experiments were carried out as directed under Methods and Procedure. The determinations for *Az. vinelandii* and *Az. chroococcum* were made once a week for a period of three weeks.

In treatment 1 (Table 17) only sucrose was added to the soil in order to have a check on treatment 2 which received both sucrose and *Az. vinelandii*. It was felt if *Az. vinelandii* were present in the soil and if it were possible to isolate it by sucrose treatment, then the error which might creep into treatment 2, because of this, might be eliminated. However, as shown in Table 17, *Az. vinelandii* did not appear in treatment 1.

Treatment 2 (Table 17), in which 1.5 percent sucrose and *Az. vinelandii* were added to the soil, show definitely that this organism may be isolated from soil containing from one organism for every 0.16 grams of soil to one organism for every 12.5 grams of soil.

Treatment 3, to which only *Az. vinelandii* was added, illustrates clearly the difficulties involved in isolating this organism from the untreated soil even if it is originally present in quantities as high as six organisms per gram of soil. Only in two instances from

the 600 and 1,000-gram treatments was it possible to isolate Az. vinelandii at the end of the first week. The duplicate treatments for the same soils did not show the presence of Az. vinelandii. Az. chroococcum, which was originally present in the soil, appeared in all subtreatments of treatment 3.

Treatment 1 (Table 18), in which the soil received only cinnamic acid (0.5 percent), was used as a check on treatment 2. It is evident from treatment 1 that Az. vinelandii was not present in the original soil or, if it was present, it was in such small numbers as to make its isolation impossible.

Treatment 2 (Table 18), in which cinnamic acid (0.5 percent) and Az. vinelandii were added to the soil, illustrates the value of this chemical in stimulating the growth of this organism and in making its isolation possible, even if the organism is present in the soil in quantities as low as one organism for every 12.5 grams of soil.

Although the amount of sucrose used was three times as great as that of cinnamic acid, the average Az. vinelandii counts were greater in the case of cinnamic acid treatment than in the case of sucrose treatment (Table 19).

Table 19--Averages of *Az. vinelandii* Counts. Series 7, Treatment 2

Treatment	*Az. vinelandii* in millions
0.5% cinnamic acid	2.065
1.5% sucrose	0.907

TABLE 20--AVERAGE NUMBER OF MICROORGANISMS FOR THE ENTIRE INCUBATION PERIOD

Treatment	Molds	Bacteria and Actinomyces	Treatment	Molds	Bacteria and Actinomyces
Check	23.2*	12.68**	1.0% fructose	109.0*	53.5**
1.0% mannite	67.8*	128.1**	2.5% fructose	361.6*	213.8**
3.0% mannite	57.0*	309.0**	1.0% raffinose	77.5*	57.3**
5.0% mannite	1.4**	161.1**	2.5% raffinose	116.8*	188.4**
1.0% glycerol	48.6*	141.0**	1.0% lactose	50.7*	64.7**
3.0% glycerol	286.8*	297.6**	2.5% lactose	81.1*	144.7**
5.0% glycerol	3.4**	151.2**	1.0% inulin	622.5*	67.1**
1.0% methyl alcohol	45.9*	32.5**	2.5% inulin	756.8*	218.8**
3.0% methyl alcohol	19.1*	35.5**	1.0% sucrose	178.6*	57.5**
5.0% methyl alcohol	4.1**	27.3**	2.5% sucrose	360.8*	201.8**
1.0% ethyl alcohol	280.8*	49.1**	1.0% maltose	169.1*	41.6**
3.0% ethyl alcohol	55.5*	24.9**	2.5% maltose	846.6*	228.6**
5.0% ethyl alcohol	53.7*	11.8**	1.0% starch	85.5*	83.9**
1.0% hexyl alcohol	4.8*	3.4**	2.5% starch	251.6*	150.2**
3.0% hexyl alcohol	984	3.7**	1.0% dextrin	64.4*	49.3**
5.0% hexyl alcohol	474	4.7**	2.5% dextrin	165.0*	221.7**
1.0% n-propyl alchool	12.2*	24.5**	1.0% phenol	2.1*	97.0*
3.0% n-propyl alcohol	38	27.9**	2.5% phenol	1.6*	2.3*
5.0% n-propyl alcohol	6	7.0**	1.0% thymol	683	325.0*
1.0% amyl alcohol	3.1*	18.4**	2.5% thymol	73	465.8*
3.0% amyl alcohol	228	11.9**	1.0% benzene	3.4*	30.96**
5.0% amyl alcohol	566	5.7**	2.5% benzene	1.1*	37.5**
1.0% glucose	33.1*	42.1**	1.0% toluene	95.4*	28.24**
2.5% glucose	3.5*	157.8**	2.5% toluene	385.9*	23.3**
1.0% galactose	129.6*	34.4**	1.0% pyrogallol	47.3*	17.5**
2.5% galactose	533.8*	142.6**	2.5% pyrogallol	3.2**	17.7**
1.0% mannose	109.0*	62.9**	1.0% phloroglucinol	1.3**	56.9**
2.5% mannose	361.7*	231.7**	2.5% phloroglucinol	3.3**	49.1**

*Numbers in thousands
**Numbers in millions

TABLE 21--AVERAGE NUMBER OF MICROORGANISMS FOR THE ENTIRE INCUBATION PERIOD

Treatment	Molds	Bacteria and Actinomyces
1.0% resorcinol	1.1**	79.0*
2.5% resorcinol	1.2*	134.6*
1.0% quinol	4.7*	3.5**
2.5% quinol	31.4*	3.8**
1.0% tannic acid	401.8*	43.9**
2.5% tannic acid	1.69**	90.2**
1.0% KH phthalate	17.3*	71.3**
2.5% KH phthalate	14.4*	259.4**
1.0% salicylic acid	1.8**	50.3**
2.5% salicylic acid	1.4**	169.5**
1.0% gallic acid	1.2**	30.4**
2.5% gallic acid	7.9**	86.9**
1.0% cinnamic acid	246.4*	62.7**
2.5% cinnamic acid	354.2*	161.1**
2.0% sodium benzoate	4.5**	68.3**
3.0% sodium benzoate	4.38**	12.8**
5.0% socium benzoate	1.27**	8.4**
2.0% calcium benzoate	1.48**	15.87**
3.0% calcium benzoate	944.7*	13.57**
5.0% calcium benzoate	678.5*	17.74**
2.0% benzoic acid	2.1**	9.02**
3.0% benzoic acid	3.5*	8.46**
5.0% benzoic acid	1.7*	5.17**
1.0% K oxalate	29.9*	27.28**
2.5% K oxalate	13.4*	148.5**
1.0% K malate	46.87*	75.28**
2.5% K malate	108.3*	261.0**

Treatment	Molds	Bacteria and Actinomyces
Check	23.2*	12.68**
1.0% Ca lactate	48.52*	21.35**
2.5% Ca lactate	52.6*	44.3**
1.0% K lactate	153.2*	47.8**
2.5% K lactate	2.3**	220.75**
1.0% Ca tartrate	23.4*	26.2**
2.5% Ca tartrate	29.65**	
1.0% K tartrate	44.35*	26.2**
2.5% K tartrate	678.3*	260.4**
1.0% Ca citrate	25.3*	19.69**
2.5% Ca citrate	11.98*	26.49**
1.0% K citrate	25.3*	35.48**
2.5% K citrate	11.98*	26.48**
1.0% Ca palmitate	20.9*	35.4**
2.5% Ca palmitate	26.3*	30.3**
1.0% K palmitate	429.7*	34.3**
2.5% K palmitate	142.4*	76.3**
1.0% Ca propionate	885.8*	10.1**
2.5% Ca propionate	1.6**	5.2**
1.0% K propionate	1.2**	26.9**
2.5% K propionate	4.2**	24.7**
1.0% Ca butyrate	655.0*	38.3**
2.5% Ca butyrate	1.3**	17.1**
1.0% K butyrate	7.6**	6.1**
2.5% K butyrate	40.1**	55.4**
1.0% K acetate	791.7*	31.4**
2.5% K acetate	2.2**	95.7**

* Numbers in thousands

** Numbers in millions

PART 2

Series 1

It was the purpose in Part 2 of this paper to study the distribution of *Az. vinelandii* in some Colorado soils and waters. The methods of determination were based on the results obtained from Part 1 of this paper and on the previous work on this subject (37). In all cases the object was to stimulate the development of *Az. vinelandii* to such an extent as to make the isolation of this organism possible by the agar-plate method.

The soils in series 1 were collected in the vicinity of Fort Collins in the fall of 1939. The pH and the Azotobacter content of these soils were determined in the manner described under Methods and Procedure.

After the preliminary determinations the soils were subjected to the agar-plate and the enrichment-culture methods, previously described, for the isolation of *Az. vinelandii*. In both cases sodium benzoate (0.2 percent) was used as the source of carbon. In the first method a one to ten dilution of soil was used, and in the second method ten grams of soil were placed in eighty milliliters of media.

Negative results were obtained with the two methods in all but two cases, Nos. 11 and 12, Table 22. The experiments for these two soils were repeated a month later with the same results. This time, however, only the agar-plate method was used with the following concentrations of sodium benzoate: 0.002, 0.02, 0.05, 0.3, 0.5, and 1.0 percent. After seven days of incubation, Az. vinelandii colonies were found on the plates containing the following concentrations of sodium benzoate: 0.02, 0.1, 0.3, and 0.5 percent. Sodium benzoate in quantities of 1.0 percent appears to be too high, and 0.002 percent too low.

Isolation of Az. vinelandii from soils Nos. 11 and 12 by the use of the agar-plate method and the enrichment-culture method was attempted again in March of 1940 but with negative results. Negative results were also obtained in the summer of 1940.

TABLE 22--DISTRIBUTION OF AZOTOBACTER* IN SOILS SAMPLED IN THE VICINITY OF FORT COLLINS

Soil No.	Soil Type	pH	Land Uses	Az. per gram of moist soil
1	Larimer loam	6.95	Virgin pasture	0
2	Larimer loam	7.58	Orchard	50
3	Fort Collins loam	7.20	Sugar beet field	140
4	Fort Collins clay loam	7.58	Wheat field	90
5	Weld loam	7.30	Sugar beet field	120
6	Cass clay loam	7.60	Corn field	40
7	Weld fine sandy loam	7.82	Wheat field	110
8	Cass silt loam	7.55	Corn field	30
9	Greeley fine sandy loam	7.52	Corn field	10
10	Cass fine sandy loam	7.58	Corn field	50
11	Fort Collins loam (Ag. Exp. Sta. Farm)	7.63	Wheat field	260
12	Fort Collins loam (Lawn, College Campus)	7.20	Lawn	110

*Determinations by agar-plate method with sucrose as source of carbon.

Series 2

The soils in series 2 were collected from various parts of eastern Colorado in May of 1940. After the preliminary determinations, given in Tables 23 and 24, the soils were treated by the enrichment-culture

TABLE 23--DISTRIBUTION OF AZOTOBACTER IN SOILS SAMPLED IN EASTERN COLORADO

Soil No.	Soil	Locality	Land Use	pH	Azotobacter* per Gram of Moist Soil
13	Fort Collins loam	Pierce	Grazing	7.52	0
14	Decker loam	Nunn	Grazing	7.20	0
15	Nunn sandy loam	Nunn	Grazing	7.30	0
16	Kaota silty clay loam	East of Carr	Grazing	7.60	0
17	Stoneham silt loam	West of Gault	Grazing	7.73	0
18	Cheyenne gravelly loam	East of Gault	Cultivated	7.40	10
19	Cheyenne loam	Hereford	Cultivated	7.90	40
20	Rosebud loam	North of Kauffman	Grazing	7.40	0
21	Rosebud loam	East of Vim	Cultivated	7.43	0
22	Laurel silty clay loam	South of Peetz	Grazing	8.38	2,100
23	Dune sand	East of Sterling	Idle land	7.30	0
24	Otis loamy sand	East of Sterline	Cultivated	6.50	0
25	Denova sandy loam	North of Hextun	Cultivated	7.28	140
26	Dawes silt loam	Paoili	Cultivated	6.35	10
27	Goshen silty clay loam	South of Wray	Cultivated	7.48	300
28	Keith silt loam	North of Vernon	Grazing	7.00	20
29	Colby silt loam	South of Newton	Cultivated	7.88	200
30	Scott clay loam	North of Burlington	Idle land	7.54	130
31	Weld silt loam	West of Cole	Grazing	7.51	0
32	Prowers silt loam	North of Wiley	Grazing	8.02	10
33	Baca silty clay loam	South of Verdun	Grazing	8.16	0
34	Wind blown ares	Edler	Idle land	7.82	0
35	Denver silty clay loam	West of Alcreek	Grazing	8.14	10
36	Colby fine sandy loam	North of Branson	Grazing	8.16	0
37	Not mapped	East of Trinchera	Grazing	8.21	10
38	Not mapped	East of Trinidad	Grazing	8.50	0
39	Organic soil	East of La Veta	Woods	7.21	0

*Determined by agar-plate method and enrichment culture method

TABLE 24--DISTRIBUTION OF AZOTOBACTER IN SOILS SAMPLED IN EASTERN COLORADO

Soil No.	Soil	Locality	Land Use	pH	Azotobacter* per Gram of Moist Soil
40	Saline soil	Blanca	Idle land	9.45	2,060
41	Saline soil	West of Blanca	Idle land	9.50	3,050
42	Sand	Great Sand Dunes	Idle land	8.65	10
43	Saline soil	North of Alamosa	Idle land	10.18	0
44	Saline soil	North of Alamosa	Idle land	9.23	320
45	Saline soil	North of Corlett	Idle land	10.00	6,500
46	Saline soil	North of Center	Cultivated	9.65	8,000
47	Minnequa silty clay loam	Penrose	Grazing	8.80	0
48	Not mapped	Colorado Springs	Cultivated	7.55	1,600
49	Not mapped	Buttes	Grazing	7.70	0
50	Not mapped	East of Colorado Springs	Cultivated	7.55	1,600
51	Not mapped	East of Colorado Springs	Cultivated	8.00	20
52	Not mapped	East of Colorado Springs	Cultivated	7.42	50
53	Not mapped	East of Colorado Springs	Grazing	8.12	0
54	El Paso loamy sand	North of Falcon	Grazing	7.70	0
55	Cass sandy loam	Eastonville	Grazing	7.73	0
56	Falcon sandy gravelly loam	East of Black Forest	Cultivated	7.40	0
57	Falcon sandy gravelly loam	East of Black Forest	Grazing	7.37	0
58	Falcon sandy loam	East of Black Forest	Grazing	7.52	0
59	Falcon sandy loam	East of Black Forest	Grazing	7.22	0
60	Wabash sandy clay loam	Black Forest	Idle land	6.10	10
61	Wabash sandy clay loam	Black Forest	Idle land	6.08	10
62	Not mapped	Black Forest	Pine woods	6.02	0
63	Podzolic soil	Black Forest	Pine woods	6.52	10
64	Not mapped	West of Black Forest	Grazing	6.58	0
65	Not mapped	Black Forest	Pine woods	6.53	0
66	Neville silty clay loam	South of Denver	Idle land	7.01	0
67	Table Mountain loam	South of Denver	Idle land	5.28	0

*Determined by agar-plate method and enrichment culture method

method and the soil-enrichment method as described under Methods and Procedure.

The data on the general Azotobacter population , determined by the agar-plate method with sucrose as the source of carbon, show that out of 55 soils tested only 25 contained Azotobacter. The pH, however, was favorable for Azotobacter development in all soils but one, No. 67 (Table 24). From Table 24 it is evident that several of the soils from the San Luis Valley had high Azotobacter counts. These soils are alkaline in nature with high pH values. Because of the small number of samples collected it would be hazardous to make any predictions concerning the factors that may influence Azotobacter distribution. It is of interest to note that Azotobacter was found in the pdozolic soil and in the organic soil of the Black Forest (Tables 23 and 24).

In the enrichment-culture method sodium benzoate (0.2 percent) and sucrose (2.0 percent) were used as the sources of carbon. The latter was used as a check on the former. In both cases ten grams of soil were added to sixty milliliters of media. Examination of the treatments by the agar-plate method showed that Az. vinelandii was not present.

The soil culture method likewise proved unsuccessful. In this case both benzoic acid (2.0 percent) and calcium carbonate (2.0 percent) were added to the

soil, the latter to keep the soil from becoming too acid. Several soils were also treated only with benzoic acid. Determinations for Az. vinelandii, once every two weeks for a period of six weeks showed that where benzoic acid and calcium carbonate were used the pH remained above 7.0 throughout the entire incubation period. However, where benzoic acid alone was used, the pH dropped below 6.0 except in a few instances where the soils were alkaline (Nos. 40, 41, 43, 44, 45, and 46, Table 24). Az. chroococcum multiplied rapidly in treated soils, except where the soil reaction became too acid. In that case the organism disappeared entirely. Az. vinelandii was not isolated from soil under any of these treatments.

Series 3

Soils numbered 68 to 174 inclusive, Tables 25, 26, and 27, were collected from various rotation plots located at the Experiment Station at Akron, Colorado. The rotation program for each plot is listed, the first-named crop being that raised in 1939, the second-named crop being that raised in 1940, and so on. The pH and the Azotobacter counts determined by the agar-plate method with sucrose as the source of carbon are also given.

It is evident from the tables that the soil reaction was sufficiently high in all cases for Azoto-

TABLE 25--DISTRIBUTION OF AZOTOBACTER IN SOILS FROM THE EXPERIMENT STATION AT AKRON

Soil No.	Plot No.	pH	Azotobacter per Gram of Moist Soil	1939 Crop
68	568 A	7.56	<10	Summer wheat
69	568 B	6.62	<10	Fallow
70	568 C	6.75	<10	Winter wheat
71	267 A	7.23	<10	Fallow
72	267 B	7.70	<10	Winter wheat
73	268 A	7.90	10	Fallow, manured
74	268 B	7.18	<10	Winter wheat
75	252 A	7.05	<10	Winter wheat
76	252 B	6.96	0	Corn
77	251 A	7.05	0	Winter wheat
78	251 B	7.52	10	Corn, manured
79	593 A	6.68	<10	Summer fallow
80	593 B	7.75	<10	Winter wheat
81	42 A	7.35	20	Alfalfa
82	42 B	7.30	35	Alfalfa
83	42 C	7.05	<10	Alfalfa
84	42 D	6.95	<10	Oats
85	42 E	7.02	<10	Corn
86	42 F	7.08	<10	Spring wheat
87	81 A	6.65	0	Winter wheat
88	81 B	7.75	<10	Fallow
89	81 C	6.92	0	Oats
90	81 D	7.00	<10	Corn
91	31 A	7.52	<10	Wheat
92	31 B	7.00	0	Sweet clover
93	31 C	6.95	<10	Oats
94	31 D	7.15	0	Corn
95	32 A	7.23	<10	Oats
96	32 B	7.20	<10	Sweet clover
97	32 C	7.02	0	Spring wheat
98	32 D	7.18	<10	Corn
99	58 A	6.92	0	Oats
100	58 B	7.16	<10	Spring wheat
101	58 C	6.61	20	Sorghum

*Determined by agar-plate method and enrichment culture method with sucrose as source of carbon.

TABLE 26--DISTRIBUTION OF AZOTOBACTER IN SOILS FROM THE EXPERIMENT STATION AT AKRON

Soil No.	Plot No.		pH	Azotobacter* per Gram of Moist Soil	1939 Crop
102		A**	7.08	10	Winter wheat, continuous
103		B	6.20	<10	Winter wheat, continuous
104		C	6.82	<10	Summer fallow
105		D	7.11	0	Winter wheat
106		E	7.01	<10	Winter wheat, continuous
107		F	7.00	<10	Winter wheat, continuous
108	20	A	7.15	<10	Oats
109	20	B	6.72	<10	Winter rye
110	20	C	6.55	<10	Winter wheat
111	20	D	7.30	<10	Corn
112		A***	6.90	30	Barley, continuous
113		B	6.65	10	Barley, continuous
114		C	7.10	<10	Summer fallow
115		D	6.95	0	Barley
116		E	7.35	0	Barley, continuous
117		F	6.35	10	Barley, continuous
118	26	A	7.00	<10	Winter wheat
119	26	B	6.88	0	Winter rye
120	26	C	8.12	<10	Oats
121	26	D	7.05	0	Corn
122	97	A	6.30	0	Winter wheat
123	97	B	6.82	0	Peas
124	97	C	6.30	<10	Oats
125	97	D	6.90	0	Corn
126		A****	7.42	<10	Oats, continuous
127		B	6.92	10	Oats, continuous
128		C	6.90	<10	Summer fallow
129		D	7.12	<10	Oats
130		E	6.92	0	Oats, continuous
131		F	7.30	0	Oats, continuous
132	28	A	7.22	0	Oats
133	28	B	7.12	<10	Fallow
134	28	C	7.00	<10	Winter wheat
135	28	D	6.73	<10	Corn

*Determined by agar-plate method and enrichment culture method with sucrose as source of carbon.
**Winter wheat series
***Barley series
****Oats series

TABLE 27--DISTRIBUTION OF AZOTOBACTER IN SOILS FROM THE EXPERIMENT STATION AT AKRON

Soil No.	Plot No.	pH	Azotobacter* per Gram of Moist Soil	1939 Crop
136	4 A	6.72	<10	Oats
137	4 B	6.85	<10	Spring wheat
138	4CC	7.25	<10	Corn
139	A**	7.40	20	Spring wheat, continuous
140	B	6.80	10	Spring wheat, continuous
141	C	7.45	10	Summer fallow
142	D	7.42	0	Spring wheat
143	E	7.45	10	Spring wheat, continuous
144	F	6.98	<10	Spring wheat, continuous
145	G	7.92	<10	Spring wheat, continuous
146	A***	6.60	<10	Sorghum, continuous
147	B	7.10	<10	Sorghum, continuous
148	95 A	6.38	0	Oats
149	95 B	6.82	<10	Peas
150	95 C	6.98	<10	Winter wheat
151	95 D	6.86	0	Sorgo
152	92 A	7.30	<10	Barley
153	92 B	7.02	<10	Peas
154	92 C	7.52	<10	Winter wheat
155	92 D	7.00	<10	Corn
156	5 A	7.52	0	Winter wheat
157	5 B	6.65	<10	Oats
158	5 C	8.12	10	Fallow
159	8 A	8.05	<10	Oats
160	8 B	7.25	<10	Wheat
161	8 C	7.75	<10	Fallow
162	51 A	7.12	<10	Barley
163	51 B	7.10	<10	Winter rye
164	51 C	7.26	30	Winter wheat
165	51 D	7.28	30	Corn
166	Corn A	6.80	<10	Corn, continuous
167	Corn B	6.92	20	Corn, continuous
168	Corn C	6.72	<10	Summer fallow
169	Corn D	6.62	0	Corn
170	Corn E	6.42	0	Corn, continuous
171	Corn F	7.30	<10	Corn, continuous
172	Corn G	6.92	<10	Corn, continuous
173	A****	6.75	0	Kafir corn
174	B	6.42	<10	Kafir corn

*Determined by Agar-plate method and enrichment culture method with sucrose as source of carbon.

**Spring wheat series

***Sorghum series

****Kafir corn series

bacter growth. However, out of 107 samples examined 81 contained Azotobacter and 26 were negative. In all cases Azotobacter counts were small, the highest being 35 organisms per gram of soil.

The soils were examined for Az. vinelandii by the enrichment-culture method and the soil-enrichment method. In the first case ten grams of soil were used, 0.2 percent of sodium benzoate serving as the source of carbon. In the second case 2.0 percent of benzoic acid and 2.0 percent of calcium carbonate were added to 150 grams of soil, instead of the usual 200, and were incubated for a period of six weeks. Examination for Az. vinelandii was made at regular intervals by the usual methods. Neither method resulted in the isolation of Az. vinelandii from these soils.

Series 4

The soils in series 4 were collected in October of 1940 from various plots on the Agronomy farm of the Experiment Station at Fort Collins. The soils were treated for Az. vinelandii by the following three methods previously described: Agar-plate method, solution-culture method, and soil-enrichment method. In all cases four sources of energy were used: Glucose, sodium benzoate or calcium benzoate, calcium butyrate, and cinnamic acid.

TABLE 28--DISTRIBUTION OF AZOTOBACTER IN SOILS FROM THE EXPERIMENT STATION AT FT. COLLINS

Soil No.	pH	Azotobacter* per Gram of Moist Soil			
		Glucose 2.0%	Sodium benzoate 0.2%	Calcium butyrate 0.1%	Cinnamic acid, 0.1%
175	7.40	900	360	530	20
176	7.80	100	230	130	10
177	7.60	160	130	150	15
178	7.72	600	1,200	1,600	200
179	7.68	23	10	20	0
180	7.79	70	65	95	10
181	7.82	40	40	66	20
182	7.72	20	20	25	0
183	7.90	40	50	83	13
184	7.93	80	40	62	0
185	7.65	20	10	16	0
186	7.70	33	30	185	27
187	7.65	10	10	20	0
188	7.48	<10	0	0	0
189	7.67	20	<10	13	0
190	7.62	<10	<10	<10	0
191	7.72	<10	0	13	0
192	7.04	25	23	53	0
193	6.82	<10	0	10	0
194	7.51	710	0	10	0
195	7.55	10	10	13	0
196	7.62	20	0	40	0
197	7.45	20	0	30	0
198	6.70	<10	0	20	0
199	7.48	10	0	10	0
200	6.58	60	130	80	0
201	6.88	0	120	0	0
202	7.43	0	20	0	0
203	7.00	200	220	190	0
204	7.72	180	140	600	0
205	7.61	10	20	25	0
206	7.48	150	110	130	0
207	7.42	300	180	220	0
208	7.45	30	40	50	0
209	7.16	20	30	40	0
210	7.75	50	40	35	0
211	7.40	50	30	<10	0
212	7.62	0	0	0	0
213	7.52	100	100	120	0
214	7.45	0	0	0	0
215	7.40	10	10	10	0
216	7.48	10	0	0	0
217	7.60	30	0	00	0
218	7.40	80	30	40	0
219	7.25	20	<10	15	0
220	7.00	40	25	35	0

*Determinations by agar-plate method.
The figures given are for Az. chroococcum.

TABLE 29--DISTRIBUTION OF AZOTOBACTER IN SOILS FROM THE EXPERIMENT STATION AT FT. COLLINS

Soil No.	pH	Azotobacter* per Gram of Moist Soil: Glucose 2.0%	Sodium benzoate 0.2%	Calcium butyrate 0.1%	Cinnamic acid, 0.1%
221	7.12	85	80	100	0
222	7.18	10	0	<10	0
223	7.12	70	65	120	00
224	7.40	20	0	0	0
225	6.90	75	50	40	0
226	6.92	<10	0	<10	0
227	6.95	60	400	35	0
228	6.92	40	40	30	0
229	7.08	30	10	0	0
230	7.30	30	0	25	0
231	7.22	20	<10	0	0
232	7.92	20	10	0	0
233	8.05	600	800	1,300	0
234	8.01	110	130	180	0
235	7.80	20	50	60	0
236	7.88	60	120	300	0
237	7.55	0	0	0	0
238	7.20	<10	<10	50	0
239	7.80	280	900	950	0
240	8.05	0	90	10	0
241	7.95	<10	0	0	0
242	6.04	30	0	0	0
243	7.80	40	30	70	0
244	7.53	30	40	20	0
245	7.52	1,600	310	1,100	0
246	7.78	20	20	10	0
247	8.20	10	0	10	0
248	8.42	0	0	10	0
249	7.80	60	20	40	0
250	7.46	40	10	20	0
251	7.65	10	10	20	0
252	7.50	30	20	120	0
253	7.58	10	20	10	0
254	7.47	20	0	10	0
255	7.55	110	60	60	0
256	7.52	40	<10	10	0
257	7.63	600	270	380	0
258	7.58	50	55	70	0
259	8.30	0	0	0	0
260	7.60	10	<10	<10	0
261	7.52	<10	<10	<10	0
262	7.88	20	<10	0	0
263	7.95	10	10	30	0
264	7.60	20	20	30	0
265	7.35	<10	0	20	0
266	7.68	<10	<10	<10	0

*Determinations by agar-plate method
The figures given are for Az. chroococcum.

In the agar-plate method the compounds just mentioned were used in the following quantities: Glucose 2.0 percent, sodium benzoate, 0.2 percent, calcium butyrate, 0.1 percent, and cinnamic acid, 0.1 percent. The pH of the media were adjusted to 7.6 before sterilization. Thirty-two plates were prepared for each soil, eight plates for each medium, using one to ten and one to one hundred dilutions. The results obtained are presented in Tables 28 and 29. Since colonies of Az. vinelandii were not obtained, only Az. chroococcum and possibly some Az. beijerinckii are listed under "Azotobacter". Of the 92 samples tested Azotobacter appeared 84 times on glucose-agar, 68 times on sodium benzoate-agar, 77 times on calcium butyrate-agar, and only 8 times on cinnamic acid-agar. In many instances, however, Azotobacter counts were greater on sodium benzoate-agar and calcium butyrate-agar than on glucose-agar.

After seven days the colonies on glucose-agar were from four to twenty millimeters in diameter, watery and milky in appearance, and in some cases brown in color. The colonies on sodium benzoate and calcium butyrate-agar were one to six millimeters in diameter and dark brown to black in color. No pigment formation was evident in case of colonies on cinnamic acid-agar. These colonies were two to ten millimeters in diameter and slightly milky in appearance.

The same four sources of carbon were used in the enrichment culture method: Glucose, 2.0 percent, sodium benzoate, 0.4 percent, calcium butyrate, 0.3 percent, and cinnamic acid, 0.2 percent. Thirty milliliters of media, with pH adjusted to 7.6 before incubation, and five grams of soil were used in all cases. After six days of incubation the treated soils were examined for Az. vinelandii by the usual sucrose-agar plate method. All treatments gave negative results.

The soil culture method was carried out as described under Methods and Procedure. In place of the usual 200 grams of soil only 150 grams were used and the following compounds added: Glucose, 2.0 percent, calcium benzoate, 1.5 percent, calcium butyrate, 1.5 percent, and cinnamic acid, 1.5 percent. The treated soils were examined for Az. vinelandii over a period of six weeks but without positive results.

Series 5

The soils in series 5 were collected from various parts of southern and western Colorado in September of 1940. After the usual pH and Azotobacter content determinations (Table 30) the soils were treated by the soil-enrichment method for Az. vinelandii.

In this case the source of carbon was cinnamic acid (2.0 percent). Because the samples were small, it was not possible to duplicate the treatments as in pre-

vious cases. Az. vinelandii was not isolated from these soils.

It is evident from Table 30 that out of 17 soil samples examined only 9 contained Azotobacter.

TABLE 30--DISTRIBUTION OF AZOTOBACTER* IN SOILS SAMPLED IN SOUTHERN AND WESTERN COLORADO. SERIES 5

Soil No.	Location	Land Use	pH	Az. per gram of moist soil
267	West of Alamosa	Idle land	6.88	0
268	West of Alamosa	Idle land	7.50	200
269	West of Alamosa	Idle land	7.70	800
270	West of Alamosa	Alkali brush land	9.62	0
271	West of Alamosa	Alkali brush land	9.67	0
272	West of Alamosa	Sandy brush land	8.72	<10
273	West of Alamosa	Idle land	9.90	0
274	West of Alamosa	Idle land	9.58	<10
275	Monte Vista	Potato field	8.28	<10
276	Monte Vista	Potato field	7.82	700
277	North of Monte Vista	Artesian pasture	8.54	300
278	South of Ackman	Cultivated	7.00	600
279	South of Ackman	Cedar forest	7.25	0
280	North of Dolores	Aspen forest	6.52	0
281	South of Steamboat Springs	Cultivated	6.10	<10
282	North of Craig	Cultivated	6.12	0
283	North of Craig	Cultivated	6.10	0

*Determinations by agar-plate method

Series 6

Because the distribution of Az. vinelandii in soils proved to be so disappointing, it was deemed ad-

visable to examine some Colorado waters in the hope of finding Az. vinelandii there. As described under Methods and Procedure, three media were used: (a) The modified Wenzl's sucrose medium, (b) the modified Wenzl's sodium benzoate medium, and (c), the Winogradsky's ethanol medium.

From Table 31 it is evident that the reaction of the original water samples was favorable for the growth of Azotobacter in all cases but one. The beaver pond sample had a pH of 5.72. However, there were many samples whose reaction was well above the limiting pH value for Azotobacter but did not show Azotobacter development.

In some cases quantitative distribution of Azotobacter is given. This was determined by the agar-plate method with sucrose as the source of carbon. A zero in such cases indicates (Table 31) that there is less than one Azotobacter cell per milliliter of water, since that was the lowest dilution used. However, the organism was present in the water samples Nos. 19 and 23 as illustrated by the fact that one hundred milliliter treatments gave positive results (Table 31).

Series 7

The identity of Azotobacter strains isolated from soils and waters was established by the use of the method proposed by Winogradsky (49). Since this work was concerned primarily with Az. vinelandii, little attention

was given to the identification of Az. beijernickii which is listed in many cases as Az. chroococcum.

It is seen in Table 31 that it was not possible to isolate Az. vinelandii from waters which had received sodium benzoate as a source of carbon. With ethyl alcohol, however, Az. vinelandii was isolated in seven instances and with sucrose in four instances. The pH of the treatments before isolation showed that they were all above 6.0.

TABLE 31--DISTRIBUTION OF AZOTOBACTER IN WATER SAMPLES

Water No.	Source of Sample	Az. per cc. of water	pH	Azotobacter Treatment a	b	c
1	Poudre River, Ft. Collins		7.65	chroococcum	chroococcum	chroococcum
2	Terry Lake		7.70	----------	----------	----------
3	Long Pond		7.82	chroococcum	chroococcum	chroococcum
4	Eaton Ditch		7.85	chroococcum	chroococcum	chroococcum
5	Lindenmeier Lake		7.87	chroococcum	chroococcum	chroococcum
6	Spring water		7.80	----------	----------	----------
7	Ditch water, Ft. Collins		7.76	chroococcum	chroococcum	chroococcum
8	Irrigation Ditch, Ft. Collins		7.87	----------	----------	vinelandii
9	Sewage Ditch, Ft. Collins		7.98	vinelandii	chroococcum	vinelandii
10	Poudre Lake		6.43	----------	----------	----------
11	Beaver Pond, Hidden Valley		5.72	----------	----------	----------
12	Bear Lake		6.00	----------	----------	----------
13	Glacier Creek		6.35	----------	----------	----------
14	Thompson River, at entrance		7.68	----------	----------	----------
15	Thompson River, below Estes Park		7.02	----------	----------	----------
16	Thompson River, North Fork entrance		7.55	chroococcum	chroococcum	chroococcum
17	Thompson River, Mouth of Canyon		7.30	----------	----------	----------
18	Thompson River, Loveland		7.18	chroococcum	chroococcum	vinelandii
19	Boyd Lake	0	8.61	chroococcum	chroococcum	----------
20	Boyd Lake group	18	8.50	chroococcum	----------	chroococcum
21	Loveland Lake	4	8.22	chroococcum	----------	chroococcum
22	Irrigation Ditch, Loveland	1	8.15	chroococcum	chroococcum	chroococcum
23	Irrigation Ditch, Ault	0	8.30	vinelandii	chroococcum	vinelandii
24	Irrigation Ditch, Ault	32	8.10	chroococcum	chroococcum	vinelandii
25	Irrigation Ditch, Ault	29	8.02	chroococcum	chroococcum	chroococcum

TABLE 31--DISTRIBUTION OF AZTOBACTER IN WATER SAMPLES (Cont'd)

Water No.	Source of Sample	Az. per cc. of water	pH	Azotobacter Treatment a	b	c
26	Irrigation Ditch, Ft. Collins	1	7.90	chroococcum	chroococcum	vinelandii
27	Irrigation Ditch, Ft. Collins	25	7.95	vinelandii	chroococcum	vinelandii
28	Irrigation Ditch, College Campus	1	8.27	vinelandii	chroococcum	chroococcum
29	Irrigation Ditch, near Denver*			chroococcum	chroococcum	vinelandii

*Irrigation ditch canvass was used in this case in place of water sample.

DISCUSSION

The isolation of Az. vinelandii is dependent upon the presence of this organism in soils and waters regardless of its quantitative distribution in the two natural media mentioned. It is evident that if this organism is found in the soils and waters in large numbers, then its isolation becomes not too difficult a problem. However, if the organism is rare, then the probability of its isolation by ordinary means is lessened. The scarcer the organism is, the more difficult becomes the problem and the less significant the result, for under such conditions more and more soil has to be used in order to isolate the organism. And under such conditions of scarcity arises the question as to the practical value of Az. vinelandii to its habitat and of the importance of its relationship to the more abundant organisms in soils and waters. If the organism is found in the soil only occasionally, then one is left in doubt as to the real habitat of this organism.

For the isolation of Azotobacter from soils numerous media have been proposed (1,25,45). The underlying principle in all these media is the absence of nitrogen compounds, the presence of source of available carbon, and the presence of certain other essential ele-

ments, of which phosphorus is the most important. Under such conditions the isolation of Azotobacter is not at all difficult if the organism is present in quantities of one cell per ten grams of soil. However, difficulties increase when the organism is present only in quantities of one cell per one hundred grams of soil. When the organism is present in quantities of one cell per thousand or ten thousand grams of soil, then working out the problem becomes impractical and the value of the organism doubtful.

Az. chroococcum is widely distributed in soils and its isolation may be made from a limited quantity of soil. As is evident from the literature (4,23,49, 50), Az. vinelandii has been isolated only in several instances. It was felt that if Az. vinelandii is present in the soil in quantities of at least one cell per one hundred grams of soil, then its isolation might be accomplished by stimulating its development in the soil to such an extent that it might finally appear on agar plates.

The use of benzoates by Az. vinelandii was shown by Winogradsky (48). Later it was demonstrated that benzoates may be used in isolating Az. vinelandii from soils by growth stimulation (37). In this paper the value of various organic non-nitrogenous compounds as growth stimulants for this organism was determined.

The value of monohydric alcohols, ethyl, methyl, n-propyl, amyl, and hexyl, in stimulating Az. vinelandii growth in soils does not appear promising. Not only do these compounds depress Az. vinelandii growth but that of Az. chroococcum also, as is evident from Tables 1 and 2. Winogradsky has shown that ethyl alcohol is readily used by pure cultures of Az. vinelandii (49). Katznelson (22) found that 0.5 percent ethyl alcohol acted favorable on Azotobacter development in the soil but that 1.0 percent supressed the growth of this organism.

That ethyl alcohol is valuable in the isolation of Az. vinelandii from waters is evident from Table 31. In this case only 0.5 percent of ethyl alcohol was used. The possibility exists that 1.0 percent of ethyl alcohol for soil treatments was too high. The lowest quantities of other monohydric alcohols used (1.0 percent) were possibly also too high for, with the exception of methyl alcohol, they did not act favorably on mold development (Figs. 1, 2, and 3). Amyl, n-propyl, and hexyl alcohols were likewise poor sources of carbon for bacteria and Actinomyces, while ethyl alcohol (1.0 percent) and methyl alcohol (3.0 percent) were stimulating. (Figs. 4, 5, and 6).

In contrast to this, the trihydric alcohol glycerol and the hexahydric alcohol mannite showed un-

usual capabilities for stimulating growth. In stimulating Az. vinelandii development mannite became more effective with increasing concentration. The 5.0 percent treatment gave higher counts than the 1.0 percent or the 3.0 percent treatment (Table 14). Glycerol, however, in concentration of 3.0 percent was more effective than mannite in 5.0 percent concentration. The slow development of Az. vinelandii (Table 1) seems to indicate that this organism does not utilize glycerol directly to any great extend but possibly depends on the decomposition products of this compound. The organism responsible for glycerol decomposition seem to be bacteria, Actinomyces, and to some extent molds (Figs. 2 and 5). An interesting fact was observed with molds in the case of 1.0 percent glycerol treatment where the highest mold counts were found after one week, after which time they begin to decrease until the eighth week when they show the upward trend again. This was just the opposite of the effect produced by 1.0 percent glycerol treatment on Az. vinelandii counts in which case the highest counts were found after six weeks (Table 1).

In the soil it appears that glycerol (3.0 percent) was more effective than mannite (5.0 percent) in stimulating Az. vinelandii growth although Löhnis and Pillai (26) place mannite before glycerol as a source of carbon for pure culture of Azotobacter. For pure cultur-

es this is probably true, but in soils the decomposition products obtained from glycerol appear to be more effective than mannite.

The majority of the carbohydrates used proved effective in stimulating Azotobacter development. They may be divided into three general groups: The most effective group (glucose, galactose, and raffinose), the intermediate group (mannose, fructose, sucrose, maltose, and dextrin), and the least effective group (starch, lactose, and inulin). In almost all instances carbohydrates in higher concentration (2.5 percent) gave better results than in lower concentration (1.0 percent). However, the increase of Az. chroococcum was slightly better than for Az. vinelandii (Table 14). This indicates that carbohydrates such as glucose, galactose, and raffinose were just as good sources of carbon for Az. chroococcum as for Az. vinelandii. Although these sugars may well be used to stimulate Az. vinelandii development in soils, the ideal source of carbon would be a compound which stimulates Az. vinelandii but not Az. chroococcum.

The use of phenol, thymol, resorcinol, pyrogallol, phloroglucinol, benzene, and toluene can not be considered beneficial in any way to Azotobacter. The fact appears to be that these chemicals were injurious to Azotobacter, bringing about the elimination of this

organism at the very start (Table 15). However, this may not mean that the decomposition products are not effective. Had Az. vinelandii been added after the molds, bacteria, and Actinomyces had sufficient time to bring about the decomposition of these compounds, the results might have been different. That many of these compounds bring about a condition in the soil known as "partial sterilization" of the soil, is evident from the figures illustrating the distribution of molds and bacteria and Actinomyces in treated soils (Figs. 19, 20, and 21). Quinol (2.5 percent), toluene (1.0 percent, 2.5 percent), and resorcinol (1.0 percent) depressed mold development at first but were stimulating later in the incubation period. Quinol (1.0 percent, 2.5 percent), pyrogallol (1.0 percent, 2.5 percent), Toluene (1.0 percent, 2.5 percent), benzene (1.0 percent, 2.5 percent), and phloroglucinol (1.0 percent) had the same effect on bacterial development. While further studies with these chemicals may result in some interesting facts, at present it may be said that they are of no value in Azotobacter isolation.

Of the aromatic acids tested the hydroxy carboxylic acids, salicylic and gallic, were found to be of no value, while tannic acit (2.5 percent) was more stimulating to Az. chroococcum than to Az. vinelandii (Table 15). Of the aromatic carboxylic acids tested the potas-

sium salt of phthalic acid did not serve the purpose, while the calcium salt of benzoic acid proved stimulating.

It is of interest to note that Azotobacter was completely absent in benzoic acid and sodium treated soils (Table 16). The reason for this is, perhaps, to be found in soil reaction. It seems that the benzoic acid treatment resulted in too low a pH and sodium benzoate treatment in too high a pH for Azotobacter growth. The calcium benzoate treatment, on the other hand, gave a pH value which was favorable for Azotobacter (Table 33). The results obtained by Reuszer (37) are just the opposite. Az. vinelandii was isolated from sodium benzoate and benzoic acid treatments, but not from calcium benzoate treated soils. It is possible that the alkali content of the soils used prevented the pH value in the benzoic acid (2.5 percent, 5.0 percent) treatments from going below 6.0.

That benzoic acid brings about an acid condition in the soil is further illustrated in Part 2, Series 2 (p.128). All soils in that series were being tested for Az. vinelandii by the soil-enrichment method, which included the addition of benzoic acid (2.0 percent) and calcium carbonate (2.0 percent). Some soils were treated only with benzoic acid in addition to the treatment just mentioned. Although Az. vinelandii was

not found in any of the soils in this series, nevertheless the soils which had received two chemicals showed a pH above 6.0 and the presence of Az. chroococcum. The soils treated only with benzoic acid were extremely acid in reaction and completely free of Az. chroococcum, except in a few cases where the soil was sufficiently alkaline to overcome the acidity resulting from benzoic acid.

The most favorable compound to Az. vinelandii growth stimulation appears to be cinnamic acid. It is apparrent from Table 15 that if cinnamic acid in concentrations of 1.0 percent or 2.5 percent is used, the organism develops to such an extent as to make its isolation possible on agar plates without any interference from Az. chroococcum. However, it is possible to isolate both Az. vinelandii and Az. chroococcum from agar plates if the soil is treated with only 0.5 percent cinnamic acid, as is shown in Table 18. Apparently high concentrations are more suited for Az. vinelandii than for Az. chroococcum.

In the soil, however, the decomposition products of cinnamic acid were probably used by Azotobacter as the source of carbon. Bacteria and Actinomyces appeared to be more instrumental in decomposing this compound into products suitable for Azotobacter than were molds. The molds developed slowly at first, in-

creasing rapidly after the eighth week (Fig. 27). Bacteria and Actinomyces developed rapidly during the first four weeks, indicating rapid decomposition of cinnamic acid (Fig. 29). Az. vinelandii numbers increased slowly at first, reaching their maximum at the beginning of the sixth week when the bacterial numbers begin to fall off (Table 8). Under such conditions the value of the microbial population of the soil becomes evident. Such results could not have been obtained with sterile soils.

Of the various aliphatic acid tested several proved promising. The calcium salts of lactic and tartaric acid (2.5 percent) were highly stimulating to Az. vinelandii and Az. chroococcum (Table 16). The potassium salt of malic acis and the calcium and potassium salts of citric acid, although of some value, were not as effective as the first two compounds mentioned. The potassium salt of oxalic acid was entirely unsatisfactory (Table 16).

Of the fatty acids tested the calcium salt of butyric acid was highly efficient (Table 16). In comparison with cinnamic acid, the 1.0 percent calcium butyrate treatment gave higher Az. vinelandii count than the 1.0 percent cinnamic acid treatment. The 2.5 percent calcium butyrate treatment gave a lower Az. vinelandii count than either the 1.0 percent calcium butyrate or the 2.5 percent cinnamic acid treatment. How-

ever, there was one difference of importance between the two sources of carbon. From the calcium butyrate treatment both Az. chroococcum and Az. vinelandii were isolated, while from cinnamic acid treatment only Az. vinelandii were secured. Other treatments which showed high Az. vinelandii counts were calcium and potassium propionate, and calcium butyrate. Potassium acetate and potassium and calcium palmitate were of little importance.

An interesting fact is brought out in treatments containing the potassium salts of aliphatic acids. As a rule the 2.5 percent concentrations of potassium salts gave lower Az. vinelandii counts than the 1.0 percent concentrations. The difference was possibly due to increased alkalinity in these soils because of the higher potassium content (Table 16). In all cases potassium salts in quantities of 2.5 percent gave much higher soil reaction than in quantities of 1.0 percent (Figs. 40, 41, and 48).

The question as to whether the potassium or the calcium salt is preferred by Azotobacter seems to be largely dependent on the acid used, With calcium lactate Az. vinelandii was absent in 1.0 percent treatment but the total gain in the 2.5 percent treatment was over a million and a half organizms (Table 16). Potassium lactate gave Az. vinelandii counts which ran only

into thousands. With potassium tartrate the 1.0 percent treatment gave much higher counts than the 2.5 percent treatment. The opposite is true with calcium tartrate. With citric acid the calcium salt, 2.5 percent, gave just as high Az. vinelandii counts as the 1.0 percent potassium salt treatment, while with the propionic acid the 1.0 percent potassium salt gave higher counts than did the 2.5 percent calcium salt treatment. Potassium butyrate was more effective than calcium butyrate.

An interesting contrast in bacterial activity is afforded by some aromatic and aliphatic compounds. The salts of such aliphatic acids as propionic, acetic, palmitic, butyric, oxalic, citric, malic, tartaric, and lactic offer in most instances a readily available source of carbon for the general soil flora. In those cases the bacterial population rises rapidly and begins to decline when the compounds are exhausted. The effect of such aromatic compounds as gallic acid, salicylic acid, quinole, toluene, phloroglucinol, and benzene on the bacterial population of the soil is somewhat different. The bacterial population is at first depressed because of the toxic effect of the compounds themselves. After a time a stimulating effect is evident, resulting partly from evaporation of the volatile compounds and partly from the decomposition of these compounds by the limited soil flora capable of acting on them. Some compounds re-

mained depressing throughout the incubation period (Figs. 22, 23, 31). For such treatments the incubation period should be extended.

It is evident from the examination of the various organic groups used that certain compounds are more effective than others. The aliphatic monohydric alcohols tested were of no value, while the trihydric alcohol glycerol and the hexahydric alcohol mannite proved effective. Of the various carbohydrates tested glucose, galactose, and raffinose were promising. Of the various aromatic compounds used cinnamic acid appeared to be the best. Also effective in Az. vinelandii growth stimulation were the calcium salt of butyric acid, the calcium salts of lactic and tartaric acid, and the calcium and potassium salts of propionic acid.

In comparing the effectiveness of various chemicals used certain factors must be taken into consideration which may be helpful in understanding the limitations of the results obtained. The length of the incubation period is of considerable importance. Better results would have been obtained had the incubation period been extended to twenty or more weeks and the determinations made weekly. Data obtained in such manner would have been more suitable for statistical analysis than the data on hand. However, because of the equipment and time involved, such an undertaking was not pos-

sible.

There is one fact which must not be overlooked when comparing Azotobacter counts for alcohol treatments with other sources of carbon used. The incubation period for alcohol treatments was only eight weeks while for treatments with other compounds, ten weeks. Also of importance is the fact that not all the series were inoculated with exactly the same number of Az. vinelandii cells, as is shown by check treatments (Tables 14, 15, and 16).

The differences in checks between series may also be due, in part, to the soil itself. The soils for each series were not sampled at the same time or at the same place, although they were from the same field. Differences in soil heterogeneity were probably of little effect in treatments which proved depressing to Az. vinelandii for the effect of the treatments was probably sufficiently severe to overcome any advantages that may have been gained through more favorable soil conditions or through the presence of larger numbers of Az. vinelandii. However, these differences were of some importance where the treatment was favorable for Azotobacter development.

Of importance also is the competition between Az. chroococcum and Az. vinelandii for the source of carbon. Although Az. chroococcum was not added to the soils

undergoing treatments, it nevertheless varies somewhat in numbers from series to series as is shown in Tables 14, 15, and 16. In all series the Az. chroococcuum content, as averaged for the entire incubation period, is greater than Az. vinelandii content. It would be expected that in the presence of sources of readily available carbon the organism present in largest numbers would develop most rapidly.

The number of Az. vinelandii cells that must be present in the soil before the soil-enrichment method can be effective is of prime importance. An answer to this question was attempted here. The results indicate (Tables 17, 18) that it is very difficult to isolate Az. vinelandii from untreated soil by means of the agar-plate method, even if the organism was originally present in quantities of six cells for every gram of soil. The isolation of Az. vinelandii from soils, however, may be accomplished with ease if an available source of available carbon such as sucrose or cinnamic acid is added to the soil. In that case Az. vinelandii may be isolated from soil in which it was originally present in quantities of from one cell for every 0.16 grams of soil to one cell for every 12.5 grams of soil. The value of the soil-enrichment method, therefore, becomes evident. If the method succeeds for one Az. vinelandii cell for every 12.5 grams of soil, then there is a strong probability that it will succeed where there is only one cell

for every one hundred or two hundred grams of soil.

The ease with which Az. vinelandii is isolated from treated soils and the difficulties met in isolating it from untreated soils clearly illustrate the advantage of the treated soil over the untreated. The value of the soil-enrichment method and of the two similar methods agar-plate method and enrichment-culture method, was tested by the examination of various soils for Az. vinelandii.

Of the 283 soils examined 210 contained Az. chroococcum and only two contained Az. vinelandii. Expressed on a percentage basis the figures are 74 percent and 0.7 percent respectively. Of 29 water samples examined, 19 or 65.5 percent, contained Az. chroococcum and 9, or 31 percent, contained Az. vinelandii. It therefore appears from the limited number of samples examined that Az. vinelandii is more commonly distributed in waters than in soils. Winogradsky (49) has emphasized the fact that Az. vinelandii is a water organism and that search for it should be made in clear waters. Muddy waters and soils, in his opinion, should be examined for Az. chroococcum.

Studies relating to Az. vinelandii distribution in waters appear to be absent in scientific literature. It therefore becomes evident from the results obtained that a more thorough investigation of waters is needed

in order to determine the extent of Az. vinelandii distribution in nature. In such determinations the value of ethyl alcohol as a source of carbon has been demonstrated.

Under such conditions of limited distribution of Az. vinelandii in soils examined, it no longer becomes necessary to evaluate the importance of this organism in the nitrogen economy of the soil. There exists the possibility that in some soils Az. vinelandii may be common. Lipman (25), although he gives no figures, states that Az. vinelandii is widely distributed in the arable soils of New Jersey.

SUMMARY

The investigation was undertaken to determine the value of various non-nitrogenous organic compounds added to unsterilized soils as stimulants for Azotobacter vinelandii, and to obtain a method or methods which might be used for demonstrating the presence of Az. vinelandii in soils. It was also the purpose of this work to investigate the presence of Az. vinelandii and Az. chroococcum in some Colorado soils and waters.

The compounds tested for Az. vinelandii stimulation were:

1. Aliphatic alcohols--methyl alcohol, ethyl alcohol, hexyl alcohol, amyl alcohol, n-propyl alcohol, mannite, and glycerol.

2. Carbohydrates--glucose, galactose, mannose, fructose, lactose, inulin, sucrose, maltose, dextrin, and starch.

3. Aromatic compounds--quinol, benzene, toluene, phenol, resorcinol, pyrogallol, phloroglucinol, thymol, tannic acid, potassium hydrogen phthlate, salicylic acid, gallic acid, cinnamic acid, sodium benzoate, calcium benzoate, and benzoic acid.

4. Salts of aliphatic acids--Calcium lactate, potassium lactate, calcium tartrate, potassium malate,

calcium citrate, potassium citrate, potassium oxalate, calcium palmitate, potassium palmitate, calcium propionate, potassium propionate, calcium butyrate, potassium butyrate, and potassium acetate.

The effects of these treatments on Az. vinelandii, Az. chroococcum, molds, bacteria and Actinomyces, and soil reaction were determined periodically.

For the purpose of studying the distribution of Az. vinelandii and Az. chroococcum, soil and water samples were collected from various parts of Colorado. In determining the distribution of Az. vinelandii in soils, one or more of the following three methods were employed: Agar-plate method, enrichment-culture method, and soil-enrichment method. Glucose, benzoic acid, calcium benzoate, sodium benzoate, calcium butyrate, or cinnamic acid was used as a source of carbon. In testing water samples the enrichment-culture method was employed with sucrose, sodium benzoate, and ethyl alcohol as sources of carbon.

The isolated Az. vinelandii strains were identified by examining the cultures for cyst formation as proposed by Winogradsky.

CONCLUSIONS

1. Of the aliphatic alcohols tested mannite and glycerol appear to be best suited as stimulants for Az. vinelandii and Az. chroococcum in unsterilized soils. Methyl alcohol, ethyl alcohol, hexyl alcohol, amyl alcohol, and n-propyl alcohol, within the limits of the concentrations used, were of no value.

2. Of the carbohydrates tested, the highest increase in Az. vinelandii numbers was obtained with 2.5 percent glucose. Galactose (2.5 percent) was next in importance, followed by raffinose (2.5 percent). Mannose, fructose, sucrose, maltose, and dextrin fall in the intermediate group as Az. vinelandii stimulants in soil, while lactose, inulin, and starch were of little importance.

3. Of the aromatic compounds quinol, benzene, toluene, phenol, resorcinol, pyrogallol, phloroglucinol, thymol, potassium hydrogen phthlate, salicylic acid, and gallic acid in concentrations of 1.0 percent and 2.5 percent were of little or no value as stimulants of Az. vinelandii or Az. chroococcum in the soil. Tannic acid and calcium benzoate were better than any of these, but sodium benzoate and benzoic acid were ineffective, probably because of soil reaction. Cinnamic acid, however, proved

to be very effective; the 2.5 percent treatment gave the highest count of all the treatments.

4. High Az. vinelandii counts were obtained with calcium lactate and calcium tartrate. The compounds belonging to this group which were also effective are potassium lactate, potassium tartrate, potassium malate, calcium citrate, and potassium citrate. Potassium oxalate proved ineffective as a stimulant of Az. vinelandii in soil.

5. Of the fatty acids tested the calcium salt of butyric acid (1.0 percent) gave the greatest increase in Az. vinelandii numbers. Other salts which were highly effective are calcium propionate, potassium propionate, and potassium palmitate and potassium acetate.

6. It is possible to isolate Az. vinelandii from soil by means of a soil-enrichment method when there is at least one cell for every 12.5 grams of soil.

7. As a general rule the bacterial population of the soil was more stimulated by the presence of available sources of carbon, such as carbohydrates, than by the presence of such compounds as quinol, benzene, toluene, phloroglucinol, and benzene depressed bacterial development at first but produced a stimulating effect later in the incubation period. In such cases conditions approaching "partial sterilization" of the soil were ob-

served.

8. Of the 283 soils examined, 210, or 74 percent, contained Az. chroococcum and only two, or 0.7 percent contained Az. vinelandii. Of the 107 soils examined from the Experiment Station at Akron, 81, or 75.7 percent, contained Az. chroococcum, while of the 93 soils examined from the Experiment Station at Fort Collins, 85, or 91.4 percent, contained Az. chroococcum and one, or 1.1 percent, contained Az. vinelandii.

9. Of 29 water samples examined, 19, or 65.5 percent, contained Az. chroococcum and 9, or 31 percent, contained Az. vinelandii.

10. Ethyl alcohol (0.5 percent) seems to be better suited as a source of carbon than sucrose or sodium benzoate in the isolation of Az. vinelandii from water.

11. From the limited number of soils tested in the State of Colorado it seems that the soil is not the natural habitat of Az. vinelandii.

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