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J. L. Harper.

ABSTRACT OF THESIS

An investigation of the interaction of soil micro-organisms with special reference to the study of the bacterial populations of plant root systems.

Experiments and a critical review of the literature of the technique of soil microbiology have shown that the agar plate method for the estimation of soil microbes may lead to severe errors.

(1) The number of micro-organisms developing from a soil innoculum to colonies on agar media, and which are counted and used to estimate the soil population, do not always all develop. Of those organisms which can reproduce or grow on the medium provided, a proportion is not represented if the colonies are too crowded. The relationship between amount of soil innoculum and the number of colonies developing from it, is not then linear. When the development of some colonies is suppressed in this way, microbial populations estimated from the plate counts may be severely underestimated. The late developing colonies in particular are suppressed when the density of colonies on plates is high.

The suppression of the development of microbial types into colonies is differential, and so changes in the degree of crowding of colonies on agar plates may bring

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about changes in the proportional representation of specific groups of microbes. The proportional representation of actinomycetes for example, is depressed at high densities, and yellow chromogens may be depressed or proportionately increased in different populations. It is shown that both the quantitative and qualitative effects of plating density can be attributed to microbial interaction, and that these effects are very significant within the conventional range of plating densities 70-200 colonies per 9 cm. diameter plate.

It is shown that there is a general relationhsip between the proportional representation of microbial types and the estimated total population of soils in the experimental results reported by a number of investigators. Many of the differences between rhizospheres and soil populations which have been claimed by investigators, and which show this relationship can be interpreted as plating density artifacts and it is pointed out that until many of the rhizosphere experiments which have been reported have been confirmed, making due allowance for or excluding the density effect, the results must be suspect.

Statistical methods have been used to show differential rhizosphere effects (in the presence of the density effect) between three fen plants growing in peat, and between banana varieties. There is a correlation between the rhizosphere flora of the banana varieties examined and resistance to

Panama disease. Within the genetic pairs Congo - Gros Michel, and Silk Fig - Guindy, the susceptible variety supports a larger microflora on its roots than the resistant. The power of the varieties to support a varied microbial flora is correlated with the size of population supported on root systems in the field.

Antibacterial and antifungal substances were isolated from the rhizomes of a number of banana varieties, but no correlation with disease resistance could be found.

An investigation of the interaction of soil micro-organisms with special reference to the study of the bacterial populations of plant root systems

JOHN L. HARPER

Department of Agriculture,

University of Oxford.

In the course of an investigation of those micro-organisms which live on the root surfaces of ten plants, it was observed that the technique used was seriously biassing the results; the classical 'plating' technique for the enumeration of soil micro-organisms had been widely used by previous investigators of root surface microbes and so it was obviously desirable to determine to what extent the method used was reliable and so to test the validity of the conclusions which previous workers had drawn from the use of this technique. The six parts of this thesis are as follows:

I. A review of the methods available for the investigation of soil micro-organisms, and especially of their use in the study of the 'rhizosphere' flora of plants.

II. Plating density: the effects of microbial interaction on the determination of the numbers of microbes in soil.

III. Plating density: the effect of microbial interaction on the determination of systematic and physiologic categories of organisms in soil.

IV. Experiments and a review of the literature concerning the 'rhizosphere' in relation to microbial interaction and plating density.

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II. Internal factors for disease resistance and antibiotics in banana roots and rhizomes.

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AN INVESTIGATION OF THE INTERACTION OF SOIL MICRO-ORGANISMS WITH ESPECIAL REFERENCE TO THE STUDY OF THE BACTERIAL POPULATIONS OF PLANT ROOTS.

JOHN L. HARPER

Department of Agriculture

University of Oxford.

PART I.

A review of the methods available for the investigation of soil micro-organisms, and especially of their use in the study of the 'rhizosphere' flora of plants.

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PART I.

A. General account of methods

Introduction.

In an experimental science, an attempt is made to disturb a natural system, and by considering the type of disturbance caused, to gain some knowledge of the thing disturbed. This peculiar character of experiments makes it necessary to attempt the separation of those qualities of an experimental result or observation which reflect the supposed essential nature of the thing observed, from those qualities imposed by the nature of the experiment or the act of observation.

There exist several methods for both observation of and experimentation upon the population of soil micro-organisms, and the picture of the population given by each method is very particular and peculiar to that method. Each method gives only a very partial idea of the nature and activity of the population studied, and most of the methods employed are so specific, that it is not easily possible to relate the results obtained from one to those obtained from another. Each method allows only a limited understanding of the soil system to be gained: each method

reflects only a very special facies of the subject.

Most of the methods of soil biology have been used at one time or another in the study of that part of the soil influenced by the proximity of the roots of higher plants, which Hiltner (1904) has called the rhizosphere (rhizosphäre). It is intended in this first part of the thesis to describe the various experimental techniques which are available for the study of soils and to show for each, how it has been used in rhizosphere studies and how far the type of results obtained are characteristic of the method used and of the subject investigated. This will be followed by a detailed discussion of the methodology of the most commonly used experimental technique in soil microbiology, the plate count and a critical analysis of the experiments which various authors have made in attempts to determine the validity of this method. This critical investigation suggests two possible ways in which the technique of this method may seriously bias the estimates of soil populations obtained by its use, and these are studied experimentally and described in Parts II and III of the thesis. In Part IV the effects shown in Parts II and III are discussed in relation to the possible bias they may have on studies of the rhizosphere flora, and

experiments and critical surveys of the rhizosphere literature are presented to illustrate this bias. In Part V a study of the rhizosphere flora of banana varieties is described, in which an attempt has been made to remove the effect of the errors previously studied.

(a) The Plate Method.

The study of soil microbiology has grown out of the science of medical bacteriology and the two studies therefore hold much in common. For example, the 'plate' method for the isolation and enumeration of micro-organisms, was described by Koch in 1881 and is of fundamental importance in both medical and soil bacteriology. In a classic paper, he describes the method and discusses its use not only for the studies of pathogenic organisms for which it was designed (the paper is entitled 'Zur Untersuchung von pathogenen Organismen') but also in studies of atmospheric contamination, water bacteriology and soil studies. Soil and medical microbiology therefore received this technique simultaneously.

There is evidence that Miquel (1879 and 1885) had used a solid medium for bacteriological studies before Koch, but it was the work of Koch that stimulated the great body of bacteriological investigation by the use of the

plating method which fills the literature of soil and other microbiological studies at the end of the nineteenth century and still persists in the twentieth.

Koch used a solidified nutrient medium which could be exposed in a suitable receptacle to the air, e.g. in hospitals, to collect a sample of aerial contaminants on its surface. If the contaminants were capable of growing on the solid medium provided, they would be expected to grow, in time, into colonies visible to the naked eye. A count made of the colonies on such a plate, would then give an index of the extent of the aerial contamination. The method was modified by Koch and subsequent workers to give estimates of the soil microflora. For this, a soil sample was diluted and spread over a solidified medium after it had become a gel, or was suspended in the medium while it was still molten and then poured with the medium into dishes. The Petri dish was developed in 1887 and greatly facilitated the use of solid gel media for microbiological studies.

Nutrient broths or bouillons were well known before the use of solidified media by Koch and Miquel, but the great advantage of solid media over broths for the estimation of microbial populations was that the solid medium maintained each original contaminant separate, allowing

it to develop to a visible colony in spatial isolation; so that the original contaminants could be counted - a feat which was virtually impossible by normal use of liquid media, (see however 'Dilution Method'). Gelatine, the original gel to be used as the solidifying medium for plate counts, suffers from one very severe disadvantage. It is a protein and is itself a nutrient for many of the micro-organisms isolated from soil. Many of these organisms secrete proteolytic enzymes, which liquefy the medium around the developing colonies and this leads to the amalgamation of neighbouring colonies and to rapid liquefaction of the whole mass of gelatine on a plate: the colonies then of course lose their spatial separation and cannot be counted. Hiltner and Störmer (1903) developed a technique for treating this problem, which involved touching each colony with a stick of silver nitrate, as soon as it became visible and started to liquefy; this stopped further activity of the colony and its enzymes. Cunningham (1913) tested this method and found that it stopped liquefaction but that the silver nitrate, diffusing from the touched spot, was liable to inhibit the development to visible size of neighbouring slow growing colonies. It is obviously not a practicable technique to use in large scale plate experiments.

The development of the use of agar-agar in place of gelatine obviated these difficulties. The bacteriological use of agar was discovered by Frau Hesse*, wife of one of Koch's early co-workers, who obtained samples from Batavia through friends in Holland where it was used for culinary purposes, Bulloch (1938). Washed agar contains little nutrient material of use to bacteria or fungi, although unless very thoroughly washed, limited growth is supported by agar media in the absence of other nutrients. Agar is itself hydrolysed by an enzyme gelase, produced by some bacteria e.g. Bacillus gelaticus, a deep sea organism (Gran (1902), Waksman and Bavendamm (1931). There are however very few organisms capable of liquefying agar, and the dangers of liquefication are much less than for gelatine. Apart from its obvious value for the estimation of proteolytic bacteria, gelatine has now been completely eclipsed by agar for soil investigations. Fellers (1916) lists many papers on the use of agar in soil microbiological studies.

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* Fellers (1917) is at fault in attributing the discovery of the use of agar to Koch in 1881. Koch makes no reference to the use of agar in his famous paper of 1881 on the plate method, for the use of agar was not developed until 1882. Fellers also states that the use of peptone in media was due to Edington (1886) but this was in fact first used by Koch in 1881, as also was white of egg as a clarifying agent, for which Fellers cites Biondi (1887) as originator.

(iii) Silicic acid gel.

Silicic acid gel was tested by Koch (1881) and found less satisfactory than gelatine for bacterial counts. It has however been extensively used by French soil microbiologists, notable Winogradsky and his associates of the Institut Pasteur. Winogradsky (1925) gives his reasons for using silicic acid gel as follows "En fait de milieu solide, la tâche qui s'impose ici est de créer un milieu microbiologique reproduisant les conditions spéciales au sol, lequel est une masse minérale riche en colloïde, pauvre en substance organique et en azote", and "Il y a des cas exceptionnels pourtant où l'emploi de la gélose ne presente rait pas d'inconvenients". The preparation of silica gel has been developed by Pochon and Tchan (1948). The method is particularly favourable for the isolation and investigation of autotrophic organisms, but is ill adapted to the estimation of bacterial numbers. As normally employed, the silicic acid gel is infiltered with some nutrient, grains of soil 'ensemencements', are placed on the gel, and after incubation, the organisms growing out from the particles are studied. Silica gel has not been used in rhizosphere studies.

(iv) Other gels.

Blood sera and pectic acid have been used as solidifying agents for nutrient media, but not in soil studies.

(v) General summary of the plating method

Owen (1915) has defined the conditions under which the plate technique may be useful in soil studies as follows, "To the extent therefore that a culture medium admits of the development upon it of all the species of bacteria occurring in the product to be analysed, and to the extent also which this aggregate development represents the same numerical proportions between the different species as existed in the material analysed, its value as a medium for quantitative bacteriological determinations depends".

Owen has listed the main disadvantages of the method as follows:

(1) It is difficult to provide artificial conditions as favourable as the environment for the development of the organisms.

(2) "..... it often happens that the development of a certain species of bacterium upon a culture medium, renders it unsuited for others, owing either to the changes which are wrought in its composition, or to the secretive products which may exercise a toxic action on the other species".

(Owen is one of the few writers who has recognised the possibility that some organisms on agar plates may suppress the development of others, thus vitiating the plate count as an unbiassed estimate of part of the soil population).

(3) Different species, present in one sample may have opposite requirements for development, both of which cannot possibly be satisfied on one agar plate; e.g. obligate aerobes and anaerobes, and types to which organic nitrogenous compounds are essential or inhibitory.

(4) The physical texture of the medium may be unsatisfactory for growth (e.g. Mycobacterium tuberculosis fails to make good growth on solidified media). This effect may be responsible for the failure of the plating method to give estimates of the micropopulation as high as the dilution method in which liquid media are used, (see Topping (1938) and discussion under 'Dilution method'). It is to be presumed from the discussion of unpublished experiments by Taylor and Lochhead (1938) that they obtained good growth of more soil organisms on semi-solid soil extract agar (i.e. 0.3% agar) than on normal fully solid agar (1% agar), and this may be another effect of physical texture.

Further criticisms of the plate method have been made by Thornton and Gray (1934) who emphasise that in plate counts, any colony may represent a single cell or a clump of cells in the sample of soil dilution which was inoculated into the agar. The effects of prolonged shaking of a sample on the estimate of its microbial population have

been considered by Waksman (1922a) for soil and by Wilson (1935) for milk, who showed that counts from a sample increased with the period for which it was shaken. It is clear, however, that even prolonged shaking cannot disperse all clumps, and the plate method must therefore underestimate the populations. Thornton and Taylor (1935) state that the plate count may represent only about 2% of the total bacterial population of a soil sample.

Despite all these criticisms, the plate method continues not only to be used, but to be the most often used means for estimating the microbial populations in soils. Undoubtedly the technique of the method is extremely elegant and this may be the great attraction which has kept the method in use. The estimate that it gives of a soil population must be of those dispersed or dispersive units of organisms in the population which are capable of growing on those nutrients provided in the medium, to // colonies visible to the naked eye under the peculiar physical conditions, e.g. pH, osmotic pressure, oxygen tension and temperature of the gel medium.

(vi) The plate method in rhizosphere studies

The plate method has been very widely used in rhizosphere studies. Hiltner (1904) himself derived the concept of the rhizosphere from plate studies of the soil

microflora. He noted a stimulation of the soil microflora in the neighbourhood of plant roots. Other investigators using the plate method have confirmed Hiltner's observations e.g. Löhnis (1926) and Smith (1928) working before 1929 in what may be called the pre-Starkey period. (Creuzberg (1928) is outstanding among rhizosphere investigators in finding no stimulation of the bacterial flora by plant roots). Smith (1928) used a particularly interesting modification of the plating technique, in which had been added crystal violet to the innoculated soil suspension, to give a concentration of 1 part in 100,000. This was sufficient to prevent the growth of actinomycetes and Gram + bacteria, and yet still allow free growth of B. radiobactar: this organism was found to be most abundant near the roots of legumes, becoming almost completely absent at a distance of 1 foot from the plant. Velich (1903) using soil extract agar for plating was able to demonstrate higher numbers of the organism Clostridium gelatinosum in the environs of sugar beet roots than distant from them.

Starkey used agar media for some of his rhizosphere studies. For his first experiments (1929a) he used albumin agar, mannite agar and glycerol-nitrate-soil agar, (the latter modified to contain 1 part of crystal violet in 100,000 parts of the medium), as plating media for rhizosphere

studies. He found an increasing rhizosphere effect for plants as they approached their maturity with a subsequent fall off from maturity to death, as measured by the plate counts of rhizosphere soil and soil distant from roots, plated on albumin agar media. The use of the glycerol-nitrate-soil medium with crystal violet enable him to detect that the greatest proportional increase in the populations was in that Gram negative group of organisms which he assigns to B. radiobacter. The categories of soil microbes which he was able to assess on these media were (a) total number of bacteria on each medium, (b) total numbers of the B. radiobacter group on nitrogen free mannite agar and (c) on soil-glycerol-nitrate agar, (d) the numbers of actinomycetes and (e) filamentous fungi. It is interesting that the estimate of B. radiobacter is strongly influenced by the nature of the medium and the dilution of the sample used for plating. In Table I some estimates of the group made on the two media are taken from Starkey's paper (1929a).

TABLE I

Plate counts of bacteria from plant rhizospheres obtained by plating 1 ml. aliquots of diluted samples on two media. (From Starkey 1929a).

(Plants 44 days old)

Media Plant Rhizosphere	Nitrogen free mannite agar Diln. 1/100,000	Glycerol-nitrate agar Diln. 1/10,000
Fallow	9.8	5.4
Oats	6.6	7.8
Corn	12.8	6.8
Beans	13.6	19.8
Potatoes	11.2	7.8
Table beet	10.2	8.4
Mangold	20.8	14.0
Rape	31.8	466.0
Sweet Clover	7.2	11.4

Starkey points out the obvious limitations of the plate method for rhizosphere studies. However, in the fourth paper of the series (1931) he again uses the plate method, this time to determine the influence of "proximity to roots on abundance and activity of micro-organisms". He was

able to show for the rhizosphere of beet, mangold, sweet clover, corn and beans that the greatest abundance of micro-organisms was on the root surface and that there was a sudden fall in the microbial population from the root surface to the adjacent soil. The number of organisms fell off with the distance from the roots. This was also found by Clark (1940) for the rhizosphere of cotton. A qualitative change in the microbial populations of the rhizosphere was shown by the plate method in the work of both Starkey and Clark. Starkey points out that actinomycetes and filamentous fungi are not so much affected by plant roots as bacteria, and Clark records a sharp increase in both actual counts and proportions of 'dye tolerant' bacteria in the rhizosphere and the depression of a spore forming bacillus (see Table II).

TABLE II (from Clark 1940)

Qualitative and Quantitative effects of proximity of soil to roots on the microbial population of soil.

	Total * bacteria	Dye * tolerant bacteria	Ratio $II_{/I}$	Spores * of bacillus	Ratio $III_{/I}$
Soil 10-15 cm. distant from roots	52.6	7.4	0.141	17.0	0.323
Soil 5-10 cm. distant from roots	47.9	9.0	0.188	18.0	0.376
Soil 2.5-5 cm. distant from roots	45.6	7.7	0.165	17.0	0.373
Soil 0.5-2.5 cm. distant from roots	54.7	15.0	0.274	11.0	0.203
Soil 0 - 0.5 cm. distant from roots	129.9	91.3	0.702	13.7	0.105
Root surface scrapings	510.0	440.0	0.863	0.1	0.001

* Numbers in hundred thousands per gram. of air dry soil or root surface scrapings.

Krassilnikov (1934) has used agar media for the investigation of rhizosphere floras. He has examined the persistence of Azotobacter in the rhizospheres of wheat and maize on specialised media for the growth of Azotobacter, and he was able to show that this organism was rapidly eliminated from

the rhizosphere of growing plants. This observation was confirmed by Clark (1948). Krassilnikov ascribed this to the toxicity of root excretions. In a later paper Krassilnikov et al. (1936a) describe a detailed survey of the microbial flora of rhizosphere and non-rhizosphere soils by the use of the plate method, and recognise the following groups which they found worth recording; (a) total number, (b) non sporers, (c) spore formers, (d) mycobacteria, (e) micrococci, and (f) cellulose bacteria, which latter group was estimated on a special medium. Sometimes the developing colonies were divided into types which could easily be distinguished from the morphology of their cells and colonies - e.g. non sporing types were divided to 'Proteus', 'fluorescens' and 'other non sporing' types and the spore formers were divided to 'mycoides' and 'other sporing' types. It is characteristic for the qualitative results from the use of the plate method to be in terms of easily recognisable morphological features of the colonies, or relatively easily determined microscopic characters such as presence or absence of spores. Krassilnikov et al. (1936a) report great increases in the total numbers of organisms in the neighbourhood of the roots, 'dozens and hundreds of times greater than in the control soil'. They found two peaks of rhizosphere activity (cf. Starkey, (1929a) who

described only one peak) the first during stalk formation in wheat and head formation in sunflowers, the second associated with fruit bearing. A qualitative difference was observed between the rhizosphere and control soils but little difference was found between the rhizosphere of different species of plant. The main difference found were increases in non spore formers and mycobacteria with a depression of spore formers in the rhizosphere. P.fluorescens and B. dentrificans were preferentially stimulated in the rhizosphere.

Lochhead and his associates have used the plate method for intensive studies of the rhizosphere floras of cultivated plants. In these studies they have plated diluted rhizosphere and non-rhizosphere soils on to soil extract agar.

Taylor and Lochhead (1938) described the following groups of organisms isolated from soil in this way.

I. Short rods, Gram positive.

II. Short rods, Gram negative.

III. Short rods, Gram variable.

IV. Short rods changing to cocci. (B. globiforme)

V. Coccoid rods, Gram positive.

VI. Cocci, Gram positive or negative.

VII. Long rods, non spore forming.

VIII. Spore forming rods.

and subdivided these according to certain physiologic characters, e.g. gelatine liquefaction, nitrate reduction. This was an extension of a classification proposed and used by Topping (1937 and 1938), of the types of organisms developing on agar plates from soil samples. The classification was used for rhizosphere studies by Lochhead (1940) who found, like Krassilnikov and Starkey, a predominance of Gram negative rods in the rhizosphere, with a depression of the percentage incidence of cocci, spore formers and Gram positive bacteria. It was found that the rhizosphere effect as manifested in the increase in total numbers of organisms above the normal soil population, and in qualitative effects as described above, was greater for tobacco and flax varieties susceptible to, than for those resistant to Thielaviopsis basicola and Fusarium lini respectively. In further attempts to take the classification of rhizosphere and soil organisms beyond that based on the morphology and staining properties of cells and colonies which they had previously used, West and Lochhead (1940a), isolated by the plate method using soil extract agar, 100 organisms from rhizosphere and 'control' soils and determined their nutritive requirements. For this purpose they determined the turbidity developed after incubation of the various isolations in the following broth media: a basic glucose salt medium (a) alone, or (b) with amino nitrogen, (c) with

amino nitrogen and growth factors and (d) enriched with yeast extract. It was found that a much higher proportion of the organisms isolated from the rhizosphere required amino acids and growth substances than of those from control soil, and the authors refer to 'the more fastidious organisms of the rhizosphere'. It was found that the organisms isolated from disease resistant flax and tobacco showed less rhizosphere effect, as measured by the nutrient demand tests, than those from susceptible varieties. West and Lochhead (1940b) have developed a parameter of the bacterial populations, the 'Bacterial Balance Index', which is an attempt to express numerically the qualitative rhizosphere effects. The bacteria isolated from a soil by the plate method on soil extract agar, are subcultured in Difco yeast extract broth, and their growth is subsequently measured in the four nutrient solutions described previously. The isolated cultures are classified into groups as follows.

A. Bacteria capable of synthesising all substances for their maximal growth from constuents of a glucose-nitrate-salts medium.

Gram positive and Gram variable bacteria	Gp. 1
Gram negative 'fluorescens' types (producing pyocyanin)	Gp. 2
Other Gram negative bacteria	Gp. 3

B. Bacteria requiring the addition of cysteine to glucose-salts medium for maximum growth.

Capable of submaximal growth in glucose-salts medium. Gp. 4

Unable to grow in glucose salts medium. Gp. 5

C. Bacteria requiring the addition of cysteine plus alanine, protine or asparagine for maximum growth.

Capable of submaximal growth in glucose-salts medium. Gp. 6

Unable to grow in glucose salts medium. Gp. 7

D. Bacteria requiring the addition of cysteine plus thiamin and/or biotin for maximum growth.

Capable of submaximal growth in glucose-salts medium. Gp. 8

Unable to grow in glucose-salts medium . Gp. 9

E. Bacteria incapable of growth in any above synthetic media, requiring the presence of unidentified substances in soil extract or yeast extract. Gp. 10

The Bacterial Balance Index is obtained by taking percentages (to the nearest whole number) of the organisms examined, which fall into these groups and group 3 is assigned a negative value and groups 5, 7 and 9 are given positive values: these four values are than summated to

give the Bacterial Balance Index. It was considered that the other groups were not useful indicators, of changes in the rhizosphere populations and they were ignored in the computation of B.B.I's. The authors claim to show a seasonal change in B.B.I. and that the rhizosphere flora has a higher B.B.I. than control soil. However, as used by West and Lochhead, there must be very great inaccuracy in the calculation of each B.B.I., which is, in the form used by these authors, the sum of the percentage representation of four groups of bacteria in a total of less than fifty isolations examined. The variance of these determinations must be enormous, and has not been determined. There is no reason to believe any of the B.B.I's. to be significantly different from each other until the error involved in their determinations has been calculated.

Lochhead and Chase (1943) have developed media for determining in greater detail the relative nutrient demands of the various organisms isolated from soil on 'non-selective' media, but this more complex system has not been used for making comparisons of soil and rhizosphere floras. Katznelson and Richardson (1943) have used the Bacterial Balance Index for studies of the tomato rhizosphere and control soils and the changes brought about in

these by soil sterilisation. The index was lower in rhizosphere than in control solid 'though not strikingly so'. West and Hildebrand (1941) however, found that on soils on which strawberries were grown, a low index was associated with incidence of a factor causing brown root rot in this crop. Katznelson and Chase (1944) showed that soil amendments, e.g. dextrose, brought about considerable qualitative changes in the soil population. They also used the Bacterial Balance Index. A summary of the investigation by Lochhead, Timonin and their co-workers is given in a review by Katznelson, Lochhead and Timonin (1948) which also includes a general survey of rhizosphere literature up to 1948. Further reviews of this literature can be found by Harley (1949), Clark (1949) and Garrett (1950).

Much other work has been done on rhizosphere floras using the plate method, but the uses described above, illustrate the form of results which may be expected from the use of this method.

(c) The Direct Method

(1) Original form.

In 1918, Conn described a direct method for the determination of the numbers of bacteria in soil. This consisted in a modification of a technique of Breed (1911), for the direct counting of the bacteria in milk, which was itself a modification of earlier methods for counting bacteria in faeces, sewage and milk, the first use of the technique being probably by Eberle (1896). In Conn's technique a soil dilution is prepared, and 1/100 cc. of this is spread on a slide over an area of 1 cm^2. After drying, the smear is stained with Rose Bengal to differentiate organisms, which stain pink, from the soil, which does not stain, and counts of the numbers of bacteria are made under a microscope. Estimates of the mean count per microscope field, with knowledge of the extent of dilution of the smeared sample, and of the relative areas of the smear and of a microscope field, are used to estimate the microbial population. Winogradsky (1925) modified Conn's method, so as to exclude from the smear, most of the heavy inorganic particles of soil which sometimes obscure the microbes. He washed each soil sample three times, allowing it to stand for 30 seconds after shaking in water, and conserved the supernatant liquid at each stage. The supernatant liquid was then centrifuged and gave a

sediment containing almost all the micro-organisms from the sample. For staining the films, he used erythrosine which he claimed to give clearer staining than Rose Bengal.

The essential faults of the method have been exposed by Winogradsky (1925) and Thornton and Gray (1934). Winogradsky states "La faiblesse de la méthode réside dans le fait qu'il n'est pas possible d'isoler les organismes qu'on voit et qu'il n'y a pas moyen d'étudier leau activite", but holds that the method allows investigation of, 1. "la quantité générale des germes visibles au microscope" and 2. "les pullulations qui ont lieu dans le sol sous l'influence d'engrais azotes". Koffman (1928) has described a somewhat similar method for the examination of microflora and microfauna, in which the soils are fixed, stained and mounted in Canada balsamm in hollowed slides. This technique is complex and, while it may be good for such soil inhabitants as protozoa, is obviously less satisfactory than Conn's and Winogradsky's methods. A technique for preparing microtome sections of soil has been devised by Harper (see Appendix III) but this is only really easy with completely organic soils, and in these the micro-organisms are often obscured by the dense dark soil.

There is frequently a remarkable disparity between the estimates of bacterial abundance made by the plate count

and by the direct method. For example Halvorson and Ziegler (1933) investigating meat pickling fluid, obtained estimates of the bacteria present of 500,000 - 1,000,000 per ml. by direct count, but only 10,000 per ml. by plating.

Breed (1911) in the first account of the use of the method for milk, gives the following figures for comparison with the plate count.

TABLE III (from Breed 1911)

Plating Method	Direct Count	
Average of 2 plates	Each individual counted as one	Clumps counted as one
37,500	1,345,000	325,000
77,000	2,950,000	730,000
51,000	3,250,000	495,000
59,500	4,070,000	570,000
50,000	7,115,000	1,110,000
24,500	4,360,000	850,000

It is clear from these data, that even when allowance is made for the development of a whole clump of organisms into a single colony, which must necessarily happen with

clumps which are plated, the direct method gives by far the higher value. This disparity between the two methods is normally, however, even more apparent for soils than for milk, for the organisms present in the latter are usually all able to grow on the medium provided. Buice (1934) for example found the ratio of the direct count to the plate count for milk samples to be roughly unity, varying from 0.01. to 1.70. In estimates of soil micro-populations however, there is a very great variety of bacteria present in a sample, and it is less likely that all will be able to develop on any one medium.

(ii) The ratio method.

Thornton and Grey (1934)have criticised the direct method for the estimation of microbial abundance, in the form used by Conn and Winogradsky. They point out that there is a very considerable error in the estimates,because they are based in Conn's method on pipetting .01 ml. of suspension of soil, which cannot be done accurately, and in Winogradsky's method on the weighing of a soil film of 0.1 mg. which is also likely to be inaccurate.

Thorton and Grey show that if a suspension of organisms is distributed at random throughout a soil smear, then the distribution of numbers of organisms viewed in sample microscope fields, should fit a Poisson distribution. They

showed however that the organisms in a soil smear were not distributed at random, and that for this reason the mean of the microscope field counts could not be a reliable estimate of the microbial population.

A method was adapted from Wright (1912), in which the two difficulties described above were eliminated. This consisted in mixing a known weight of the sample soil with a known volume of a suspension of indigo particles of about 500,000,000 particles/cc. The mixture is diluted with agar and spread on a microscope slide. Erythrosine is used to stain the bacteria, and the ratio of bacteria to indigo particles is counted for each randomly sampled microscope field. It was found by Thornton and Gray (1934) that the ratios from replicate microscope field were distributed at random, and the sampling technique was therefore regarded as valid. In comparisons of this technique with plate counts made on Thornton's medium (1922), the ratio method gave estimates of soil populations 117-1730 times as great as the plate method.

It is of considerable interest that the metabolic activity of soil is more closely correlated with the direct count than with the plate count. For example, Jenson (1936) found a significant correlation of carbon dioxide evolved

per gm. of soil, with the direct count made by the ratio method, but no correlation between the plate count and CO_2 evolution for the same soil.

Jensen (1934b), (who had used the ratio technique with Thornton and Gray) finds no fault with the method, apart from making the change back to Rose Bengal as stain. Topping (1938) however, found considerable difficulty in using the method and lists the following objections to it.

(a) Stained organisms dwindle in size to beyond the limits of visibility.

(b) Bodies appear which are pink at one focus and brown at another and so are of uncertain nature.

(c) Large masses are present which may be clumps, and sheathed compact clumps are uncountable.

(d) Adsorption of bacteria by soil is more pronounced in some soils than in others.

(e) It is difficult to obtain indigo particles of uniform size and free from clumping.

The chief objections which can be raised to the use of any form of direct method in soil investigations are that, (a) it gives no information about the precise location of the organisms, which information is given by the 'buried slide' method;

(b) it gives no information about the activities of the organisms, which disadvantage it holds in common with the 'buried slide' method.

(c) it is by no means clear that only living organisms are counted, and it may be held that the inclusion of dead organisms is responsible for the much higher estimate of the population given by the direct count than by the plate method. However Strugger (1948) has recently applied to soil microbiology, the techniques developed by Strugger (1940) and Strugger and Hilbrich (1948) for differentiating between living and dead bacteria by special staining methods. These methods involve the use of a fluorescent dye, acridinorange, which fluoresces green at the concentrations to which it is absorbed by living cells, and red at the concentrations to which it is absorbed by dead cells. Moreover, the dye is an intra-vital stain, and there is therefore no theoretical reason why the stained cells should not be picked off a slide with a micro-manipular and their culture attemped. The use of the fluorescence microscope for examination of bacteria in soil may prove valuable for rhizosphere studies.

(d) <u>The Buried Slide Method</u>

This method is essentially the use of a glass slide, immersed in soil, which can subsequently be examined for microbes stuck to its surface. After some period in contact

with the soil, micro-organisms make contact with the glass, grow on it and stick to it. Subsequent fixing and staining of the soil film on the slide gives a permanent record of the microbial activity in the soil. This technique was devised independently by Cholodny (1930) and Rossi (1934).

(1) Rossi-Cholodny:

(The 'Bodenplatten' or 'Aufwuchsplatten methode').

A cut is made in the soil surface with a sharp knife, and into the cut is placed a clean microscope slide, and the soil is pressed around the insertion to made a close contact between soil particles and glass. The slide is covered with surface soil from the surroundings, and is left in position for 1-3 weeks. After this period the slide is carefully removed by digging, so that there is no smearing of the surface. The slide is dried, fixed and stained and examined microscopically. A picture, is obtained by this method, of the 'physiognomy' of the soil system - a picture of the spatial arrangement of the organisms in the soil, and in particular, the abundance of fungal development can be assessed far more satisfactorily in this way than by the plate method. Rossi described in a series of birthday essays to Stoklasa (1923), a somewhat similar technique and has priority in publication. A detailed description of the method has also been published in English, Rossi and Ravenna et al. (1937b).

In the Rossi method, a slide is pressed momentarily against a bared vertical soil surface, removed and subsequently dried, fixed and stained. It is thus free from the objection which might be made to Cholodny's method, that disturbance of the soil, and long rest of the slide in the disturbed soil, made the method give a picture of selected organisms only, those in fact which grow on glass surfaces in disturbed soils.

(ii) Modified methods of Conn etc.

Conn (1932) adapted the Rossi-Cholodny technique for use in experimental studies of the soil microflora. The only essential change which he made is that the soil is placed in small glass tumblers which must destroy the original soil organisation, in this way however the conditions of the soil may be changed or maintained at any given conditions. Slides are left immersed in the soil for 5 - 7 days only. Ziemiecka (1935) made a further modification of Conn's method, for use in the experimental determination of the types of organisms which are stimulated by specific organic additions to the soil. The substances to be tested are smeared or spotted onto clean slides which are then buried in tumblers as in the Conn method. After various time intervals the slides are removed, and the micro-organisms fixed and stained. (It is obviously

important in this type of experiment to use some untreated slides as controls; this Ziemiecka failed to do). Jensen (1934b) compared the Rossi-Cholodny method modified by Conn, with a plate count made of the same soils. Effects of soil amendments were shown by both methods both in qualitative and quantitative comparisons. It is concluded that the two methods used together compensate each other's disadvantages. Jensen criticises the Rossi-Cholodny method on the following grounds:-

(i) It does not give cultures of the organisms observed.

(ii) Most organisms cannot be determined simply by morphology.

Criticisms (i) and (ii) are both of course applicable to any direct method for observing the soil microflora.

(iii) "....we cannot tell whether the picture, seen on the slides has been produced soon after the slide was placed in the soil, or just before it was removed" (This is surely to be found by comparing slides made after a range of days immersion in the soil, or comparing the results obtained by the use of the original Rossi method with those from the Cholodny method).

(iv) "....the distribution of organisms on the slides is always too irregular to admit of reliable counting".

Jensen has made a further modification of the Rossi-Cholodny method and used it for the quantitative determination of fungus mycelium in soil. On slides prepared by the Rossi-Cholodny method modified by Conn, replicate microscope fields were examined for presence or absence of fungus mycelium, also the number of fungal spores was counted, but as Jensen points out, the distribution of spores over a Rossi-Cholodny slide is not random, and so the mean is not a valid estimate of the population. He does not, however, point out that the percentage of field with fungus present is misleading for the same reason. Percentage absence can be used as a measure of the density of a population distributed at random, for the number of replicate samples bearing no representatives of the subject investigated, represents the first value in the Poisson series, e^{-m}; $m.\ e^{-m}$; $\frac{m^2}{2!}\ e^{-m}$; etc. Therefore, if percentage absence (or 100 - % absence = % presence) is known, m, the mean density can be determined, but only if the distribution is Poisson. Percentage of fields containing mycelium is not therefore a valid index of fungus density when the distribution of fungus on the samples is not random.

(111) <u>The buried slide method in rhizosphere studies</u>

Starkey (1938) has used the Rossi-Cholodny method for rhizosphere studies, where the method has shown itself

to be extremely elegant. Confirmation was obtained of the observations made by the plate method, that the greatest abundance of organisms was directly upon the root surfaces, particularly about root hairs. The population of bacteria was primarily of coccoids, which is of especial interest in view of the demonstration by Lochhead (1940) by the plating method, that rods predominated in the rhizosphere flora. Linford (1942) has used a modification of this method for rhizosphere investigations, and has published photographs similar to Starkey's, of microbes congregated on plant root surfaces, obtained by the use of an observation box in which plants are grown and in which some of the roots develop against small observation windows made of cover slips.

Timonin (1940b) has noted a predominance of short and long rods in the rhizosphere together with coccoid forms. The buried slide method showed no qualitative difference between the rhizosphere of varieties of flax resistent and susceptible to wilt in the qualitative nature of organisms but quantitative differences were noted.

(e) Metabolic methods

(i) The methods of Remy and Löhnis

Most of the methods for investigating soil microbes have been ways of counting them, or of isolating them and

studying their activity in the highly artificial conditions of liquid or agar culture. Ziemiecka's modification of the Rossi-Cholodny plate was an attempt to investigate microbial action on specific subtrates within the milieu of a relatively undisturbed soil. However, the methods of Remy (1902 a,b,c,d,) were the first to be employed in the investigation of the metabolic activity of a soil as a whole. The Remy method consisted essentially in the addition of a soil sample to a liquid medium containing some metabolite, with the aim of finding what types of action the soil had upon this substance. Chemical analysis of the medium after a period of incubation gave a measure of the reaction undergone. In 1909-10 a controversy arose between Fischer (1909 and 1910) and Löhnis (1909) over the respective merits of the plate method and the Remy method, for the investigation of soil microbes and Conn (1910), supporting Fischer, described the conditions of Remy's experiments as "....an unknown number of unknown organisms under conditions which are totally new to the bacteria.....". The comparison of the plate method and the Remy method was not of course, really sensible, for one was a measure of the numbers of certain viable organisms, whereas the other was a measure of the metabolic potentialities of a soil under specific conditions. The two methods are to a great extent complementary.

The major criticisms of the Remy method are as follows:-

(i) When a soil is placed in a solution of some substance, the physical and chemical conditions of the soil are so grossly changed, that the micro-organisms in the system cannot be expected to behave in the same way as they would in a normal undisturbed soil. (But of course one cannot investigate a normal undisturbed soil).

(ii) That the presence in the soil sample of just one individual organism capable of multiplying and carrying out a reaction with the added subetance, may in time give as strong a reaction with or as complete a transformation of the substance, as a soil sample initially containing many millions of that organism. The method then tells what a soil can do, but this need be no indication of what it does do under natural conditions, and it is significant that Stevens et al. (1909 a and b) have shown that nitrification and ammonification go on at very different rates in soils and solutions of soil. The early usage of Remy's method is well reviewed by Workes and Lipman (1907).

Brown (1913a) added strong solutions of proteins and other organic nitrogenous compounds to large samples of soil - so that the soil texture was not unduly disturbed - e.g. 10 ml. of metabolite solution added to 100 gm. soil - and after incubation, determined the extent of breakdown of

the metabolite. Experiments of this sort are widespread in the literature. However it is not readily possible to compare two soils by this method, because the conditions may have changed considerably in the course of the experiment. Only when a soil process can be measured dynamically, can the decomposition of metabolites usefully be compared. This can be achieved by sampling, over a series of time intervals from the beginning of the experiment, replicate soils to which a metabolite has been added, or by measuring heat changes if a thermal reaction is involved in the destruction of the metabolite, e.g. Newman and Norman (1943), or if gas changes are involved these may sometimes be followed manometrically, Lees (1949b) or by a continuous flow gas absorption method.

(ii) The methods of Lees and Quastel

Lees and Quastel (1944) and Lees (1949) developed the biochemists technique of tissue perfusion, for use in the investigation of the dynamics of soil processes, and have been able to compare soils in this way, and to investigate the dynamics of soil processes under conditions which depart relatively little from those of normal soil. When a soil reaction can be investigated dynamically, it is often possible to extrapolate back to the start of the experiment and so to compare 'untreated' soil systems.

An example of the use of metabolic methods in rhizosphere studies is found in the work of Martin (1949). The

Lees-Quastel method was used and he was able to demonstrate that soils from around the roots of different species of grass showed significantly different nitrifying powers.

(f) The Dilution Method

The enormous discrepancies frequently found between estimates of mixed microbial populations made by the direct and the plate methods have led many authors, e.g. Halvorson and Ziegler, to suppose that many bacteria will not develop to colonies on solid media. If this be so, the great advantage of the plate method in effecting spatial separation of developing colonies is lost by the intolerance of the solid medium by some organisms. Hiltner and Störmer (1903) developed a method for estimating soil organisms which did not involve the use of solid media. A soil is diluted many times and samples of various dilutions are taken into a substrate for bacterial growth. From a determination of the lowest concentration of a soil to give bacterial growth when innoculated into the substrate, it is possible to calculate the number of organisms present in the original sample. Löhnis (1905) in a comparison of the estimates of bacterial numbers by the gelatine plate and the dilution method found 1,270,000 and 3,750,000 bacteria per gm. by the gelatine plate and dilution methods respectively.

The mathematical foundation for the interpretation of results obtained from the use of the dilution method has been studied by Halvorson and Ziegler (1933).

If random samples are taken from a soil dilution, then the numbers of samples containing given numbers of organisms should accord with a Poisson distribution. Where m is the mean number of organisms per sample, the e^{-m}, me^{-m}, $\frac{m^2}{2!}.e^{-m}$; etc. represents the proportion of the samples with 0, 1, 2, etc. organisms. If a soil dilution be sampled and replicate aliquots removed from it to tubes of a liquid nutritive medium, the proportion of tubes in which no growth occurs should give an estimate of e^{-m} from which m can be calculated. A full mathematical treatment of this percentage absence test is given by Halvorson and Ziegler (1933) The presumptive coli test used in milk and water pollution studies is an example of the dilutions and % absence method in common use.

Topping (1938), has used this method for the estimation of soil bacteria, and has compared the estimates of a soil population made by plate counts, and the dilution method using sterile soil as the medium for bacterial growth. Table IV is taken from this study by Topping (1938).

TABLE IV

A Comparison of Yeast extract agar, Yeast extract broth and sterilised soil in estimating the numbers of micro-organisms in soils.

Soil No.	Plate Count	Dilution Method			
	Yeast extract agar	Yeast extract broth		Soil	
		2 weeks	5-6 weeks	2 weeks	5-6 weeks
Incubation at 22°C.	2 weeks				
A	43.0	79.2	130.0	28.8	115.0
B	28.8	81.8	81.8	19.3	49.3
C	21.0	47.4	125.0	11.6	74.2
D	8.4		15.0		11.02
E	8.0		39.8		19.5
			3 weeks in incub.		3 weeks in incub.
F	41.0		74.2		19.3
G	33.0		39.9		21.4

Higher estimates of the population were given by the use of broth than agar, but there was little difference between soil and broth as media for a dilution count. Very much higher estimates of the population are obtained in the dilution tests by incubation for 5 - 6 than for 2 weeks,

and it may be that the increased estimate is due to the increased chance of contamination under prolonged incubation. The dangers of contamination in the use of the dilution method are great, and it is absolutely essential to have a control series of tubes to each experiment in which each control tube is exposed to every risk of contamination to which the experimental series is exposed.

Glathe and Cunningham (1933) used the dilution method for estimating anaerobes in manure and found that it gave higher estimates than the plate count.

The method has its greatest value in the enumeration of the protozoan population of soils, Cutler, Crump and Sandon (1922). For soil bacteria it is more tedious than the plate method and gives lower estimates than the direct method.

The dilution method in rhizosphere studies

Starkey (1929a) innoculated dilutions of rhizosphere soils from various plants, into tubes of a nitrogen-free medium, and attempted to assess the rhizosphere effect in terms of the number of nitrogen fixing organisms in soil and rhizosphere. However, too few replicates were used to enable proportional absence, e^{-m}, to be calculated accurately for even one soil, and comparison was clearly impossible.

Katznelson (1946) used the dilution method to assess the numbers of organisms of types known not to flourish on

normal nutrient agard - e.g.

Algae in sodium nitrate solution.

Nitrifying bacteria in ammonium sulphate solution ~~bacteria~~.

Ammonifying bacteria in 1% gelatine and salts.

Denitrifying bacteria in asparagine nitrate solution.

Cellulose decomposing bacteria in Omelianesky's medium.

This is one of the only ways available for estimating these organisms but it is not possible from the data given, to discover how Kalznelson obtained the results which he presents or to judge their significance. It is very important that in the recording of results based on statistical methods, sufficient data be given to enable the significance of the results to be judged.

(f) The Soil Plaque method

The soil to be studied is mixed with some substance on which the soil may act, e.g. starch. The soil is added to Petri dishes and pressed down to give a smooth surface. The dish is placed in a damp atmosphere and colonies of micro-organisms, developing in the soil upon the added materials, are detected either by the direct microscopic method or by observation of colonies growing on the soil surface.

This method is due to Winogradsky (1925) and its use has been mainly limited to the French school of micro-

biologists at the Institut Pasteur. The technique is described in full in Pochon and Tchan's manual (1948).

Wilson J.K. (1937) has used the method in attempts to develop colonies of Azotobacter. He concluded that they could be produced from almost any soil. The method has not been used for rhizosphere studies as it is not sufficiently sensitive to detect soil differences; its chief value lies in its power of multiplying a few specific microbes, e.g. Azotobacter, which are then available as sources of cultural material.

All of the methods described in this paper have been used for microbiological studies of soil, but specialised methods for the investigation of soil fungi, e.g. La Touche (1948) have been omitted from this review.

Not only does the method used determine to a great extent the form of the results of an investigation, but the results may also vary with the way in which a method is employed. The plate method is particularly liable to give misleading results through misuse, and ways in which this may happen will be described in the next section.

PART I. B

The methodology of the plate count.

(a) Introduction

The plate count is not only limited in its applications for the reasons discussed earlier, but it is also capable if mishendled, of giving results which are misleading even ~~to~~ in the very restricted sense in which plate count data can be acceptable. It is proposed here briefly to review the investigations of the technique of the plate method.

(b) The Statistical treatment of plate count data

(i) General method

The process of investigation of soil microbes must necessarily involve sampling. The number of organisms present in soil is so vast that any procedure must be of profound value which will indicate the amount of sampling necessary for a reasonably accurate estimate of the subject considered to be made. More important however, is that when sampling is as vital a part of investigations as in soil microbiology, it is necessary to know how much faith can be placed in estimates made of the microbiological population of a soil from the examination of relatively insignificant samples. The careful use of statistical methods for the design of experiments and their analysis enables both these requirements to be met.

The statistical treatment of plate count data has been studied by a number of investigators. Johnstone (1919) describes a statistical treatment for the plate count data obtained in the routine examination of mussels for faecal contamination. The sampling procedure here adopted is very simple. Five sample mussels are opened into a flask of sterile water, are shaken in the diluent, and samples of the diluent are plated directly onto McConkey's nutrient agar. The variation between replicate plates made in this way gives an estimate of the variability of any one sample, and so of the validity of the single sample and the mean for estimating the whole microbial content of the five examined mussels. He realised that the distribution of plate counts which he obtained was not Gaussian, and erroneously supposed that the mean and standard error were not therefore valid parameters by which to compare populations. It remained for Fisher et al. (1922) to show that a Poisson distribution is expected for plate count data and that the mean, although not necessarily the most probable value, is with its variance a real and useful parameter of the plate population.

Budinov (1910) made one of the first attempts at statistical treatment of plate count data. Waksman (1922a) appears to accept Budinov's treatment in his own account of the use of statistical methods for the analysis of plate count data from soil studies.

The extent of variation between samples is expressed by the sum of the squares of deviations of individual observations from their mean divided by the number of observations - 1. This statistic, the 'variance' of the observations, is an estimate of a property of the population sampled. This variance divided by the number of observations gives a further statistic, the variance of the mean of the observations. The so called 'standard errors' of the observations and the mean can be obtained by taking the square roots of the two variance estimates.

Thus* Mean = $\bar{x}$ = Sx/n

Variance of observations = $V_x = \frac{S(x - \bar{x})^2}{n - 1}$

Number of observations = n

Degrees of freedom = n - 1

Variance of mean = $V_{\bar{x}} = \frac{S(x - \bar{x})^2}{n(n - 1)}$

Standard error of observations = $V_x = S_x$

Standard error of mean - $V_{\bar{x}} - S_{\bar{x}}$

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* N.B. The terminology and symbols employed by Waksman have been transformed where possible into the modern conventional form used by Fisher (1946)

Waksman (1922a) uses two other statistics which have since dropped out of use; these are the coefficient of variability, C.V., and the 'most probable error'.

$$\underline{\text{Coefficient of variability}},\ C.V. = \pm \frac{Sx}{\bar{x}} \cdot 100$$

$$\underline{\text{Most probable error}}.\ E_m = \pm\ Sx.\ 0.6745$$

The most probable error gives a measure of limits of variability within which 2/3 of the observations should fall if the distribution is normal.

Waksman (1922b) makes and presents a large number of observations for which he has calculated the statistics described but, for several reasons, the use which he makes of them is less than it could be, and some of the usage is not valid under the particular circumstances of his experiments.

The peculiar value of the calculations of the statistics described, is that they should enable a judgement to be made, and the justification for the judgement to be assessed, as to whether two sets of observations differ significantly or not. It should be possible from the statistics to determine the chance of obtaining the two sets of results which are being compared from one sampled population when the difference between the compared sets, and the variability of each set has been determined. The *t* test, devised by Student, allows this to be done and so gives a purely objective measure of the justification for what must anyhow be a subjective

decision. Waksman (1922b) does not use such a test, and in fact some of the difference which he assesses to be real, are found not to be very significant when the test is applied.

However, there is a more fundamental error in Waksman's use of statistics. This will be illustrated in considerable detail because of its very real importance in the application of statistics to microbiological studies.

(ii) "The error of the wrong replicate".

Johnstone (1919), in his very early use of statistical method in plate count studies, has estimated the error in counts of faecal contaminants of mussels. Five mussels were sampled and opened into 250 ml. of sterile water. After shaking, 1 ml. samples of the diluted mussel fluid were taken to McConkey's agar in Petri dishes and the developing colonies were counted. The mean of the replicate plate counts should give an estimate of the numbers of those organisms in the sample of mussel washings, which are capable of growing to colonies on McConkey's agar. The results obtained give information about the variation between sub-samples from the mussel dilution. This can give an estimate and allow assessment of the accuracy with which we know the total number of organisms which were present in the five mussels and which were capable of growing on the McConkey

agar. But, of course, the variation between these replicate plate counts is in no sense an estimate of the variation between individual mussels. All the bacteria estimated could have come from one of the five mussels in the sample, or the five mussels could have been quite unrepresentative of the whole batch of mussels from which the sample was taken. It is very important that this rather obvious feature of the relevance of error determinations be fully appreciated, for in soil microbiological studies it is more complex, and there has been wide misuse of the statistics in consequence. The example of Johnstone's mussels is quoted because it is the simplest form of the fallacy. In the normal procedure of soil microbiology, there is intercalated between sampling the soil, and taking dilution subsamples into agar, a long process of dilution, which is made necessary because of the vast numbers of soil organisms which are present in the size of soil sample required for easy and accurate weighing. This process of dilution is liable to lead to very severe errors in the estimation of the bacteria in the original soil sample. A typical technique for the sampling of a soil microflora would be as follows. A 10 gm. sample of soil is taken from the field, and is suspended in a litre of some diluent, for example sterile water. From this 1 in 100 suspension, a sample of 10 ml. is taken

to 90 ml. of diluent to give a dilution of 1 part in 10,000. 10 ml. of the latter is taken to a further 90 ml. of diluent and this process is repeated until a 'suitable' dilution for plating is obtained. From the final dilution, a series of 1 ml. samples is taken with a pipette into some sort of molten agar in Petri dishes and after incubation the developing colonies are counted. In such a procedure, variation can occur at the following stages:

(a) <u>between replicate soil samples</u>; Unless the extent of variation between replicate samples is known the extent to which the 10 gm. sample is representative of the soil sampled, cannot be assessed.

(b) <u>between replicate dilution series, prepared from any one soil sample</u>.

This sampling error obviously arises at every step in the preparation of a dilution series, and if a series of progressive dilutions is used, such as that described, error is liable to become cumulative at each stage.

(c) <u>between replicate plates prepared from the 'satisfactory' dilution of each sample</u>.

(d) <u>between sample areas on one agar plate</u>. It frequently happens in the use of the plate method, that the number of colonies of micro-organisms developing on an agar plate is too high for an accurate count to be made with the

naked eye. A sampling procedure is then adopted for the estimated of the colonies on the plate. This consists either in counting the colonies in a number of random microscope fields on the plate, or counting the colonies in some of the spaces of a 'counting plate', an apparatus for dividing the plate into a number of equal sized areas.

In the type of study presented by Waksman (1922b), the plates have been replicated and the variation between plate counts has been used as an estimate of the error of the whole sampling procedure. In fact, such an error estimates the variability of the plate counts and the extent to which we know the microbial content of the ultimate dilution. In a microbiological study of a soil, it is obviously required to compare the estimates obtained by <u>replication of the whole of the sampling procedure</u> that separates the soil system on the one hand from the plate count on the other. There are few cases in which this has been done. A good example of correct replication is given in a paper by James and Sutherland (1939a) in a test of technical error which will be considered later.

An interesting example of this very characteristic error in statistical treatment of plating data, is the comparison by Waksman (1922b) of the estimation of a soil microflora

by using a 1/1000 soil dilution for plating, counting colonies on one plate with a counting plate, and using a 1/100,000 dilution and preparing replicate plates. The following data are given in Table V.

TABLE V (from Waksman (1922b)

Dilution of soil sample	Counting method	Mean $\bar{x}$	Most probable error		Coefficient of variability
			Obs. x	Mean $\bar{x}$	C. V.
1;100,000	Total count on replicate plates	73,68	1.36%	1.01%	15.09 ± 0.97%
1:1,000	Counting plate used on one plate	33.6	3.06%	1.03%	35.4 - 2.18%

The errors given here however cannot be used for comparing the two methods because the error of (a) is the measure of variation between plate counts from replicate samples of one 1:100,000 dilution and (b) is a measure of the variation between replicate fields on one agar plate. In order to compare the two methods it would be necesary to prepare a

number of 1 in. 1000 and 1 in. 100,000 dilutions from some initital common dilution, e.g. 1/100, and to estimate the mean and variance of the population of that dilution which the methods hold in common by repeated use of method (a) and of method (b); the methods could then be compared by the t. test.

(iii) *Fit to the Poisson series as a test of the validity of the plate count.*

In 1922, Fisher, Thornton and Mackenzie pointed out that the counts on replicate plates prepared from a soil dilution *should* fit a Poisson distribution. They point out that the conditions required for a Poisson distribution are as completely fulfilled in the case of the plate count as with counts of blood corpuscles in the replicate haemocytometer fields which were studied by Student and found to conform to the Poisson distribution. The form of the Poisson distribution, e^{-m}; $m.e^{-m}$; $\frac{m^2-m}{2!}.e$; $\frac{m^3}{3!}.e^{-m}$; etc. has been discussed already with reference to determination of density of micro-organisms by presence and absence tests.

In the test for goodness of fit of replicate plate counts from a soil sample to the Poisson series, a further property of the series is used, $\bar{x} = V_{\bar{x}}$ (the mean of a series of replicate samples from a Poisson series equals the variance of the observations).

For a test of goodness of fit to a Poisson distribution to be made, a series of sets of paralell plates must be prepared, and for each set the chi-square is calculated as follows $\bar{x}^2 = \frac{S(x - \bar{x})^2}{x}$. For any given number of degrees of freedom, the probability of occurrence of the various values of x^2 can be found. A goodness of fit test is performed as follows.

Actual number of x^2 for a particular value - theoretical number of x^2 is calculated and divided by,

Theoretical number of x^2 values, and the total of all of these x^2 values gives the final x^2 value for which a probability for goodness of fit of the data to a Poisson distribution may be obtained from tables.

Fisher, Thornton and Mackenzie (1922) examined data of Cutler Crump and Sandon (1922) which contained grouped samples from the soil microflora and which could be tested for fit to a Poisson distribution. It was shown that the majority of the sets of plates in Cutler's data had x^2 values within the limits of expectation, but occasionally individual plates or sets of plates showed excessively high x^2 values. It was shown that an organism forming spreading colonies between agar and the bottom of the Petri dish suppressed the development of colonies of other organisms on the plate - see Table VI from Fisher, Thornton and Mackenzie and later discussion.

TABLE VI

Series A. Suspension innoculated with spreaders			Series B. Control. without spreading colonies	
Plate No.	No. of colonies	Area of spreading colonies	Plate No.	No. of colonies
1	85	nil	1	95
2	79	nil	2	90
3	78	nil	3	86
4	70	nil	4	85
5	58	nil	5	85
6	60	2.25 cm^2	6	82
7	56	7.75 cm^2	7	81
8	45	27.0 cm^2	8	77
9	41	54.5 cm^2	9	73
10	39	56.5 cm^2		

x^2 Index: Plates 1-5 5.86 x^2 Index = 1.89

: 1-10 40.01

The data from Table VI are expressed in Graph IV.

Another organism forming spreading colonies was also isolated by Thornton and shown to have a similar depressing effect.

Not only have cases with excessive variance been reported, but also cases with excessively small variance, in which the plate counts in a replicate series, are closer to the mean than is expected on random sampling. In cases of both excessively small and excessively large x^2 cases, that is, of excessively constant as well as excessively variable plate counts, the mean is suspect as an estimate of the population. It is shown that the plate count data of Breed and Stocking (1920) and of Cutler, Crump and Sandon (1922) tend to fit a Poisson distribution, and it is claimed that the means which they obtain are therefore 'a direct measure of the density of the bacterial population considered ....' and '.....the accuracy of such an estimate is known with precision'.

Fisher, Thornton and Mackenzie's test has been widely used since its publication in 1922. Harmsen and Verweel found that the distribution of starch and protein decomposing organisms developing on albumen agar plates from soil innocula, was in very close agreement with the theoretical Poisson distribution. However, in estimates of the total bacteria and actinomycetes there was found to be a preponderance of high and a shortage of small x^2 values. In their words '.....there must be some factor at work which greatly increases the variation of the separate plates in most series rendering the mean more inaccurate and more unreliable".

The plate count data given by Waksman (1922c) were analysed by Harmsen and Verweel for fit to a Poisson and the results were found to deviate in the same way as Harmsen and Verweel's own. The cause of this deviation will be considered later. Other uses of the x^2 test have been made by Sutherland et al. (1938) Jensen (1934a) and Wilson and Kullman (1931).

Sutherland and James (1938) used the x^2 test for goodness of fit of plate counts to Poisson series for pure cultures of Pseudomonas fluorescens and a mixed culture of P. fluorescens and Bacterium globiforme which had been innoculated into sterile soils. They found good fit to a Poisson distribution for plate counts of both pure and mixed culture and good fit for the counts from P. fluorescens at dilutions of 1 : 2,000,000 and 1 : 10,000,000 but there is no information on the actual plate counts and plating densities from which these observations were made. This work of Sutherland and James is of considerable importance for it shows that technique need play no part in upsetting the expected Poisson distribution of plate counts. James and Sutherland (1939a) found however that with normal soils containing a mixed population of organisms there was often very severe departure of plate count data from the Poisson distribution. This departure became increasingly apparent

with the increased interval between sampling of the soil and preparation of the plates. It is apparent that it is anomalous variation in the numbers of bacteria which accounts for the high x^2 values, rather than variation in actinomyces and fungi which tend to show the Poisson distribution. A seasonal appearance of high x^2 values was noted and attributed to the presence of pin point colonies and excessive numbers of colonies of Mucoralen. A plating density of 40 - 200 colonies per plate was used in these studies. Wilson and Kullmann (1931) found many anomalies in their attempts to estimate rhizobia by the plate method. They suppose the large x^2 values given by replicate plates to be due to variations in the growth requirements of rhizobia at different growth stages, and to the gummy nature of the colonies. Jensen (1934a) investigated the validity of his plating technique by the x^2 method and found normal behavious for bacteria and fungi and barely significant departure for actinomycete counts. It is not possible to obtain directly or indirectly from the data any indication of the plating densities employed in these studies.

The use of statistics as described in the above paper necessitates a great deal of labour in the replication of treatments and sampling. It cannot however be overstressed, that without a statistical treatment of replicates, results from investigations involving much sampling must be almost meaningless.

(c) Technical Studies

The technical processes involved in sampling a soil, diluting it and plating the ultimate dilution on to agar, are so many that there are obviously large risks of error due to purely technical faults. Many attempts have been made to determine the nature and to control the extent of variations due to technical and manipulative errors.

The technical stages which require to be examined are

(1) The sampling of the soil.

(2) The dilution of the soil.

(3) The preparation of media.

The literature of technique is vast and only certain selected papers and reviews will be discussed here. Nearly all the technical problems which are encountered in soil microbiological studies are met also in studies of the microbiology of milk, and it is for the latter that the most careful and exhaustive surveys of these problems of technique have been made, and as many of the conclusions should relate as much to soil as to milk studies, they, also, have been considered here.

(1) The sampling of the soil

It is clearly necessary in the study of soil microbes to determine just what is to be sampled from a system as complex as the soil. It has been shown by many investigators

that the microbial population of a soil, changes with depths (e.g. Rippel-Baldes (1947)) and it is known from the work of the rhizosphere investigators already discussed, that there is a micro-pattern in the distribution of soil organisms, determined by the distribution of higher plants. It has also been shown, by Kubiëna (193), that there is a micro pattern in the soil flora associated with soil pore space and other properties of soil texture. The size of sample taken must relate to the problem under consideration; and the existence of the various patterns must be borne in mind in the choice of sample size. Thus, if it is a grass field to a depth of 12", it is likely that the minimal sample size in which the microflora will not be unduly influenced by vagaries of the plant population, will be of an area equal to or greater than the size of the repeating pattern of vegetation in the grass field. Samples smaller than this may be expected to show an unnecessarily large variance. The sample should then be mixed as thoroughly as possible, so that the local variations from place to place within it are destroyed - and the subsamples for dilutions should then be taken as random samples from this mixture. It is again important that the subsample removed be larger than the 'unit of pattern' of the mixed soil sample. It should be possible to determine the size of the 'unit of pattern', by plotting

the standard error of estimates made from various sample sizes; the unit of pattern should be the sample size above which the standard error does not decrease.

Wilson (1935) showed that there is a change in the number of bacteria /cc. with depth in an undisturbed churn of milk and that samples must be taken from the whole depth of the churn and subsequently shaken to destroy the vertical pattern. In soil this vertical pattern exists, and in addition a horizontal pattern determined by the pattern of vegetation past and present, and microtopographical features, and the sample must extend both horizontally and vertically to the limits of the pattern. I know of no known case in which these precautions have been taken, and the very high variance of soil microbiological estimates may be due in part to this cause. Wyant (1921) gives a table showing the weights of soil used by various investigators as samples, and the largest is 100.00 g., it being more usual to take smaller weights, e.g. 1 g. It is unlikely that even 100.00 g. constitutes a sufficiently large sample to cover the 'unit of pattern' for soils bearing vegetation. James and Sutherland (1939a) have shown that storage of a soil sample before diluting and plating, results in great divergence of the plate counts from the Poisson series, and they advise plating immediately after sampling.

(2) The dilution of the soil

There exist in soils a very large number of micro-organisms, and dilution is necessary if a sample sufficiently large to be weighed accurately is to be examined for content of micro-flora. The degree to which a soil must be diluted will depend on the number of organisms which are present in it and the proportion which will grow and form colonies on the medium provided. There is a danger that false estimates will be obtained if too many colonies are allowed to grow on a plate. The optimal density of colonies per plate to be attained by the dilution will be considered in detail later (d).

Wynt (1921) reviews the early stages in the dilution process as practised by various soil investigators, but he makes no experiments to test the various procedures. Waksman and Fred (1922) described a procedure (for the estimation of soil organisms), which they hoped would become standard. It is obviously important to distribute the organisms of a soil sample, uniformly through the diluent and this is usually accomplished by mechanical or hand shaking, of the first dilution and hand shaking of successive dilutions. However in 1923, Whittles published a paper claiming that these shaking procedures gave great underestimations of the microbial populations of the soil, and that electrically operated vibration of the first dilution resulted in counts corresponding to 10,- 87,000,000,000,000 bacteria / g. of soil. Thornton

(1923) showed that these data were obviously suspect and Smith and Worden (1925) were able to show that the sterilisation technique used by Whittles on the vibrator was inefficient and that contamination could be responsible for the whole difference in plate count which he attributed to vibration and clump disintegration. Smith and Worden found no real advantage in a vibration method over normal mechanical shaking.

Wilson (1935) describes very interesting experiments on the dilution procedures for milk. Delivery pipettes are prefered to container pipettes. A comparison of the error of delivery through 1 ml. pipettes by (a) gravity delivery, (b) gravity and contact of pipette tip with glass side of vessel at end of delivery, (c) as (b) but blowing out last drops, (d) as (b) but in contact with walls of the glass vessel all the time during delivery, (e) delivery by blowing and contact until empty, and (f) delivery from one graduated mark to a second, showed, rather surprisingly, method (e) to be the most accurate. The Ministry of Health has subsequently advised the use of this method in water analysis ( ).

Wilson advises the use of Ringer s solution for dilution and shows that metal distilled water is toxic, and glass distilled water is so ewhat toxic to milk organisms. It is shown that the distilled water used as a diluent, has both a bactericidal and a dispersive effect. The first lowers the

number of colonies, the second, by breaking up clumps, increases it, the resultant plate count is a resultant of the two and is exposed to more variables than a count from a Ringer dilution. The Ringer's solution can be used at dilutions up to 1/16. The dispersive effect of distilled water may account for the date of Waksman (1922b) which show a higher count from soil samples diluted with distilled water than with salt solutions.

Wilson has also shown that a very considerable error is introduced by the use of one pipette for more than one dilution. A number of organisms apparently cling to the walls of a pipette, to be released later when more dilute suspensions of soil are pipetted. Another unexpected effect which is shown by Wilson's experiments, is that a dilution prepared by taking 1 ml. into 90 ml. gives a 13% higher count than taking 1 ml. into 9 ml. This is apparently due to the greater ease of shaking the dilution in the former case, and results in a greater dispersion of clumps.

(3) The preparation of the medium

Almost as many media have been used as there have been investigators in the field of soil microbiology. Conn (1914), Lipman and Brown (1903) Lipman and Brown (1910) have reviewed the early studies, but it is with the work of Waksman (1922b), Thornton (1922) and Wilson (1935) and their

use of statistical methods for comparisons of plate counts, that the media were legitimately compared and researches directed to the improvement of media were possible.

Waksman (1922b) gives as the requirements of a medium that it 'be of definite chemical composition (synthetic) and must allow the development of the greatest number of organisms'. Fischer (1910b) and Lipman and Brown (1910) developed the first snythetic media which could be made up to exact chemical composition. Waksman (1922b) compare five media; albumen agar, casein agar, soil extract agar, sodium asparaginate agar and urea nitrate agar. He replicated the samples from the ultimate dilution of a soil sample and so obtained an estimate of the power of the five media to support the growth of organisms in this one dilution sample. The highest number of organisms was supported by soil extract agar, followed by casein agar, albumen agar, sodium asparaginate agar and urea nitrate agar in that order. See Table VII from Waksman (1922b).

TABLE VII

	M.N.		E.N.
Albumen agar	89.1	±	2.23
Casein agar	111.6	±	2.33
Soil extract agar (from 'rich' greenhouse soil)	121.3	±	3.22
Sodium asparaginate agar	76.4	±	1.91
Urea nitrate agar	51.3	±	1.52

Despite the results of this experiment, Albumen agar was chosen for Waksman's later experiments, for little reason but that it had been used and given satisfaction previously in his laboratory, although casein agar was shown by this experiment to be significantly better. The soil extract agar which was greatly superior to all other media in the numbers of micro-organisms which it would support, was turned down on the grounds that it was not readily duplicated.

Wilson tested various meat and vegetable extracts and found that the incorporation of yeast extracts in synthetic agar media allowed more bacteria from milk to grow and form colonies than any other extracts. Yeastrel or Marmite were recommended as additions.

Thornton (1932) endeavoured to make an agar medium which was reproducible and which allowed a maximum number of organisms to develop to colonies while suppressing the development and spread of 'spreading colonies' such as Bacillus dendroides and B. mycoides. 'For the purpose of the general bacterial count, constancy in the results obtained with a medium is by far its most important property ..... Constancy depends mainly on the following features,

(a) The composition of the medium must be constant

(b) There must be as little interference between developing colonies as possible

(c) The medium must not encourage rapid growth of fungi

(d) Its reaction must vary but slightly'.

A medium containing asparagine 0.05% and mannitol 0.1% and agar 1.5% together with mineral salts was adopted, because it suppressed spreading of B. dendroides, because manitol was shown to have the least effect in lowering the pH of the medium on autoclaving, and because comparison of five independently prepared batches showed that the medium was readily and accurately reproducible. It is emphasised by Thornton that the pH of the medium is of very great importance in determining the number of colonies which may grow on the medium, the change from pH 7.0 to 6.6 being sufficient to reduce by more than half, the number of colonies developing from an inoculated soil sample.

It is not suggested that any of the media discussed will allow all types of soil micro-organisms to grow on them - and undoubtedly for accurate comparisons of soil microflorae, if the plate method must be used, the Thornton medium is the most reasonable to use. It has certainly been most completely investigated. Nevertheless, in soil investigations the quality of non-selectivity is desirable, and it seems that soil extract agar is among the least selective media which can be found.

Organic extracts are frequently changed in the course of sterilisation and toxins or growth promoting substances may be produced as a result of autoclaving. The disadvantage of non-reproducibility of soil extract media can usually be effectively removed for a series of experiments by making a stock of the medium in considerable bulk.

Smith and Worden (1925) have shown that although the colonies of bacteria grown on soil extract agars made from different soils frequently differ in appearance, there is no significant difference in the number of colonies supported unless the soil used be very poor and sandy. They showed that soil extract agar supported a more varied microbial population than Thornton's mannite salts agar.

It was of interest of determine to what extent the extraction procedures and sterilisation procedures were responsible for the selectivity of media prepared from the extracts. It has frequently been held that heat treatment releases both bacterial nutrients and also toxins from soil organic matter, and in view of the frequent use made of soil extract as a 'non selective medium', e.g. Löhnis (1910), Topping (1938), Lochhead et al. (1940 and 1943), it was thought desirable to make an investigation to discover what type of preparation gave the most non-selective medium.

A basic medium was prepared, consisting of glucose 1 g/l., K_2HPO_4 1 g/l., KNO_3 0.5 g/l., $MgSO_4$ 0.2 g/l., $CaCl_2$ 0.1 g/l., Na Cl 0.1 g/l., $FeCl_3$ 0.01 g/l., agar agar 15 g/l. and distilled water 1,000 ml. This medium was autoclaved at 15 lbs. pressure for 20 mins. in tubes containing ca. 10 ml.

Organic extracts were prepared from Juncus inflexus roots which formed the major part of the peat underlying tussocks of this plant. The roots were cut into 2 cm. lengths and five lots of roots were weighted out. Extracts of these were prepared in five ways.

(1) The roots were ground in 100 ml. of distilled water with a pestle and mortar. They were then allowed to steep in the water at room temperature (ca. 22°C) overnight. The following morning an extract was made by hand squeezing the roots in a bag of muslin.

(2) The roots were ground in a pestle and mortar and during the grinding process 100 ml. of boiling distilled water was added. The roots were left in the liquor to cool and stand overnight. The liquor was then strained off as before, and the remains of the roots were discarded.

(3) The roots were ground in 100 ml. of distilled water in a pestle and mortar and afterwards the roots and water were heated together in a steam bath for half an hour. The mixture was then cooled and allowed to stand overnight, after which the liquid was strained off and collected as before.

(4) The roots were frozen at ca - 5°C. overnight and thawed next morning. They were then ground in a pestle and mortar with distilled water and the extract was squeezed through muslin without further standing.

(5) The roots were ground on a pestle and mortar together with 100 ml. of distilled water and the roots and extract were then autoclaved together at 15 lbs pressure for half an hour. The extract was then made as usual after all night standing, by squeezing through muslin.

Sterilisation of extracts.

Five aliquots of 20 ml. of each of the above prepared extracts were sterilised in five different ways.

(a) To the extract in a sterile tube was added 0.2 ml. of chloroform.

(b) The extract was autoclaved at 1½ atmospheres pressure for ½ hour.

(c) The extract was steamed for ½ hour on three successive days.

(d) The extract was filtered through a Seitz S.B. Sterimat.

(e) The tubes of extract were exposed to ultraviolet light for 20 minutes.

Dilution of extracts

For dilution 1, the original sterilised extract was used, and for dilution 2, one ml. of the sterilised extract was taken with a sterile pipette to a tube containing 5ml. of sterile distilled water. The third dilution was prepared by taking 1 ml. of the second dilution into 5 ml. of distilled water. The dilutions thus effected were 1/5 (original), 1/30 and 1/180.

Preparation of microflora sample

A single 1 cm. length of a root of Juncus inflexus was washed in 10 ml. of saline, and after shaking by hand, 1 ml. was taken from this to 200 ml. of saline and again shaken. Before sampling, both the extracts and the micro-flora samples were well disturbed by blowing air through them.

Procedure

Certain sterilisation techniques took longer to perform than others, and so, after an extraction had been made, the

extracts were kept in a refrigerator at 2°C. until all sterilisations had been completed. Before the experiment, the extracts were t aken into a water bath at 45°C. (this removed chloroform from treatment (a)). The tubes of agar were also placed in a water bath at 45°C. after autoclaving. Tubes were removed from the bath and to each was added 1 ml. of a root extract. Two tubes were used for each extract-sterilisation-dilution. One was poured directly into a sterile Petri dish, the other was innoculated with 1 ml. of a suspension of the washings of 1 cm. length of Juncus inflexus root.

Five extraction techniques, five sterilisation techniques and three dilutions gave seventy-five combinations in all. For each of these combinations an innoculated and uninnoculated plate was prepared (the latter as a sterility control). In addition to the 150 plates of the above experiment, five plates were innoculated with 1 ml. each of root sample washings into basic medium without the addition of extract. The numbers of colonies developed on these plates at the end of five days are presented in tabular form (Table VIII)

T A B L E VIII

ri-ation		a	b	c	d	e	Extraction Totals
traction							
1	1	397 (*)	397 (o)	595 (o)	388(o)	* (*)	Sx = 3926
	2	387 (*)	387 (o)	614 (o)	284(o)	* (*)	
	3	394 (*)	394 (o)	491 (o)	376(o)	* (*)	$\bar{x}$ = 436.2
2	1	* (*)	416 (o)	684 (o)	800(o)	* (*)	Sx = 5663
	2	* (*)	326 (o)	664 (o)	876(o)	* (*)	
	3	* (*)	371 (o)	673 (o)	843(o)	* (*)	$\bar{x}$ = 629.2
3	1	333 (2)	330 (1)	394 (o)	373(o)	1250 (*)	Sx = 3532
	2	318 (o)	403 (28)	395 (o)	440(o)	1276 (*)	
	3	345 (o)	328 (1)	362 (o)	507(o)	1147 (*)	$\bar{x}$ = 392.4
4	1	- (-)	645 (o)	389 (o)	326(o)	* (*)	Sx = 3777
	2	- (-)	801 (o)	323 (o)	360(o)	* (*)	
	3	- (-)	582 (o)	338 (o)	343(o)	* (*)	$\bar{x}$ = 419.7
5	1	273 (1)	383 (1)	323 (o)	279(1)	453 (*)	Sx = 3008
	2	338 (o)	355 (o)	343 (o)	322(o)	373 (*)	
	3	364 (o)	331 (o)	338 (o)	334(o)	304 (*)	$\bar{x}$ = 334.2
ri-	Sx	-	6149	6907	6851	-	Sx
sation tals	$\bar{x}$		409.9	460.4	456.7		$\bar{x}$

lution		1	2	3
tal	Sx	6702	6593	6611
	$\bar{x}$	446.8	439.5	440.7

ntrol ate	283	285	338	281	317

riance Vx = 681.2 $V\bar{x}$ = 130.2 $S\bar{x}$ = 11.411 D.F. = 4

( ) = Control plates, * = heavily contaminated

- = no experiment

TABLE IX

Extraction: Sterilisation data

(mean values over 3 dilutions from Table VIII.

Sterilisation	b	c	d
Extraction			
1	392.7	566.6	349.3
2	371.0	677.0	839.6
3	353.7	383.7	440.0
4	576.0	340.0	343.0
5	356.3	334.7	311.6

Fiducial limits ($P = 0.05$)m for comparison in this table

$= \pm$ 343.9

fiducial limits ($P = 0.05$) for comparison in values in this table with the mean plate count of control experiments

$= \pm$ 216.2

Significant deviations from mean control plate count underlined.

TABLE X

Analysis of variance of extraction: sterilisation: dilution data

Item	Sum of squares	Degrees of Freedom	Mean square	t.	Probability
Main effects					
Dilution	455	2	227.6	0.0098	-
Sterilisation	23,753	2	11,876.5	0.515	-
Extraction	440,900	4	11,022.5	4.79	less than 0.05
Ist order interaction					
Dilution:Sterilisation	12,765	4	3,191.2	0.14	-
Dilution:Extraction	9,780	8	1,222.5	0.05	-
Sterilisation:Extraction	194,524	8	24,315.5	1.05	-
2nd order interaction (Error)					
Dilution:Sterilisation: Extraction	368,612	16	23,028.2		
TOTAL	1,041,505	44			

Sampling error variance per plate	= 23,028.2	Standard error	= 151.9
" " variance per extraction total	= 2,558,7	" "	= 50.6
" " " " dilution total	= 1,535.2	" "	= 37.8
" " " " Sterilisation total	= 1,535.2	" "	= 37.8
" " " " extraction:sterilisation total	= 7,676.1	" "	= 87.6

It is clear that neither chloroform treatment nor exposure to ultra violet light were effective in giving sterilisation of extracts. It is probable that chloroform would be more effective if the extracts were completely clear. Analysis of variance - Table X shows that the effects of the extraction procedures differ significantly, but that there is no significant difference between the effects of the sterilisation procedures or extent of dilution of the extracts within the limits employed. Significantly higher counts were given by the boiling water extracted, Seitz filtered extracts, and the next best extract was made in the same way but was sterilised by three day steaming. It is clear from Table IX that extracts prepared and sterilised in most ways do not give a significantly higher count than the glucose salts agar alone.

(d). The density of colonies on a plate and the interection of organisms upon the medium

The complicated procedure of dilution in the preparation of a soil sample for plating, is used to bring the number of colonies which will develop upon the plate, to a 'satisfactory' number. What constitutes a satisfactory number for the 9 cm. Petri dish of common use, is a matter which has caused some discussion and stimulated much experiment. The optimal number of colonies on a plate must obviously be that number which allows easiest and most accurate estimation of those organisms in an innoculated sample which can grow to colonies on the medium provided. Antagonism, synergism, competition, lysis, and confluence of colonies are all known to occur between bacteria on agar, and all could influence the plate counts obtained in a normal soil investigation.

If such effects are operative, the intensity of the effects should change with density of colonies and so the relation between the dilution of an innoculated sample and the plate count which develops from it should not be linear.

Noyes and Grounds (1918) have used a very convenient method for detecting an effect of this sort. A number of soils were sampled from plots sown with various crops, and the samples were diluted to 1 part in 40,000 and 1 part in

400,000. 1 ml. aliquots of these two dilutions were plated into an agar medium. It was assumed that if the relation between dilution and plate count were linear, i.e. if the 1 in 40,000 dilution gave ten times as many colonies as the 1 in 400,000 dilution, that the density of colonies on both plates was not great enough to depress the count. They found that "..... the ratio between the number of colonies on plates from the 1 in 40,000 dilution and the 1 in 400,000 dilution is dependent on the number of organisms present, rather than on the cropping system or the time of year at which the samples were taken.

The averages show:

(a) that the average of all comparisons for the 1 in 40,000 dilutions was too great for satisfactory plates.

(b) that the results from the two dilutions tend to check when the number of colonies on the plate from the 1 in 40,000 bacterial dilutions is less than one hundred".

However, the suggestion that the relation between the counts from the two dilutions at less than 100 colonies per plate is linear, is not supported by analysis of Noyes and Grounds' data (see Table XII).

In Table XII the ratios of the plate counts from the 1/40,000 to those from the 1 : 400,000 dilutions from Table XI have been grouped into classes according to the 1 : 40,000

plate count. The mean ratios for each class, and the standard deviation of the ratios have been calculated and the results are presented in Graph I. There is a very clear depression of the ratios below the expected ratio of 10 in the class of 60-80 and more colonies per plate. The differences in the standard error for the various classes are mainly due to the variable numbers of observations per class.

Wakeman (1923b) has tested the linearity of the dilution: plate count relation but the errors which have been calculated are examples of the 'error of the wrong replicate', and any differences shown may be due to chance variations in the preparation of the dilution series. The results of Wakeman's experiment are presented in Graph II and III, but it is impossible to judge the significance of these results.

Rossi (1921) in a paper criticising the plate method disputes the existence of a linear relation between dilution and plate count, and goes so far as to say, 'In the majority of cases the number of germs that can be successfully developed from the soil by isolating cultures diluted with agar and gelatine, is inversely proportional to the quantity of soil infusion employed for the determination. Thus for instance, with 1/15 cc. of soil infusion, there is generally obtained a larger number of germs than with 0.1 cc". He attributes this as follows ".... there are always in the soil a few germs such as B. mycoides which, if there are but a few individuals are sufficient to hinder the development of all the other species".

TABLE XI

Data from Noyes and Grounds (1918)

Plot Supporting		Average of Triplicate Plates	Ratio	1-40,000 / 1-400,000
		1-40,000	1-400,000	
Clothing	14.11.14	69.7	8.3*	8.39
	6. 2.15	73.0	11.6	6.29
	2. 3.15	107.2	10.7*	10.01
	27. 3.15	62.0	7.7*	8.05
	15. 4.15	94.0	6.0	15.66
Millett	14.11.14	99.3	11.3*	8.78
	6. 2.15	92.0	18.6	4.95
	2. 3.15	172.7	22.7	7.61
	27. 3.15	83.3	11.4	7.74
	15. 4.15	193.3	27.0	7.15
Soya Beans	14.11.14	75.3	10.3	7.31
	6. 2.15	125.3	20.3	4.76
	2. 3.15	165.7	17.0*	9.75
	27. 3.15	112.0	12.0*	9.33
	15. 4.15	80.0	10.7	7.47
Nothing	14.11.14	62.3	8.3	7.50
	6. 2.15	83.3	10.6	7.85
	2. 3.15	82.0	19.3	4.24
	27. 3.15	113.3	11.0*	10.30
	15. 4.15	145.0	12.7	11.42
Hairy Vetch	14.11.14	109.0	8.7	12.52
	6. 2.15	163.0	20.6	7.91
	2. 3.15	225.3	36.7	6.13
	27. 3.15	175.0	16.0*	10.93
	15. 4.15	185.7	29.3	6.34
Winter Rye (Sown early)	14.11.14	122.0	8.7	14.0
	6. 2.15	226.0	31.6	7.15
	2. 3.15	245.5	13.7	17.91
	27. 3.15	114.3	16.0	7.14
	15. 4.15	200.0	27.6	7.24
Nothing	14.11.14	65.3	9.7	6.73
	6. 2.15	129.7	22.0	5.89
	2. 3.15	173.7	13.3	13.06
	27. 3.15	72.3	6.7*	10.79
	15. 4.15	110.3	15.0	7.35

TABLE XI (Cont.)

Plot Supporting	Date	Average of Triplicate Plates 1-40,000	Average of Triplicate Plates 1-400,000	Ratio 1-40,000 / 1-400,000
Winter Rye (Sown late)	14.11.14	70.0	7.0*	10.00
	6. 2.15	68.7	14.0	4.91
	2. 3.15	148.0	16.3	9.39
	27. 3.15	187.0	15.7	8.03
	15. 4.15	167.0	27.3	6.11
Crimson Clover	14.11.14	44.7	3.3	13.54
	6. 2.15	91.0	15.6	5.83
	2. 3.15	254.3	23.3	10.90
	27. 3.15	46.0	6.7	6.86
	15. 4.15	122.2	16.7	7.32
Nothing	14.11.14	62.0	6.3*	9.84
	6. 2.15	93.0	17.3	5.38
	2. 3.15	181.0	13.3	13.60
	27. 3.15	43.0	3.0*	14.33
	15. 4.15	96.0	9.7	9.89
Buckwheat	14.11.14	50.0	10.3	4.85
	6. 2.15	121.3	21.3	5.69
	2. 3.15	163.0	15.0	10.86
	27. 3.15	86.0	6.4	13.43
	15. 4.15	119.7	14.0	8.55
Natural Growth of weeds	14.11.14	54.7	5.7*	9.59
	6. 2.15	100.3	23.3	4.30
	2. 3.15	152.5	33.6*	4.53
	27. 3.15	82.3	11.0	7.43
	15. 4.15	108.0	13.7	7.88
Av. of all 60 comparisons		117.18	14.3	
Av. of all 15 comparisons*		94.4	9.4	

TABLE XII

Noyes and Grounds data grouped according to the count on the 1/40,000 dilution plate

1:40,000 dilution plate count class	Mean Ratio Count form 1:40,000 / Count form 1:400,000	Number of observations in class	Variance of mean $V\bar{x}$	Standard deviation of mean $S\bar{x}$
40-60	9.83	5	3.39	± 1.85
60-80	7.981	10	0.334	± 0.57
80-100	8.225	12	0.965	± 0.98
100-120	8.60	9	0.604	± 0.77
120-140	7.62	6	1.363	± 1.36
140-160	8.28	3	4.03	± 2.00
160-180	9.46	7	0.817	± 0.90
180-200	9.03	3	3.38	± 2.3

Thornton (1922) has also drawn attention to the suppressing effect which spreading colonies (B. dendroides) may have on the development of colonies on agar plates. Thornton's data are plotted in Graph IV. The effect described by Rossi of inverse relationship between dilution and plate count is not very well supported by data, but a somewhat similar effect has been noted by Noyes and Grounds (1918) who found that the number of colonies on a plate did actually decrease with time from 3 to 10 days incubation if

the plate count was high, but increased over the same period if the count was low, due to late developing colonies becoming visable. The data of Noyes and Grounds, Table XIII, have been plotted in Graph V to show the total number of colonies developed after 10 days as a percentage of those developed after three days.

A regression has been fitted to these data for the relationship between log. 10 day count and log 3 day count.
3 day count

This regression is plotted in Graph IV and it can be seen that the plate count which neither increases nor decreases with further incubation after 3 days is ca. 100. It is necessary to assume either physical amalgamation of colonies or lysis to account for the reduction in the plate count after three days.

It is apparent from the foregoing example, that the number of colonies developing early on a plate determine to a certain extent the number which can develop late. This is supported by an examination of some data of Waksman (1932b) from an experiment designed like that of Noyes and Grounds to determine the optimal time for incubation of agar plates. In Table XIV and Graph VI, Waksman's data have been transformed to show the relationship between the percentage of colonies developing late, and the number developing early. A regression has again been plotted and it is apparent that the regression

will pass zero, (that is to say, the plate count at which further incubation after 3 days produces no change) at ca. 154 colonies which is of the same order as this factor obtained from Noyes'and Grounds' data.

This effect, by which the number of late developing colonies is determined by the early developing, may be used to account for an apparent anomaly in Waksman's date. It was found (Waksman (1928b)) that the plate count at 5 days was 115.9 ± 6.53 and at 10 days was 172.5 ± 4.65. Although the mean number of colonies had increased the error had decreased. Yet if the colonies are distributed at random on the plates the mean of a replicate series of plate counts should equal the variance. If the 10 day count is higher than the 3 day count, the error should be correspondingly higher instead of lower. If, however, the variability manifest at the three day stage itself limits the variability of the late developers, the reduction in the error by 10 days incubation is to be expected.

If this reasoning is valid, it must follow that the distribution of colonies on the replicate plates is not that expected from a Poisson distribution. Harrison and Verweel (1936) analysed the plate count data of Waksman (1928c) which was made from 10 day counts by the same technique as was used

in the incubation period experiment just described, and found very real departures from Poisson distribution (Fisher, Thornton and Mackenzie, x^2 test.)

Harmsen and Verweel (1936) discuss the causes of departure from the expected Poisson distribution. They noted from their own experiment, that plates bearing 100 or more colonies on non selective media which were used for determining total bacteria and actinomycetes, showed great departures from the expected Poisson distribution whereas plates bearing ca. 20 colonies on selective media gave good fits to the Poisson distribution for counts of protein and starch 'disintegrators'. They also found that Wakeman's counts of actinomycetes on more selective media gave good fit to a Poisson distribution, and that the discrepancies were almost entirely due to the bacteria. Harmsen and Verweel note that antagonism between organisms on the agar plates could account for some distortion of the Poisson distribution, e.g. B. mycoides and B. dendroides (Fisher, Thornton and Mackenzie), but this explanation is discarded on the grounds that they did not find many organisms on the plates which showed antagonisms when isolated. They suggest that synergism between colonies on the plates will account for the phenomenon. They state "....the phenomenon of sybiosis" (among soil organisms) "was of much more frequent occurence than is generally assumed". It is supposed that on a plate, the

presence of an organism producing a necessary growth substance allows a number of satellite colonies to develop around it which could not develop in its absence. They found that the addition of yeast and soil extracts to the synthetic agar medium used for these investigations resulted in a much closer fit to the Poisson distribution - and they supposed that this was due to the replacement of the synergist by the organic extracts for the provision of the necessary growth factors. However if synergisms were the explanation of this phenomenon of unexpectedly high variability, then at higher densities of colonies on a plate, relatively more of the innoculated organisms should be able to develop to colonies. In fact it will be shown in Part II of this thesis that relatively fewer organisms develop at higher plating densities. There is clearly interaction between organisms on plates, but it is interference not mutual aid.

James and Sutherland (1939a and b) have also observed departures of plate count distribution from the Poisson, and have explained these by supposing antagonisms between the colonies on the plates. They confirmed the observation of Harsen and Verweel that the actinomycetes were not affected, and these showed good fit to the Poisson.

Summary

It seems then, that bacteria on replicate plates from a soil sample are liable to show unexpected variability, which is greater than that expected if they are distributed at random. The examination of some of the literature of the plate count shows that there is interference in the development of some colonies when the density on a plate is high, and that this is particularly true of the late developing colonies whose chance of reaching visible size is dependent on the density of the early developers. It might then be supposed that in soil micro-biological studies, e.g. rhizosphere studies, this interference effect might be important in biassing the estimates of the populations of bacteria. Also, as it has been shown that some bacterial types, e.g. slow growers are more affected by interference than others, it seems likely that interference occurs at normally employed plating densities, biassing the qualitative as well as the quantitative estimates of the soil populations. Experiments designed to test the nature and extent of these interference effects are described in the following parts of this thesis, together with a discussion of the types of error which this plating technique may introduce into rhizosphere studies.

TABLE XIII

Change in Numbers of Bacterial Colonies on Plates with Increasing Length of Time of Incubation

(from Noyes and Grounds (1913)

Classes of 3 day plate counts				Average Counts 3 days	10 days
Colony numbers between	3,900	and	3,000	3,905	1,630
"	2,030	and	2,100	2,030	1,610
"	1,700	and	1,300	1,750	1,003
"	1,600	and	1,700	1,600	360
"	1,500	and	1,600	1,590	960
"	1,400	and	1,500	1,456	1,021
"	1,300	and	1,400	1,330	340
"	1,200	and	1,300	1,240	320
"	1,100	and	1,200	1,135	330
"	1,000	and	1,100	1,070	940
"	900	and	1,000	910	325
"	800	and	900	852	677
"	600	and	700	659	433
"	500	and	600	541	440
"	400	and	800	450	387
"	300	and	400	340	303
"	250	and	300	279	275
"	200	and	250	228	213
"	175	and	200	195	200
"	150	and	175	163	172
"	125	and	150	139	136
"	100	and	125	115	123
"	90	and	100	95	109
"	80	and	90	83	92
"	70	and	80	72	85
"	60	and	70	65	79
"	50	and	60	54	57
"	40	and	50	43	53
"	30	and	40	35	46
"	20	and	30	22	33
"	10	and	20	14	24
"	0	and	10	7	15

TABLE XIV

The relationship between the density of colonies per plate and the proportion of colonies developing late (Graph VI)

Adapted from Wakeman (1928b). Influence of temperature and period of incubation on bacterial numbers.

25°C

5 days 'Early'	12 days Total	12 days - 5 days 'Late'	'Late' / 'Early' x100
120	164	44	36.6
84	158	74	88.0
105	172	67	63.8
139	191	52	37.4
118	197	79	66.9
128	183	55	42.9
126	152	26	20.6
142	168	26v	18.3
81	179	98	120.9
116	161	45	38.7

Mean 5 days ('Early') 115.9 S.d.$\bar{x}$. = 6.53
Mean 12 days (Total) 172.5 S.d.$\bar{x}$. = 4.65

Regression of Late/Early on Early $\bar{x}$ = 115.9
$\bar{y}$ = 53.42
b = - 1.395 P. .001

Data of Wakeman transformed to illustrate the thesis that the early developed colonies on an agar plate determine the number of colonies developing late.

PART II

Plating density: the effects of microbial interaction on the determination of the numbers of microbes in soil.

In Part I of this thesis it was emphasised that the validity of the 'plating' method for the enumeration of soil micro-organisms has never been firmly established, and that there is reason to believe that use of the method may lead to quantitative and qualitative errors in soil studies. In this part of the thesis a series of experiments is described which were designed to test the hypothesis, that the 'plating method' gives a biassed estimate of the numbers of those organisms, or clumps of organisms, present in a given soil sample which are capable of growing on a given medium.

Statistical examination of the experimental results obtained by Noyes and Grounds (1918) has shown (see Part I) that there may be a depression of the number of colonies on a plate below expectation, when the total number of colonies growing on that plate is high. Not only is there this practical demonstration of the quantitative effects of plating density, but there are many theoretical reasons to expect that as the number of colonies on a plate increases, the proportion which can be counted, changes.

The effects of crowding of colonies on a plate may be expected to be as follows. (1) There is likely to be

physical amalgamation of colonies which arise from the closely placed innocula. This effect of proximity which has been described by Breed and Dotterer (1916) and by Wilson (1935), which causes amalgamation of colonies on the agar plates at high plating densities and gives reduced estimates of milk microfloras (and presumably of other populations estimated in this way) must become increasingly important as plating density increases, and must eventually play an important part in limiting the value of the plating method. This type of interaction may be called physical interaction. Wilson (1935) has shown that with a single species Escherichia coli, the plate count estimate is not significantly biassed if there are less than 300 colonies per plate.

(2) Increasing density of the colonies of micro-organisms on an agar plate, must result in competition between colonies for necessary nutrients and growth substances, which may become exhausted before some of the slower growing types reach visible size. The curve relating dilution to plate count for competitive interaction should be of the form of Graph X. As the density increases the variance of replicate plate counts should increase owing to the larger number of colonies which are of a size at the threshhold of visibility. However, it has been shown in Part I that the variance diminishes with increasing plate count. Competitive interaction is therefore unlikely.

(3) It is reasonable to expect that antagonism should occur between members of the population on the agar medium at high densities of colonies per plate. Brian states '..... there is strong evidence that the capacity to produce antibiotics is frequent among, and characteristic of, the micro-organisms of the soil association', 'Among the bacteria two groups of closely related species are particularly concerned with the production of antibiotics - the aerobic spore formers (Bacillus subtilis - mycoides - mesentericus group) and the Pseudomanas fluorescens group. These are characteristic universally distributed soil organisms' and 'Production of antibiotics is frequent among the saprophytic actinomycetes; all those species of actinomycetes reported as producing antibiotics have originally been isolated from soil'.

Waksman (1947) has reviewed the literature on the production of antibiotics by soil organisms in culture. In view of the great abundance in soil of organisms with antagonistic properties, it is to be expected that some antagonism will be shown between organisms developing from a soil sample. It is in fact be plating diluted soil samples on agar and allowing the colonies of organisms to develop, that many antagonists have been isolated: the presence of an active antagonist is shown by the inhibition of the growth of any sensitive spreading colony. Any non-spreading colony developing on a plate, would not of course show that it was being antagonised by a neighbour, but as soon as the antibiotic

produced from a neighbour reached the necessary concentration for inhibition, would stop growth. This might be before the colony had grown to a size visible to the naked eye. If this effect occurs on agar plates prepared from normal soil dilutions, it will become increasingly apparent as the density of colonies on the plates increases. This should result in suppressing the development of some potential colonies and failure of the plate count to represent all those organisms which are capable of growing on the medium provided. This type of interaction may be called antagonistic interaction, and should also include the production of acid, by the metabolism of organisms on the plates, which may inhibit the develcpment of bacterial and other colonies (see Thornton(1922) for an account of the effect of lowered pH on the bacterial count from a soil sample). If these effects are operative, they would be expected to lead to an estimate of the soil population, lower than that which is theoretically possible from an infinitely high dilution and infinitely low plate count. It is also to be expected, that if there is antagonistic interaction on agar plate, there should be lowering of the standard error as the plate count is increased because the number of colonies developing early should to a certain extent control and limit the number developing late.

(4) It has already been suggested in Part I that some organisms may lyse colonies of other organisms, so reducing

the number of colonies on a plate with long incubation. It is conceivable that a further type of interaction may occur on agar plates in which one organism provides, in the end products of its metabolism, necessary nutrients or growth factors, making possible the growth of another organism on a medium deficient in this substance or substances. Therefore, as the density of colonies on an agar plate is increased, it might happen that some organisms develop to visible colonies which were not able to do on a sparsely populated or otherwise unpopulated plate. This type of interaction may be called synergistic interaction, e.g. that between Rhodotorula rubra and Mucor Ramannianus, neither of which will grow on synthetic media alone, but will grow in each others presence, (quoted from Rippel-Baldes (1947)).

In Graph VII and VIII, theoretical curves are presented relating plate counts to the dilution of the sample plated, for antagonistic and synergistic interaction.

In the absence of interaction, physical, competitive, antagonistic or synergistic, the relationship between the concentration of soil innocculum ~~xxxx~~, and the plate count from which it was derived should obviously be linear unless a bias be introduced into the method by contaminating colonies on the plates. The effect of this latter type of error would be to increase those estimates of a soil population made from highly diluted soil

samples and low plating densities - and can obviously be eliminated by preparing a control series of plates, exposed to all the possible contaminations to which the experiment and series is exposed, and subtracting the counts on these control plates from the experimental series. The effect of regular contamination on the plate count dilution relationship is shown in Graph IX. However, if any of these interaction effects are real, it is obviously important to determine at what dilution or plating density there is significant departure from the linear relationship between plate count and dilution, although of course all of the effects discussed are capable of acting to a limited extent at densities of more than one colony per plate. Experiments were made to test for the existence and extent of any departure from linear relationship between the extent of dilution of a soil sample and the number of visible colonies developing an an agar medium from aliquot samples of various dilutions.

Experiment I.

A tussock of Juncus inflexus was taken from a fen at Cothill near Oxford, to the laboratory and 10 g. of soil plus roots was sampled from immediately below rhizome level. This was transferred to a 1 litre Erlenmeyer flask containing ½ litre of sterile Ringer's solution at one tenth of normal strength, and the mixture was shaken for 15 minutes in a mechanical shaker. 500 ml. of Ringer/10 was then added to bring the volume of

washing fluid to 1,000 ml. and the flask was shaken for two minutes more. From this approximately 1/100 dilution, 20 ml. was taken to 80 ml. of Ringer/10 to give a dilution of 1/500. Subsequent dilutions were made as follows:

TABLE XV

Dilution procedure					Dilution effected
Dilution A					10,000 p.p.m.
" B	20 ml. of A	taken to	80 ml.	Ringer/10	2,000 "
" C	10 ml.	"	90 ml.	"	1,000 "
" D	2 ml.	"	98 ml.	"	200 "
" E	1 ml.	"	99 ml.	"	100 "

Dilutions B.C.D. and E. were replicated.

This method for the preparation of a dilution series is more satisfactory than progressive dilution, for there can be no cumulative error and all the dilutions have experienced the same shaking and should not differ in the extent to which bacterial colonies have been broken up.

After 2 minutes shaking of the dilutions by hand, 1 ml. samples were taken to tubes containing 20 ml. of augmented glucose salts agar (see Appendix I), sterile and molten at 45°C., and after rotating the tubes to mix, and sterilising the mouths by flaming, the agar was poured into sterile, 9 cm. diameter, Petri dishes. The tubes were plugged with cotton

wool and incubated, in order to determine whether any colonies developed on the walls of the tube, due to innocula left behind; there was no sign of residual colonies developing in the tubes.

The plates were incubated at 25°C. for 7 days and the colonies on each plate were then counted. It was quite impossible to obtain a direct count with the naked eye because all the plates compared bore very large numbers of colonies. An estimate of the numbers of colonies present was, however, made by counting the colonies in randomly chosen microscope fields on each plate. The field area was 7.55 mm^2 and the area of agar in the Petri dishes was 6360 mm^2. The conversion factor, Plate area/mean field area, was 842.8, and the average field count for a plate was multiplied by this factor to give the estimated plate count.

The results obtained are presented in Table XVI and in Graphs XI and XII.

TABLE XVI (see Graph XI)

The relationship between dilution of a soil sample and the number of colonies developing per plate.

Dilution of sample in p.p.m.	Soil dilution series	Plate replicates	x Total colonies counted	y No. of fields examined	$\frac{x}{y}$ Mean colonies per field	$\frac{x}{y}$ x 842.8 = z Calculated colonies per plate	Bacteria per ml. of dilution sampled estimated from z
10,000	Common to 1 and 2	a	143	7	20.4 ± 0.91	17193	16392 ± 800.9
		b	148	8	18.5 ± 0.95	15591	
2,000	1	a	37	10	3.7 ± 0.85	3118	3834.5 ± 716.4
		b	54	10	5.4 ± 1.26	4551	
	2	a	98	19	6.2 ± 0.60	5225	4888.0 ± 336.8
		b	97	18	5.4 ± 0.56	4551	
1,000	1	a	58	15	3.9 ± 0.53	3287	3245.0 ± 42.0
		b	53	14	3.8 ± 0.53	3203	
	2	a	69	15	4.6 ± 0.45	3877	3708.5 ± 168.5
		b	55	13	4.2 ± 0.72	3540	
200	1	a	37	21	1.8 ± 0.22	1517	1584.5 ± 67.49
		b	49	25	1.9 ± 0.27	1652	
	2	a	35	31	1.1 ± 0.20	952	1011.0 ± 59.0
		b	57	45	1.3 ± 0.17	1070	
100	1	a	34	48	0.71 ± 0.13	598	617.00 ± 53.7
		b	36	49	0.73 ± 0.12	615	
		c	25	42	0.59 ± 0.12	497	
		d	48	53	0.90 ± 0.15	758	
	2	a	25	44	0.57 ± 0.11	480	474.6 ± 44.0
		b	33	51	0.65 ± 0.11	548	
		c	24	51	0.47 ± 0.09	396	

TABLE XVII (see Graph XII)

The relationship between dilution of a soil sample and the estimation of the soil microflora, (from data in Table XVI

Dilution in p.p.m.	Soil dilution series	Bacteria/gm of soil
10,000	Common to 1 and 2	1,639,200 ± 80,090
2,000	1	1,917,250 ± 358,200
	2	2,444,000 ± 168,400
1,000	1	3,245,000 ± 42,000
	2	3,708,500 ± 168,500
200	1	7,922,500 ± 337,450
	2	5,055,000 ± 295,000
100	1	6,170,000 ± 537,000
	2	4,746,000 ± 440,000

It is quite clear from Graph XI that at the very high plating densities used in this experiment, there is significant departure of the curve relating dilution to plate count, from the expected straight line. The interaction is of the antagonistic type. (in Graph XII) The differences between the two dilution series are rather striking and the standard deviation of subsamples from a dilution is clearly no indication of the sampling error

involved between replicate dilutions (see Part I "Error of the wrong replicate" and discussion of Waksman's dilution/plate count data). It is obvious from Graph XII that interaction of this type is responsible for very considerable under-estimations of the soil microflora. It would seem unlikely that such a high level of plating density would ever be used in practice, but Landelout and Gilbert in 1948 were still using plating densities of >1,000 colonies per plate for comparison of the numbers of micro-organisms at various depths in soil profiles, and much of the rhizosphere data of Lochhead and associates has apparently also been derived from plating experiments at densities of >1,000 colonies per plate.

However, it is obviously more important to know to what extent variations about the more frequently used plating densities affect estimates of microbial abundance.

Experiment II

A dilution series was prepared from a soil sample shaken from the roots of linseed which had been grown for six weeks in a light sandy soil in pots. 10 g. of the soil was taken into 1 litre of Ringer's solution at one tenth normal strength. This gave a dilution of approximately 1 part of soil to 100 parts of diluent. From this dilution 1 ml. was taken with a sterile pipette into a flask containing 99ml. of sterile Ringer's solution/10. This gave a dilution of 1 part in

10,000 or 100 parts per million. From this, a dilution series was accurately prepared, using the 100 p.p.m. dilution as the stock solution for the preparation of each subsequent dilution and so again avoiding cumulative error. The dilution series was as follows: (Table XVIII)

TABLE XVIII

Dilution procedure						Dilution effected
Dilution A						100 p.p.m.
B	10 ml.	of A	taken to	90 ml.	Ringer/10	10 p.p.m.
C	20 ml.	"	"	80 ml.	"	20 p.p.m.
D	50 ml.	"	"	50 ml.	"	50 p.p.m.
E	5 ml.	"	"	95 ml.	"	5 p.p.m.
F	2 ml.	"	"	98 ml.	"	2 p.p.m.
G	1 ml.	"	"	99 ml.	"	1.p.p.m.
H	2 ml.	"	"	998 ml.	"	0.2 p.p.m.
I	1 ml.	"	"	999 ml.	"	0.1 p.p.m.

The dilution series was replicated once. From each dilution 1 ml. samples were pipetted into tubes containing 21 ml. of soil extract agar (prepared according to Fred and Waksman (1928) sterile and molten at 45°C. After rotation of the tubes to mix the samples, the contents were poured into sterile Petri

dishes and incubated at 25°C. At the end of seven days the colonies growing on the plates were counted by the naked eye; the results are given in Table XIX.

TABLE XIX

Dilution of soil sample in p.p.m.	A Dilution Series Replicate Plates a	b	c	d	B Dilution Series Replicate Plates a	b	c	d
20	190	-	-	-	185	-	-	-
10	94	99	-	-	64	106	-	-
5	108	91	-	-	84	64	-	-
2	22	21	60	41	22	30	35	26
1	39	21	14	3	15	25	73	77
0.2	-	-	-	-	12	1	4	3
	-	-	-.	-	5	0	9	9
0.1	-	-	-	-	3	2	4	5

The variation between dilution series is comparable to that between plates - so it is legitimate to consider them as one series. In Table XX is given an analysis of the error of the plate count estimates based on this treatment.

TABLE XX (see Graph VIV)

Dilution in p.p.m.	Mean Plate Count = $\bar{x}$	n	$(x - \bar{x})^2$	V_x	$V_{\bar{x}}$	$S_{\bar{x}}$
20	187.5	2	12.5	12.5	6.2	2.5
10	90.8	4	1027	342.3	85.6	9.2
5	86.8	4	995	331.6	82.9	9.1
2	32.1	8	1235	176.4	22.1	4.7
1	33.4	8	5364	766.2	95.7	9.8
0.2	6.63	8	285.9	40.8	5.10	2.3
0.1	3.5	4	5.0	1.6	0.4	0.6

In Graph XIII the relationship between dilution and plate count is plotted for this experiment and again it is seen that there is a fall away from the expected straight line, and again this is of the form expected for antagonistic interaction. These data, transformed into estimates of the microbial population of the soil sample, are plotted against the concentration of soil inoculum in Graph XIV and against the mean plate count for each dilution, in Graph XV. It is clear that dilutions giving a plating density higher than 30 colonies per plate are liable to lead to underestimation of the soil population. This is true of soil extract agar which is regarded as a non-selective medium because it provides a great range of nutrients

except dextrose

but none in great quantity. It gives slow growth of colonies and would therefore be expected to allow the least production of antibiotics, by antagonists, of commonly used media because it permits only limited colony growth. It is probable that on carbohydrate and protein media there is an even greater effect of plating density. In an experiment in which a diluted soil sample was added to glucose-sucrose-mannitol agar containing Andrade's indicator, the zones of colour due to acid production were so great after 3 days that there was normally overlapping of zones if more than 3 acid producers were present on a plate. The effect of this on the growth of slow developing colonies may be considerable. Most of the published estimates of soil micro-organisms made by the plating method have been made from plating densities which fall within the interaction range of 30 colonies upwards, and it seems certain that very misleading accounts of the populations of those micro-organisms in soils which are capable of living on specific nutrient media, have been obtained from plating studies in the past.

The transformed data of Noyes and Grounds (Part I, Graph I) suggest that interaction of approximately the same order as shown in the above experiment occurred in their experiments.

In the experiments described in this paper, there is evidence that as plating density increases, there is a tendency for the estimates of the microbial populations of soil based on the plate counts, to reach an asymptotic value. A possible

explanation for this observation, is that organisms sensitive to antagonism or to competition for some vital nutrient, are completely prevented from development on plates with more than a limiting density of colonies, and that at higher densities there is a normal linear relationship between dilution and plate count, the latter being now composed entirely of bacterial types insensitive to interaction. This would explain the tendency of the curve in the graph of the transformed data of Noyes and Grounds to rise to the theoretical value at high plating densities, for if a dilution of 1/400,000 gave a plating density high enough to prevent the growth of density sensitive types, the 1/40,000 dilution of the same soil would be expected to give 10 times as many density insensitive colonies on a plate from an aliquot sample: the population estimates made from these plate counts would then be estimates of that part of the microbial population which was insensitive to crowding. This tendency to reach an asymptotic value excludes the possibility of 'physical' interaction as the cause of the density effect.

From Graph XIV it can be estimated that the soil sample contained in each gram of soil, 35,000,000 $\pm$ 6,000,000 bacteria of a type capable of growing on soil extract agar of the type provided. But the estimate can also be made that this soil contained 9,080,000 micro-organisms $\pm$ 920,000 per grams of types insensitive to crowding on this medium. Following normal plating

technique only the latter estimate would have been made. Further evidence of the importance of interaction in the estimation of microbial abundance is given in Part III of this paper, in a consideration of the qualitative changes which are brought about by the use of the dilution plate method at high plating densities.

SUMMARY

It is shown that on soil extract agar, there is a failure of some micro-organisms to develop to visible colonies, unless the dilution of soil sample used as innocculum be so low as to give a density of 30 or fewer colonies per 9 cm. diam. plate. This effect may be responsible for very considerable under-estimations of the soil microflora by the plating method. There is evidence of the great danger of regarding the standard deviation of replicate plate counts as estimates of other phases of sampling procedure involved in estimating soil populations. ("The error of the wrong replicate").

An investigation of the Interaction between Soil Micro-organisms with especial reference to the study of the bacterial populations of plant roots.

PART III

Plating Density: the effects of microbial interaction on the determination of systematic and physiologic categories of organisms in soil.

In Part II of this paper it was shown that over the range of plating densities normally employed for the estimation of soil micro-organisms by the dilution-plate method, interaction occurs between the organisms present and that the development of a proportion of the organisms to visible colonies is inhibited. It was suggested that microbial antagonisms was responsible for this effect. Certain categories of micro-organisms are known to be especially sensitive to antibiotics produced by common soil inhabitants (Waksman (1947)). For example, the activity of both fungal and bacterial antibiotics is generally greater against Gram Positive bacteria and spore formers than against Gram negative bacteria and non-spore formers. Some organisms are known to be markedly sensitive to acidity, and on media containing carbohydrates, acid produced as a metabolic product by actively growing organisms may antagonise the development of other more slowly developing colonies.

It is likely then, that at high density of colonies per plate, the relative representation of these sensitive micro-organisms will be reduced and the percentage of insensitive types increased.

Experiments were made to determine to what extent the proportional representation of specific microbial types developed from a soil sample on agar plates is affected by the density of colonies on those plates.

Experiment I. Tussocks of Juncus inflexus were sampled from a fen at Cothill, Nr. Oxford and taken to the laboratory. Four roots were removed from the tussock and each was washed in sterile saline; dilutions of these washings were then prepared. 1 ml. samples of the diluted root washings were taken to molten glucose-sucrose-mannitol agar*, and after seven days incubation at 25°C. counts were made of the number of colonies of Bacillus mycoides and of actinomycetes on the plates. No total plate counts were made. The results are given in Table XXI and the relationships between the counts of these organisms and dilution of root samples are given in Graphs XVI and XVII.

It is clear that there is very obvious departure from expected linearity of the relationship between dilution and plate counts of these organisms. Graph XVII for B. mycoides shows this clearly and the curve is of the form which has been shown in Part II to hold for the relationship between dilution

* See Appendix I

and total plate count. Graph XVI for the actinomycetes is less constant in form and shows even more extreme departure from expectation.

The effect of the density of colonies is clearly shown by considering the ratio between bacteria and actinomycetes plotted against dilution, Graph XIX, and is seen more strikingly when this ratio is plotted against the count of B. mycoides per plate, Graph XVIII. For any one root sample the estimate obtained of the relative abundance of B. mycoides and actinomycetes is clearly related to the extent to which the sample used for plating had been diluted. It is, of course, unnecessary to suppose that interaction between B. mycoides and the actinomycetes is responsible for this effect - it is more likely that the interaction of each with the rest of the microflora of the plate gives this mutual relationship. Borodulina (1935) has in fact shown that the antagonistic inter-relations of soil actinomycetes and B. mycoides on agar media are the opposite to those which would be needed to account for the results of this experiment. Lochhead and Landerkin (1949) have shown that there are complex antagonistic inter-relationships between soil actinomycetes on agar media, and it is possible that this is responsible for part of the anomalies in actinomycete plate counts.

TABLE XXI (See Graphs XVI, XVII, XVIII, XIX)

The Relationship between the numbers of colonies of B. mycoides and Actinomycetes developing from various dilutions of the root washings of Juncus inflexus on an agar medium.

Concentration of inoculum in parts per million (p.p.m.)	Replicate root samples	Colonies of B. mycoides A.	Colonies of Actinomycetes B.	A / B
200	A	36	6	0.166
	B	50	0	0.000
	C	52	0	0.000
	D	54	10	0.185
40	A	9	5	0.550
	B	26	1	0.038
	C	19	?	-
	D	16	1	0.062
8	A	3	3	1.000
	B	5	0	0.000
	C	1	?	-
	D	3	7	2.333
1.6	A	1	7	7.000
	B	3	8	2.666
	C	3	?	-
	D	1	9	9.000
0.32	A	2	4	2.000
	B	0	7	-
	C	2	?	-
	D	0	2	-

A similar effect of plating density on proportional representation of microbial types has been observed in the

course of an investigation of that proportion of organisms present in a soil sample which is capable of actively liquefying gelatine.

Experiment II. Roots of Juncus inflexus were sampled as before, and dilutions were prepared to distribute the root washings of 1 cm. length of root between 500 ml. of diluent (nutrient broth). 1 ml. samples of the dilution were taken to nutrient gelatine* and the numbers of organisms giving active liquefaction of the gelatine were counted after 5 days incubation (see Table XXII). In Graph XX, the percentage of liquefying organisms present on a plate is plotted against total plate count. There is clearly a depression of liquefyers at high plate counts. However, in this experiment, seven separate root samples were used, and so it is possible that the relationship between percentage liquefyers and the plate count is a true reflection of a real relationship between percentage liquefyers and microbial abundance in the soil. An experiment was therefore designed to test the effect of plating density on the proportional representation of microbial types on plates obtained from a sample of a dilution of one soil sample.

Experiment III. A 10 g. sample of the roots of Juncus inflexus plus attached soil, was sampled from Cothill as described in Part II of this paper. The sample was diluted and the dilutions were plated onto augmented medium* and after 7 days incubation

* See Appendix I.

the actinomycetes were counted and expressed as percentages of the total plate count. The results are given in Table XXIII and Graph XXI. The relation between the count of actinomycetes and the dilution of the sample is of particular interest as it has a very peculiar form. On the plates prepared from the first three stages of the dilution series, the count shows the expected departure from linearity, but as the plating density increases there is an actual decrease in numbers of actinomycetes per plate.

TABLE XXII (see Graph XX)

The relationship between density of colonies per plate and the percentage representation of gelatine liquefyers.

Root Sample	Replicate Plates	Total Count	Liquefyers	Percentage Liquefyers
1	A	27	8	29.6
	B	31	10	32.2
	C	25	7	28.0
	D	-	-	-
2	A	22	13	59.1
	B	140	21	15.0
	C	41	9	21.9
	D	-	-	-
3	A	522	0	0.0
	B	382	0	0.0
	C	717	0	0.0
	D	-	-	-
4	A	960	7	0.7
	B	222	6	2.7
	C	160	4	2.5
	D	242	4	1.7
5	A	-	-	-
	B	1250	6	0.5
	C	c.a.1250	7	0.6
	D	-	-	-
6	A	816	2	0.2
	B	824	8	1.0
	C	920	5	0.5
	D	800	3	0.4
7	A	1600	1	0.1
	B	-	-	-
	C	1648	2	0.1
	D	-	-	-

There is a pronounced fall in the percentage of antinomycetes in the total plate population (a similar effect of dilution was shown by the actinomycetes in results of Experiment I, but in less convincing form).

It was desirable to test for an influence of plating density on the representation of categories of soil organisms over a narrow range of plating densities within the limits normally employed in micro-biological studies, i.e. 50-200 colonies per plate.

Experiment IV.

Linseed was sown in pots of soil and sand 1 : 1 and grown for 2 months in the garden of the Department of Agriculture, Oxford University. At the end of this period the plants were removed from the pots and after shaking the roots to remove the loosely attached soil, 10 g. of roots together with soil still remaining attached, was transferred to a flask containing 1 litre of Ringer/10 saline solution and was shaken for 15 minutes in a mechanical shaker. From this soil suspension, a dilution of 1 part of roots plus soil in a million parts of diluent was prepared by the dilution procedure described in Part II of this paper. Two dilution series A and B were prepared and from each, five 1 ml. samples were taken to each of two media, a glucose salt medium (the basic medium or Medium A) and the same medium plus amino acids and growth factors (the augmented medium or Medium B)*,

* See Appendix I.

The dilution samples were pipetted into tubes containing 20 ml. of the agar media, molten at 25°C. and these were then poured into sterile Petri dishes and allowed to set.

After seven days incubation, counts were made of total colonies per plate, actinomycetes nad yellow chromogens. The differences between the counts on the two media gave estimates of the proportions of the organisms which required growth substances or amino acids for development to visible colonies. The results are given in Table XXIV.

Regressions of the plate count on % actinomycetes, % chromogens, % increase in plate count due to augmentation of medium, % special nutrient demanders and percentage increase in chromogens due to augmentation of medium have been calculated, Tables XXV, XXVI, XXVII, XXVIII, XXIX, XXX, XXXI, and some of these results are presented graphically, Graphs XXII, XXIII, XXIV and XXV. Correlation coefficients have also been calculated for the above relationships. By means of these statistics it is possible to test for the presence of significant trends or changes in the qualitative character of the plate populations as the density of colonies per plate is changed.

Over the range of 67-95 colonies per plate on the basic medium and 72 - 115 colonies per plate on the augmented medium there is no significant relation between the plate count and percentage actinomycetes. Although there was a clear effect of plating density on the proportions of actinomycetes

represented in the plate populations of Experiment 111, the range of densities used in this particular experiment, with this particular set of rhizosphere organisms, is insufficient to show any such effect. There is also no correlation between % chromogens and the plate count on the basic medium, Table XXVII, but there is significant positive correlation between these two values on the augmented medium B., (Table XXVIII, Graph XXII).

It is obvious from the above data, that the higher the density of colonies per plate on augmented medium, the higher is the proportional representation of yellow chromogens. It is significant that that proportion of chromogens which requires added nutrients, (i.e. grow on the augmented but not on the basic medium), is significantly increasedon crowded plates, Table XXX and Graph XXIV. This observation is of very real interest in view of Harmsen and Verweel's claim that there is synergistic interaction on agar plates and that this is removed by the addition of special nutrients. It is clear from this experiment, that an interaction, which does not occur on the basic medium, is able to occur on addition of special nutrients.

There is a significant correlation between the ratio, count on the augmented medium/count on basic medium and the plate count on the basic medium, (Table XXIX, Graph XXIII) and also, the proportionate extent by which the count on the

augmented medium exceeds that on the basic medium (i.e. the proportion of special nutrient demanders), is significantly increased as the total plate count on the augmented medium is increased, Table XXXI, Graph XXV.

It is concluded from this experiment, that proportional changes in the development of microbial type from a single soil sample can be induced by changing the density of colonies per plate, and that this effect is significant within the density range of 70 - 120 colonies per 9 cm. diameter Petri dish.

Summary

The hypothesis that certain types of organism growing on agar media are depressed at high plating densities and that other types are proportionately increased is confirmed. It is seen that variations in the density of colonies per plate may have their effect on the proportional representation of types, even within the range 70 - 120 colonies per plate which is a normally employed and recommended plating density (Waksman (1922b)). It is to be expected that many investigations of qualitative differences between soil microfloras have been biassed and serious errors introduced by this previously unrecognised effect.

TABLE XXIII (see Graph XXI)

The relationship between dilution of a soil sample and the estimation of percentage actinomycetes

Dilution	parts per million	Soil dilution series	Total Plate Count	Total Actinomycetes per Plate	% Actinomycetes x	Mean % Actinomycetes per dilution class $\bar{x}$	D.F.	$S\bar{x}$
10000	p.p.m.	1 and 2 joint	17193 15591	118.0 421.4	0.68% 2.7	1.69	1	± 1.01
2000	p.p.m.	1	3118 4551	421.4 505.7	13.5 11.1	12.30	1	± 1.20
1000	p.p.m.	1	3886 3202	724.8 480.4	22.05 15.00	18.52	1	± 3.52
200	p.p.m.	1	1517 1651	396.1 303.4	26.11 18.36	22.23	1	± 3.87
100	p.p.m.	1	598 615 497 758	177.0 84.3 160.1 143.3	29.57 13.69 32.20 18.88	23.58	3	± 4.37
2000	p.p.m.	2	5225 4551	134.8 463.5	2.58 10.2	6.39	1	± 3.81
1000	p.p.m.	2	3876 3539	952.4 901.8	24.56 25.4	24.98	1	± 0.42
200	p.p.m.	2	952 1070	354.0 186.4	37.16 17.32	27.24	1	± 9.91
100	p.p.m.	2	480 547 396	118.0 151.7 33.7	24.56 27.69 8.51	20.25	2	± 5.94

Table XXIII see Graph XXI

The effect of the density of microbial colonies on agar plates on the proportional representation of microbial types from the soil sample.

Dilution series	Replicate	Media *	Plate Count Total	Actinomycetes	Chromogens	% Actinomycetes	% Chromogens	% Increase in plate count due to Nutrients	% Increase in Chromogens due to Nutrients
1	1	A	81	10	25	12.3	30.8		
1	1	B	73	6	18	8.2	24.6	96.0	- 9.58
1	2	A	67	7	13	10.4	19.4		
1	2	B	94	14	25	14.9	26.6	140.2	+ 12.8
1	3	A	70	14	15	20.0	21.4		
1	3	B	72	12	20	14.2	27.5	119.7	+ 6.9
1	4	A	71	14	16	19.7	22.5		
1	4	B	85	14	25	16.5	29.4	102.8	+ 10.6
1	5	A	76	13	20	17.1	26.3		
1	5	B	73	9	16	12.3	21.9	160.4	- 5.5
2	1	A	82	12	20	14.6	24.4		
2	1	B	115	11	42	9.6	36.5	110.8	+ 19.1
2	2	A	66	5	21	7.6	31.8		
2	2	B	107	8	34	7.5	31.7	121.0	+ 12.1
2	3	A	95	5	23	5.3	24.2		
2	3	B	110	13	34	11.8	30.9	90.1	+ 10.0
2	4	A	92	15	17	16.3	18.4		
2	4	B	102	15	45	14.7	44.0	140.2	+ 27.4
2	5	A	95	12	21	12.6	22.1		
2	5	B	115	12	43	10.4	37.4	115.7	+ 19.1

Table XXIV see Graphs XXII – XXV

* See Appendix I

TABLE XXV

Correlation between % Actinomycetes and plate count on medium A.

MEDIUM A.

Plate No.		Plate Count	Actino. Count	% Actino.
VI	1	81	10	12.3
	2	67	13	10.4
	3	70	14	20.0
	4	72	14	19.7
	5	76	13	17.1
VII	1	82	12	14.6
	2	66	5	7.6
	3	95	5	5.3
	4	92	15	16.3
	5	95	12	12.6

$\Sigma X = 795$ $\Sigma Y = 144.9$ $n = 10$

$\bar{x}_2 = 79.5$ $\bar{y}_2 = 14.49$ $\Sigma XY = 11293.3$

$\Sigma X^2 = 64361.0$ $\Sigma Y^2 = 2331.61$ $(\Sigma X)(\Sigma Y)/_n = 11519.5$

$(\Sigma X)^2/_n = 63202.5$ $(\Sigma Y)^2/_n = 2099.6$ $\Sigma xy^2 = -226.2$

$\Sigma x^2 = 1158.5$ $\Sigma y^2 = 232.0$ $b = -.1952$

$r = -226.2 / \sqrt{1158.5 \times 232.0} = -226.2 / 518.3 = -0.436$

$S\ d.y.x^2 = 232.0 - (-226.2)^2/1159 = 187.9$

$S\ y.x^2 = 187.9/8 = 23.5$

$Sb^2 = 23.5/1159 = .0203$

$Sb = \sqrt{0.0203} = \pm 0.1425$

$t = b/Sb = 0.1952/0.1425 = 1.369$ P = between .2 and .3

Regression coefficient b = -0.1952 P. .2 - .3

Correlation coefficient r = -0.463 P. >.1

TABLE XXVI

Correlation and regression between % Actinomycetes and plate count on medium B

MEDIUM B

	Plate No.	Plate Count	Actino. Count	% Actino.
I	1	73	6	8.2
	2	94	14	14.9
	3	72	12	14.2
	4	85	14	16.5
	5	73	9	12.3
II	1	115	11	9.6
	2	107	8	7.5
	3	110	13	11.8
	4	102	15	14.7
	5	115	12	10.4

$\Sigma X = 946$ $\quad \Sigma Y = 120.1$ $\quad n = 10$

$\bar{x} = 94.6$ $\quad \bar{y} = 12.0$ $\quad SXY = -11211.9$

$SX^2 = 92306.0$ $\quad SY^2 = 1526.3$ $\quad (SX)(SY)/n = 11361.5$

$(SX)^2/n = 89491.6$ $\quad (SY)^2/n = 1442.4$ $\quad Sxy = -149.6$

$Sx^2 = 2814.4$ $\quad Sy^2 = 83.9$ $\quad b = -0.053$

$r = -149.6/\sqrt{2814.4 \times 83.93} = -0.307$

$Sd.yx = 83.9 - (-149.6)^2/2814.4 = 75.95$

$sy.x^2 = 75.9/8 = 9.49$

$sb^2 = 9.48/2814.4 = 0.0037$

$sb = \pm\sqrt{0.00337} = \pm 0.058$

$t = b/sb = -0.05315/0.058 = 0.916$

Regression coefficient b = -0.053 P = .3 - .4

Correlation coefficient r = -0.307 P = > .1

TABLE XXVII

Correlation and regression between % chromogens and plate count on medium A

MEDIUM A

Plate No.		Plate Count	Chromogen Count	% Chromogens
VI	1	81	25	30.8
	2	67	13	19.4
	3	70	15	21.4
	4	71	16	22.5
	5	76	20	26.3
VII	1	82	20	24.4
	2	66	21	31.8
	3	95	23	24.2
	4	92	17	18.4
	5	95	21	22.1

ΣX = 795 $\quad \Sigma Y_2$ = 241.3 $\quad n$ = 10

$\bar{x}_2$ = 79.5 $\quad y_2$ = 24.1 $\quad \Sigma XY$ = 19079.8

ΣX^2 = 64361.0 $\quad \Sigma Y^2$ = 6000.11 $\quad (\Sigma X)(\Sigma Y)/n$ = 19183.3

$(\Sigma X)^2/n$ = 63202.5 $\quad (\Sigma Y)^2/n$ = 5822.57 $\quad \Sigma xy^2$ = -103.5

Σx^2 = 1158.5 $\quad \Sigma y^2$ = 177.54 $\quad b$ = -0.0893

$r = -103.5 / \sqrt{1158.5 \times 177.54} = -0.228$

$Sd\ y.x^2 = 177.54 - (-103.5)^2/1159 = 168.29$

$S^2 y.x^2 = 168.29/8 = 21.04$

$Sb^2 = 21.04/1159 = 0.0182$

$Sb = \sqrt{0.0182} = \pm .1349$

$t = b/Sb = 0.089/0.1349 = 0.659$ d.f. 8

Regression coefficient = b = -0.0893 $\quad P > .1$

Correlation coefficient = r = -0.288 $\quad P > .1$

TABLE XXVIII

Correlation and Regression between % Chromogens and plate count on medium B

MEDIUM B

Plate No.		Plate Count	Chromogen Count	% Chromogen
I	1	73	18	24.6
	2	94	25	26.6
	3	72	20	27.5
	4	85	25	29.4
	5	73	16	21.9
II	1	115	42	36.5
	2	107	34	31.7
	3	110	34	30.9
	4	102	45	44.0
	5	115	43	37.4

$\Sigma X = 946$ $\Sigma Y = 310.5$ $n = 10$

$\bar{x} = 94.6$ $\bar{y} = 31.0$ $\Sigma XY = 30151.3$

$\Sigma X^2 = 92306.0$ $\Sigma Y^2 = 11039.6$ $(\Sigma X)(\Sigma Y)/n = 29373.3$

$(\Sigma X)^2/n = 89491.6$ $(\Sigma Y)^2/n = 9641.0$ $\Sigma xy = 778.00$

$\Sigma x^2 = 2814.4$ $\Sigma y^2 = 398.6$ $b = +0.276$

$r = 778/\sqrt{2814.4 \times 407.3} = 0.726$

$\Sigma dy.x^2 = 398.6 - (-791.2)^2/2814.4 = 176.2$

$S_{y.x}^2 = 176.2/8 = 22.0$

$Sb^2 = 22.0/2814.4 = 0.00782$

$Sb = \sqrt{.00782} = .088$

$t = b/Sb = 0.276/.088 = 3.14$

Regression coefficient = b = + 0.276 P = .02 - .01

Correlation coefficient = r = + 0.726 P = .02 - .01

TABLE XXIX

Correlation and regression between $\frac{\text{Count on Medium B}}{\text{Count on Medium A}} \cdot 100$ and the total count on Medium A.

Plate No.		Plate Count Medium A	Plate Count Medium B	100 . B/A
I	1	81	73	90.1
I	2	67	994	140.2
I	3	70	72	102.8
I	4	71	85	119.7
I	5	76	73	96.0
II	1	82	115	140.2
II	2	66	107	160.4
II	3	95	110	115.7
II	4	92	102	110.8
II	5	95	115	121.0

$SX = 946$ $\quad SY = 1196.9$ $\quad n = 10$

$\bar{x}_2 = 94.6$ $\quad \bar{y}_2 = 119.7$ $\quad SXY = 115569.6$

$SX^2 = 92306.0$ $\quad SY^2 = 147574.3$ $\quad (SX)(SY)/_n = 113226.7$

$(SX)^2/_n = 89491.6$ $\quad (SY)^2/_n = 143256.9$ $\quad Sxy = 2342.9$

$Sx^2 = 2814.4$ $\quad Sy^2 = 4317.4$ $\quad b = +0.83246$

$r = 2342.9 / \sqrt{2814.4 \times 4317.14} = +0.672$

$Sdy.x^2_2 = 4317.4 - (2342.9)^2/2814.4 = 2367.1$

$sy.x^2_2 = 2357.1/8 = 295.8$

$sb^2 = 295.8/2814.4 = 0.105$

$sb = \pm \sqrt{0.105} \pm 0.324$

$t = b/sb = 0.8324/0.324 = 2.57$

Regression coefficient = b = +0.832 $\quad$ P .05 - .02

Correlation coefficient = r = +0.672 $\quad$ P .05 - .02

TABLE XXX

Correlation and regression of Yellow Chromomens on $\frac{B - A}{\text{Total count on B}} \times 100$ with total count on B.

Plate No.		Total B count	Yellow Chromogens count on Medium B less count on Medium A	$\frac{\text{Chromogens B-A}}{\text{Total Count on B}} \times 100$
I	1	73	- 7	- 9.58
	2	94	+ 12	+ 12.8
	3	72	+ 5	+ 6.9
	4	85	+ 9	+ 10.6
	5	73	- 4	- 5.5
II	1	115	+ 22	+ 19.1
	2	107	+ 13	+ 12.1
	3	110	+ 11	+ 10.0
	4	102	+ 28	+ 27.4
	5	115	+ 22	+ 19.1

$S\underline{X}$	= 946	$S\underline{Y}$	= 102.9	n	= 10
$\underline{x}_2$	= 94.6	$\underline{y}_2$	= 10.3	SXY	= 11081.2
SX^2	= 92306.0	SY^2	= 2173.0	$(SX)(SY)/_n$	= 9734.3
$(S\underline{X})^2/_n$	= 89491.6	$(S\underline{Y})^2/_n$	= 1058.8	Sxy	= 1346.9
Sx^2	= 2814.4	Sy^2	= 1114.2	b	= + 0.4782

$r = 1346.9/\sqrt{2814.4 \times 1114,2} = 0.7609$

$Sdyx^2 = Sy^2 - (Sxy)^2/Sx^2 = 1114.2 - (1346.9)^2/2814.4 = 469.6$

$syx^2 = 469.6/8 = 58.70$

$sb^2 = 58.70/2814.4 = 0.02086$

$sb = \sqrt{0.02086} = \pm 0.1442$

$t = b/sb = 0.4782/0.1442 = 3.318$ 8 d.f.

Regression coefficient = b = +0.4782 P .02 - .01

Correlation coefficient = r = 0.7609 P .02 - .01

TABLE XXXI

Correlation and regression between $\frac{\text{total count on B-A}}{\text{total count on B}} \times 100$ with total count on B

Plate No.	Plate Count on B.	Plate Count on A.	B - A	$\frac{B - A}{B} \times 100$
I 1	73	81	- 8	- 10.9
2	94	67	27	28.7
3	72	70	2	2.8
4	85	71	14	16.4
5	73	76	- 3	- 4.1
II 1	115	82	31	28.6
2	107	66	41	38.3
3	110	95	15	13.6
4	102	92	10	9.8
5	115	95	20	17.4

$SX = 946$ $\quad SY = 140.6$ $\quad n = 10$

$\bar{x}_2 = 94.6$ $\quad \bar{y}_2 = 17.06$ $\quad SXY = 15082.1$

$SX^2 = 92306.0$ $\quad SY^2 = 4104.72$ $\quad (SX)(SY)/n = 13300.8$

$(SX)^2/n = 89491.6$ $\quad (SY)^2/n = 1976.836$ $\quad Sxy = 1781.3$

$Sx^2 = 2814.4$ $\quad Sy^2 = 2127.9$ $\quad b = +0.6329$

$r = 1781.3 / \sqrt{2814.4 \times 2127.9} = +0.727$

$Sd\ y.x^2 = 2127.9 - (-1781.3)^2/2814.4 = 1000.6$

$S^2 y.x^2 = 1000.6/8 = 125.07$

$Sb^2 = 125.07/2814.4 = .0444$

$Sb = \sqrt{0.0444} = \pm .2107$

$t = b/sb = 0.6329/0.2107 = 3.003$

Regression coefficient = b = + 0.6329 $\quad P = .02 - .01$

Correlation coefficient = r = + 0.727 $\quad P = .02 - .01$

PART IV

Experiments and a review of the literature concerning the rhizosphere in relation to microbial interaction and plating density

(a) Experimental and statistical investigation of the problems introduced by the density effect into rhizosphere studies.

In the earlier parts of this thesis it was shown that when a soil sample is taken into nutrient agar, the development of some organisms into colonies is inhibited if the density of colonies per plate is "too high".* It was also shown that the crowding of colonies on agar plates, suppresses microbial types differentially, and that the estimates of proportional representation of such classes of organism by the plating method varies with plating density. Of course, if some part of the population changes in proportion, it is likely to bring about proportional changes in other parts of the population as well.

It was of interest to determine how far these two effects, the quantitative and qualitative bias of estimates obtained from plating data, have affected the results of rhizosphere studies made by various investigators. An experiment was therefore designed to determine what influence the plating density effect may have on studies of the rhizosphere.

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* The density of colonies at which interaction effects may bias quantitative and qualitative estimates of a soil microflora is probably very variable and dependent on the types of organism present in the population examined.

For this experiment, some plant species inhabiting a calcareous fen were used, for it was hoped that this experiment might give some indication of the extent to which a pattern in the distribution of higher plants, imposes a pattern in the soil microflora. These investigations arose, as described in the preamble to Part I, out of a study of the micro-organisms inhabiting fen peat. Experiments were therefore continued, where possible, with this soil type.

Many investigators have claimed that crop plants on tilled soils, exert a selective effect on the soil microflora. They have demonstrated this pattern, by comparing the microbial population of rhizosphere soils and control soils (i.e. unplanted soils), and also by comparing the microfloras of the roots of different varieties of plants grown in pots. The choice of fen plants for these experiments gave an opportunity to examine these general rhizosphere effects but for a natural community.

The terms 'rhizosphere' and 'control soil', have no real meaning in a fen soil which is nearly all plant material - mainly root material - at various stages of growth or death and decomposition. Here, in a broad sense, all the microbial population is rhizosphere. If a rhizosphere effect is to be looked for in such a soil, it must be shown as a differential effect between rhizospheres. The effect may, for example, be looked for, between root age, root position or root species. For this experiment root species was chosen. The peat of the fen

is waterlogged and highly organic, soil condition under which (according to Lochhead (1940)), rhizosphere effects are least manifest.

Three fen species were sampled, and the samples were chosen and mixed so that as far as possible, position and age differences were randomised within the samples before subsamples were taken.

Experiment I. Two tussocks each of Juncus subnodulosus, Deschampsia caespitosa and Molinia caerulea were sampled from Cothill, a fen basin near Oxford. From the first tussocks, three and from the second tussock two subsamples were taken, each of 10 g., and consisting of roots to a depth of 4" and the soil remaining attached after shaking. Care was taken to exclude the roots of all species but that one which formed the bulk of each sampled tussock. Each 10 g. sample was taken to 1 litre of Ringer /10, and the mixture was shaken mechanically for 15 minutes. A dilution series was prepared as described in Part II to give an ultimate dilution of 1 part of roots plus attached soil, in one million parts of diluent. Four replicate plates were taken to soil extract agar* from each dilution sample, and after 10 days incubation at 25°C. the total colonies, and also the numbers of yellow chromogens and actinomycetes on each plate, were counted. The results are presented in

* See Appendix I.

Table XXXII. The counts from replicate plates have been summated, and the total count from each set of replicate plates is presented for each sample and subsamples of the three species in Table XXXIIIA. The variation between sample tussocks is of the same order as between subsamples - and the five subsamples have therefore been considered as equivalents for the purposes of statistical analysis. The mean 'sum of four replicate plate values' has been calculated for the actinomycetes, chromogens, the percentage actinomycetes and percentage chromogens for the three species. The significance of the differences between the means for the three species have been assessed by the t test and the calculations for this are given in Table XXXIIIB. Few of the differences observed prove to be significant when tested in this way. The total numbers of yellow chromogens derived from each of the three species do however differ significantly, although the percentages of chromogens from the three species are not significantly different.

The absence of significant quantitative differences between the three fen species with respect to actinomycetes and total count is not altogether suprising. The unit of 10 g. of root plus attached soil is probably too small a sample to represent the microflora of the rhizosphere of the plants, not, probably because there is great field variation between replicate 10 g. samples of rhizosphere, but because 10 g. of roots plus

attached soil is not a just sample of the rhizosphere. The microbial population of the rhizosphere may be constant, but until it is possible to determine and to measure the amount of rhizosphere present in a given sample of root/soil mixture, it is obviously impossible to make any accurate assessment of the abundance of microbes within that rhizosphere. This problem is considered further in Part V of this thesis.

It is very interesting that this analysis of experimental results shows no qualitative differences between the microfloras of the three sampled species.

In Graph XXXII, the data from Table XXXII are plotted to show the relationship between % chromogens and the total plate count (see Part III). It is at once obvious that the percentage of chromogens is again related to plating density. The percentage of chromogens is lowest on those plates with the greatest density of colonies, and vice versa. This behaviour of the chromogenic population is the opposite to that found in the experiment described in Part III, but there is no real reason to expect that a taxonomically ill defined group such as 'chromogens', will always behave in the same way, when there is no reason to suppose that it will always have the same constituent species in its population. The most interesting feature of Graph XXXII, is that at any given plating density, the percentage of yellow chromogens from Juncus subnodulosus

appears to be lower than the percentage from the other two

species. It seems that although comparison of the mean percentage chromogens from the three species had shown no significant difference, real differences might still exist, masked by the density effect which, it has been shown in Part III, may itself determine the proportions of yellow chromogens developing per plate.

If there is a real difference between the proportion of chromogens on the roots of the three fen species, it seemed possible that it might be demonstrated, provided that the density effect were determined and 'eliminated' from the calculations first. This was done, by plotting the regression of percentage chromogens on plating density for each species, and then testing for the significance of the differences between the regressions by the method of fiducial limits., see Snedecor (1946). Essentially this method consists in fitting a curve or straight line to a set of data so that the squares of the distance from the various points to the line is at a minimum for the given type of curve. In this particular experiment such a 'regression' line can be fitted to the plate count/percentage chromogen data for each of the three species, (fiducial limits are a method of estimating the significance of the distance apart of two such lines). The distance apart of the two lines is one way of judging difference between the sets of data; another way is by comparison of the slopes of the regressions, which in this

experiment are a measure of the intensity of interaction or the measure of the effect that plating density had on the estimation of percentage yellow chromogens for the particular populations of soil microbes which were examined.

In Part III of this thesis, linear regressions were fitted to % chromogen and plate count data. Over the narrow range of plating densities considered in that experiment, a linear regression seems adequate, but as the range of plating densities is increased, the relation between plating density and the proportional representation of types becomes clearly curvilinear and a log./log. transformation converts the data to a linear form; the justification for the use of this form of transformation, before calculation of regression, will become more apparent in the later parts of the thesis.

The linear regressions of log. percentage chromogens on log. total plate count for the three species are plotted in Graphs XXVI, XXVII, XXVIII with fiducial limits calculated to include the relevant range of plating densities. The calculations for these regressions and for the fiducial limits, are given in Tables XXXIV, XXXV, and XXXVI. The application of the t test for significance of the regression coefficient, b, shows significant regression of percentage chromogens on plate count for Deschampsia caespitosa and for Juncus subnodulosus, but not for Molinia caerulea. The regression coefficients for the three species are not significantly different, and this absence of

significant difference suggests that the interaction between chromogens and the rest of the plate population, is of the same nature for the three species. However, although there is neither significant difference between mean percentage chromogens on the roots of the three species, nor between the intensity of chromogen interaction as found in the three estimates of b, yet the fiducial limits fitted to the regressions for these data show clear and significant differences between the percentage chromogens on Juncus subnodulosus and the other two species over the median range of plating density. Significant differences were not shown by the other statistical tests because the density effects masked the rhizosphere differences. This method of regression analysis however still shows no difference between the percentage chromogens from the rhizospheres of Deschampsia caespitosa and Molinia caerulea.

An analysis was made in the same way for the total plate counts and percentage actinomycetes from the rhizosphere plates of three species, see Tables XXXVII, XXXVIII, and XXXIX, and Graphs XXIX, XXX and XXXI. There is significant regression of % actinomycetes on plate count for Juncus subnodulosus and Deschampsia caespitosa, but no significant differences can be detected between the species, either with respect to b or by setting fiducial limits to the regressions.

It must be emphasised that although comparison of the rhizosphere floras of the three species described here

without making allowance for the effects or plating density must be very unreliable, yet the statistical method used here is not entirely legitimate. There is present in the treatment used here, a form of the common error of the wrong replicate, discussed in Part I. This error arises in the present analysis because each plate has been treated as an independent sample, both for the rhizosphere sample and also for the plating density, but in fact, the plates are not independent samples but are subsamples of dilutions of independent rhizosphere samples. For the statistical treatment just described to be applied with complete justification, each plate must represent an independent rhizosphere sample. if this requirement be fulfilled, there must be as many dilution sequences as plates, and sufficiently wide range of plating densities represented by plates to allow the regression coefficients to be calculated with accuracy. If these conditions are fulfilled the experiment will reach such dimensions that it becomes virtually impossible in normal research studies.

However the experiment offers a demonstration of differences between the rhizosphere populations of fen plants and it is reasonable to suppose that the distribution of micro-organisms other than chromogens is affected by the species of plant under which a sample of the microflora is taken.

TABLE XXXII

Plate Counts and Percentages Occurrence of Actinomycetes and

subnodulosus, Molinia caer

		Molinia caerulea					Deschampsia cae	
		Total Plate Count	Actinomycetes Total	%	Yellow Chromogens Total	%	Total Plate Count	Actinomycete Total
		112	10	8.92	30	26.78	126	7
		75	7	9.33	30	40.00	117	9
	1	82	6	7.31	23	28.04	130	10
		91	4	4.39	21	23.08	135	11
		120	18	15.00	27	22.50	25	3
		135	21	15.55	32	23.70	26	7
A	2	124	12	9.67	25	20.16	28	8
		115	6	5.22	35	30.43	37	8
							58	13
							77	7
	3						75	11
							63	6
		86	6	9.70	30	34.88	49	9
	1	51	3	5.88	21	41.17	49	6
		80	5	6.25	18	22.50	108	22
		74	6	8.11	15	20.27	38	6
B		41	5	12.19	14	34.15	(46)	13
		63	7	11.11	24	38.09	53	8
	2	54	8	14.81	18	33.33	50	3
		54	8	14.81	10	18.52	36	3
						$\bar{x}$		
						28.60		

XXXII

cetes and Chromogens form Rhizosphere Samples of Juncus

inia caerulea and Deschampsia caespitosa

mpsia caepitosa					Juncus subnodulosus			
inomycetes		Yellow Chromogens		Total Plate Count	Actinomycetes		Yellow Chromogens	
otal	%	Total	%		Total	%	Total	%
7	5.55	13	10.32	33	9	27.27	8	24.24
9	7.69	8	6.84	30	7	23.33	9	30.00
10	7.69	28	21.54	28	6	21.43	4	14.28
11	8.15	22	16.29	27	8	29.62	4	14.8
3	12.00	7	28.00	17	5	29.41	7	41.18
7	26.92	13	50.00	30	4	13.33	12	40.00
8	28.57	10	35.71	9	4	44.44	2	22.22
8	21.62	8	21.62	30	2	6.66	5	16.66
13	22.41	21	36.21	42	14	3.33	9	21.42
7	9.09	23	29.87	40	7	17.50	5	12.50
11	14.66	33	44.00	21	7	33.33	19	46.00
6	9.52	26	41.26	50	12	24.00	13	26.00
9	18.36	11	22.44	132	5	3.79	13	9.84
6	12.24	10	20.40	84	5	5.95	7	8.33
22	20.37	27	25.00	106	5	4.72	12	11.32
6	15.79	23	60.53	113	6	5.31	10	8.85
13	28.26	11	23.91	21	4	19.05	3	14.28
8	15.09	19	35.85	25	1	4.00	3	12.00
3	6.00	18	36.00	24	4	16.66	3	12.50
3	8.33	13	36.11	17	3	17.65	3	17.64
			30.09					21.20

TABLE XXXII

Plate Counts summed over replicate plates and percentage occ

samples of Juncus subnodulosus,

		Molinia caerulea					Deschampsia c	
		Total Plate Count	Actinomycetes Yellow Chromogens Total			Total Plate Count	Actinomycete	
			Total	%	Total	%		Total	%
A	1	360	27	7.5	104	28.9	508	37	7.
	2	494	57	11.5	119	24.1	116	26	22.
	3						273	37	13.
B	1	291	20	6.9	84	28.9	244	43	17.
	2	212	28	13.2	66	31.1	185	27	14.
$\bar{x}$		339.3 ± 59.78	33.00 ± 6.49	9.77 ± 1.53	93.25 ±11.55	28.25 ± 1.47	265.20 ± 66.38	34.00 ± 3.26	15 ±2

E XXXIII

tage occurrence of actinomycetes and chromogens from rhizosphere

dulosus, Molinia caerulea and Deschampsia caepitosa.

ampsia caepitosa					Juncus subnodulosus			
nomycetes	Yellow Chromogens		Total Plate		Actinomycetes	Yellow Chromogens		
al	%	Total	%	Count	Total	%	Total	%
7	7.3	71	14.0	118	30	25.4	25	21.2
6	22.4	38	32.7	86	15	17.4	26	30.2
7	13.6	103	37.7	153	40	26.1	46	30.1
3	17.6	71	29.1	435	21	4.82	42	9.6
7	14.6	61	32.9	87	12	13.8	12	13.8
4.00	15.10	68.8	29.28	175.8	23.60	17.50	30.20	20.98
3.26	±2.48	±10.46	± 4.06	± 65.98	± 5.13	±3.94	±10.98	± 4.18

TABLE XXXIII (b)

Species sampled	Difference between means	Standard deviation of difference	D.F.	t	Probability
Total Plate Count					
Molinia-Deschampsia	74.1	±89.3	7	0.83	.4 - .5
Molinia-Juncus	163.5	±89.0	7	1.84	.1 - .2
Deschampsia-Juncus	89.4	±93.6	8	0.95	.3 - .4
Total Actinomycetes					
Molinia-Deschampsia	1.0	± 7.26	7	0.14	.9
Molinia-Juncus	9.4	± 8.27	7	1.14	.3 - .4
Deschampsia-Juncus	10.4	± 6.07	8	1.71	.1 - .2
Total Chromogens					
Molinia-Deschampsia	24.45	±15.6	7	1.57	.1 - .2
Molinia-Juncus	63.05	±15.9	7	3.96	.01 - .001
Deschampsia-Juncus	38.60	±15.2	8	2.53	.02 - .05
Total % Actinomycetes					
Molinia-Deschampsia	5.33	± 2.91	7	1.83	.1 - .2
Molinia-Juncus	7.73	± 4.23	7	1.83	.1 - .2
Juncus-Deschampsia	2.40	± 4.65	8	0.52	.6 - .7
Total % Chromogens					
Molinia-Deschampsia	1.03	± 4.31	7	0.24	.8 - .9
Molinia-Juncus	7.27	± 4.42	7	1.64	.1 - .2
Juncus-Deschampsia	8.30	± 5.83	8	1.42	.1 - .2

TABLE XXXIV

The regression of percentage yellow chromogens on total plate count for rhizosphere samples of Molinia caerulea

Log. Total Plate Count X		Log. Percentage Chromogens Y		XY	
SX	= 30.450	SY	= 23.072		
$\bar{x}$	= 1.903	$\bar{y}$	= 1.442	n	= 16
SX^2	= 58.312	SY^2	= 33.466	SXY	= 43.796
$(SX)^2/_n$	= 57.950	$(SY)^2/_n$	= 33.264	$(SX)(SY)/_n$	= 43.908
Sx^2	= 0.362	SY^2	= 0.202	Sxy	= - 0.112

$b = Sxy/Sx^2 = -0.310$

$Sdy.x^2 = 0.202 - (0.112)^2 / 0.362 = 0.167$

$sy.x^2 = 0.167 / 14 = 0.012$

$sy.x = \pm\sqrt{0.012} = \pm 0.109$

$sb^2 = 0.012 / 0.362 = 0.033$

$sb = \pm\sqrt{0.033} = \pm 0.181$

$t = b/sb = 1.711$ $P = 0.1 - 0.2$

Fiducial limits at t .05 = 2.13

= Standard error of Y = $\pm sy.x.\sqrt{1/n + x^2/Sx^2}$. 2.120

at x = 0.0 = $\pm$ 0.053

= $\pm$ 0.3 = $\pm$ 0.129

= $\pm$ 0.6 = $\pm$ 0.236

TABLE XXXV

The regression of percentage yellow chromogene on total plate count for rhizosphere samples of Juncus subnodulosus

Log. Total Plate Count X		Log. Percentage Percentage Y		XY	
SX	= 31.709	SY	= 25.122		
$\bar{x}$	= 1.585	$\bar{y}$	= 1.256	n	= 20
SX^2	= 51.787	SY^2	= 32.653	SXY	= 39.173
$(SX)^2/n$	= 50.273	$(SY)^2/n$	= 31.555	$(SX)(SY)/n$	= 39.829
Sx^2	= 1.514	Sy^2	= 1.097	Sxy	= - 0.656

$$b = Sxy/Sx^2 = -0.433$$

$Sdy.x^2 = 1.097 - (0.656)^2 / 1.514 = 0.813$

$sy.x^2 = 0.813 / 18 = 0.045$

$sy.x = \pm\sqrt{0.045} = \pm 0.213$

$sb^2 = 0.045 / 1.514 = 0.030$

$sb = \pm\sqrt{0.030} = \pm 0.173$

$t = b/sb = 2.508$ P = .02 - .05

Fiducial limits at $t_{.05} = 2.09$

= Standard error of $Yxt = \pm sy.x\sqrt{1/n + x^2/Sx^2} \cdot 2.09$

at x =	0.0	= ± 0.099
	= ± 0.3	= ± 0.146
	= ± 0.6	= ± 0.242

TABLE XXXVI

The regression of percentage yellow chromogens on total plate count for rhizosphere samples of Deschampsia caespitosa

	Log. Total Plate Count X	Log. Percentage Chromogens Y	XY
Σ	= 35.197	= 28.604	
$\bar{x}$ / $\bar{y}$	= 1.760	= 1.430	n = 20
Σ^2	= 63.003	= 41.886	ΣXY = 49.751
$(\Sigma)^2/n$	= 61.941	= 40.905	$(\Sigma X)(\Sigma Y)/n$ = 50.339
s^2	= 1.061	= 0.980	Σxy = -0.588

$b = \Sigma xy / \Sigma x^2 = -0.554$

$s_{dy.x}^2 = 0.980 - (0.588)^2 / 1.061 = 0.654$

$s_{y.x}^2 = 0.654 / 18 = 0.036$

$s_{y.x}^2 = \pm\sqrt{0.036} = \pm 0.191$

$s_b^2 = 0.036 / 1.061 = 0.034$

$s_b = \pm\sqrt{0.034} = \pm 0.185$

$t = b/s_b = 2.994$ P = .01 - .001

Fiducial limits at $t_{.05} = 2.09$

= Standard error of Y .t $= \pm s_{y.x}\sqrt{1/n + x^2/\Sigma x^2} \cdot 2.09$

at x = 0.0 = ± .089

= ± 0.3 = ± .146

= ± 0.6 = ± .218

TABLE XXXVII

The regression of percentage actinomycetes on total plate count for rhizosphere samples of Molinia caerulea

Log. Total Plate Count X		Log. Percentage Actinomycetes Y		XY	
ΣX	= 30.450	ΣY	= 15.431		
$\bar{x}$	= 1.903	$\bar{y}$	= 0.964	n	= 16
ΣX^2	= 58.313	ΣY^2	= 15.331	ΣXY	= 29.326
$(\Sigma X)^2/n$	= 57.950	$(\Sigma Y)^2/n$	= 14.882	$(\Sigma X)(\Sigma Y)/n$	= 29.367
$s x^2$	= 0.363	$s y^2$	= 0.449	$s xy$	= - 0.041

$$\underline{b = s xy / s x^2 = -0.0114}$$

$s dy.x^2 = 0.449 - (0.041)^2 / 0.363 = 0.444$

$s y.x^2 = 0.444 / 14 = 0.0317$

$s y.x = \sqrt{0.0317} = \pm 0.178$

$s b^2 = 0.0317 / 0.363 = 0.0873$

$s b = \sqrt{0.0873} = \pm 0.295$

$t = s/sb = 0.0386$ P = >.9

Fiducial limits at $t_{.05} = 2.12$

= Standard error of Y .t = $\pm$ sy.x $\sqrt{1/n + x^2/\Sigma x^2}$ x .2.12

at x = 0 = $\pm$ 0.094

= 0.3 = $\pm$ 0.210

= 0.6 = $\pm$ 0.403

TABLE XXXVIII

The regression of percentage actinomycetes on total plate count for rhizosphere samples of Deschampsia caespitosa

Log. Total Plate Count X		Log. Percentage Actinomycetes Y		XY	
ΣX	= 35.197	ΣY	= 22.366		
$\bar{x}$	= 1.760	$\bar{y}$	= 1.118		
Σx^2	= 63.002	ΣY^2	= 25.997	ΣXY	= 38.800
$(\Sigma X)^2/n$	= 61.941	$(\Sigma Y)^2/n$	= 25.012	$(\Sigma X)(\Sigma Y)/n$	= 39.361
Σx^2	= 1.061	Σy^2	= 0.985	Σxy	= - 0.561

$b = \Sigma xy / \Sigma x^2 = - 0.529$

$\sigma dy.x^2$	= $0.985 - (0.561)^2 / 1.061$	= 0.0689
$\sigma y.x^2$	= $1.061 / 18$	= 0.038
$\sigma y.x$	= $\pm \sqrt{0.038}$	= $\pm$ 0.196
σb^2	= $0.038 / 1.061$	= 0.036
σb	= $\pm \sqrt{0.036}$	= $\pm$ 0.190
t	= $b/\sigma b$ = 2.783	P = .01 - .02

Fiducial limits at $t_{.05}$ = 2.09

= Standard error of Y x t = $\pm\ \sigma y.x \sqrt{1/n + x^2/\Sigma x^2}$. 2.09

at x =	0.0	= $\pm$ 0.092
	= $\pm$ 0.3	= $\pm$ 0.150
	= $\pm$ 0.6	= $\pm$ 0.254

TABLE XXXIX

The regression of percentage actinomycetes on total plate count for rhizosphere samples of Juncus subnodulosus

Log. Total Plate Count X		Log. Percentage Actinomycetes Y		XY	
SX	= 31.709	SY	= 22.436		
$\bar{x}$	= 1.585	$\bar{y}$	= 1.122		
SX^2	= 51.787	SY^2	= 27.676	SXY	= 34.647
$(SX)^2/_n$	= 50.273	$(SY)^2/_n$	= 25.169	$(SX)(SY)/n$	= 35.571
Sx^2	= 1.514	Sy^2	= 2.507	Sxy	= -0.924

$b = Sxy/Sx^2 = -0.610$

$Sdy.x^2 = 2.506 - (0.924)^2 / 1.514 = 1.942$

$sy.x^2 = 1.942 / 18 = 0.107$

$sy.x = \pm\sqrt{0.108} = \pm 0.327$

$sb^2 = 0.108 / 1.514 = 0.071$

$sb = \pm\sqrt{0.071} = \pm 0.267$

$t = b/sb = 2.286$ $P = .02 - .05$

Fiducial limits at $t_{.05} = 2.09$

= Standard error of Y x t $= \pm sy.x\sqrt{1/n + x^2/Sx^2} \cdot 2.09$

at x = 0.0 = $\pm$ 0.153

= $\pm$0.3 = $\pm$ 0.250

= $\pm$0.6 = $\pm$ 0.463

(b) Discussion of the literature of the rhizosphere in relation to the effects of plating density in soil microbiological studies.

Hiltner (1904) found that the soil immediately adjacent to the roots of higher plants was richer in micro-organisms than soil which was distant from the roots. This quantitative difference between rhizosphere and 'control' soils determines, that if dilutions of the soils are plated onto agar media and the dilutions are prepared on a weight for weight basis, that the rhizosphere plates will carry a greater density of colonies than the control soil plates. If the effects of plating density which have been described in Parts, I, II, III and IV of this thesis are of general occurrence, it is to be expected that the populations of colonies on the plates will be quantitatively and qualitatively affected by the density of colonies developing on these plates. This density effect is bound to be far more extreme on the densely populated rhizosphere plates than on the less populated control soil plates. It is to be expected that qualitative differences will be imposed between the rhizosphere and control soils by these density differences (see Part III and IV). It remains to determine whether there is any reason to believe that these qualitative differences which various investigators have found between rhizosphere and control soils, reflect

anything but the density induced consequences of the numerical richness of the rhizosphere.

It is sometimes possible to examine this hypothesis because several authors have reported their experimental results of rhizosphere investigations in such a form that the qualitative differences which they have found between the microflora of soil and rhizosphere can be plotted graphically against the estimates of microbial abundance for the same populations. When the experimental methods have involved plating the soils and rhizosphere samples at the same dilutions, the estimates of microbial abundance are clearly directly proportional to the actual plate counts from which they were derived. It is possible from such graphs to see how far the qualitative differences between the populations studied can be interpreted as artifacts of plating density.

A good example of rhizosphere investigations to which this test can be applied, is found in the work of Clark (1940). This investigator studied the quantitative and qualitative changes in the rhizosphere of higher plants, with increasing distance from the root surface. Some of the results of this experiment are quoted in Table II, Part I, of this thesis, and in Graph XXXIII the proportions of 'dye-tolerant' and 'spore forming' bacteria estimated from the rhizosphere populations, are plotted against the estimates of microbial abundance for

The same samples (the latter being apparently roughly proportional to the plate counts from which the estimates were derived). The proportional representation of the two microbial types is related to the distance from a root surface, is related to the estimated microbial population of the sampled soil and is also related to the plating density at which the samples were studied. The data provide no means by which any of these three related phenomena can be shown to be causal, (although distance from the root is probably at least indirectly causal). However, the experiments reported in the earlier parts of this thesis showed that the plating density alone can produce qualitative effects of this kind. In another experiment of Clark (1940), the same classes of bacteria were studied and his data show the same relationship between their proportional representation and the estimates of bacterial abundance in the soils from which they were derived, although the problem investigated was different.

Many of the rhizosphere investigations of Lochhead, Timonin and Katznelson and their colleagues in Canada, can be interpreted in the form of graphs relating the proportional representation of microbial types to microbial abundance in the sampled soils, and just as with Clark's studies, the qualitative changes in soil microflora can be shown to be related to the presence of the root systems of higher plants, just as closely related to the estimates of microbial abundance in the sampled soils and also related to the probable density at which the populations were plated. Selections from these investigations are presented

in Graphs XXXIV - XL. There is no reason to believe that any of the qualitative differences which these authors have found between the microfloras of roots and control soil, between roots of different species of plants, and between the rhizospheres of plant varieties resistant and susceptible to disease, are anything but the products of interaction of quantitatively different populations on agar plates.

The Bacterial Balance Index of West and Lochhead (1940) is another qualitative character of rhizosphere and soil microfloras which will clearly be heavily biassed by the differences in plating density which must occur, if the compared microbial populations are quantitatively different and are plated at the same dilutions. This criticism must be added to the criticisms of this method made in the first part of this thesis.

Often it is impossible to obtain any information from the published results of rhizosphere investigations, about the order of and variation in plating density employed in the investigations. In these cases it is impossible to calculate, or prepare a graph to show, the relationships between microbial abundance in soil and microbial abundance on agar plates, and the proportional representation of microbial types.

In most of the examples quoted above, it is possible to interpret the data as showing a regular relationship between microbial abundance in the soil and proportional representation of types in the soil. Although the density effect has been

shown to act on plates, there is no reason to suppose that it always does so, or that experimentally obtained differences between microfloras in respect of qualitative characters, are not a normal consequence of the same type of interaction occurring, not on plates, but in the soil, with the same effects on the plating data. However, if rhizosphere and soil are compared at equal plating densities, it is possible to eliminate the plating density effect and to expose any real soil differences. This can be done with the experimental data of Katznelson and Richardson (1943 and 1948). When the proportional representation of actinomycetes in the microbial populations which they examined, is plotted against the estimated total population of bacteria plus actinomycetes, it is found that unlike the data from other workers, the points for the rhizosphere data lie separate from the soil data on a quite distinct curve. In the second paper, (1948) the authors compared the rhizosphere floras of healthy soils and soils in which strawberries had been grown and which had developed a brown root rot. The authors found no differences between the healthy and the root rot soils, and only record the experiments with root rot soils. To the soil was added, 3% strawberry plant tissue or 2.5% dry blood, 2.5% oat straw. Acetic acid to pH 4.0 - 4.5 or the soil was sterilised by steam treatment or left untreated as control. Investigations of strawberry rhizosphere

and control samples from the treated soils were made after 30, 100 and 220 days. The results are plotted in Graph XLI, and the independence of the rhizosphere and soil samples is quite apparent. It is quite clear that there is no simple relationship between the total microbial population in the sampled soils and the percentage of actinomycetes, (compare graph of Timonin's data). The separation of the rhizosphere and control soil data in Katznelson's work is apparently due to the plating of rhizosphere and control soils at different dilutions, (see also Graph XLII). The authors make little reference to procedure in either the 1943 or 1948 paper, but it is noted in the 1943 paper that the dilution of the rhizosphere soil was ten times greater than for the control soil samples. If a correction is made so that the ordinates of the graphs become proportional to the actual plate counts which must have been obtained, the points tend to lie around one curve. Graph XLIII. One point, marked X in the graph is clearly anomalous but it is possible that this sample was diluted ten times more than the remaining rhizosphere samples, in which case it would occupy a normal position on the graph. Other evidence that these authors diluted the rhizosphere samples to ten times the extent of the soil samples is obtained from a consideration of the form of the soil and rhizosphere data given by these authors., see Table XL. The estimates of actinomycetes for the soils and rhizospheres,

and the estimates of total bacteria are made to the same number of significant figures and the third significant figure for the rhizosphere samples if always zero but for the soil samples is always an integer. This in itself confirms that the rhizosphere samples were diluted ten times as much as the soil samples.

Log. - Log. regressions have been fitted to the percentage actinomycete - 'plate count' relationships, and it is found that there is barely significant difference between the samples. Moreover, what significantdifference is shown places the percentage actinomycetes at any given plating density, higher for the rhizosphere samples than for the control soil. This is also suggested by the data of Graph XLIII. It is noteworthy that this is the opposite to the normal condition found by Lochhead and his associates, and it seems likely that the density effect has been responsible for the impression that actinomycetes in rhizospheres were in lower proportion than in soil when the opposite may be the case.

Summary

It has been shown that the effects of interaction between micro-organisms on agar plates, which were described in earlier parts of this thesis may obscure real differences between soil microfloras. A statistical method is described by which the interaction effects may be determined and the masked rhizosphere

effects may be assessed. It is shown that the rhizosphere effects described by other workers, can be interpreted as the consequences of microbial interation, and that many qualitative differences which have been reported may in fact be artifacts.

TABLE XL (from Katznelson and Richardson 1948)

Influence of soil treatment on numbers of bacteria and actinomycetes in root rot soil and in strawberry rhizospheres.

Soil treatment	Bacteria	Actinomycetes	% Actinomycetes
Control soil	5.4	5.0	8.47
Strawberry tissue	9.0	14.0	13.46
Dried Blood	120.0	5.0	0.415
Oat Straw	28.6	15.0	4.98
Acetic acid	11.2	5.0	4.27
Steam	-	-	-
Control soil	2.6	2.3	0.81
Strawberry tissue	2.7	2.1	0.72
Dried Blood	19.2	0.3	0.16
Oat Straw	11.5	1.6	1.37
Acetic acid	7.1	1.6	2.20
Steam	11.8	1.0	0.84
Control soil	5.8	1.0	1.69
Strawberry tissue	4.2	4.5	9.67
Dried Blood	21.9	0.1	0.045
Oat Straw	29.3	3.0	1.013
Acetic acid	9.2	3.0	3.16
Steam	22.8	5.0	2.145
Control Soil	270	100	3.58
Strawberry tissue	330	110	3.23
Dried Blood	650	1	0.015
Oat Straw	670	40	0.593
Acetic acid	180	60	3.22
Steam	500	70	1.38
Control Soil	180	150	7.69
Strawberry tissue	270	170	5.92
Dried Blood	400	10	0.243
Oat Straw	750	850	10.17
Acetic acid	50	50	9.09
Steam	300	150	4.76

PART V

A study of the rhizosphere of some banana varieties

In the summer of 1949 an opportunity was offered by the University Department of Agriculture, for the author to visit the West Indies, and facilities for microbiological research were offered by the Director of the Colonial Microbiological Research Institute, Port of Spain, Trinidad.

A research programme was drawn up which allowed continuation of the type of microbiological study of the rhizosphere of high plants described in earlier parts of this thesis, and at the same time, was directed to one of the major problems in West Indian agriculture, the Panama disease of bananas. A general account of the disease and studies of the resistance of banana varieties to Panama disease is given in Appendix II.

There have recently been many demonstrations of the presence in soils of micro-organisms which are capable of antagonising plant pathogens in culture. For example, Meredith (1944) (1) has shown that there are present in banana soils many actinomycetes which are antagonistic to *Fusarium oxysporum-cubense* in culture. He found however no correlation between the resistance of soil type to the development of the disease and the abundance of the antagonists. In a few cases it has been demonstrated that the introduction of an antagonist to

a soil, sterile except for the presence of a plant pathogen, depresses the incidence of the disease in plants subsequently grown in the soil. Weindling (1946) and Brian (1949) have reviewed much of the work on microbial antagonism to plant pathogens. The greatest claims for the significance of microbial antagonists to soil borne pathogens have been made by Russian workers, notably Novogrudskij (1936-7), who claims that the bacterisation of seeds of linseed with cultures of organisms which have been shown to lyse Fusarium lini in culture, greatly reduces the incidence of disease in the developing seedlings.

The establishment of a root disease in a crop is frequently dependent on soil conditions. This subject has been reviewed by Garrett (1944). Frequently the addition of organic matter to soils heavily infected with some root disease organism, has resulted in a depression of the incidence of the disease, supposedly because of antagonism to the pathogen by other soil organisms notably soil saprophytes which have been stimulated by the soil amendments. The growing plant is itself responsible for very considerable soil amendments in the form of decaying root cap cells, root hairs and the cortex of older roots, together with possible root excretions from living cells. Organic soil amendments bring about a change in the soil microflora, and it is therefore to be expected that the population of micro-organisms on the root surfaces of plants should resemble that of an organically manured soil. Lochhead and his co-workers have shown this to be true and have

made an intensive study of the effects of plants in modifying the soil microflora, effects which they have shown to be both quantitative and qualitative, (reviewed by Katznelson, Lochhead and Timonin (1948)).

Not only is the soil microflora changed by the presence of plant root systems, but different plants may influence the soil population in different ways. The effect of this change on soil pathogens has been shown by Dey (1946) who has shown that the intercropping of peas with Sorghum reduced the incidence of Fusarium udum in the peas. Garrett [illegible], reviews those cases in which the growth and subsequent ploughing in of a crop may reduce the period of rotation necessary for the control of soil borne diseases. Katznelson et al. [illegible] review their own work on the differences between the rhizospheres of resistant and susceptible varieties of flax and tobacco to the disease caused by Fusarium lini and Thielaviopsis basicola respectively. The resistant varieites support a smaller microbial population in their rhizosphere than the susceptible varieties.

Experimental

The experiments to be described were designed to detect differences between tne rhizosphere flora of banana varieties, and to discover to what extent the differences which were found were related to the nutrient content of the underground parts

of the varieties tested. For these experiments, banana varieties resistant and susceptible to Panama disease were chosen to test the hypothesis that there is some correllation between disease resistance and the rhizosphere population. The organisms isolated from the rhizospheres were also tested for the production of antibiotics to Fusarium oxysporum cubense in culture.

The banana plants used in these experiments were kindly provided from experimental plots at the Imperial College of Tropical Agriculture, Trinidad, B.W.I.

Experiment I. The rhizosphere of banana varieties.

Two suckers were taken from each of the following banana varieties in the field.

Gros Michel Silk (Both susceptible to Panama disease)

Congo Guindy (Both immune to Panama disease)

The suckers were cleaned of attached soil and the roots were removed. The suckers were then planted in individual 10" pots containing a loamy soil. Two pots were filled with soil but no banana was planted, this was to act as a control experiment.

After one month active growth of new roots had taken place and from each pot a 10 gm. sample of roots plus attached soil was taken into one litre of Ringer's solution at one tenth of normal strength. The flasks were shaken in a mechanical shaker for ten minutes. From each flask 1 ml. of suspension

was taken into 99 ml. of sterile Ringer/10 to give a dilution of 1 part of the washings from 1 gram of root sample in 10,000 parts of diluent. After shaking, a 1 ml. sample was taken from the above dilution to a further flask containing 99 ml. of Ringer/10, to give a final dilution of 1 part in 1,000,000. From this final dilution, samples of 2.0 ml., 0.6 ml., and 0.2ml. were pipetted into tubes containing 20 ml. of molten soil extract agar at 45°C. and after rotation of the tubes to mix the innoculum with the agar, the mixture was poured into sterile Petri dishes. Four banana varieties and the control soil were sampled each being replicated at the pot level, and from the 1/1,000,000 dilution of the root washings, samples were taken to give three levels of plating density, each of these ultimate samples being replicated three times.

The roots from which the rhizosphere samples were taken were dried and weighed.

The colonies which developed on the agar plates were counted, see Table XLI. The counts from the plates inoculated with 2.0 ml. were discarded as they were too high to be counted accurately.

Variety of banana			Volume of 1/1,000,000 dilution per plate (1) 2.0	(2) 0.6	(2) - $\bar{x}$	(3) 0.2	(3) - $\bar{y}$	Total difference of observations from mean soil count for each variety. (2) - $\bar{X}$ + (3) - $\bar{Y}$	Weight of Root	Estimated total bacteria per gm. root (2) - $\bar{x}$ + (3) - $\bar{y}$ 4.8 0.00,0001
Gros Michel		1		33	(17)	15	(8)			
	A	2		51	(35)	11	(4)			
		3		55	(39)	18	(11)	220	0.327	140,000,000
		1		42	(26)	22	(15)			
	B	2		36	(20)	21	(14)			
		3		36	(20)	18	(11)			
Congo		1	127	33	(17)	11	(4)			
	A	2	130	25	(9)	10	(3)			
		3	123	27	(11)	13	(6)			
		4	61	20	(4)	8	(1)	105	0.180	121,458,000
	B	2	86	46	(30)	6	(-1)			
		3	75	32	(16)	12	(5)			
Silk Fig		1	89	21	(5)	5	(-2)			
		2	71	16	(0)	9	(2)			
		3	75	26	(10)	7	(0)			
		1	49	28	(12)	4	(-3)	39	0.102	79,538,000
	B	2	71	26	(10)	7	(0)			
		3	75	26	(10)	7	(0)			
Guindy		1	83	21	(5)	9	(2)			
	A	2	81	17	(1)	4	(-3)			
		3	83	13	(-3)	9	(2)			
		1	122	52	(36)	6	(-1)	108	0.315	71,250,000
	B	2	111	34	(18)	21	(14)			
		3	131	45	(29)	15	(8)			
Soil		1	86	8		3				
	A	2	56	12		8				
		3	68	14		7				
		1	62	14		11				
	B	2	61	29		6				
		3	61	18		5				

$\bar{x}$ = 16 $\bar{y}$ = 7

Discussion of results.

The expression of bacterial counts from rhizosphere samples presents great difficulty. Lochhead and his co-workers have expressed their results in terms of the number of bacteria and other organisms per gram of soil washed off the root system of a plant which has been pulled from the soil and shaken. This method suffers from the difficulty that the amount of soil which remains attached to a root system which has been shaken is greatly affected by the stickiness of the soil, varying from soil to soil and from one soil moisture content to another. Ideally the expression of rhizosphere effect should take into account the amount of root present and perhaps also the area of root surface which in most cases is extremely difficult to measure. In the final column of Table XLI the number of organisms developing per plate has been summed for each variety and for the controls. The count obtained from the control has been subtracted from the rhizosphere counts, and the difference has been corrected to give the total estimated difference between the count from ten grams of control soil and that from 10 grams of root plus attached soil. This has then been divided by the dry weight of roots. The order of the results is not of course changed by the subtraction of the control soil count, although the differences would be somewhat reduced if this was not done. In this form, the figures in the final column of Table XLI represent the increase in numbers of micro-organisms

brought about by the presence of 1 gm. dry weight of roots in the soil sampled.

There is an outstanding difference between the numbers of organisms supported by the root system of the genetically related pair, Congo and Gros Michel and those of the Silk Guindy pair. In each pair it is noteworthy that the resistant variety supports a smaller rhizosphere flora than the susceptible. This is in accordance with the findings of Lochhead et al. with flax and tobacco.

The plate counts are sufficiently low for interaction to be relatively insignificant. The only effect of interaction in this experiment would be to reduce but not to charge the order of the differences.

Experiment II. The interaction between the rhizosphere flora of banana varieties and their growth on media prepared from the varieties.

Agar media were prepared from the rhizomes of Congo, Gros Michel, Silk and Guindy bananas as described in Part I of this paper. Soil extract medium was prepared as described above and a synthetic medium, glucose-marmite peptone agar* was also prepared. Soil extract medium was prepared as previously described.

Roots of bananas were sampled in the field, one 10 gm.

* see Appendix I.

sample being taken from Congo, Gros Michel, Silk Fig and two samples of soil taken intermediate between the rows of banana plants. Insufficient roots could be obtained from the Guindy plants to make a sample. Dilutions of the samples were prepared in the same way as in experiment I. 2.0 ml., 0.6 ml., and 0.2 ml., samples were taken from the 1.1,000,000 dilutions of each soil and root sample onto each type of agar medium. After seven days the colonies which had developed on the plates were counted and the plate counts are presented in Table XLII. These results are shown in histograms, Figs. 1 and 2.

Discussion of results.

The variation between counts for media and varieties is so great that it was considered advisable to transform the figures to logarithms for an analysis of variance. The analysis is given in Table XLIII. It is clear from the analysis that the varieites differ significantly in their rhizosphere populations, but as no attempt has been made to correct for root weight this may probably be ignored. However, it is quite clear that the media are selective on the rhizospheres and the interactions between banana variety and media are significant. There is therefore evidence that it is through the varying nature of the soil amendments made by the banana varieties that differences between the rhizosphere populations are developed. Interactive effects varying with plating density should be

Plate Counts on Medium/Root sample interaction experiment

Root Samples	Media: ml. of 1:1,000,000 Dilution/Plate	Gros Michel	Congo	Silk	Guindy	Soil extract	Glucose-Peptone-Agar	Marmite	Dilution Totals	Totals
Gros Michel	2.0	37	55	18	12	54	82		258	
	0.6	11	23	7	3	22	43		109	407
	0.2	5	13	2	2	5	13		40	
Congo	2.0	233	222	44	20	164	268		951	
	0.6	32	35	15	9	53	109		253	1301
	0.2	8	19	5	2	24	39		97	
Silk	2.0	80	118	57	32	80	185		552	
	0.6	10	24	5	2	17	50		108	742
	0.2	9	13	8	0	7	45		82	
Soil A	2.0	40	87	10	3	93	108		341	
	0.6	8	12	7	2	18	77		124	506
	0.2	1	4	3	1	15	17		41	
Soil B	2.0	34	26	15	19	30	65		189	
	0.6	4	5	1	0	17	35		62	259
	0.2	1	3	1	0	2	1		8	
TOTAL		513	659	198	107	601	1140		3215	3215

TABLE XLIII

Analysis of variance of Medium/Root Sample/Dilution interaction experiment.

(Analysis of the logs of values in Table II)

Main effects	Sum of squares	D.F.	Variance	Variance ratio	Probability
Variety of root sampled	5.70	4	1.425	35.09	less than .001
Type of medium	12.22	5	2.444	60.19	less than .001
Dilution	16.08	2	8.041	198.05	less than .001
1st order interaction					
Variety/Medium	1.053	20	0.0526	1.295	-
Variety/Dilution	0.628	8	0.0526	1.933	.05 - .01
Medium/Dilution	0.755	10	0.0755	1.859	.05 - .01
2nd order interaction					
Variety/Medium/Dilution = Error	1.626	40	0.0406		
TOTAL	38.062	89			

removed in this treatment as in the dilution and dilution-media and dilution-variety interactions.

In Table XLIV the counts are expressed as the percentage of those developing on the soil extract medium for each sample. In this way the sample errors and main differences between the root and soil samples have been eliminated and only interactions and medium effects remain. These are shown graphically, Graph XLV.

TABLE XLIV

	Gros Michel	Congo	Silk	Guindy	Soil
Gros Michel	65.4	112.3	33.3	20.9	100.0
Congo	113.2	114.5	26.5	12.4	100.0
Silk	95.2	149.0	67.3	32.7	100.0
Soil A	37.3	81.7	15.9	4.7	100.0
Soil B	79.6	69.3	34.7	38.7	100.0

Experiment III. Antagonistic organisms in the rhizosphere of Congo and Gros Michel bananas.

After counting the colonies obtained in experiment II, the plates were flooded with glucose-marmite peptone agar, molten at 45°C. which had been heavily seeded with Fusarium oxysporum-cubense. Occasional actino-mycetes showed antagonism to the development of the fungus, demonstrated by rings of

inhibition of the fungus in the agar above the colonies, but there was no correllation between banana variety and abundance of these organisms.

A further test for the presence of antagonists to the fungus was made. Dilutions of the root washings of Congo and Gros Michel bananas were plated onto soil extract agar. After seven days, 20 colonies were picked from random sectors on the plates and innoculated into small tubes containing yeast extract broth. From each plate 20 bacterial colonies were sampled and from a replicate series of plates actinomycetes were transferred to broth.

After ten days the broth was tested for antibiotic activity against Fusarium oxysporum-cubense. Each tube of extract was boiled for two minutes to effect partial sterilisation and four drops from a microdropper were placed in a hole cut with a cork borer in agar heavily seeded with spores of the fungus in Petri dishes. The results are given in Table XLVI. There is a high proportion of the bacteria isolated from the roots of Congo which are active, but only one antagonist was isolated from the roots of Gros Michel. At high plating densities the antagonist seemed to be suppressed, ~~see Graph XLVI~~. No actimycetes showed any activity. The following counts were obtained fromantagonistic bacteria.

TABLE XLV

Banana variety	Plate Count	Antagonists in 20 isolated	% Antagonists
Gros Michel			
Root sample A(a)	186	1	5.0
(b)	49	0	0.0
B(a)	39	0	0.0
Congo			
Root sample A(a)	35	11	55.0
(b)	138	6	30.0
(c)	98	4	20.0
(d)	303	0	0.0
B(a)	43	2	10.0
(b)	224	0	0.0
(c)	215	3	15.0
C(a)	54	5	25.0

The antagonistic organism was a bacterium which was later taken into pure culture and the isolations were compared. It failed to produce antibiotic in subsurface colonies although in surface colonies was extremely active against Fusarium oxysporum-cubense. Further investigations of this organism and of other fungistatic bacteria are being made at the Colonial Microbiological Research Institute, Port of Spain, Trinidad.

SUMMARY

The rhizosphere flora of banana varieties differs quantitatively from variety to variety. The comparison of rhizospheres is difficult, because the form of expression of

the results may take many forms, none of them completely satisfactory. On the method adopted here, the two genetic pairs of banana, Gros Michel - Congo and Silk Fig - Guindy differ widely in the rhizosphere flora which they support, the former pair supporting the greater number of micro-organisms. Within each pair, the highest number is found in the variety susceptible to Panama disease. The nutrient requirements of rhizosphere floras is related to the sap of the banana variety from which they have been derived.

A bacterium strongly antagonistic to Fuarium oxysporum-cubense was isolated in high numbers from the rhizosphere of of the Panama disease resistant Congo, although this was virtually absent from the susceptible Gros Michel.

PART VI

GENERAL SUMMARY

1. The quantitative effects of plating density on the estimation of microbial abundance.

It has been shown in this thesis, both by examination of the published work of other investigators and by original experiments, that the plating method may lead to severe underestimation of the microbial populations of soils. This has been shown (i) by a reinterpretation of the studies of Noyes and Grounds and Waksman, on the number of colonies developed on a soil plate after successive periods of incubation. It can be shown from the experimental results obtained by these authors, that the number of colonies developing early on a plate, limits and suppresses the development of the slower growing types, and that this effect becomes progressively greater as the density of colonies per plate is increased. There may in fact be a reduction in a plate count with long incubation - due to amalgamation of growing colonies, or perhaps to lysis or phage activity. The plate count remains unchanged after 3 days incubation if it is ca. 150 colonies per cm. diameter plate - and presumably a much lower count is needed if the full complement of slow growing colonies is to become apparent.

(ii) Re-examination of other work of Noyes and Grounds, shows that the ratio between the counts from two dilutions of

a soil sample, differing in concentration by tenfold, is seldom itself ten, and is significantly lower than ten if the greater plating density is more than 60 - 80 colonies (per 9cm. plate).

(iii) Experiments have shown that the expected linear relationship between dilution of a soil sample and plate count, is not obtained unless the plate counts are relatively low, (30 - 60 colonies per 9cm. plate). This departure from linearity is true over the density ranges 200 - 10,000 colonies per plate and also 40 - 200 colonies per plate.

The extent to which this effect may influence estimates of the microbial population of the soil must depend on the intensity of the effect, and that in turn is likely to vary from soil to soil, and from one nutrient agar to another. If, as has been suggested, the production of antibiotics and the change of reaction of the medium by the earliest colonies to develop, is the factor causal in this plating density effect, then the greater the nutrient content of the medium, the more likely is the density effect to be severe.

In estimating a microbial population from a soil, it is necessary to determine at what plating density there is departure from linear relationship between dilution and plate count - or at what stage this becomes significant relative to sampling error. This precaution should be taken for each new soil type examined and for each new medium which is used.

In evaluating the significance of published estimates of soil micro-floras, any of these which are based on plate counts of more than 100 should be suspected of being underestimates. Ratios of quantitative estimates between soil types, e.g. the ratio of microbial numbers in rhizosphere and control soils are liable to be underestimates. This is particularly true when a rhizosphere ratio of 10 or more is claimed and when the two compared soils appear to have been plated at the same concentration of soil innoculum per plate.

2. <u>The qualitative effects of plating density on the estimation of categories of micro-organisms</u>.

The suppression of development into colonies of the full complement of bacterial colonies from a soil sample on agar, is unlikely to affect all categories of organisms equally. Whether the effect be due to antibiotic production, or acid production or lysis, it is unlikely to affect all microbial types equally. In fact, it has been shown to affect late developers in particular, which are not likely to be a random selection from the population. There is then, theoretical expectation of a change in proportional representation of microbial types with changes in plating density. This has been shown to be true for actinomycetes, chromogens and special nutrient demanders. There is then, a special problem involved in the comparison of microbial

populations with respect to the proportional representation of types within the populations. It is necessary, either to compare the populations at plating densities so low that interaction does not become significant, or so that the effects of density are likely to affect the compared samples equally. A statistical technique which allows this latter to be done accurately is described in Part IV, but it is tedious both in the magnitude of experiment required and the extent of calculation demanded. The use of considerably larger Petri dishes than the standard 9 cm. diameter, would allow lower density of colonies per plate to be employed, and yet sufficient colonies be obtained to give satisfactory estimates of proportional representation of types. There is no reason to believe that qualitative changes could occur on plates without coupled quantitative changes, (except for the hypothetical case of balance and antagonistic and synergistic interaction on the plates). The test for the limiting density of colonies described for quantitative estimates, is therefore likely to be valid for qualitative estimates also.

3. The interpretation of qualitative differences between micro-floras exposed by the plating method - especially differences between rhizosphere floras and control soils.

It was shown in Part IV that by varying the density at

which a soil sample was plated, significant differences in the qualitative expression of the micro-flora of that sample could be obtained. Proportional or qualitative differences recorded between populations are therefore suspect if the plating density is high (e.g. >100 per 9 cm. plate), and particularly if the density is likely to have been different for the compared samples. This suspicion arises particularly in comparisons of rhizosphere and control soils, where there is often suspicion that the two soils, quantitatively different in populations, have been plated at the same densities. Seldom is sufficient description of technique given in published rhizosphere studies to enable antifacts of plating density to be separated from real qualitative differences.

The quantitative and qualitative results of rhizosphere studies are suspect until they have been confirmed with allowance made for the plating density effect. The regular relationship between proportional representation of microbial types, and the total estimated population, which is so pregnently found, can in most cases be interpreted equally well as true rhizosphere effect or a regular antifact, the consequence of plating density differences.

4. Demonstrated rhizosphere effects of fen plants and banana varieties.

It was shown in an experiment described in this thesis,

that there is a significant difference between the percentage of chromogenic bacteria in the rhizosphere of Juncus subnodulosus to the percentage on the roots and rhizosphere soil of Molinia caerulea and Deschampsia caespitosa. This difference was obscured in the plate count results, but was exposed by a statistical method which enabled correlations with the plate count to be assessed and eliminated from the comparison. This seems to be the first demonstration of a rhizosphere effect in natural communities, and under the peculiar highly organic wet conditions of fen peat, under which rhizosphere effects are expected to show very little.

Significant differences were found between the quantitative response of the rhizosphere flora of banana varieties in development on media prepared from these varieties. The proportion of a rhizosphere population which media from the four varieties could support, was roughly proportional to the bacterial flora supported by these same varieties in the field. The greater part of the plating density effects, was removed in these experiments, in the form of first order interactions in an analysis of variance.

It is clear that although the plating density effect may be responsible for artifacts in the form of false rhizosphere effects, real qualitative and quantitative differences are imposed on a soil micro-flora by the presence of root systems, and these can still be exposed by the plating method.

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Appendix I.

Media used in experiments described in this thesis.

I. Glucose-Sucrose-Mannitol agar.

Glucose	3 g.
Sucrose	3 g.
Mannitol	3 g.
Peptone	5 g.
Agar	15 g.
Distilled water	1000 ml.

The carbohydrates and peptone are dissolved in the water, and the agar is then stirred in and the mixture steamed u til the agar is dissolved. The pH is adjusted to 6.8, and the medium poured into tubes, plugged and autoclaved at 1½ atm. for ½ hour.

II. Nutrient broth.

Marmite	3 g.
Peptone	5 g.
Distilled water	1000 ml.

Dissolve ingredients in the distilled water. Bring the solution t to the boil. Adjust reaction to 7.2 Filter through cotton. Dispense int tubes. Sterilise at 1½ atm. for ½ hour

Glucose-Marmite-Peptone agar (G.M.P. is made by adding 10 g. of glucose to medium II before dissolving the agar.

III. Nutrient gelatine.

Marmite	3 g.
Peptone	5 g.
Gelatine	150 g.
Distilled water	1000 ml.

Mix the ingredients in distilled w water. Bring to the boil. Adjust reaction to pH 7.2. Dispense into tubes. Sterilise in an Arnold for 30 min. on three successive days.

IV. Glucose salts agar. Basic medium after Lochhead. (A)

Glucose	1.0 g.	Dissolve ingredients in the distilled water and bring to the boil. Adjust pH to 6.8, tube plug and autoclave at 1½ atm. for ½ hour.
K_2HPO_4	1.0 g.	
KNO_3	0.5 g.	
$MgSO_4$	0.2 g.	
$CaCl_2$	0.1 g.	
NaCl	0.1 g.	
$FeCl_3$	0.01 g.	
Distilled water	1000 ml.	
Agar	15.0 g.	

V. Augmented medium. (B)

As IV but with addition of

Asparagine	0.5 g.	Dissolve these ingredients in addition to those of IV before steaming and adjusting pH.
Aspartic acid	0.1 g.	
Cysteine	0.1 g.	
Glycine	0.1 g.	
Alanine	0.1 g.	
Thiamin	0.1 gamma	
Biotin	0.0005 gamma	
Beta-alanine	0.1 gamma	
Nicotinic acid	0.1 gamma	
i-inositol	0.1 mg.	

VI. Soil extract agar.

Mix 1 Kg.of loamy soil with 1 litre of distilled water and autoclave the mixture . Add ca. 10 g. of calcium carbonate and filter through two filter papers, repassing the extract until a clear filtrate is obtained. (In Part V is described a procedure for clearing the extract by centrifuging, this type of cleared extract was only used in the banana rhizosphere experiments). To the cleared extract is added 0.5 g. of K_2HPO_4 and 15 g. of agar. The mixture is now steamed until the agar is dissolved , the pH is adjusted to 6.8 , the medium is tubed, plugged and autoclaved at 1½ atm. for ½ hour.

APPENDIX II

Internal factors for disease resistance and antibiotics in banana roots and rhizomes

In the course of a brief stay in Trinidad, some experiments were performed in an attempt to discover the nature or cause of the resistance shown by certain varieties of banana to Panama disease.

The Nature of the disease.

Panama disease of bananas is caused by a fungus, Fusarium oxysporum - cubense, which normally enters the banana plant through the root bases, grows through the rhizome or sucker ('bulb') to the pseudostem and leaf bases, causing a progressive wilting of the leaves in acropetal succession and death of the plant. Wardlaw has investigated the course of infection of the susceptible Gros Michel variety of banana by the Panama disease organism, and has emphasised that "the disease is soil-borne and infection always proceeds from the roots and rhizomes upwards to the pseudostem and leaves".(1) Attempts to innoculate even the very susceptible Gros Michel through the petioles and pseudostems have been unsuccessful. Once entry has been accomplished through the root bases, there seems no barrier to further invasion, the fungus growing through the sucker or 'bulb' into the pseudostem and to the petioles.(1)

The cause of resistance in certain varieties must

therefore be looked for in the roots and rhizomes, and in this appendix, those experiments are reported which were designed to test the hypothesis that an internal factor conferring disease resistance is present in resistant varieties of banana.

The nature of resistance to soil bone disease.

Martin(2) has reviewed much of the literature on the nature of resistance to soil borne pathogens. Brown, Brooks and Bawden(3) discuss the causes of the resistance shown by plants to facultative, obligate and virus parasites respectively. Forms which resistance has been known to take are among the following.

(a) Resistance has been ascribed to cork formation, e.g. resistance of potato varieties to common scab, Actinomyces scabies, and Powdery scab, Spongospora subterranea is claimed to be correlated with rapid rates of isolation of the pathogen by cork formation(4). Tisdale has claimed that resistance of flax varieties to Fusarium lini is also correlated with the rate of cork formation(5).

(b) It has been demonstrated that resistant varieties of plants frequently contain a substance toxic to the pathogen, which is absent from the susceptible varieties or present in smaller amounts. Reynolds,(6) showed that the HCN containing glycoside, linamarin, was present in greater amounts in flax plants resistant to Fusarium lini than in the

susceptible varieties and that Fusarium lini is extremely sensitive to HCN poisoning. He also found other toxic substances present in the resistant plants. Angell, Walker and Link,(7) found protocatechuic acid in the outer scales of onion varieties resistant to onion smudge, Colletotrichum circinans. Irving G.W.Jr.(8) has isolated a fungicidal substance 'tomatin' from tomato plants and has shown that this substance disappears from susceptible varieties of tomato on infection by Fusarium bulbigenum var. lycapersiis but remains present in the resistant varieties. Rubin, Artskovskaya and Proskurnikova(9) have claimed that resistance of potatoes to infection by Phytophthora infestans is determined at least in part by oxidative changes in the contained phenols.

(c) It has also been suggested that susceptibility may be due to the presence of an internal chemical factor in the susceptible varieties which is essential or stimulant to the pathogen, but which is absent or in smaller amount in the resistant varieties(10).

It seemed of interest to determine whether any of these three forms of resistance could be found in banana varieties which are known to be resistant to Panama disease. Wardlaw has shown that infection of bananas by the Panama disease organism does not take place through wound surfaces on the very susceptible Gros Michel banana, and that infection is only through active young roots. It seemed therefore unlikely

that the rate of cork formation is the effective form of resistance in resistant varieties of banana. The experiments to be described were therefore limited to tests for the presence of toxic substances in banana varieties and for the presence of growth stimulating substances and necessary nutrients for the fungus.

In these experiments several varieties of banana were used, in particular the variety Gros Michel, which is economically very important and very susceptible to Panama disease and the genetically allied variety, Congo, which is immune to Panama disease. The varieties used are classified below according to cytology, parthenocarpy and disease resistance. I am indebted for these data to Mr. N.A. Simmonds of the Banana Research Scheme, Imperial College of Tropical Agriculture, Trinidad.

All are parthenocarpic representatives of Eumusa spp., M. acuminata Colla. or the interspecific hybrid M. acuminata Colla x M. Balabisiana Colla.

Putative Parentage	Musa acuminata Colla	Musa Balbisiana Colla x Musa acuminata Colla
Susceptible to Panama disease	Gros Michel (3x)	Silk (3x)
Immune to Panama disease or virtually so	Congo (3x) I C I (4x) I C 2 (4x)	Guindy (2x)
Ploidy	2x (2n = 2x = 22) 3x (2n = 3x = 33) 4x (2n = 4x = 44)	

Experimental

(1) The growth of Fusarium oxysporum cubense on banana rhizomes

Suckers of Gros Michel and Congo bananas were taken from field plots at Imperial College of Tropical Agriculture and were washed, first with water then with soap and water, and a layer of the cortex about 1 cm. thick was then removed with a flamed scalpel. The surface was again washed with tap

water and the rhizome was soaked in a 1% solution of Aretan (soluble organo-mercuric fungicide) for 5 minutes. It was then washed throughly in several successive changes of sterile distilled water, and a further 1 cm. layer was cut off from the whole surface with a flamed scalpel. The rhizome was then flooded with absolute alcohol, of which excess was drained off and the remainder was rapidly burned off. The surface of the sucker was removed once more, and was then cut into slices 0.5 cm. thick from which discs were cut with a 1.0 cm. sterile cork borer. These discs showed no signs of contamination when left for 10 days in a damp atmosphere in sterile Petri dishes.

Fresh discs were streaked with a spore suspension of Fusarium oxysporum-cubense. Rapid growth of the fungus was obtained on discs of both Congo and Gros Michel rhizome. There was no difference in the rate of spread of the fungus or in spore production on the discs of the two varieties.

Fresh discs of Congo and Gros Michel rhizomes were cut as before and placed in Petri dishes in 1 cm. holes cut with a sterile cork borer in a glucose-marmite-peptone agar medium previously seeded with Fusarium oxysporum-cubense spores. Even growth occured over all the medium and spread over the discs of both varieties. There was no stimulation or depression of growth in the medium by either variety of banana.

(2) The growth of Fusarium oxysporum-cubense on agar media prepared from banana rhizomes.

Agar media were prepared from the rhizomes of Gros Michel(S) and Congo(I), Silk Fig(S) and Guindy(I)

(S) = Susceptible to Panama disease

(I) = Immune to Panama disease

The media were prepared as follows.

The outer cortex was cut away from selected banana rhizomes. The remainder was cut into small cubes and ground in a Waring blender with twice the weight of distilled water. The pulped product was squeezed through muslin and was centrifuged at 25,000 R.p.m. in a Sharples vertical centrifuge - repassing the extract until a clear liquid was obtained. After standing at room temperature for 12 hours, the extract was doubled in volume by addition of distilled water. Agar was added to 3½%weight/volume and the medium was steamed for one hour. The pH was adjusted to 6.3 and the medium was autoclaved at 15 lbs for half an hour. The extracts were characteristically coloured when prepared as media. Silk Fig and Guindy media were pink, the colour deepening on exposure of the agar to air; Gros Michel and Congo grey, the Gros Michel becoming slightly pink but not approaching the colour of the other varieties. A peculiarity of the media was that on remelting in a water bath at 100°C, the agar of the

resistant varieties took nearly half an hour longer to melt (i.e. twice as long) as the susceptible varieties. This is a correlation with resistance whereas colour of medium is a correlation with supposed parentage.

M. acuminata medium not red	M. balbisiana x M. acuminata medium red	
Congo	Quindy	Resistant Agar hard to melt
Gros Michel	Silk Fig	Susceptible Agar easy to melt.

The medias were poured into sterile Petri dishes and holes of 1 cm. diameter were cut in the medium with a cork borer. Into these holes were placed discs of 1 cm. diameter of glucose-marmite peptone agar on which Fusarium oxysporum-cubense was growing rapidly. The agar was also innoculated with spores of Fusarium oxysporum-cubense by pin-point innoculation. The growth rate of the fungus was measured as rate of increase in diameter of the developing colonies. The growth rate was the same on all medias. The rhizomes of the bananas prepared in this way did not differ with respect to any growth stimulating or growth inhibiting substances.

(3) The effects of extracts of banana rhizomes on the growth of microorganisms especially Fusarium oxysporum-cubense.

An experiment was performed in which banana rhizomes of Congo (I) and Gros Michel (S) were extracted with equal weights of distilled water in a Waring Blender. Extracts which had been partially sterilised by boiling for 2 minutes and untreated extracts were tested for activity against Fusarium oxysporum-cubense in culture, by dropping four spots of the extracts into holes cut with a 1.0 cm. cork borer in an agar plate of Glucose-marmite-peptone agar, heavily inoculated with Fusarium oxysporum-cubense spores. (The Wilkins-Fleming test method for determination of antibiotics.) Both the heated and untreated extracts of Congo rhizomes frequently, but not inevitably, gave rings of inhibited growth of the fungus, mainly inhibition of aerial growth, but no inhibition was obtained from the Gros Michel extracts. The inhibition by the Congo extracts was traced to the presence of an antagonistic bacterium which was later isolated from the test extract and shown to have antibiotic activity against Fusarium oxysporum-cubense in culture. It is interesting that it was only from Congo extract that this type of inhibition was obtained; the significance of fungistatic organisms has been considered in Part V of the thesis.

In a further experiment extracts were completely sterilised. An extract of the rhizome sap from Congo and Gros Michel was made by freezing 73 gm. of rhizome and extracting in a Waring blender after thawing, with 75 ml. distilled water. The extract was then cleared by centrifuging at 3500 r.p.m. and sterilised with chloroform for 4 hours. The extracts thus obtained were sterile and gave no inhibition of Fusarium oxysporum-cubense on G.M.P. agar.

Roots of the following varieties were examined for anti-fungal and antibacterial substances in alcoholic extracts.

Congo	(I)	and Gros Michel	(S)
Guindy	(I)	and Silk	(S)
I.C.I.	(I)	and I.C.2.	(I)

Extracts were prepared as follows.

44 gm. of roots were extracted in a Waring Blender in 200 ml. of 95. alcohol. The mixture was allowed to extract overnight. The mixture was then filtered to separate the pulp from the extract and the liquid was evaporated at reduced pressure in a water bath at temperature 60 - 80°C. to 1 ml. Three millilitres of Methyl alcohol was then added to maintain the extracts sterile. On the following day the extracts were again evaporated down to remove the Methyl alcohol and the extracts were made up to 10 ml. with distilled water. These extracts were tested against B. subtilis and Fusarium oxysporum-cubense. The saps had no activity against either

of these organisms. The extracts were again evaporated to 2 ml. and tested against B. subtilis, B. solanacearum (isolated from Solarum tuberosum from American type culture collection), Mycobacterium phlei and Fusarium oxysporum-cubense, by the Wilkins-Fleming plate method.

This time considerable rings of inhibition were observed. The following results were obtained. See Tables I and II.

TABLE I

Antibiotic spectra of banana root extracts. 1st DAY

Inhibition Ring diameter in mm. inclusive of 10 mm. hole

Micro-organism	B. subtilis	B. solanacearum	Fusarium	Myc. phlei
BANANA				
Gros Michel	21	19	12.5	15
	20.5	19	12	15
Congo	24	21	14	20
	25	21	12	19
Silk	18	17.5	16	(10)
	17	15	15	(10)
Guindy	17	(10)	16	(10)
	18	(10)	19	(10)
I.C.I.	18	13	20	14
	18.5	14	20	15
I.C.2.	14	(10)	13.5	(10)
	18	(10)	13	(10)

TABLE II

Antibiotic spectra of banana root extracts 3rd DAY

Micro-organisms	B. subtilis	B. solanacearum	Fusarium	Myc. phlei
BANANA				
Gros Michel	17 17	16.5 17	(10) (10)	14.5 13.5
Congo	21 22	18 18	(10) (10)	19 19
Silk	14 14	13 14	(10) (10)	(10) (10)
Guindy	13 14.5	(10) (10)	(10) (10)	(10) (10)
I.C.I.	14 15	13 12	(10) (10)	14 15
I.C.2.	12 13	(10) (10)	(10) (10)	(10) (10)

Inhibition of all organisms by all extracts was observed on the first day after plating except Guindy and I.C.2. against B. solanacearum and Silk, Guindy and I.C.2. against Mycobacterium phlei. On the third day the Fusarium oxysporum plates showed no inhibition, the fungus having grown over the first day inhibition rings.

The Significance of change in Ring diameter

In a number of cases where banana root extracts have

been plated out on to media containing a test organis, there is a wide inhibition zone on the first dey and a lowered diameter on subsequent days. To a great extent this seems to be characteristic of the organism under test rather than of the test solution.

For example, on the first day after plating Fusarium oxysporum cubense and testing the series of banana root extracts against it, inhibition rings were obtained. On the third day, there was heavy overgrowth of the inhibition rings and by the fourth day after plating there was no significant ring left. Possible explanations for this phenomenon are - (a) The organism slowly inactivates the antibiotic: (b) The antibiotic substance is unstable or volatile: (c) There are present in the culture, forms which are not inactivated by the antibiotic and which spread and become apparent in the cleared zone, becoming apparent late because they are present in a very small proportion of the population. It is very noteworthy that the colonies which appear in the inhibition zone are usually discrete and separate from the main mass of mycelium spreading from the rest of the plate. This suggests that the growth on the cleared zone is from isolated spores and is not overgrowth from the fungus outside the inhibition ring.

In the case of B. subtilis and B. solanacearum there

is a clear difference in behaviour. B. solanacearum maintains the original ring size but B. subtilis shows rapid over-growth and then this is again from small discrete colonies. It would appear that this case can only be explained if the antibiotic active against B. solanacearum is equally active against all the population, but that the B. subtilis cultures are more variable.

There is evidence from these results that there can be extracted the roots of banana more than one antibiotic substance. Congo extract inhibits the growth of both B. subtilis and M. phlei, Silk inhibits the growth of B. subtilis but not that of M. phlei. It seems likely therefore that the extracts of Silk contain an antibiotic which is not present in Gros Michel, Guindy or I.C.2. It is conceivable that the antibiotic present in Silk, Guindy and I.C.2. is present in Gros Michel, Congo and I.C.1., but there is no evidence from the experiment to enable this to be decided. A similar argument can be put forward in the case of Guindy and I.C.2. for it seems certain that the root extracts of Gros Michel, Congo, Silk and I.C.1. contained a substance active against Bacterium solanacearum, while Guindy and I.C.2. did not contain such a substance. All the extracts were active against B. subtilis and the activities although not the same for the varieities, run parallel for each variety tested. There is therefore evidence that a

substance or substances which can be called A is present in all the banana roots. A further substance is present in the roots of Gros Michel, Congo, Silk and I.C.1., which is absent from the remainder, this can be called B. A further substance active against Mycobacterium phlei is present in the roots of Gros Michel, Congo and I.C.1., this substance can be called C. The antibiotic spectra of the varieties tested were therefore:

Gros Michel	A B C	Congo	A B C
Silk	A B	Guindy	A
Guindy	A	I.C.1.	A B C
		I.C.2.	A

Although these alcoholic extracts of roots gave inhibition of Fusarium oxysporum-cubense, there is no complete correlation between the presence of the antibiotic and resistance, Congo(I) showed more activity than Gros Michel(S), Guindy(I) showed a little more than Silk(S), but Silk(S) gave a higher activity than Congo(I), and I.C.2.(I), and a good resistant strain contained less than any of the susceptible varieties. The extraction of a substance by alcohol which is not obtained by water extraction, may be evidence that it is a product of the extraction procedure rather than present in vivo. This is also suggested by the absence of toxicity on first evaporations of the alcoholic extract.

Alcoholic extracts of the rhizomes of banana varieties

prepared as above for roots had no inhibiting effect on Fusarium oxysporum cubense, and little varietal difference in antibiotic activity against B. Subtilis, B. solanacearum and Mycobacterium phlei.

Conclusions

There is no evidence that there is present in the roots or rhizomes of bananas any stable substance which prevents the growth of the Panama disease organism. Discs of living tissue and media prepared from rhizomes of resistant and susceptible varieties of banana supported free and equal growth of the pathogen. Alcoholic extracts of banana roots show antibiotic activity including activity against Bacillus subtilis, Bacterum solanacearum, Mycobacterium phlei and ephemeral activity against Fusarium oxysporum cubense. There are varietal differences in the quantity and type of antibiotic produced, but these show no correlation with disease resistance.

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APPENDIX III

A Method for Preparing Microtome Sections of Soils

In view of the many shortcomings of the Rossi and Cholodny methods for the investigation of the distribution of microbes in soils (see Part I), it was desirable to find some more direct method for the observation of microbes in situ in the soil. Clearly the most satisfactory form that this could take, would be a thin section of the soil which could be stained and subsequently examined microscopically. A section prepared in this way, would be expected to show the distribution of the various soil components of organic nature, and the relation of the distribution of microbes to that of living and dead organic matter. An attempt was made to prepare microtome sections of soil.

Cores of peat were taken from peat samples brought to the laboratory. The cores were taken in cork borers, $\frac{1}{2}$ inch in diameter. (It was noticed that unless the cork borers were very sharp there was a distortion of the sample due to the catching of stout fibrous roots on the cutting edge and the subsequent pulling of the root out of position. However, when sharp borers were used with a rapid movement, this distortion was reduced).

It was found that the peat cores, when placed in tubes of fixative, immediately swelled and disentegrated. It was found that by pouring a 2% solution of agar over the block immediately after cutting and paring it down to a layer of about 1/8 inch

thick over the surface of the block, after it had cooled, the block could be held in its original shape and passed through fixatives and embedding solutions easily.

The cores were taken vertically from the soil to a depth of one inch, and removed from the cork borer by means of a smaller cork borer bearing a small cork mounted on its end. After coating in agar, the blocks were immersed in Helly's solution in a flask at reduced pressure for 24 hours. The core was then removed and washed in running tap water for 24 hours, or until there was no further diffusion of the yellow colouring of the chromate into the surrounding washing medium. The core was then passed up through 20%, 40%, 60%, 80%, 95%, and absolute alcohol, and then on to alcohol/chloroform mixtures 3/1, 2/2 and 1/3 and then to pure chloroform. To the core in pure chloroform, was added solid paraffin wax, a little each day until no more would dissolve. The core was then placed in an open dish and covered with molten wax and the whole was placed in an oven at 52°C. This was now left until there was no more evaporation of chloroform from the dish. The block was placed in a small paper box and fresh clean wax added, the whole was then rapidly cooled by immersion in cold water.

The blocks were trimmed and seated on a microtome section bearer; a sledge microtome was used because the blocks were too large to be fitted into a rotary or rocking type of instrument.

It was found on attempting to cut sections that there was

a large number of small sand grains in the peat which quickly ruined the knives. It was therefore necessary to remove the sand grains. An attempt was made to remove the sand grains by soaking the cut surface of the block in hydrofluoric acid. This was found to be effective in removing the exposed grains, but the sections were always tightly rolled when cut and would not unroll in water when warmed. This coiling occurred at both low and high temperatures and humidities. Also, although the acid was effective in removing the exposed grains, there was always the danger of cutting a grain immediately beneath the surface which had been protected from the acid by a thin layer of wax.

The few sections which have been made have been stained with crystal violet or with erythrosin. The latter stain seemed to be more satisfactory, and micro-organisms could be seen in the sections. Sections were cut at 12 - 20 microns, which is obviously too thick for accurate observations on the distribution of micro-organisms. There seems reason to believe that with an improved knife and use of hydrofluoric acid, useful sections of peat could be made.

One of the main disadvantages of cutting microtome sections of peat embedded in wax, was the frequent presence of sand grains in the peat. It seemed possible that this difficulty might be overcome if the peat were first embedded in a material of hardness comparable to the sand grains, and if sections were then prepared by a fossil sectioning technique. To test the

possibilities of this method a portion of the stem of Equisetum giganteum was taken; it was known that this stem was heavily silicified and that it was not normally possible to cut microtome sections of the material because of the heavy silica content. A plant stem was taken for this test and not a peat core in order to avoid the initial difficulties of breakdown of the peat core and because it was necessary to compare the appearance of a section cut in sodium silicate with fresh sections cut freehand with a razor.

The stem was taken from 50% alcohol and so was roughly fixed. It was washed in distilled water for 24 hours to remove traces of alcohol and was then transferred to a 1% solution of sodium silicate (commercial water glass). At 24 hour intervals the solution was changed and the stem was passed through 2%, 4%, 6%, 12%, 25%, 50%, 75%, to 100% water glass. The material was then placed in a tube of the 100% solution and when it had become less viscous after warming it was poured into a cardboard tray, and sodium silicate was added until the stem was well covered. The box was then placed in a 90°C. oven for a week.

A second stem was carried through the embedding process in the same way but the silicate was hardened by placing it in a calcium chloride dessicator until the block had set to a glass-like consistency. The latter method was in general the simpler and was less likely to distort the tissues. The dessication

method was therefore adopted for use in later experiments.

The blocks set quite hard with much the same refractive index as glass. A specimen was taken to the Department of Geology, University of Oxford, where it was kindly arranged that sections should be cut and ground. It was found that the material was easy to cut and polish although it was brittle, but that after a short time of grinding the material became sticky. Sections were however prepared, and these were distorted as compared with the hand sections. There was a swelling of the cell walls probably brought about by the strong alkalinity of the sodium silicate.

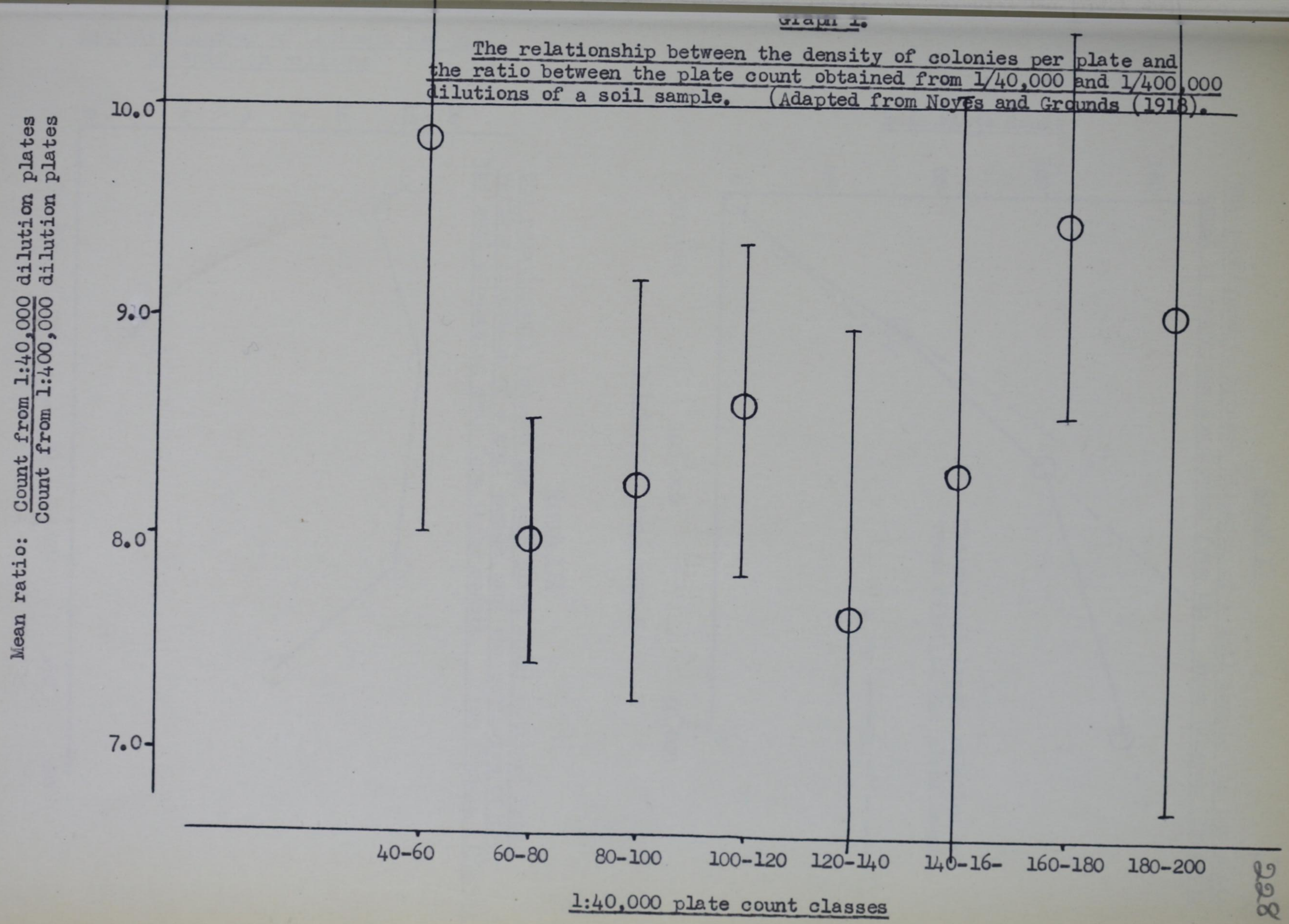
An attempt was made to embed material of peat coated in agar in the same way as described for wax embedding. It was found that much smaller changes of concentration of sodium silicate than of alcohols were necessary to avoid a splitting of the agar coat. There was a formation of deep brown pigment in the silicate solution when the peat was added. If a split occurred in the agar coating of the blocks, there was an almost immediate exudation from the block of the brown pigment which emerged in a steady stream from the crack. It was observed that when a block was allowed to remain in a split state in the silicate for the usual period of 24 hours, and was then transferred to a stronger solution, there was an immediate repetition of the exudation. This suggests that a reaction

between the peat and the silicate takes place and that the extent of the reaction is determined by the concentration of the silicate. This type of behaviour would be characteristic of the formation of a sodium salt of a humic acid. It is seen from the above observations that peat embedding in sodium silicate is not a satisfactory method for preparing sections of peat, or at least that the method adopted here is not satisfactory.

It has been suggested that peat could conveniently be embedded from chloroform into bakelite, following a technique used for embedding rock fragments before sectioning. This technique remains to be tried.

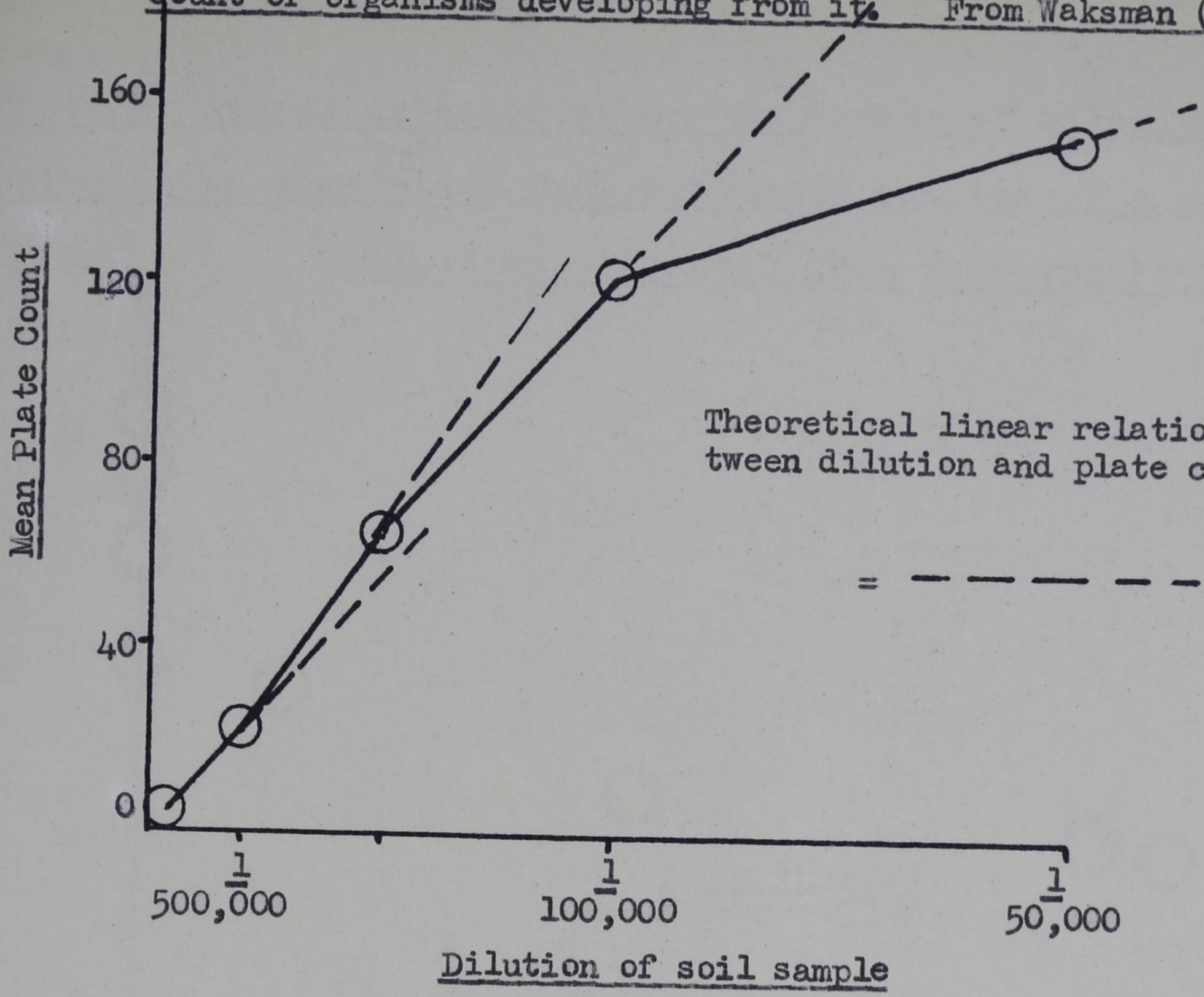
Graph 1.

The relationship between the density of colonies per plate and the ratio between the plate count obtained from 1/40,000 and 1/400,000 dilutions of a soil sample. (Adapted from Noyes and Grounds (1918).



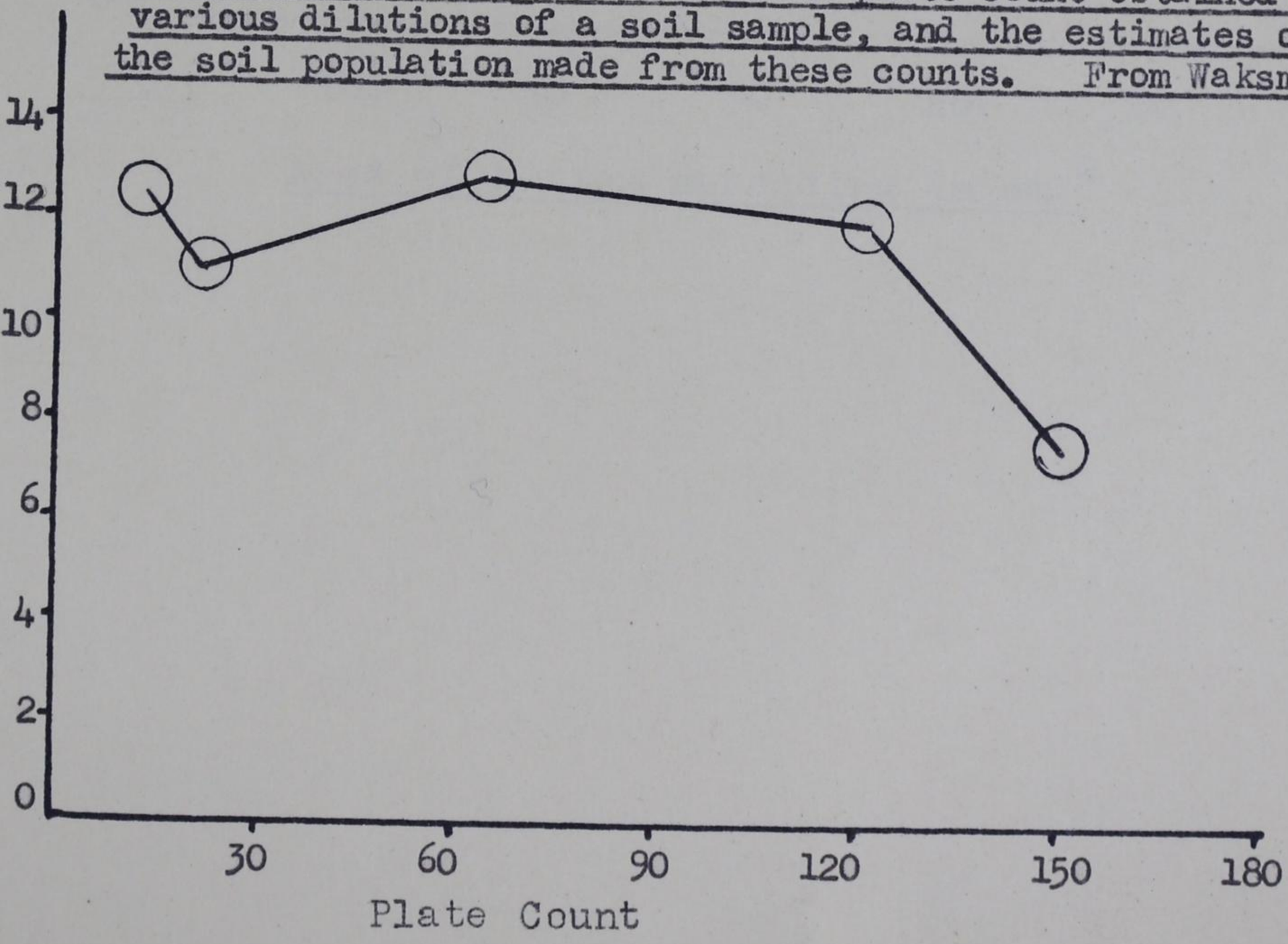
Graph II

The relationship between dilution of a soil sample and the plate count of organisms developing from it. From Waksman (1922-6).



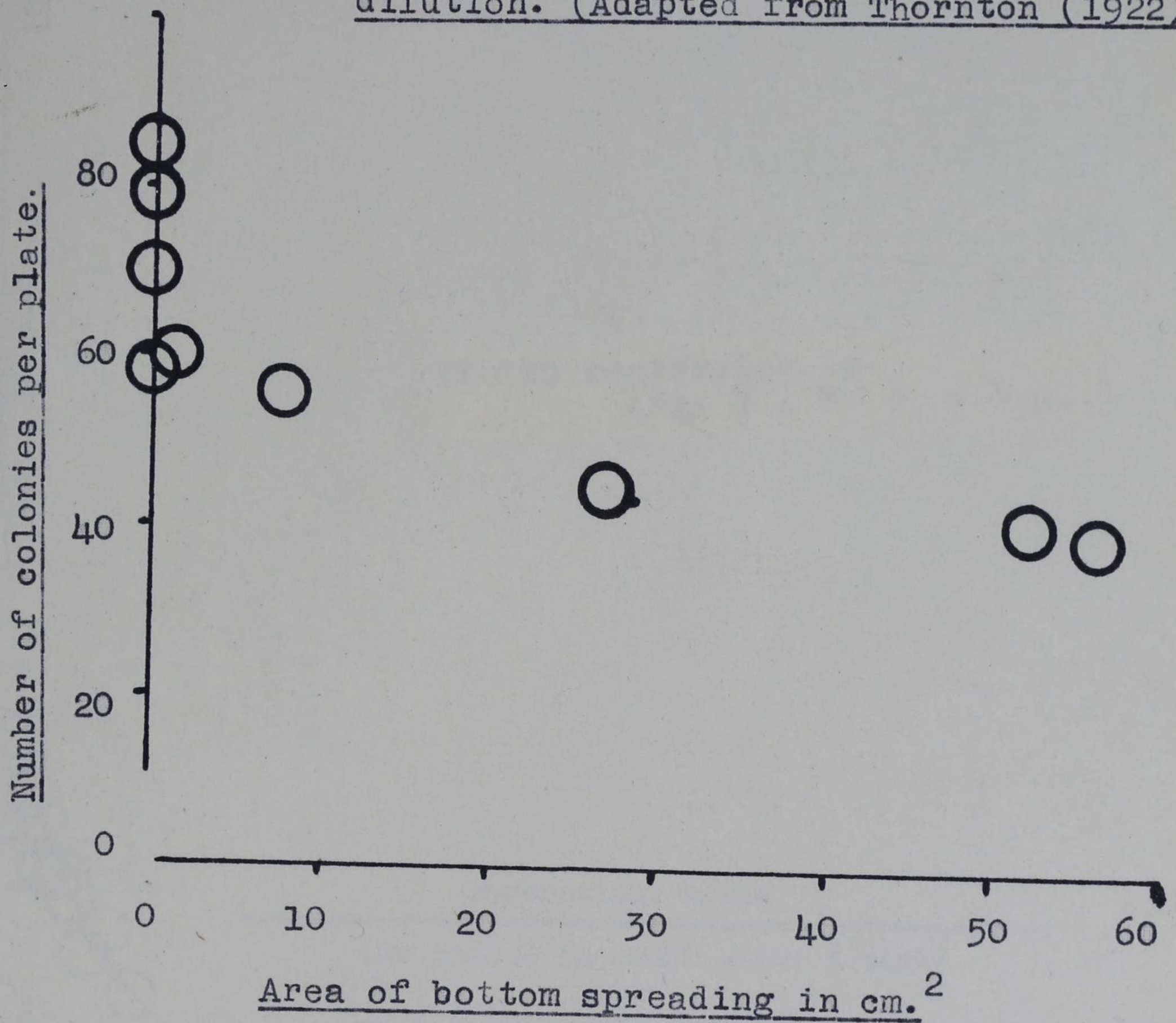
Graph III

The relationship between the mean plate count obtained from various dilutions of a soil sample, and the estimates of the the soil population made from these counts. From Waksman (1922-6)



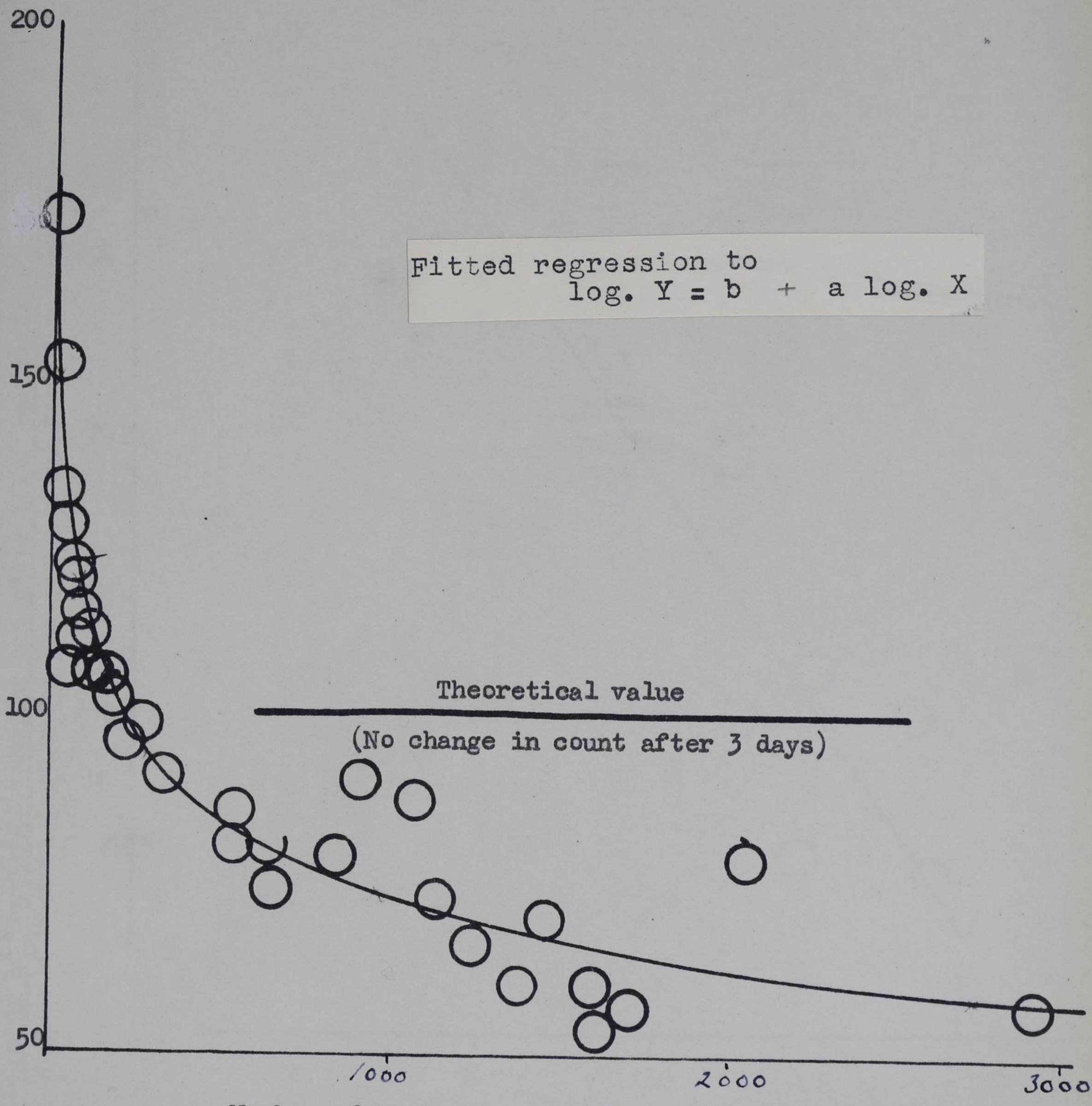
Graph IV. (see Table VI.)

The effect of apreading colonies on the number of colonies developing on an agar plate from replicate samples of a soil dilution. (Adapted from Thornton (1922)).



Graph V (See Table XIII).

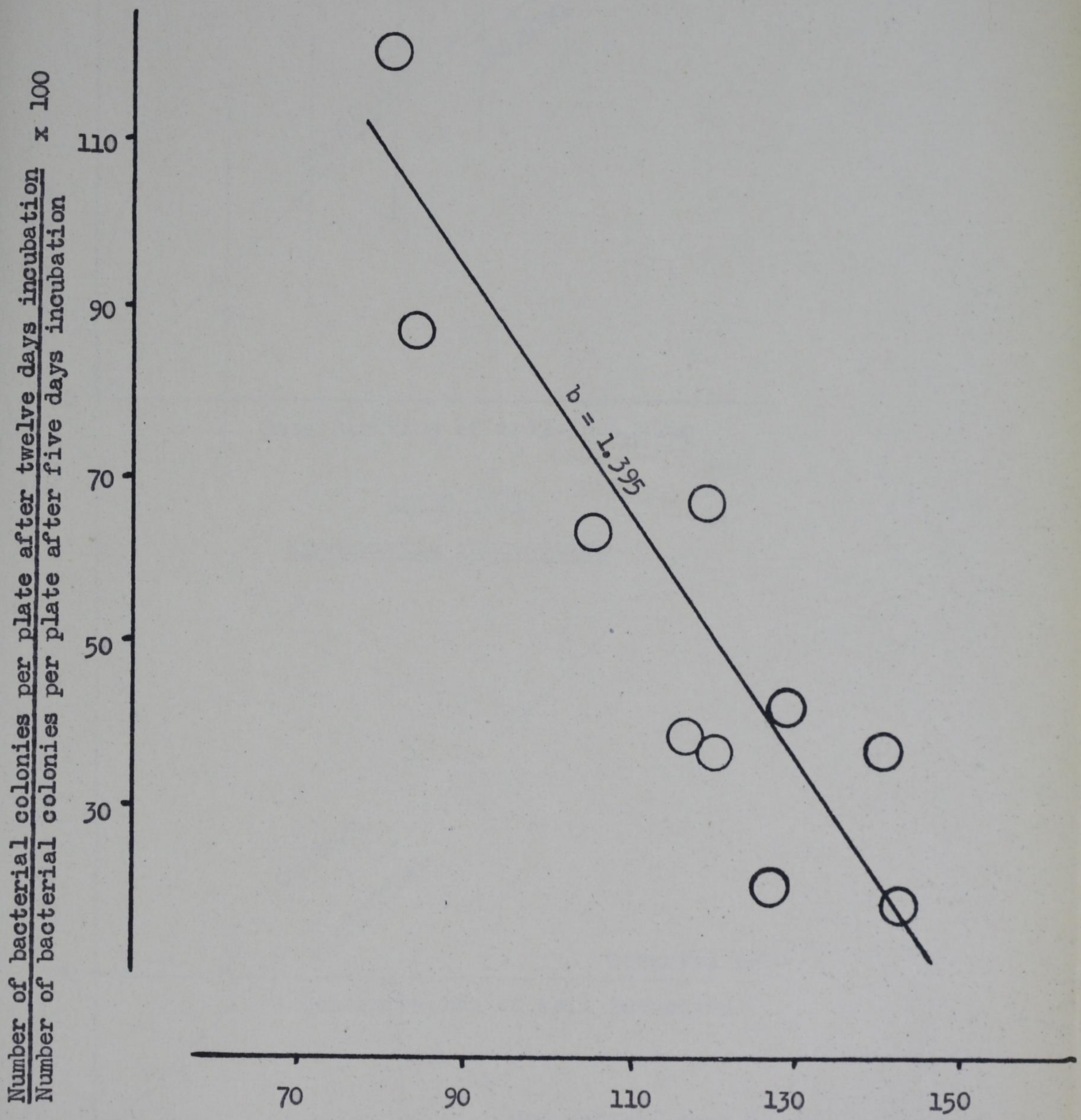
The relationship between the density of colonies per plate and the proportion of colonies developing late. (Adapted from Noyes and Grounds (1918)).



Fitted regression to
log. Y = b + a log. X

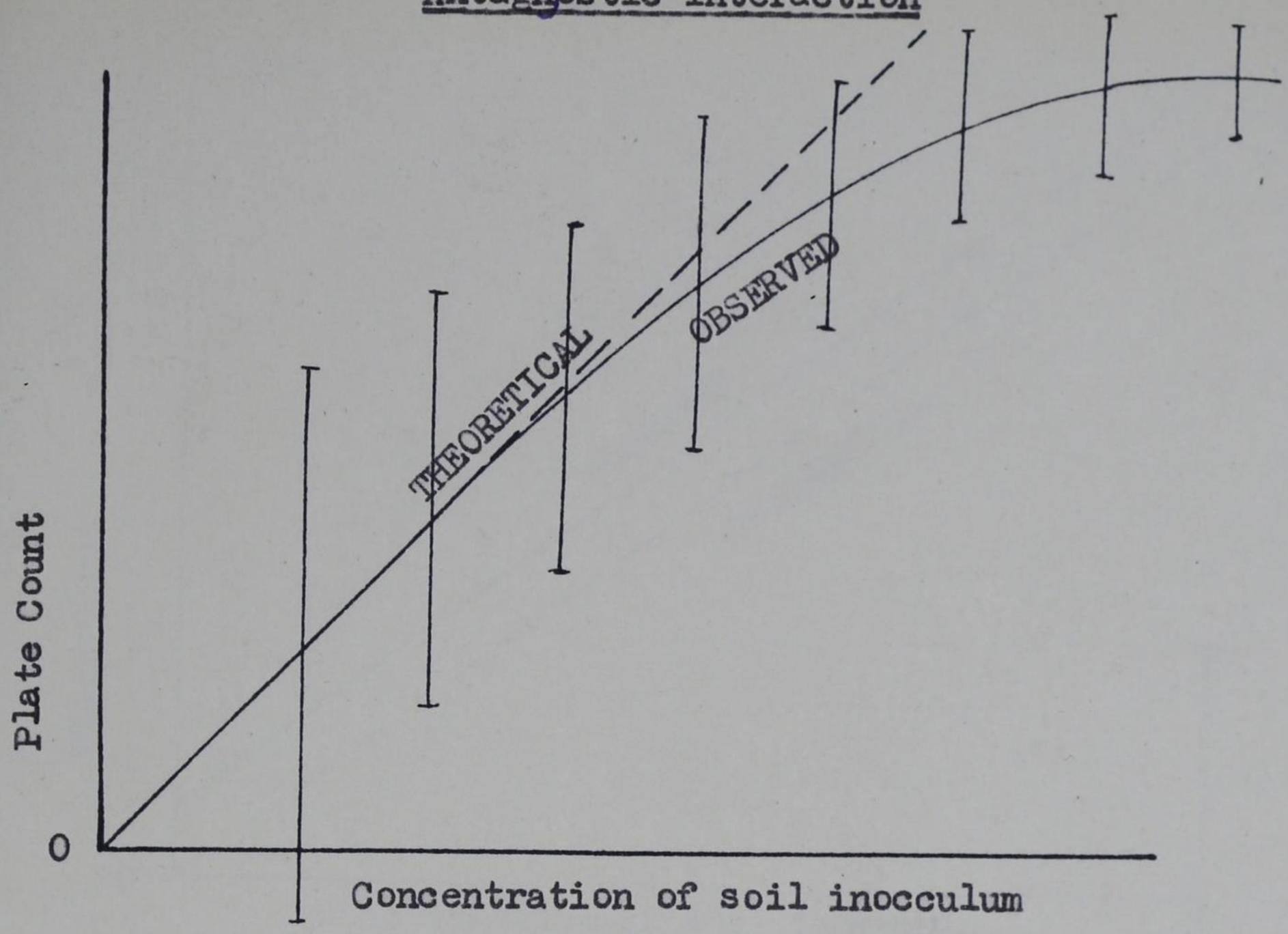
Graph VI (See Table XIV)

The relationship between the density of colonies per plate and the proportion of colonies developing late. (Adapted from Waksman (1922-3)).



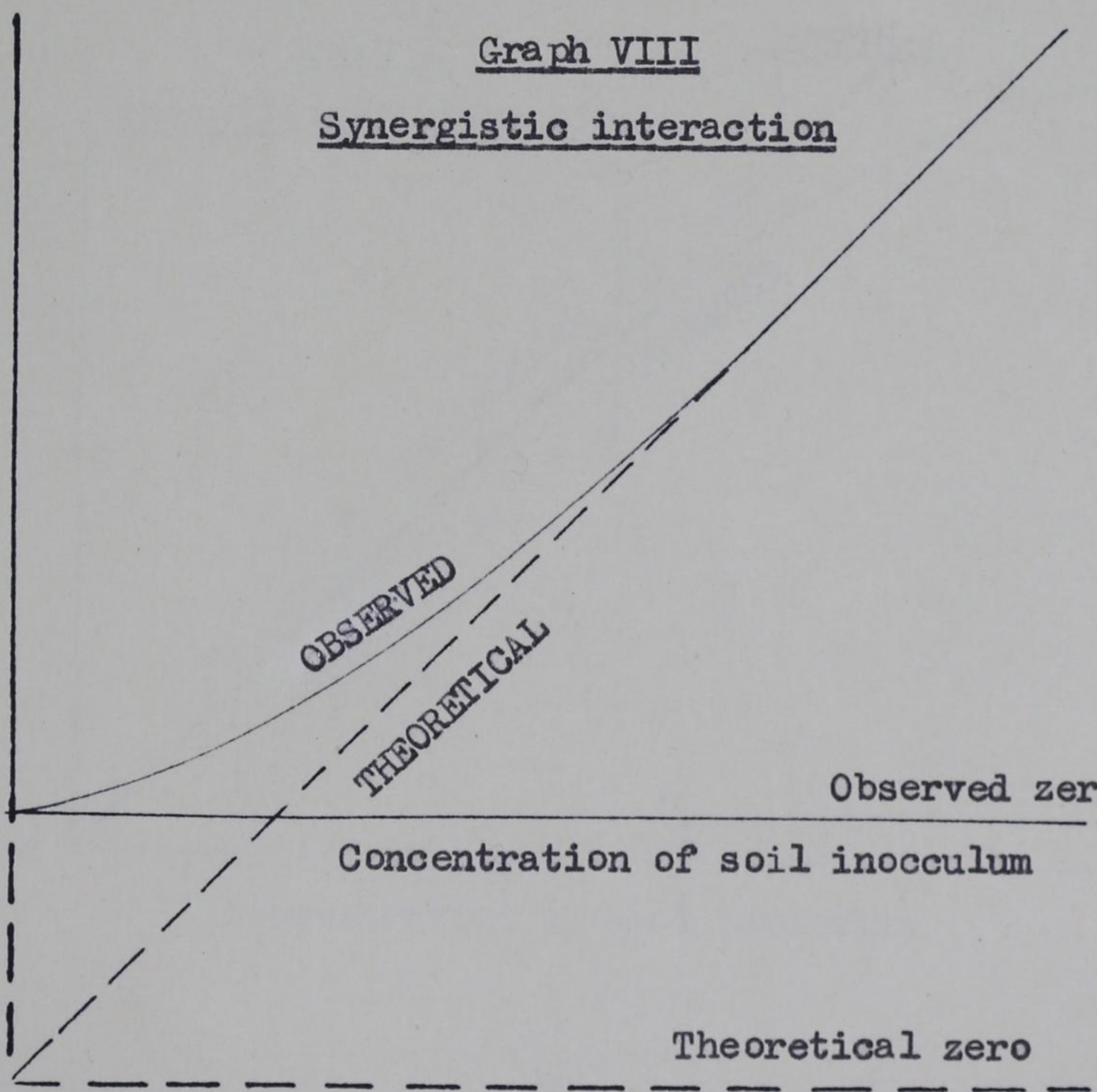
Graph VII

Antagnostic interaction



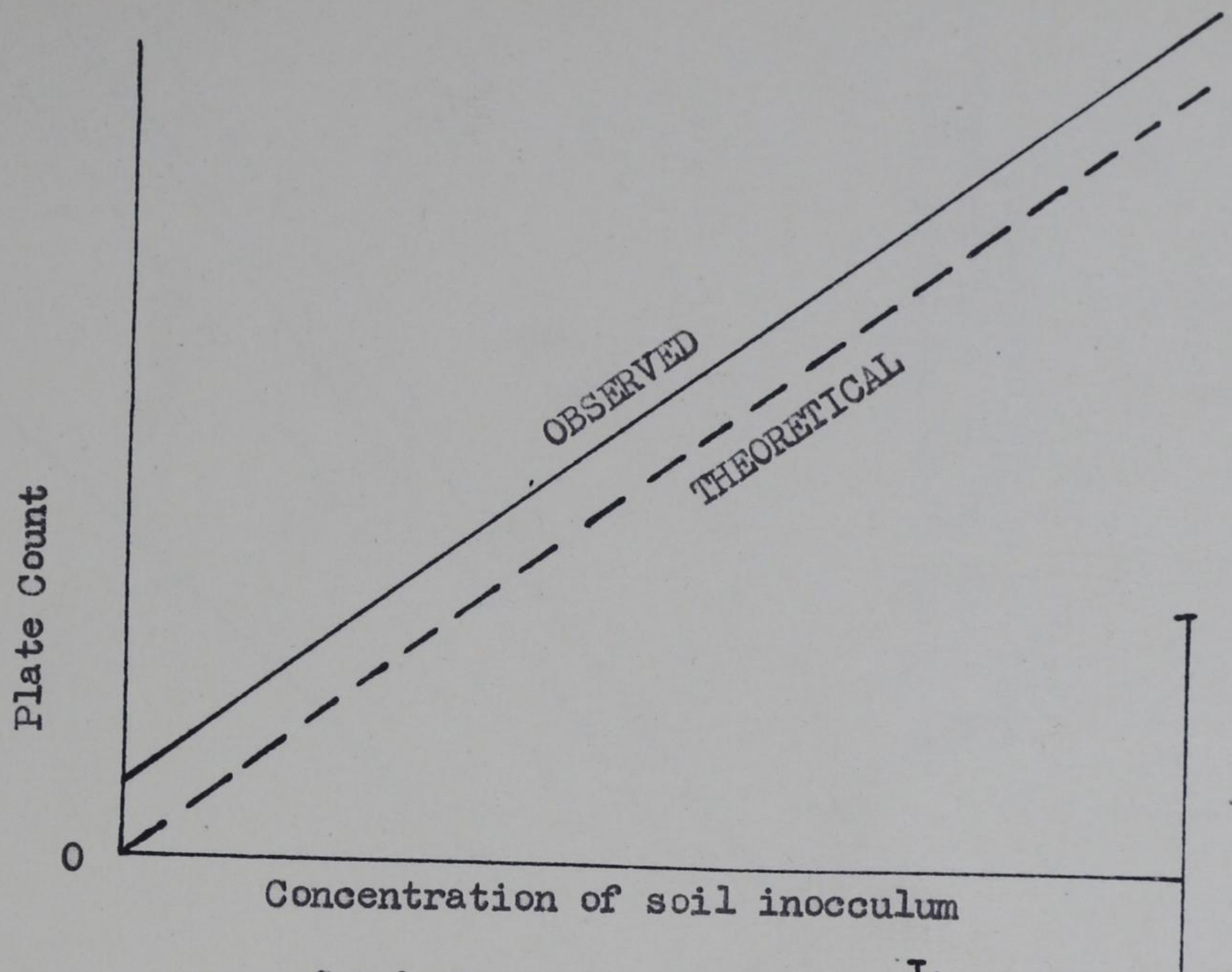
Graph VIII

Synergistic interaction



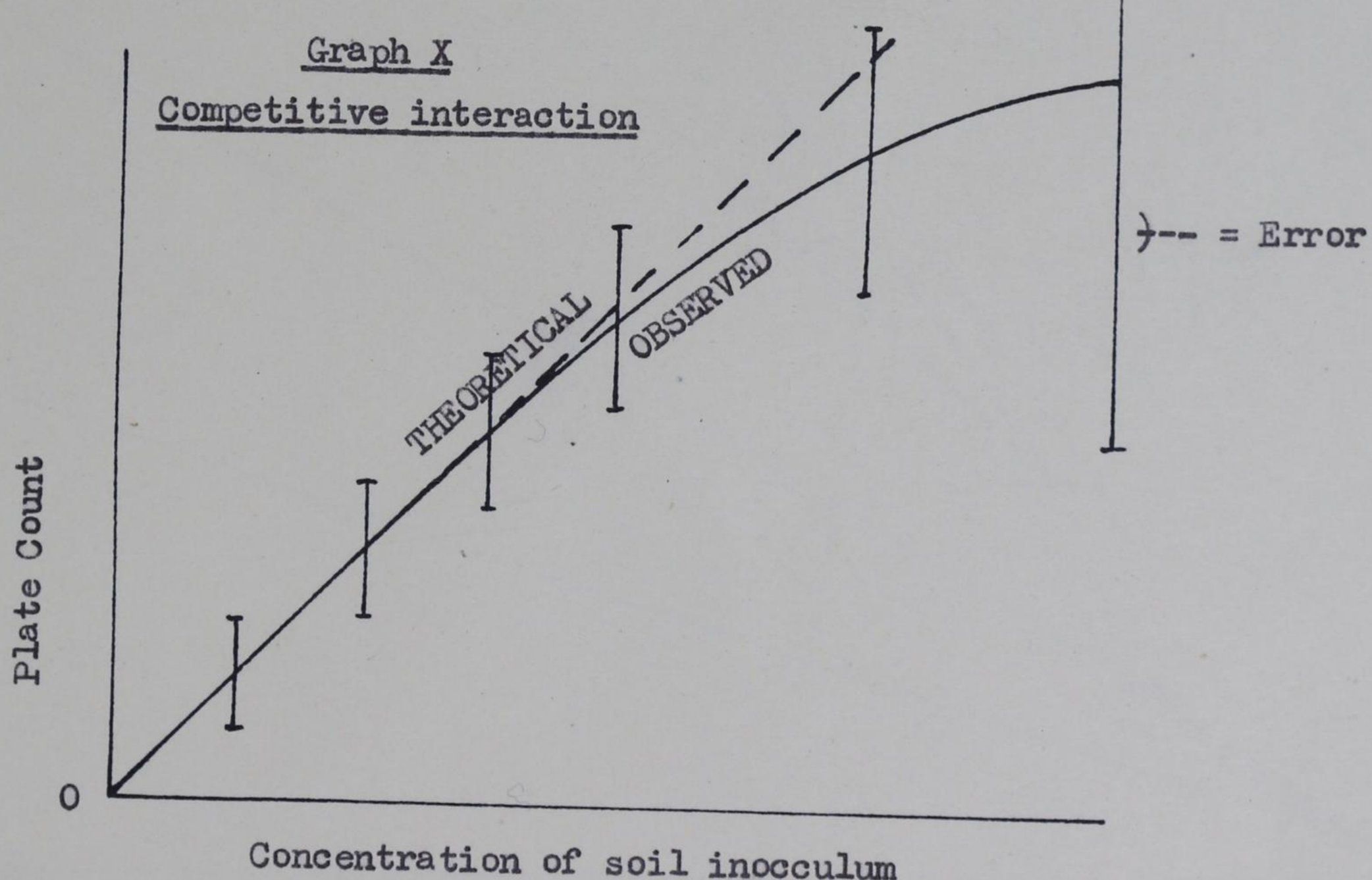
Graph IX

Contamination



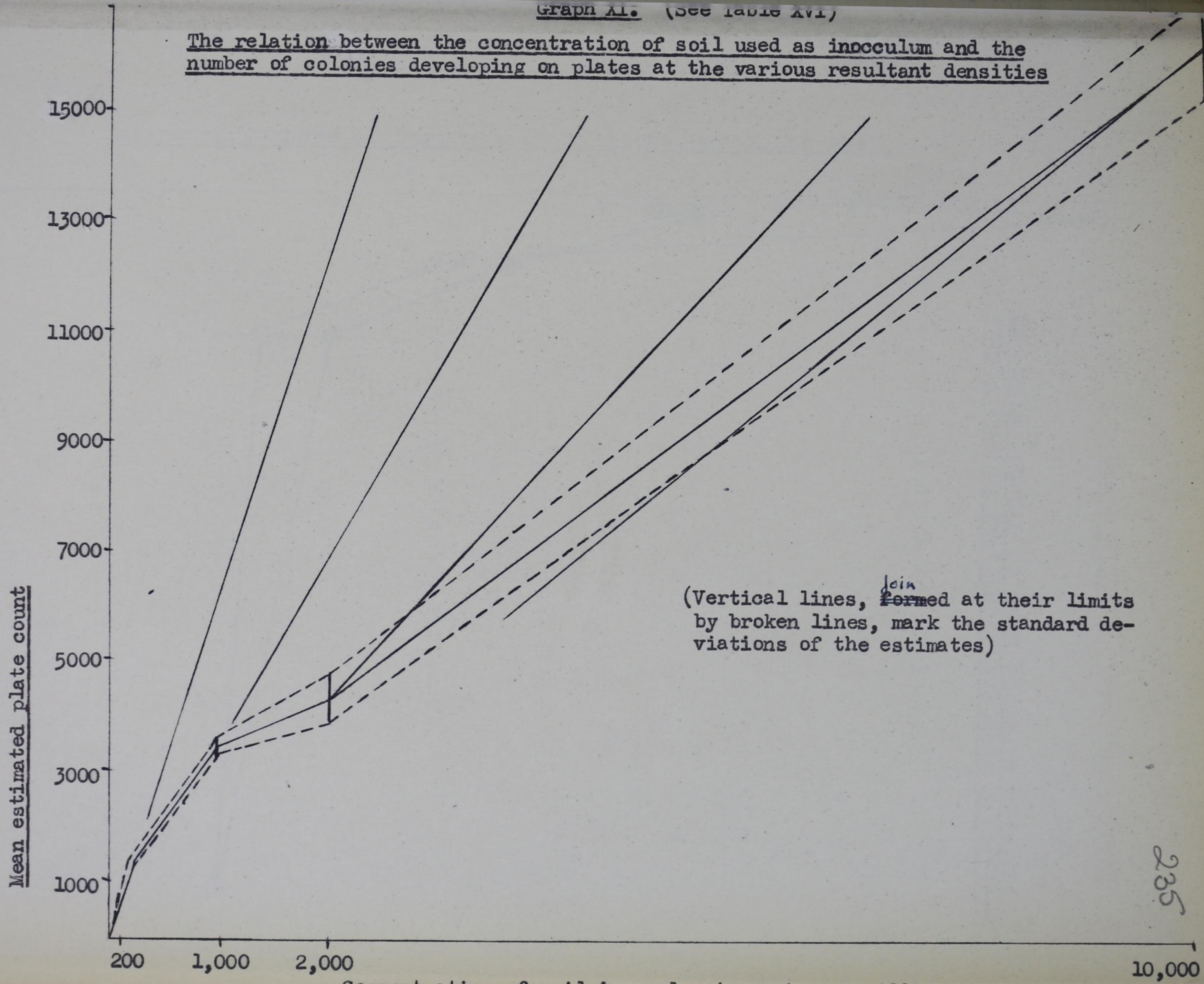
Graph X

Competitive interaction



Graph XI. (See Table XVI)

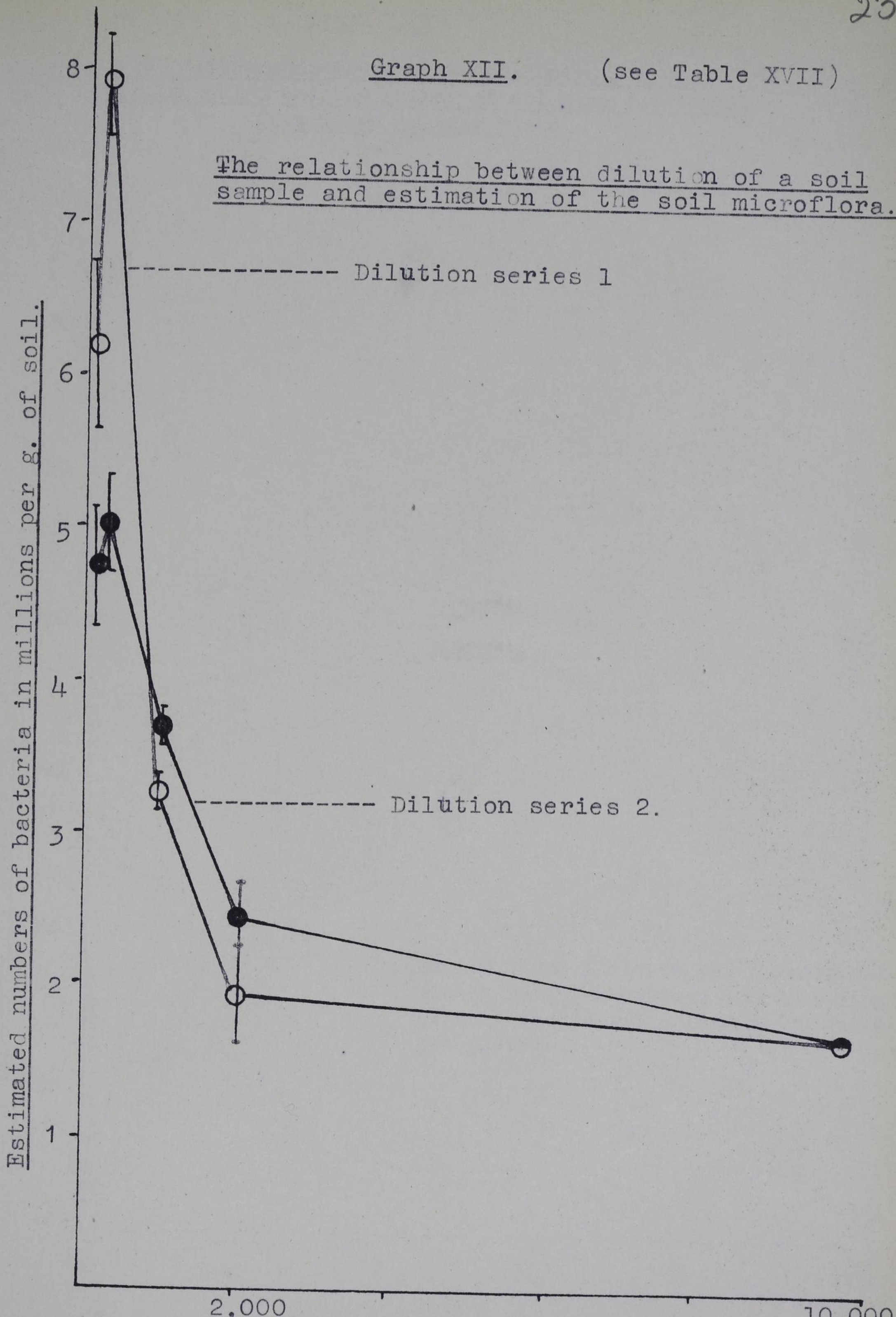
The relation between the concentration of soil used as inocculum and the number of colonies developing on plates at the various resultant densities



(Vertical lines, ~~formed~~ join at their limits by broken lines, mark the standard deviations of the estimates)

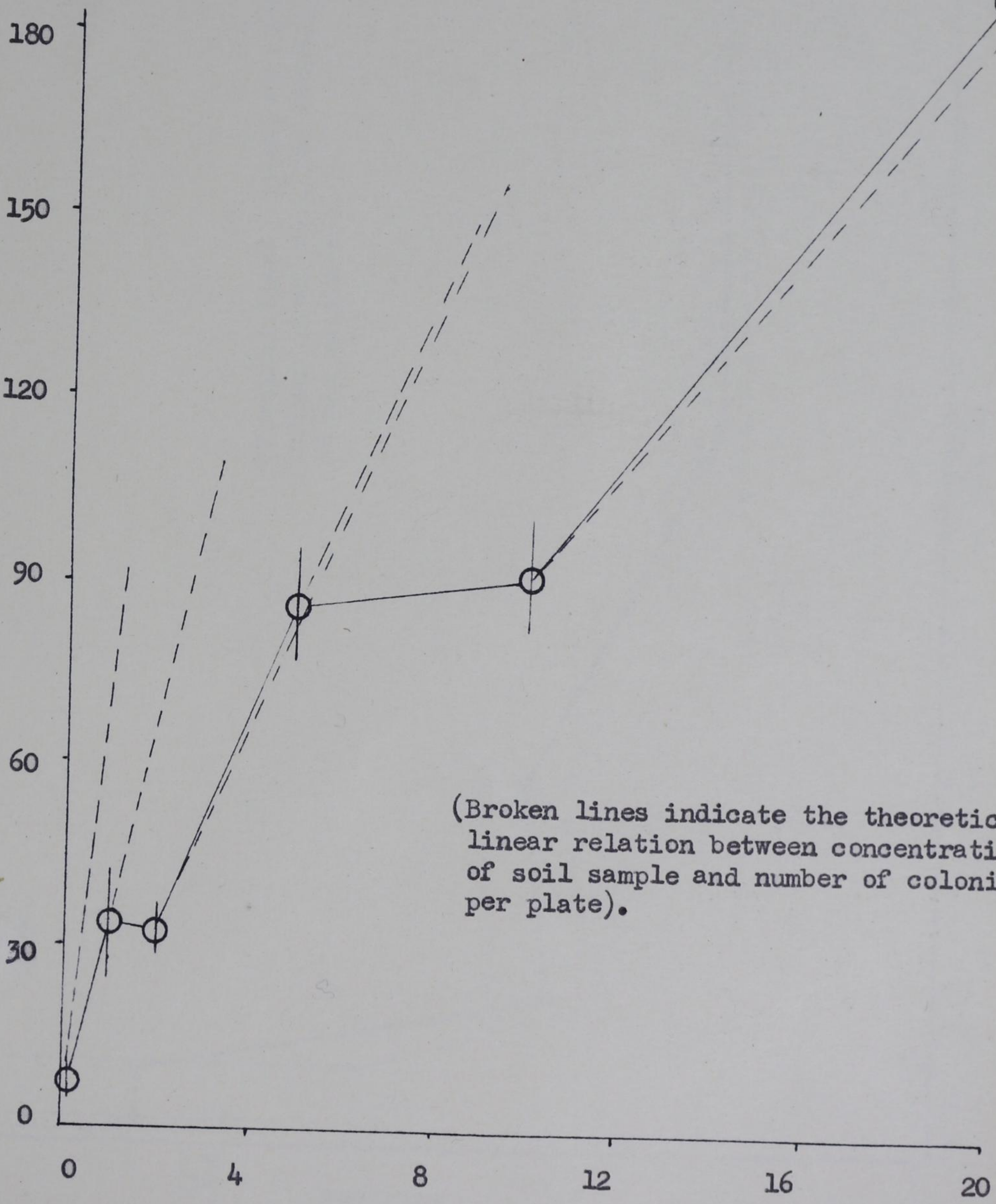
Graph XII. (see Table XVII)

The relationship between dilution of a soil sample and estimation of the soil microflora.



Graph XIII

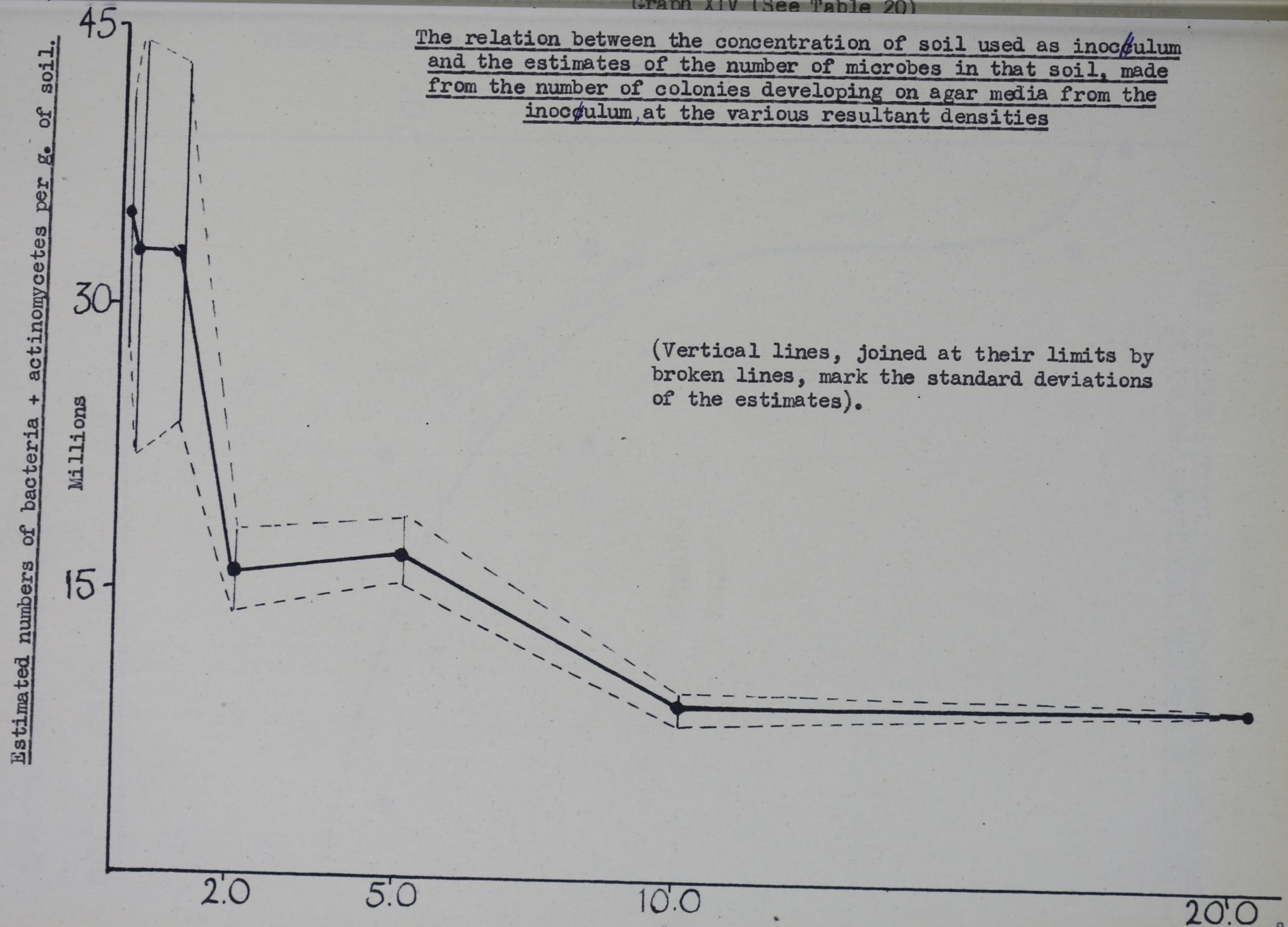
The relationship between the concentration of a soil inoculum and the number of colonies developing from it on an agar plate



(Broken lines indicate the theoretical linear relation between concentration of soil sample and number of colonies per plate).

Graph XIV (See Table 20)

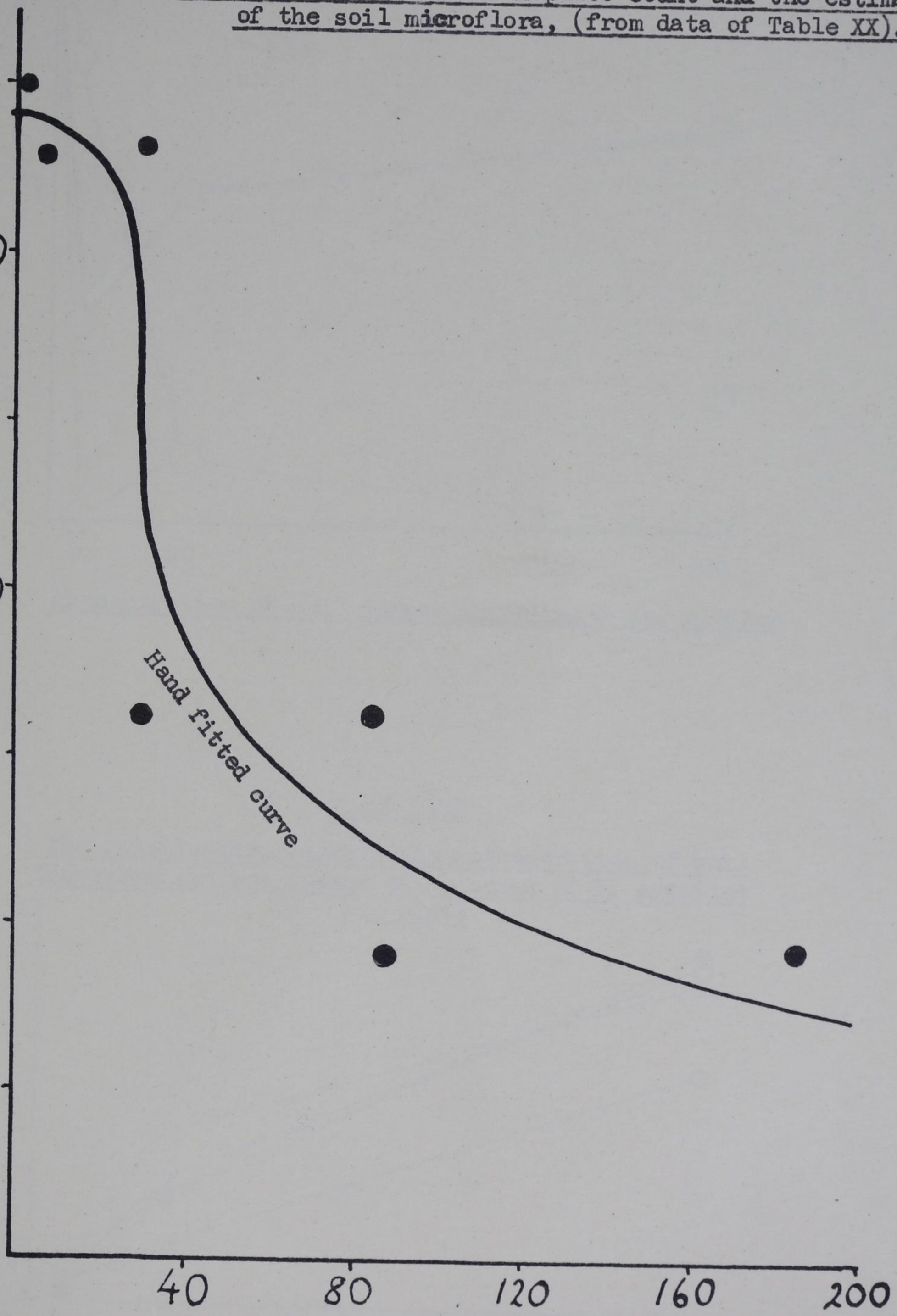
The relation between the concentration of soil used as inoculum and the estimates of the number of microbes in that soil, made from the number of colonies developing on agar media from the inoculum, at the various resultant densities



(Vertical lines, joined at their limits by broken lines, mark the standard deviations of the estimates).

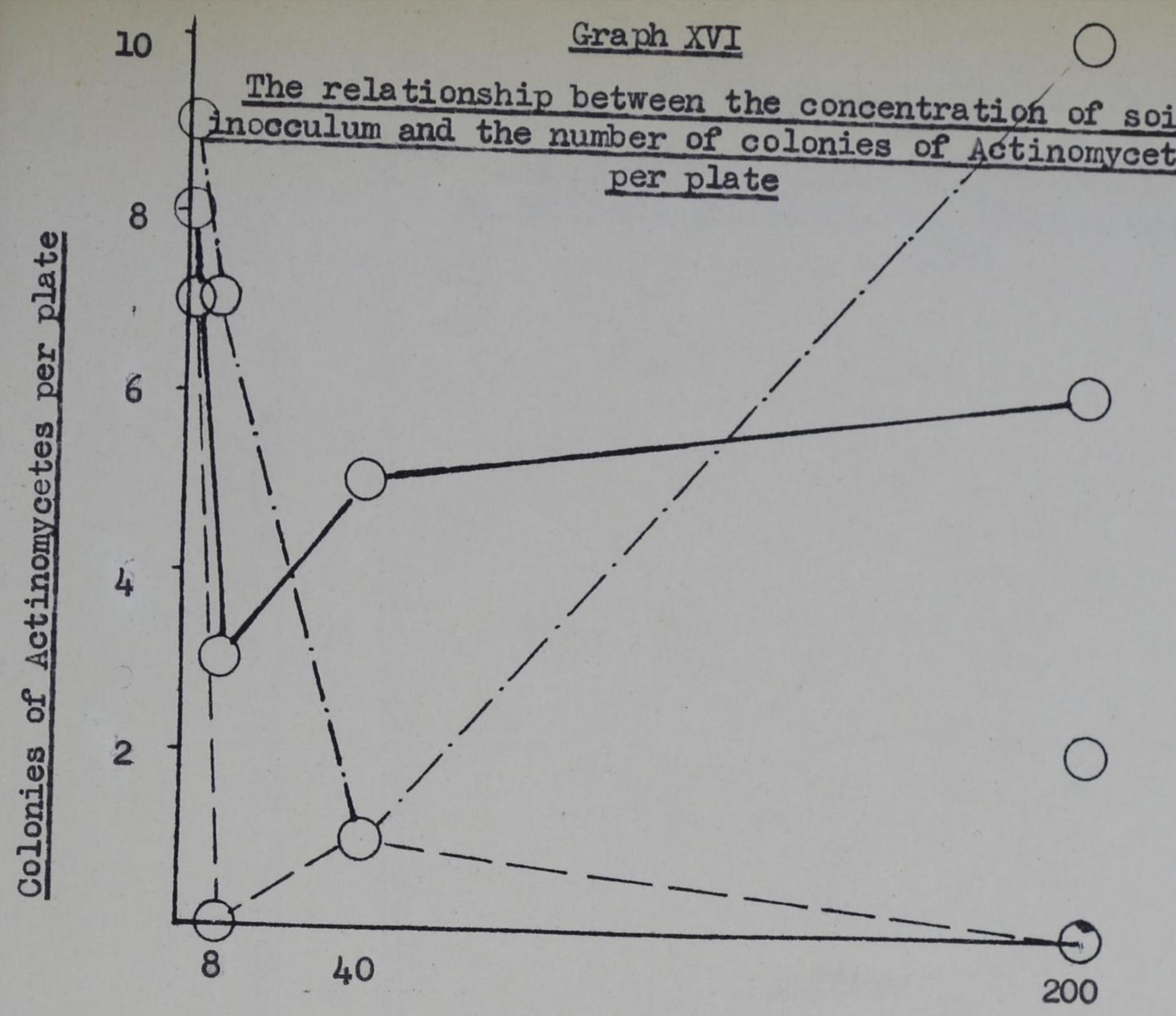
Graph XV

The relation between mean plate count and the estimates of the soil microflora, (from data of Table XX).



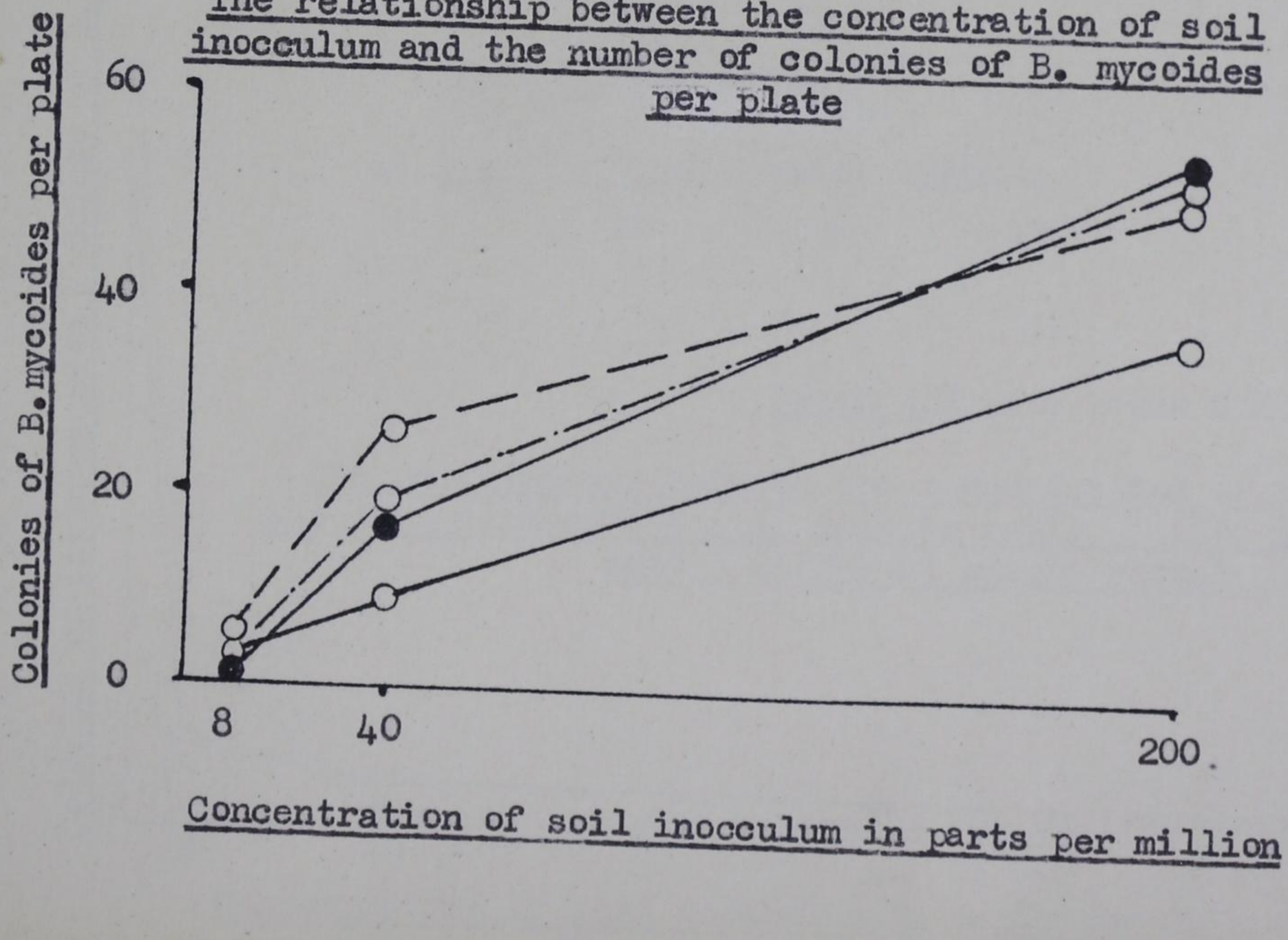
Graph XVI

The relationship between the concentration of soil inocculum and the number of colonies of Actinomycetes per plate



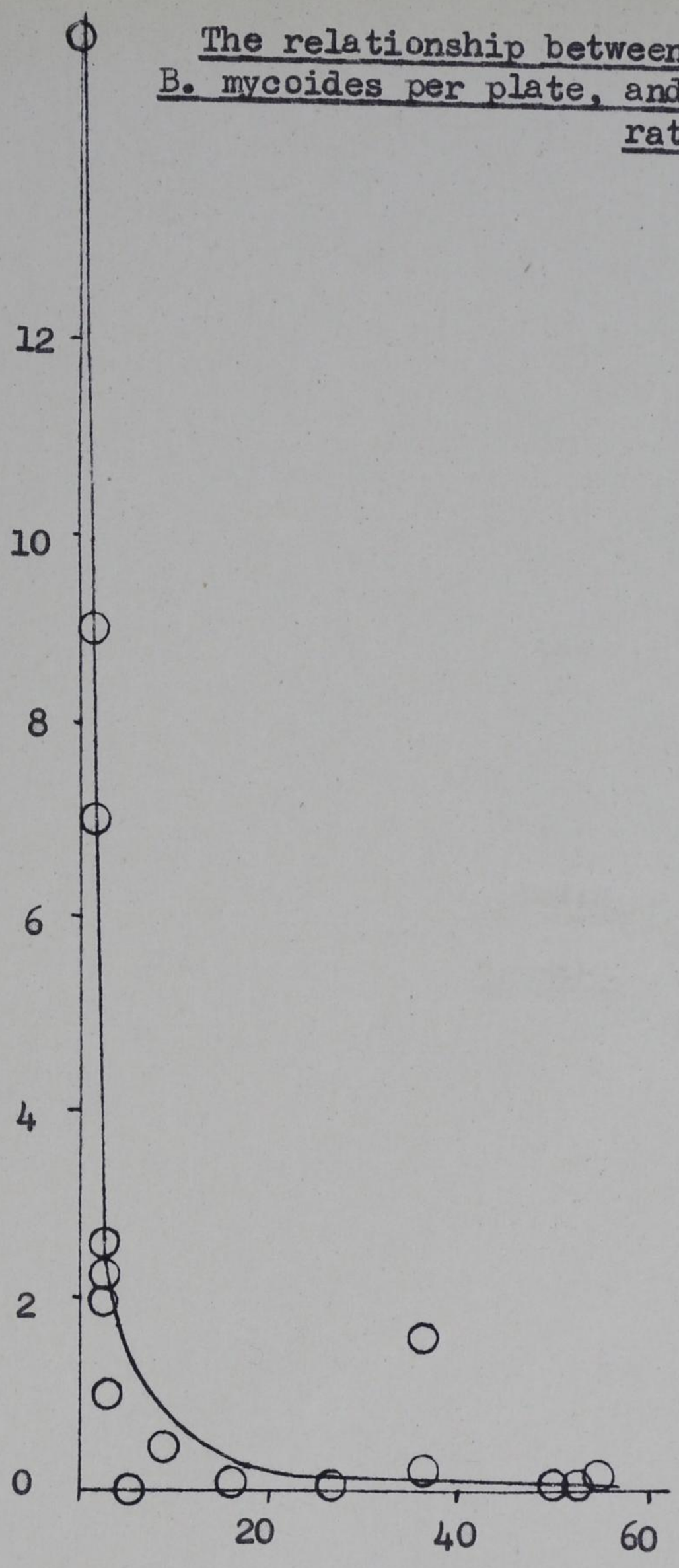
Graph XVII

The relationship between the concentration of soil inocculum and the number of colonies of B. mycoides per plate



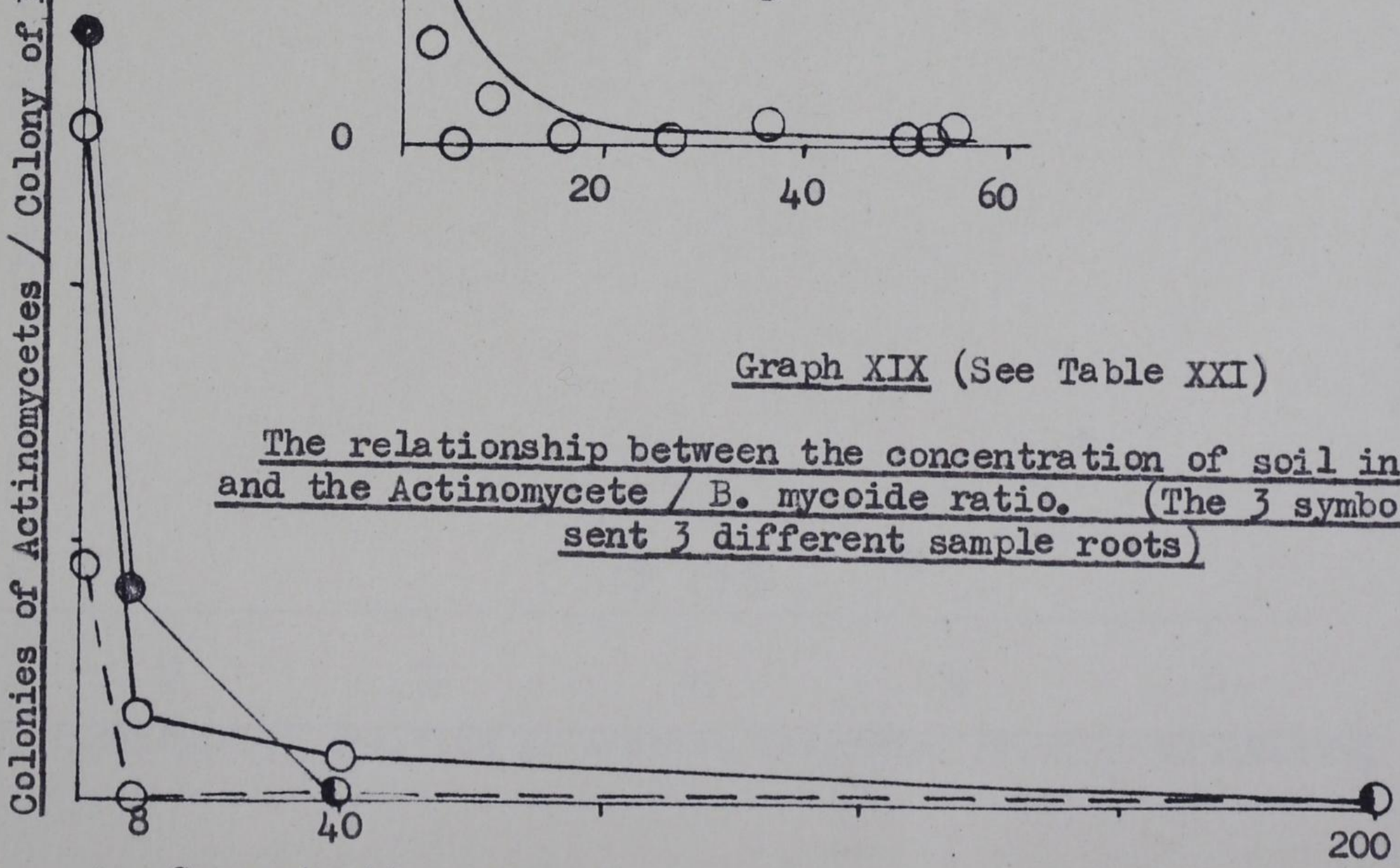
Graph XVIII (See Table XXI)

The relationship between the number of colonies of B. mycoides per plate, and the Actinomycete / B.mycoide ratio



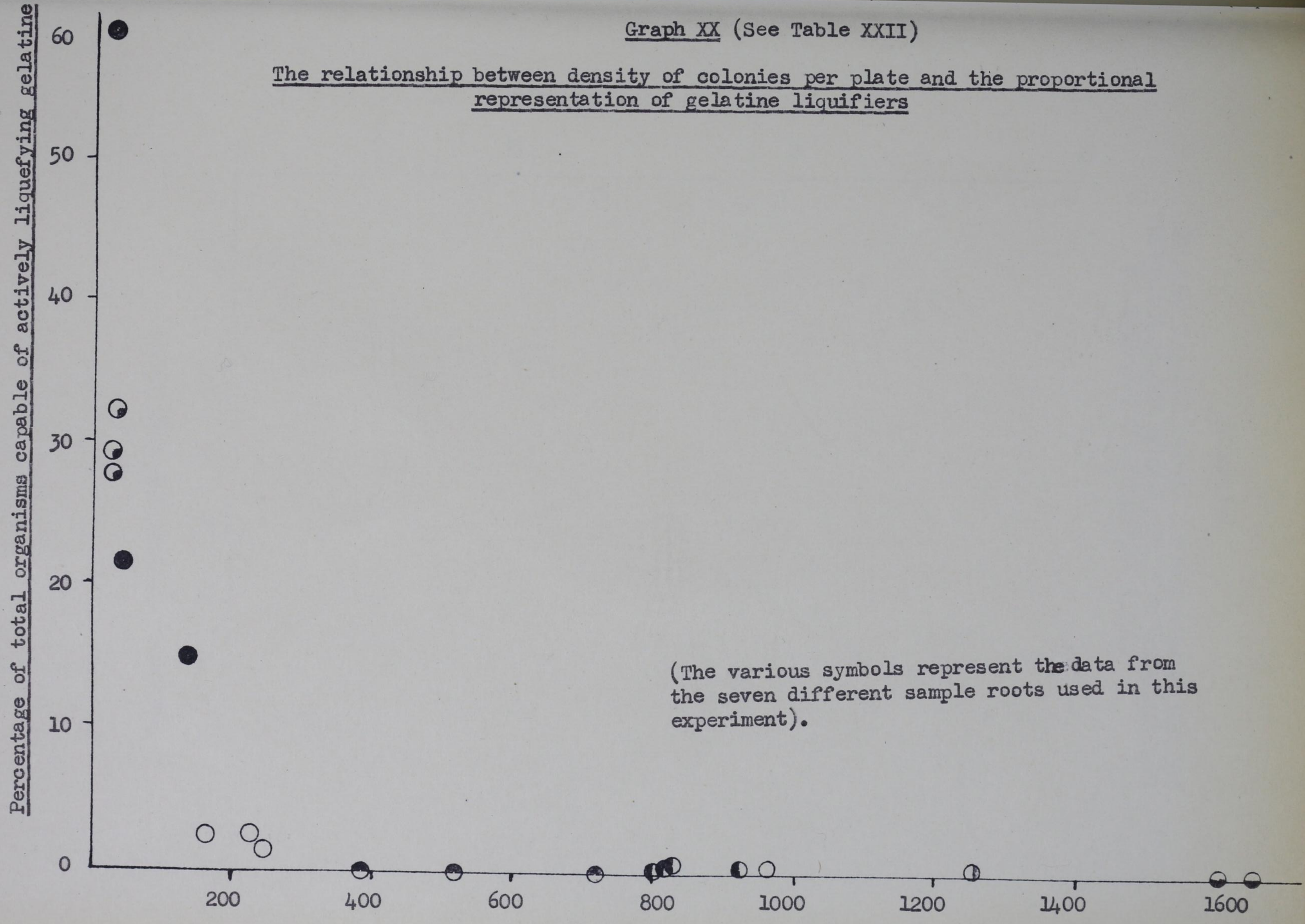
Graph XIX (See Table XXI)

The relationship between the concentration of soil inocculum and the Actinomycete / B. mycoide ratio. (The 3 symbols represent 3 different sample roots)



Graph XX (See Table XXII)

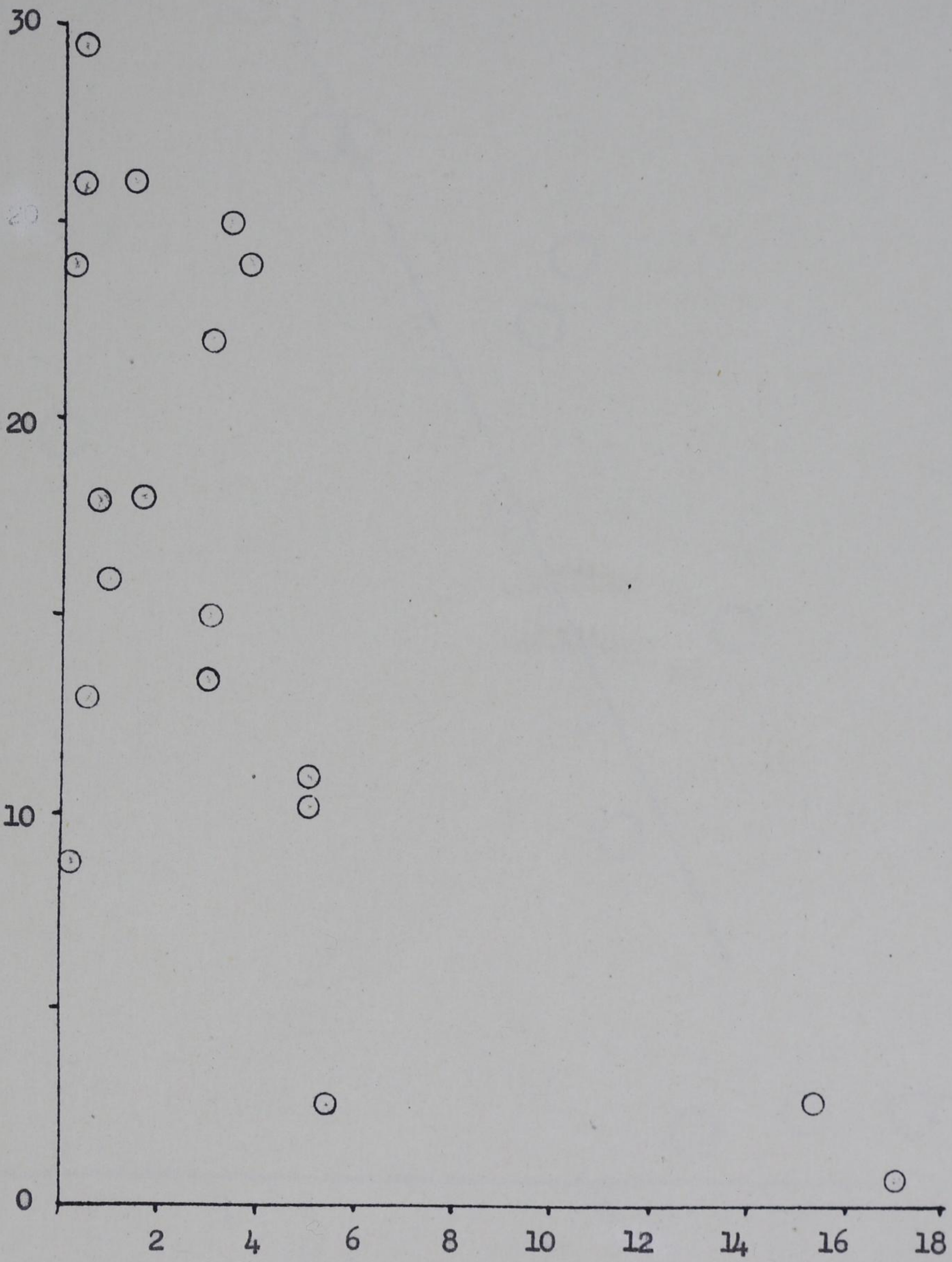
The relationship between density of colonies per plate and the proportional representation of gelatine liquifiers



(The various symbols represent the data from the seven different sample roots used in this experiment).

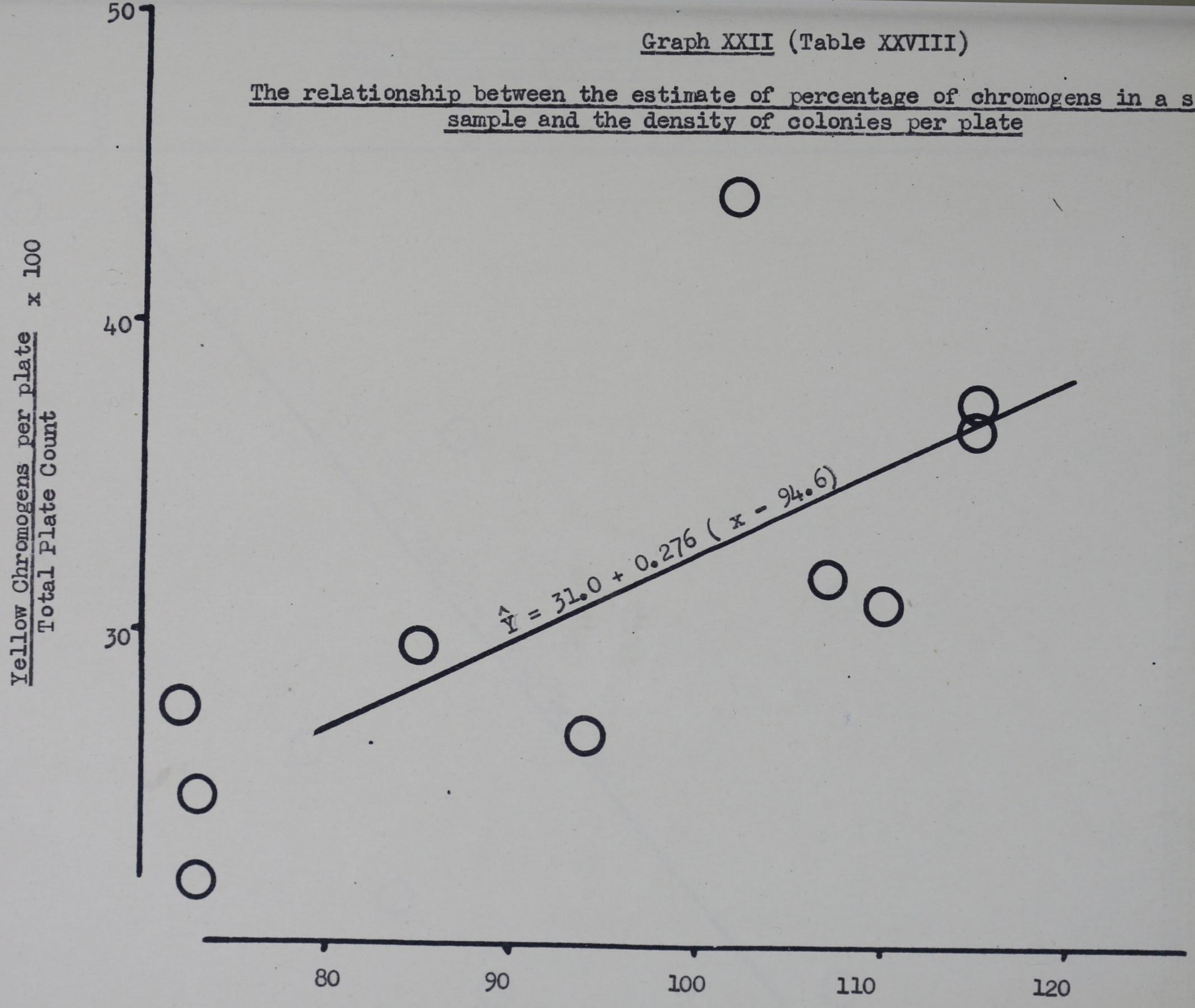
Graph XXI

The relationship between plating density and the estimation of the proportions of actinomycetes in the microbial population of a soil



Graph XXII (Table XXVIII)

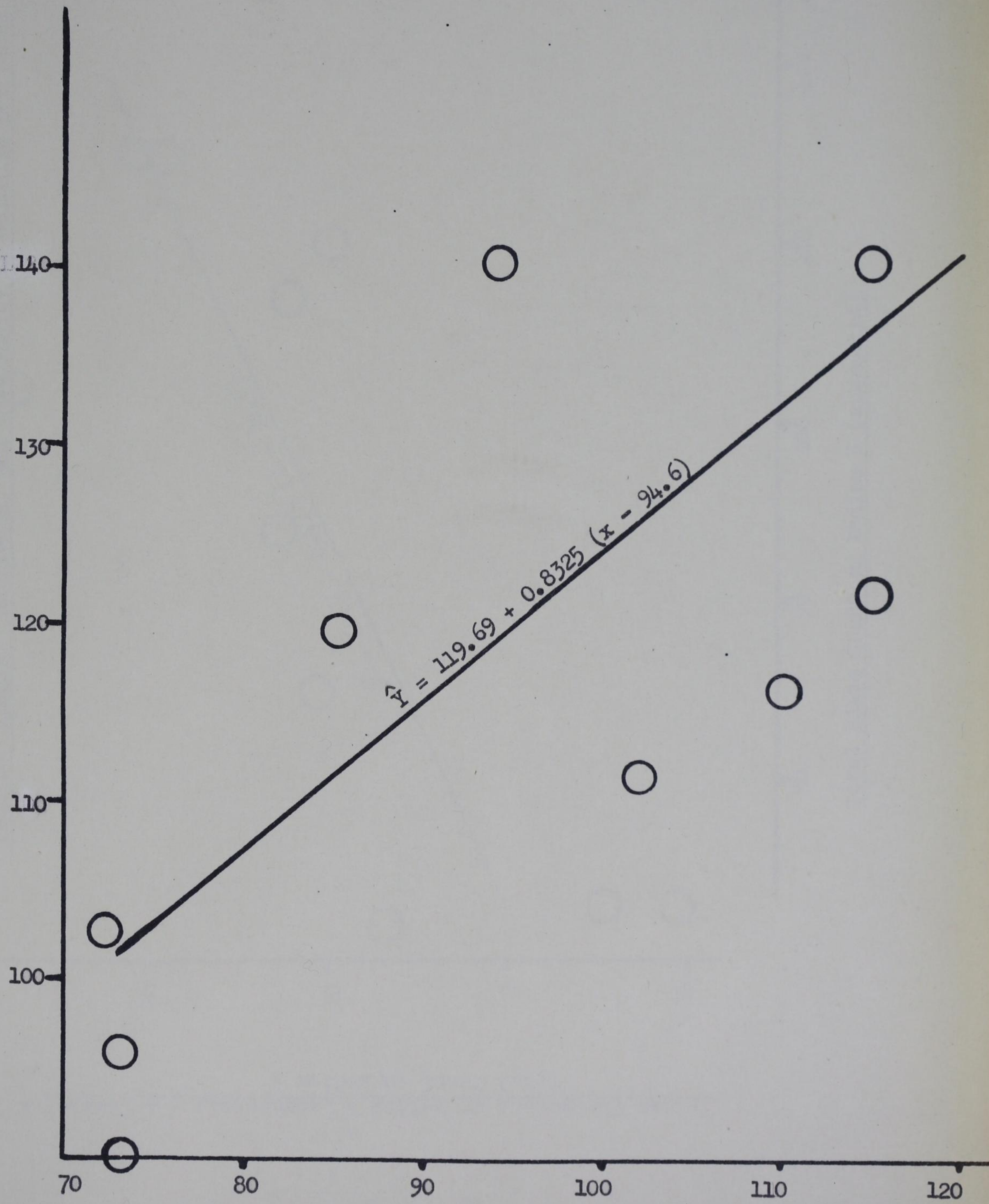
The relationship between the estimate of percentage of chromogens in a soil sample and the density of colonies per plate



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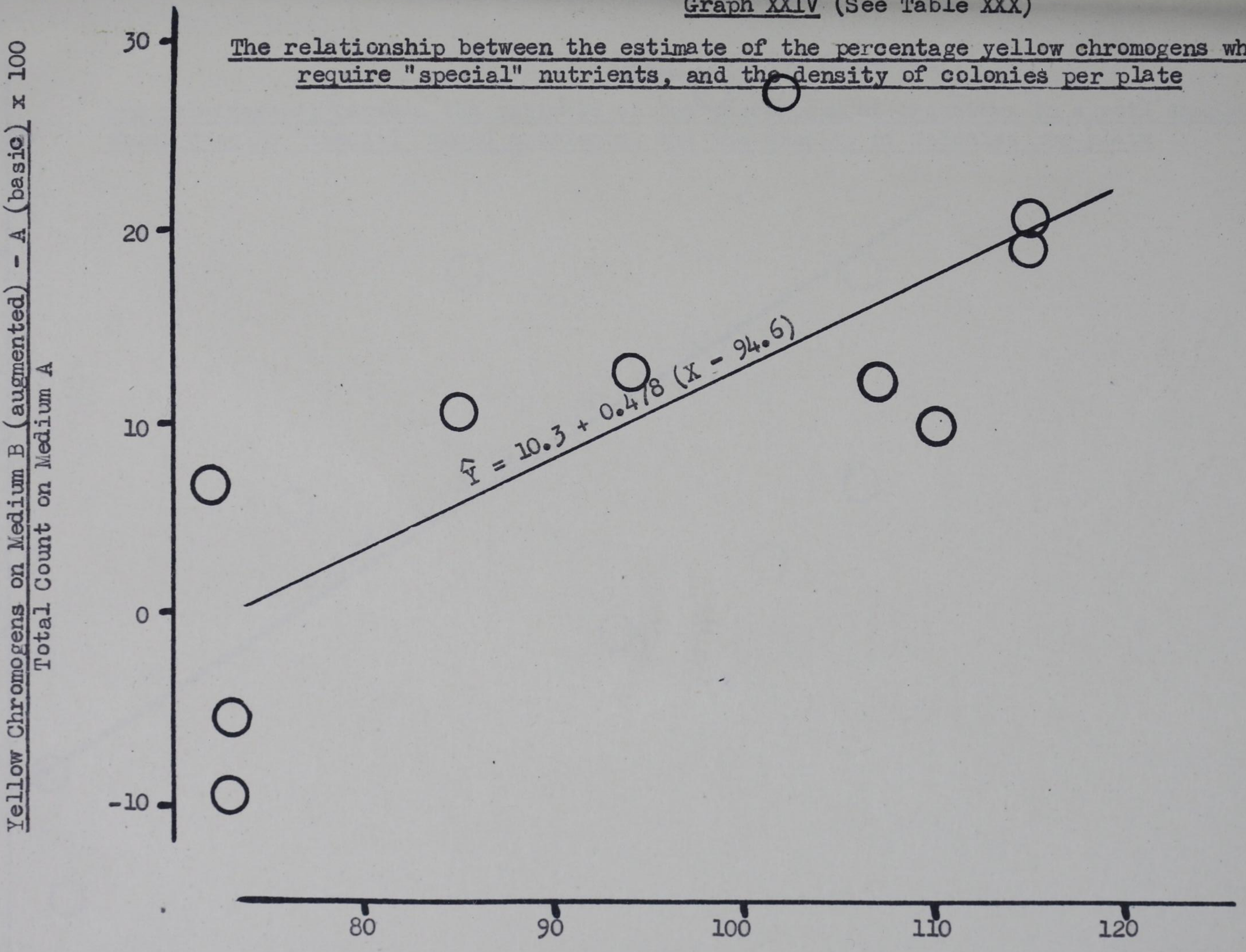
Graph XXIII (See Table XXIX)

The relationship between the ratio of the plate count from a soil sample on media A and B and the density of colonies per plate



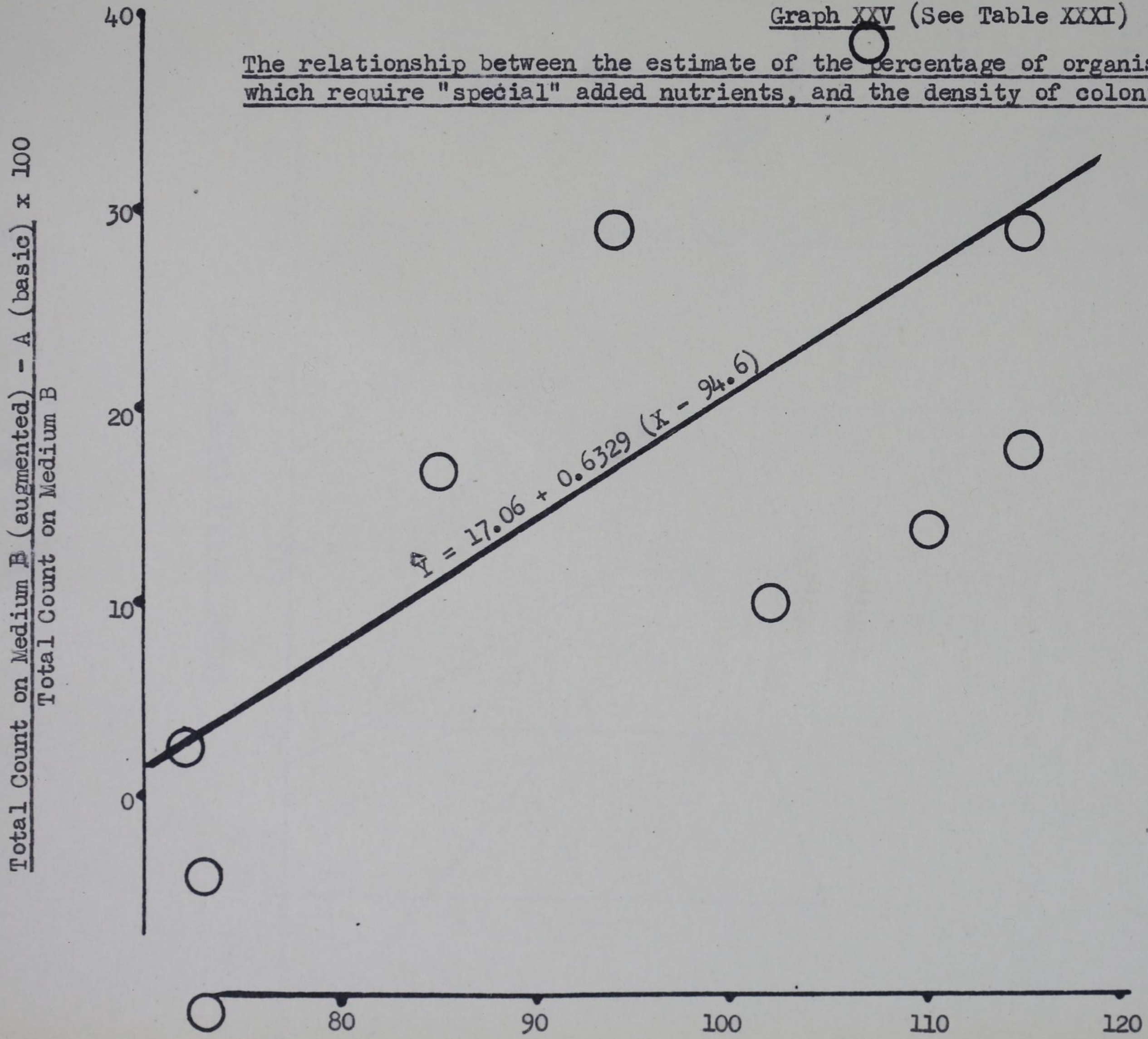
Graph XXIV (See Table XXX)

The relationship between the estimate of the percentage yellow chromogens which require "special" nutrients, and the density of colonies per plate



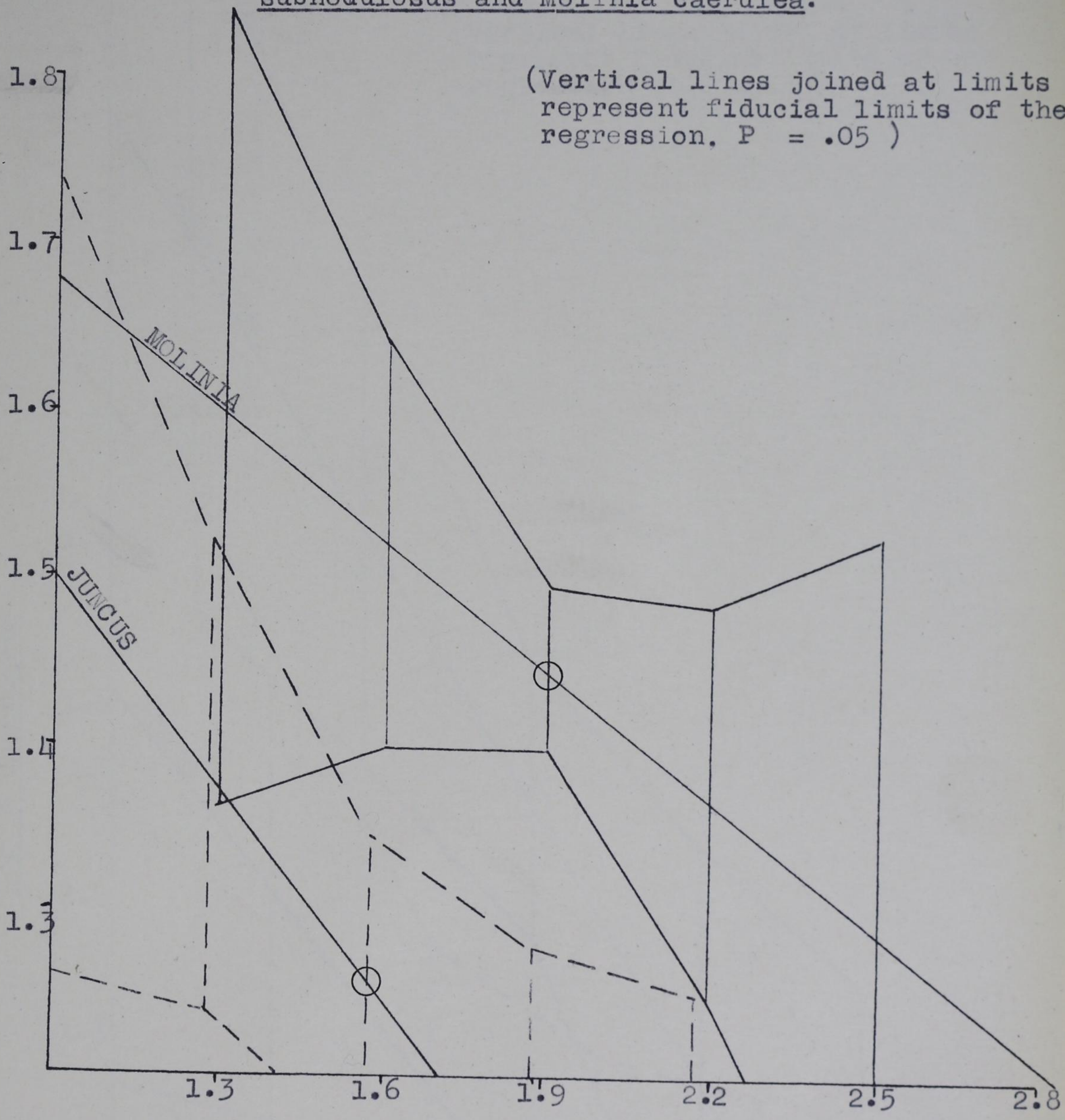
Graph XXV (See Table XXXI)

The relationship between the estimate of the percentage of organisms in a soil sample which require "special" added nutrients, and the density of colonies per plate



Graph XXVI. (See Tables XXXIV and XXXV)

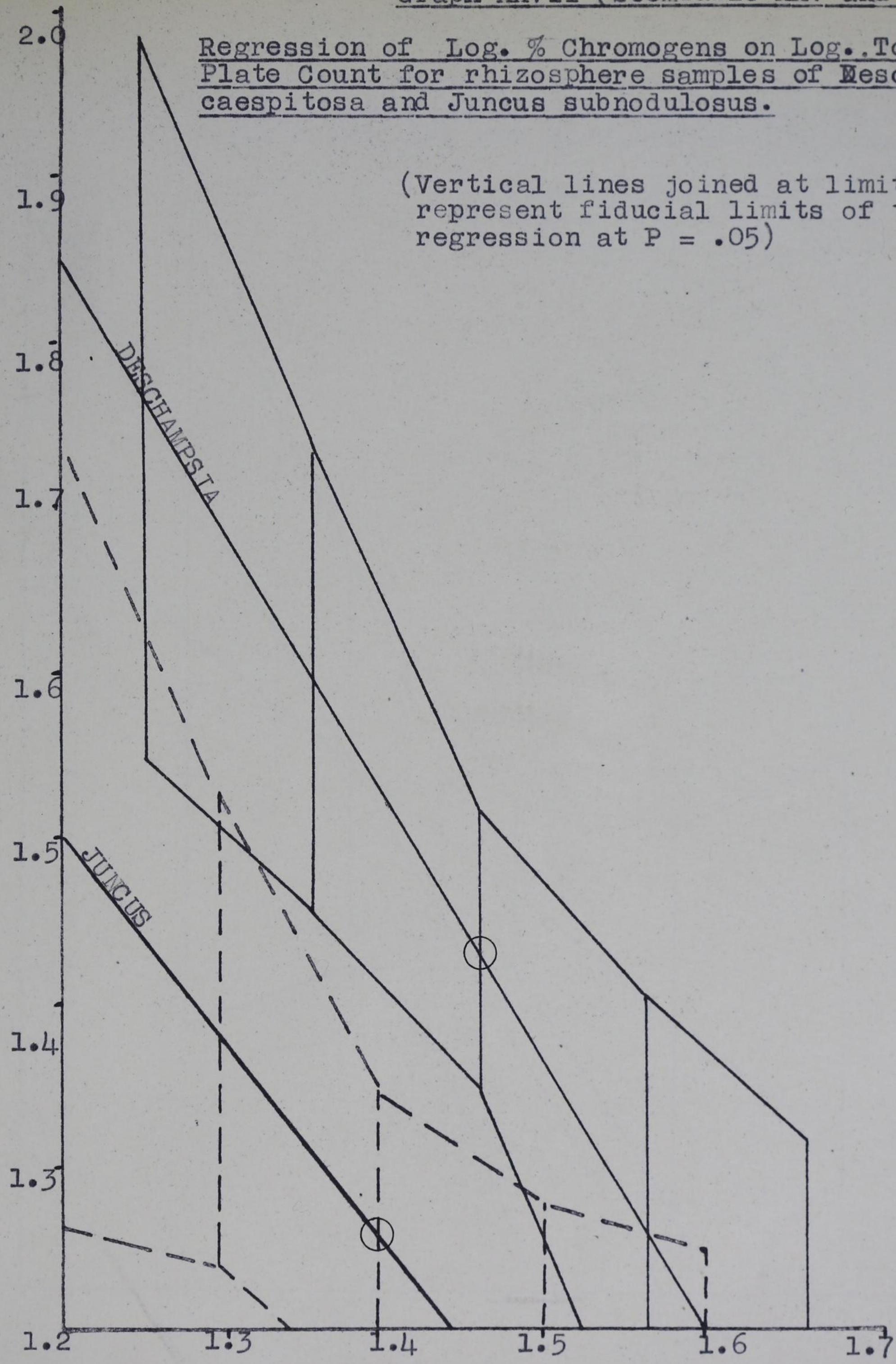
Regression of Log. % Chromogens on Log. Total Plate Count for rhizosphere samples of Juncus subnodulosus and Molinia caerulea.



Graph XXVII (see Table XXV and XXVI)

Regression of Log. % Chromogens on Log. Total Plate Count for rhizosphere samples of Deschampsia caespitosa and Juncus subnodulosus.

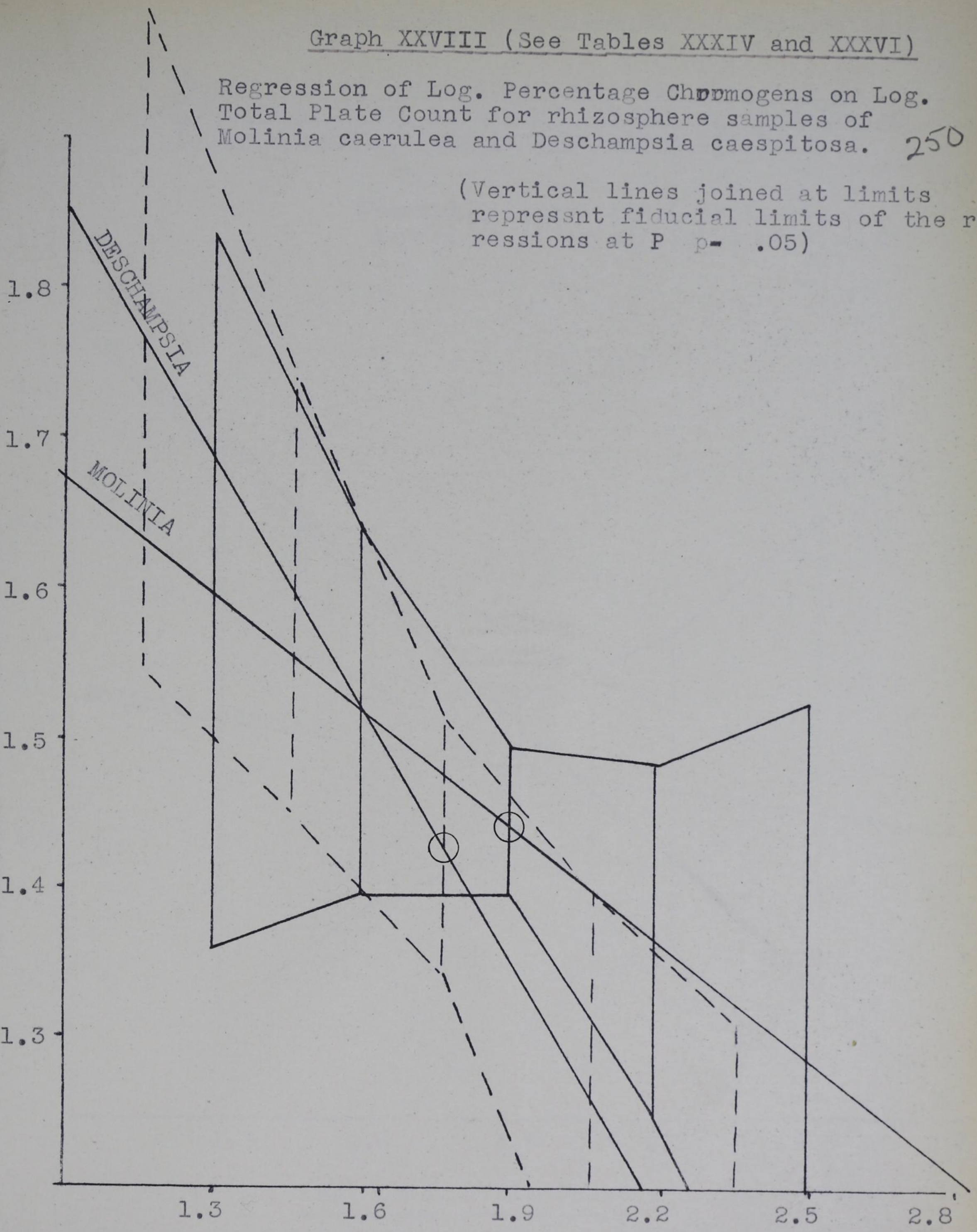
(Vertical lines joined at limits represent fiducial limits of the regression at P = .05)



Graph XXVIII (See Tables XXXIV and XXXVI)

Regression of Log. Percentage Chromogens on Log. Total Plate Count for rhizosphere samples of Molinia caerulea and Deschampsia caespitosa. 250

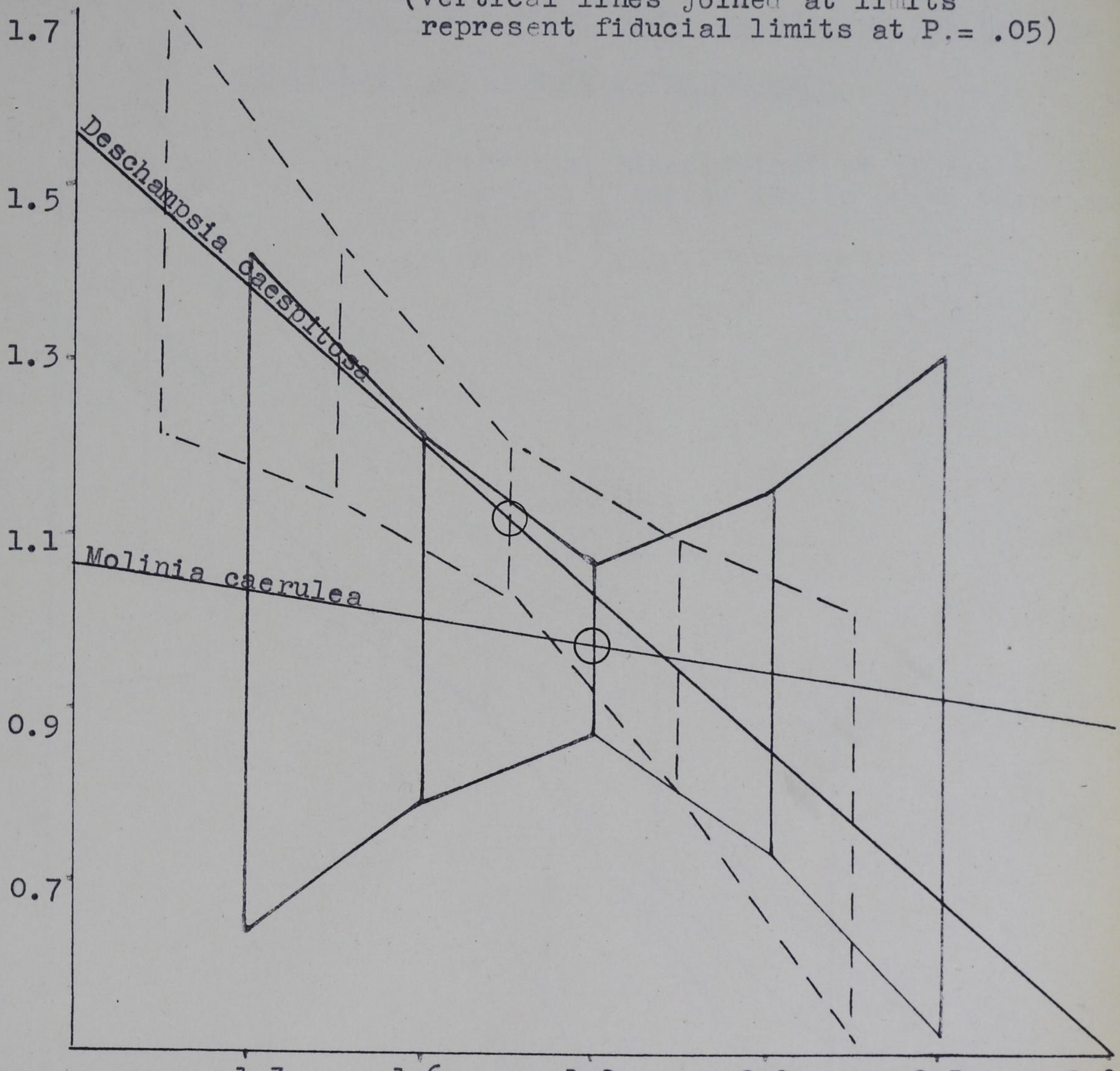
(Vertical lines joined at limits repressnt fiducial limits of the regressions at P p= .05)



Graph XXIX. (See Table XXXVII and XXXVIII)

Regression of Log. percentage actinomycetes on Log. Total Plate Count for rhizosphere samples of Molinia caerulea and Deschampsia caespitosa.

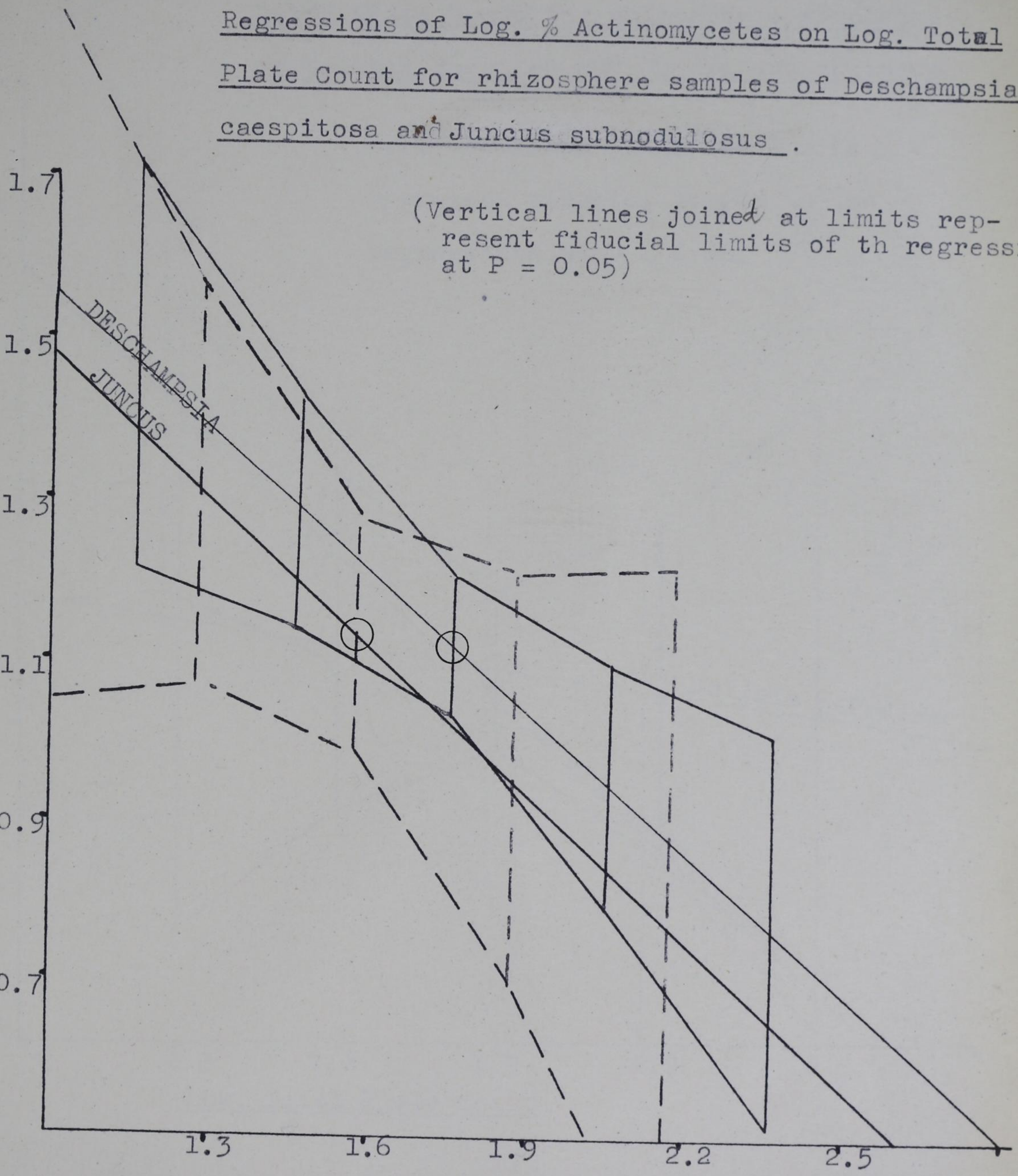
(Vertical lines joined at limits represent fiducial limits at P.= .05)



Graph XXX

Regressions of Log. % Actinomycetes on Log. Total Plate Count for rhizosphere samples of Deschampsia caespitosa and Juncus subnodulosus .

(Vertical lines joined at limits represent fiducial limits of th regressions at P = 0.05)

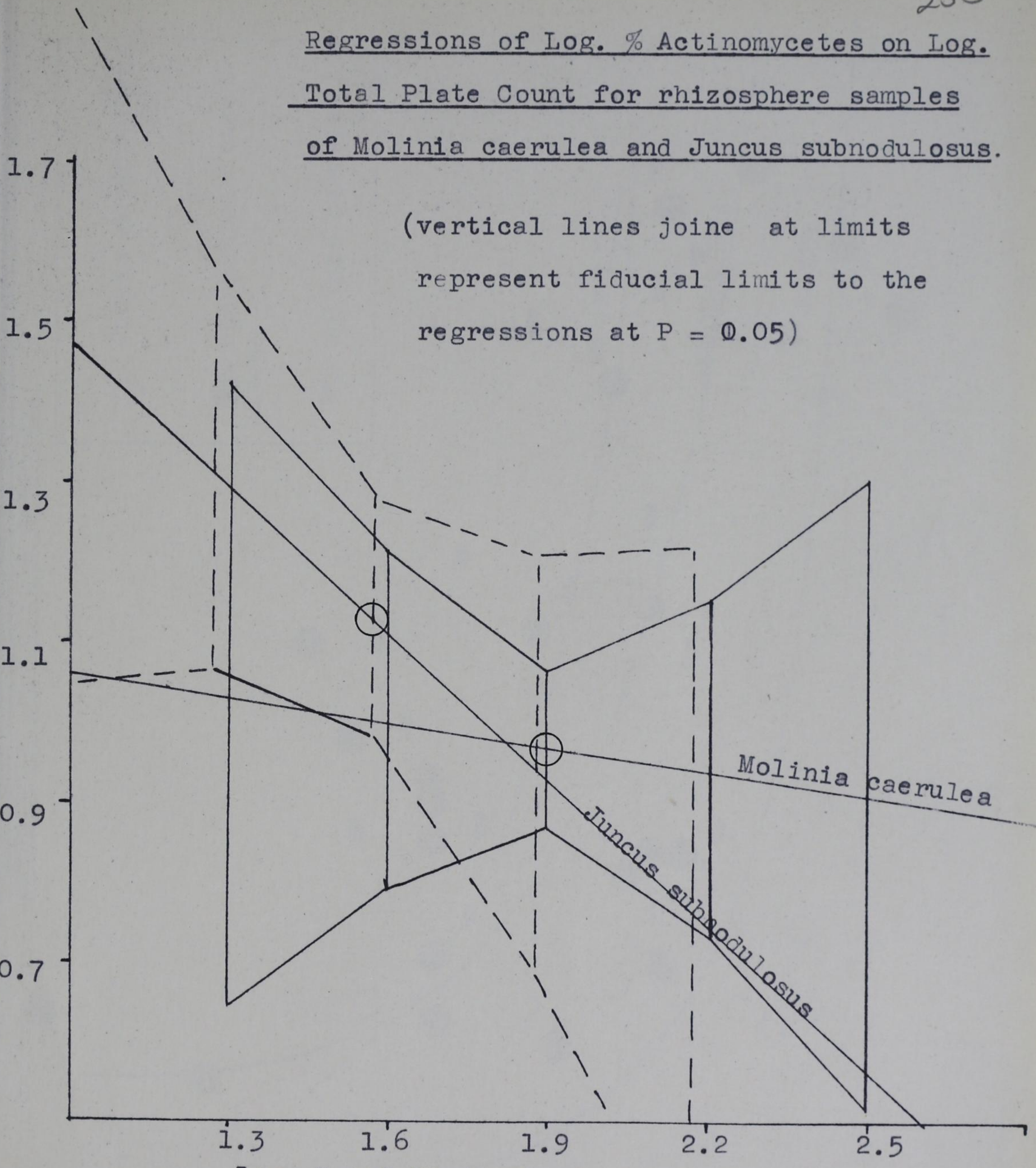


Log. Total Plate Count

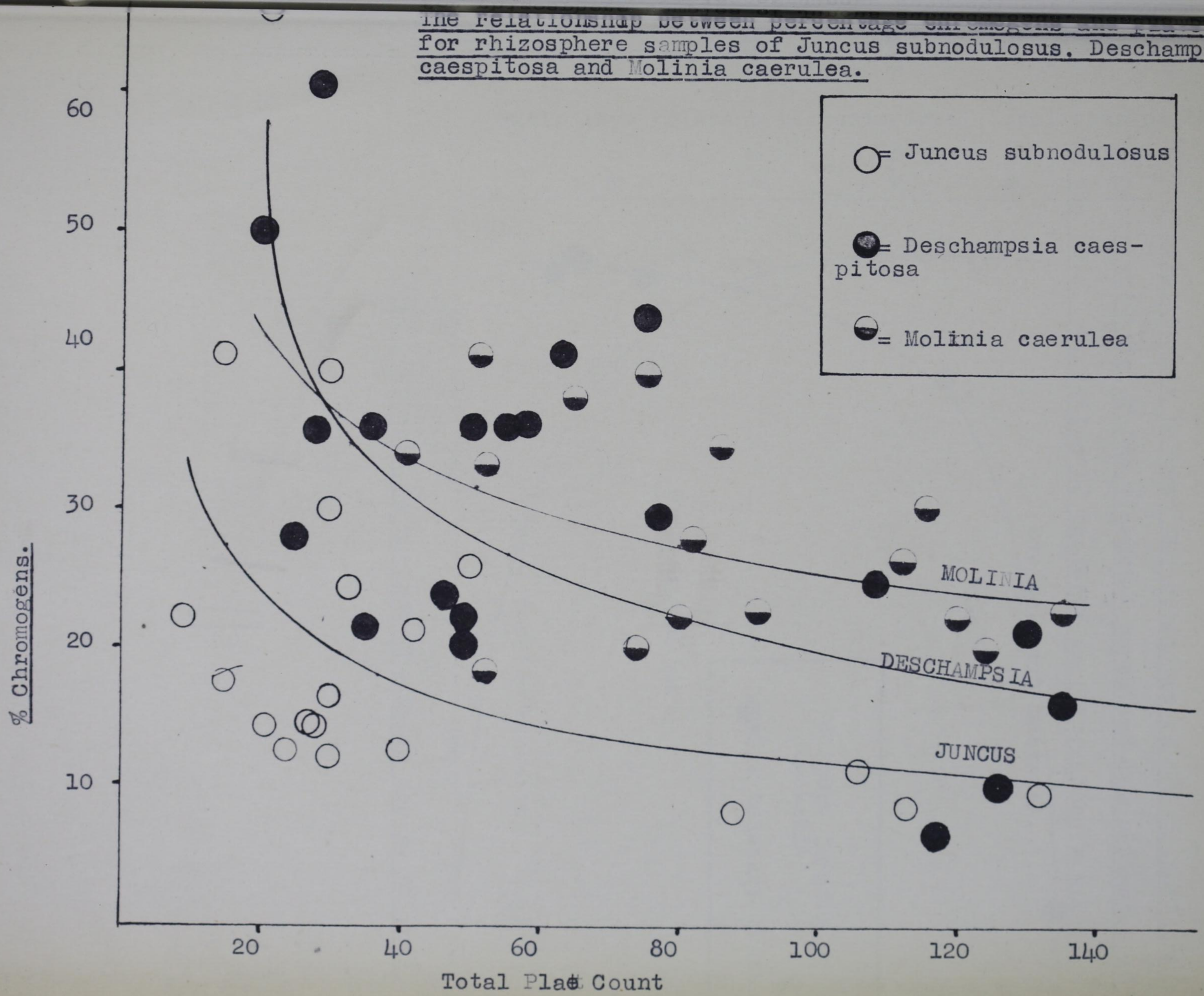
Graph XXXI. (See Tables XXXVII and XXXIX)

Regressions of Log. % Actinomycetes on Log. Total Plate Count for rhizosphere samples of Molinia caerulea and Juncus subnodulosus.

(vertical lines joine at limits represent fiducial limits to the regressions at P = 0.05)



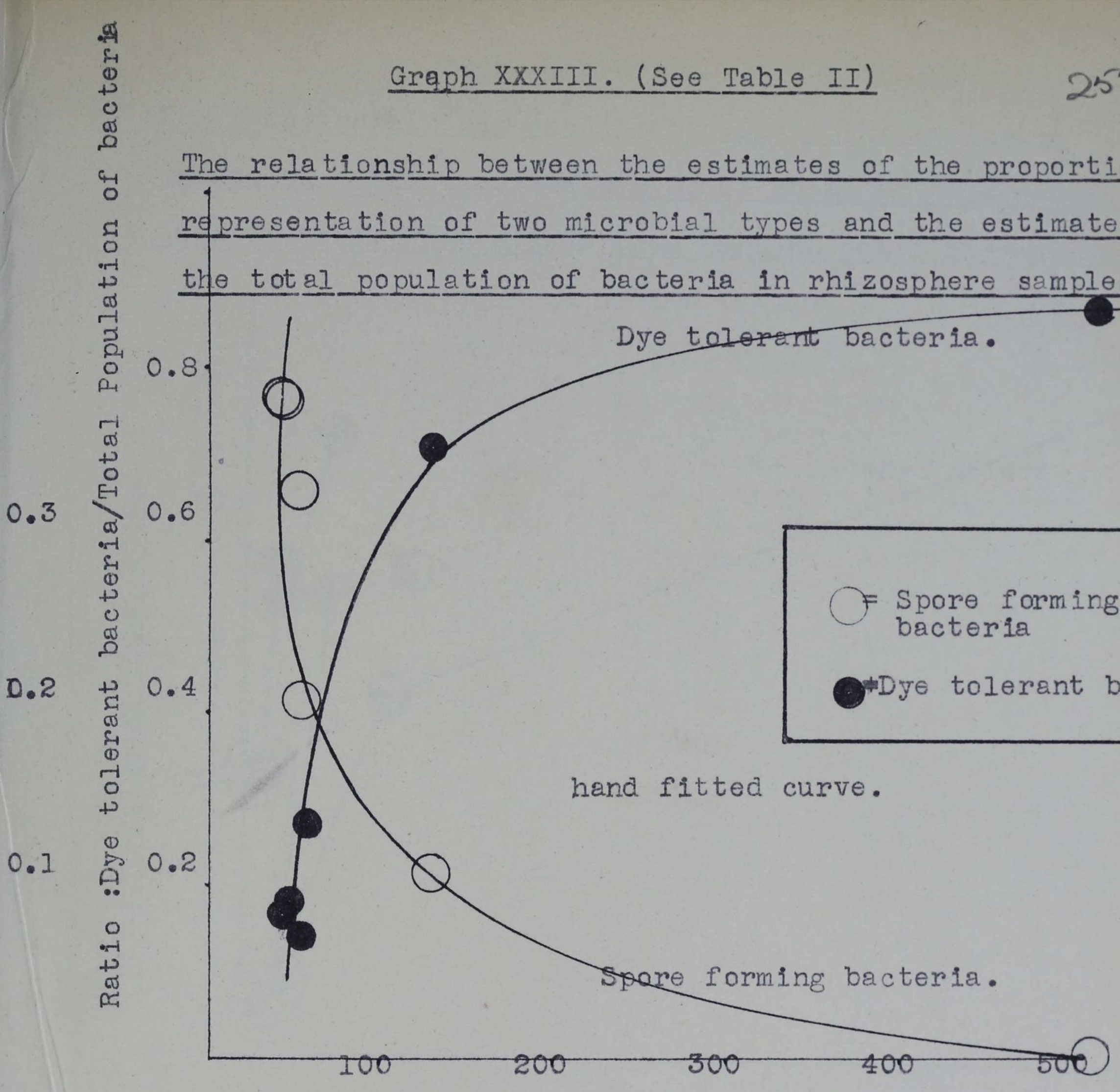
The relationship between percentage chromogens and plate count for rhizosphere samples of Juncus subnodulosus. Deschampsia caespitosa and Molinia caerulea.



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Graph XXXIII. (See Table II)

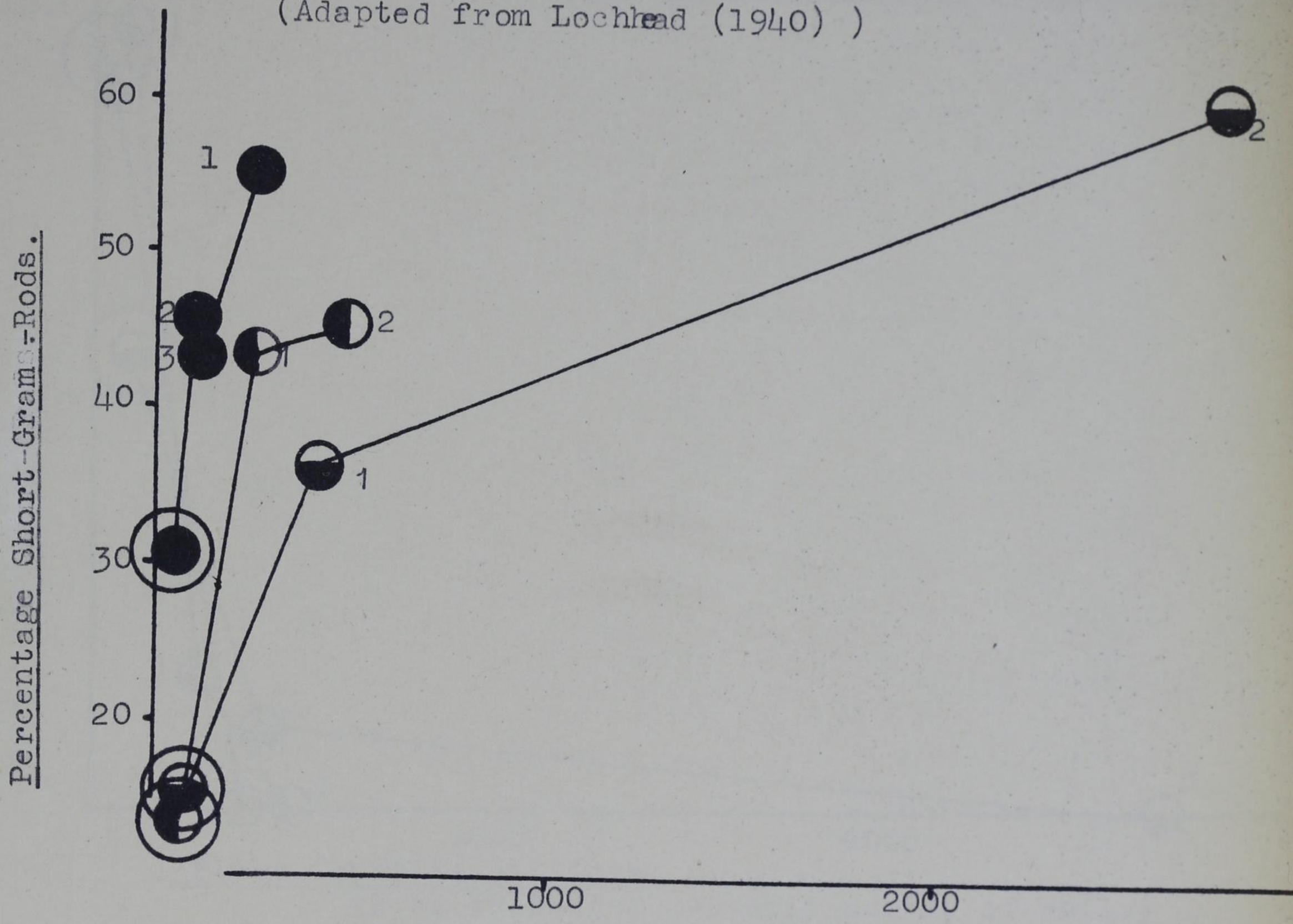
The relationship between the estimates of the proportional representation of two microbial types and the estimates of the total population of bacteria in rhizosphere samples.



Graph XXXIV

The relationship between 'plate count'(the estimated number of microorganisms per g. of soil) and the percentage of short Gram - rods from various soils and rhizospheres.

(Adapted from Lochhead (1940))



●=Rotation Plots
1.Clover, 2.Mangels
3.Oats.

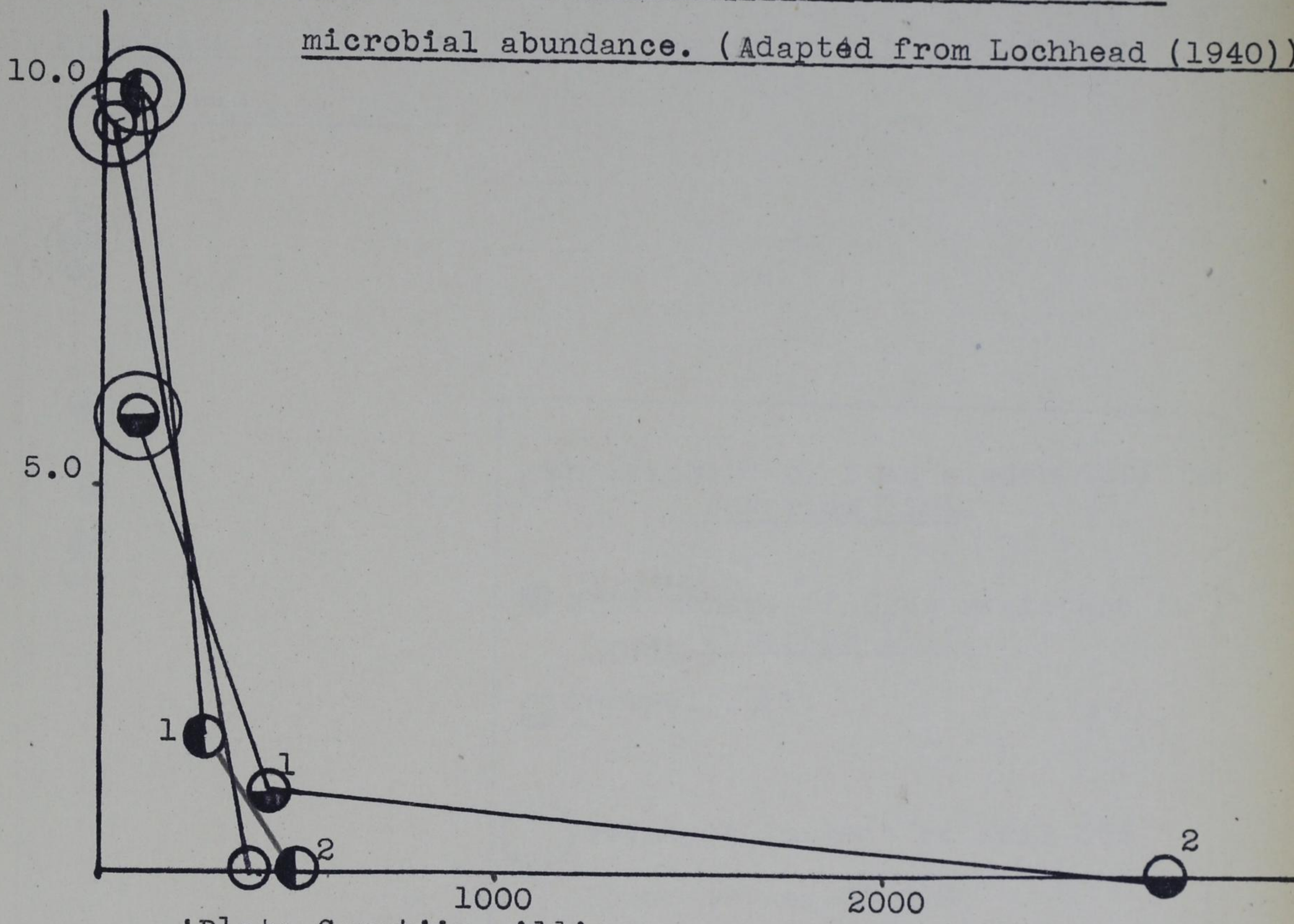
◐=Tobacco Soil
1.Resistant) to Thielaviopsis basicola
2.Susceptible)

◒=Flax Soil.
1Resistant) to Fusarium lini
2.Susceptible)

○=Control Soil.

Graph XXXV

The relationship between the estimates of % spore formers in a soil or rhizosphere sample and the estimate of total microbial abundance. (Adapted from Lochhead (1940))



○ = Corn

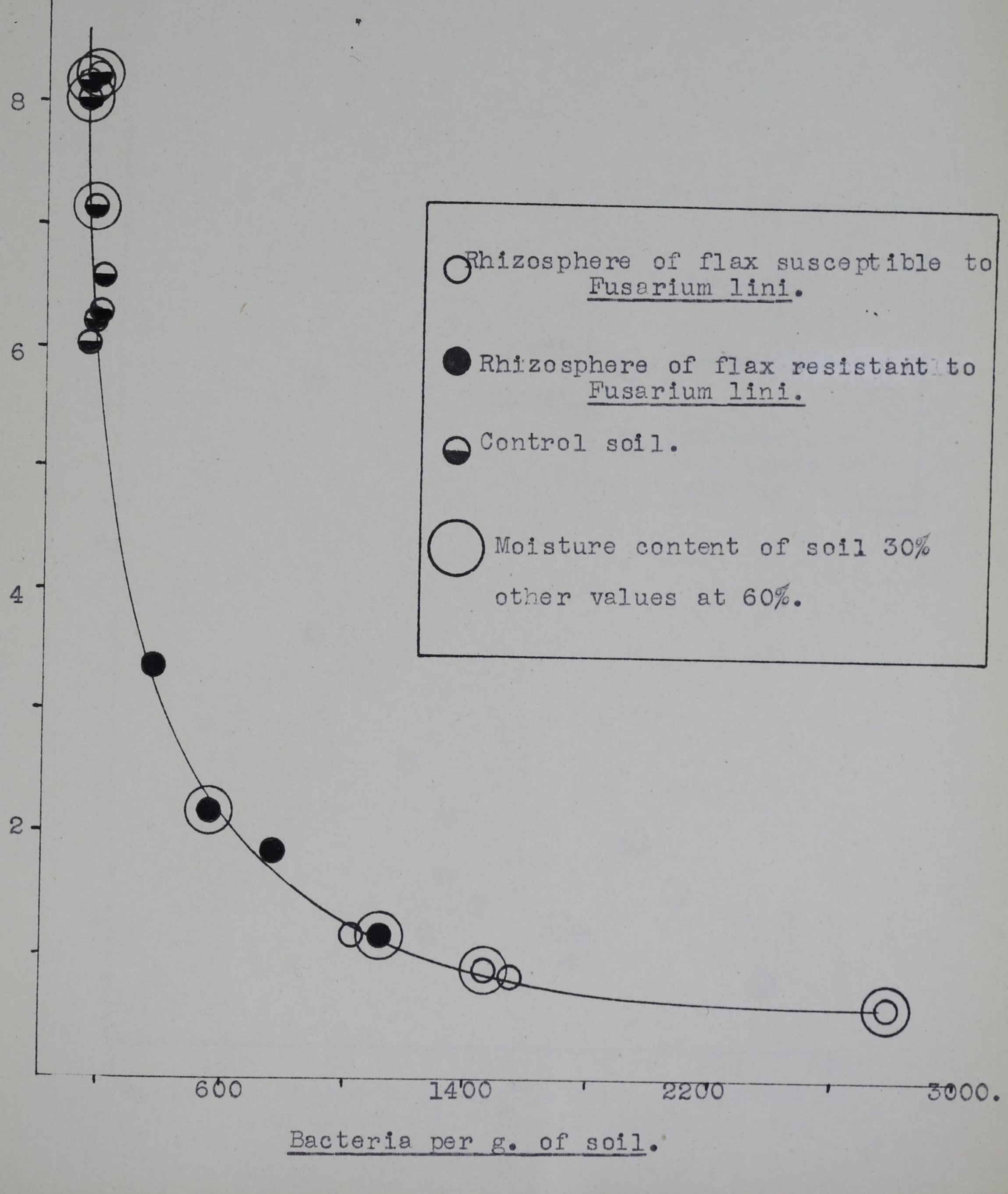
◐ = Tobacco
1. Resistant)
2. Susceptible) to Thielaviopsis basicola

◔ = Flax
1. Resistant)
2. Susceptible) to Fusarium lini

○ = Control Soil

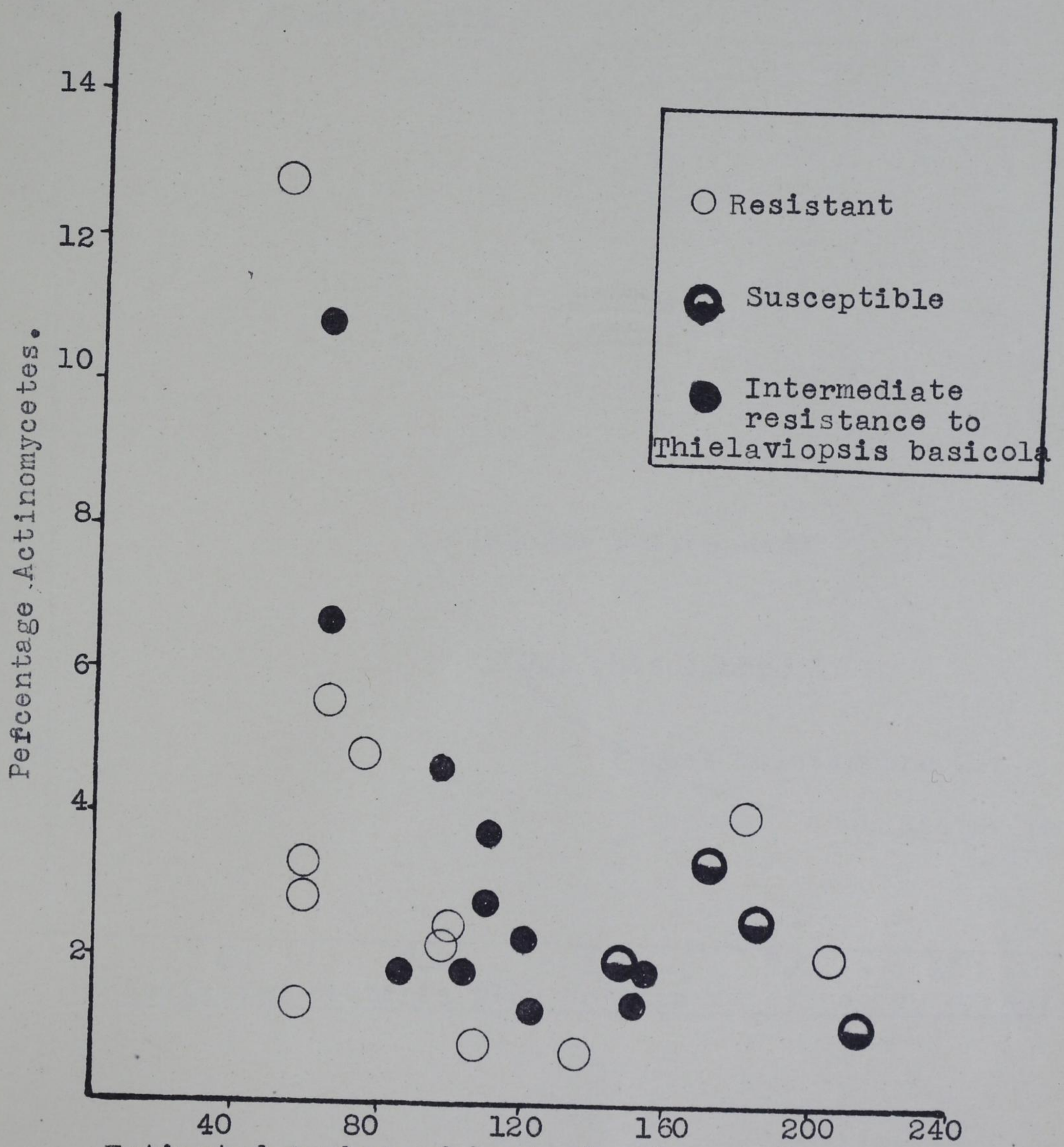
Graph ~~XXXV~~ I

.The relationship between estimates of the percentage of actinomycetes in rhizosphere and soil samples and the abundance of bacteria in the same samples.(Adapted from Timonin 1940)



Graph XXXVII.

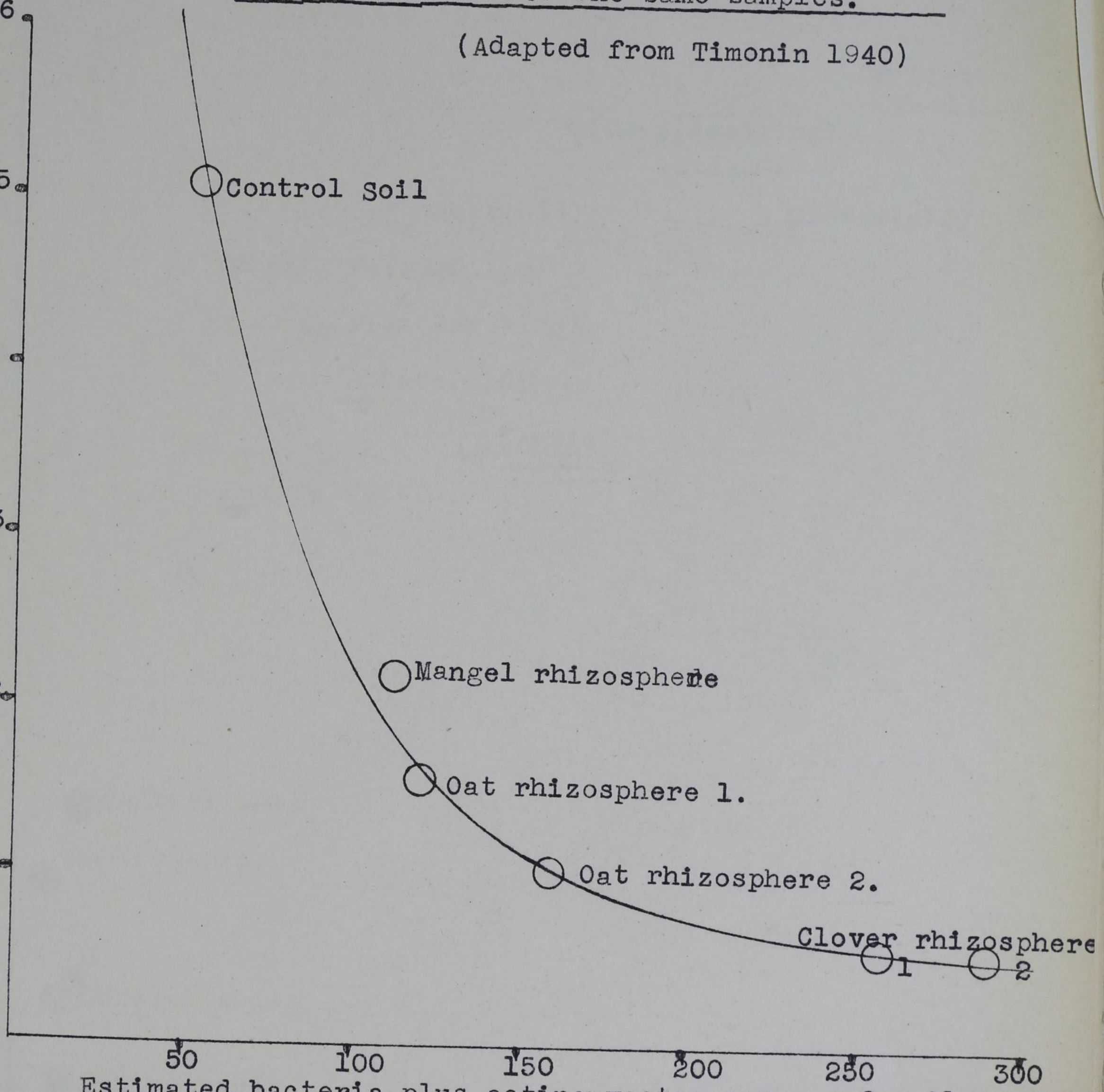
The relationship between percentage actinomycetes estimated for various soil and rhizosphere samples, and the total microbial flora for the same samples.(Adapted from Timonin 1940



Graph XXXVIII.

The relationship between percentage actinomycetes estimated for various soil and rhizosphere samples and the total microbial flora for the same samples.

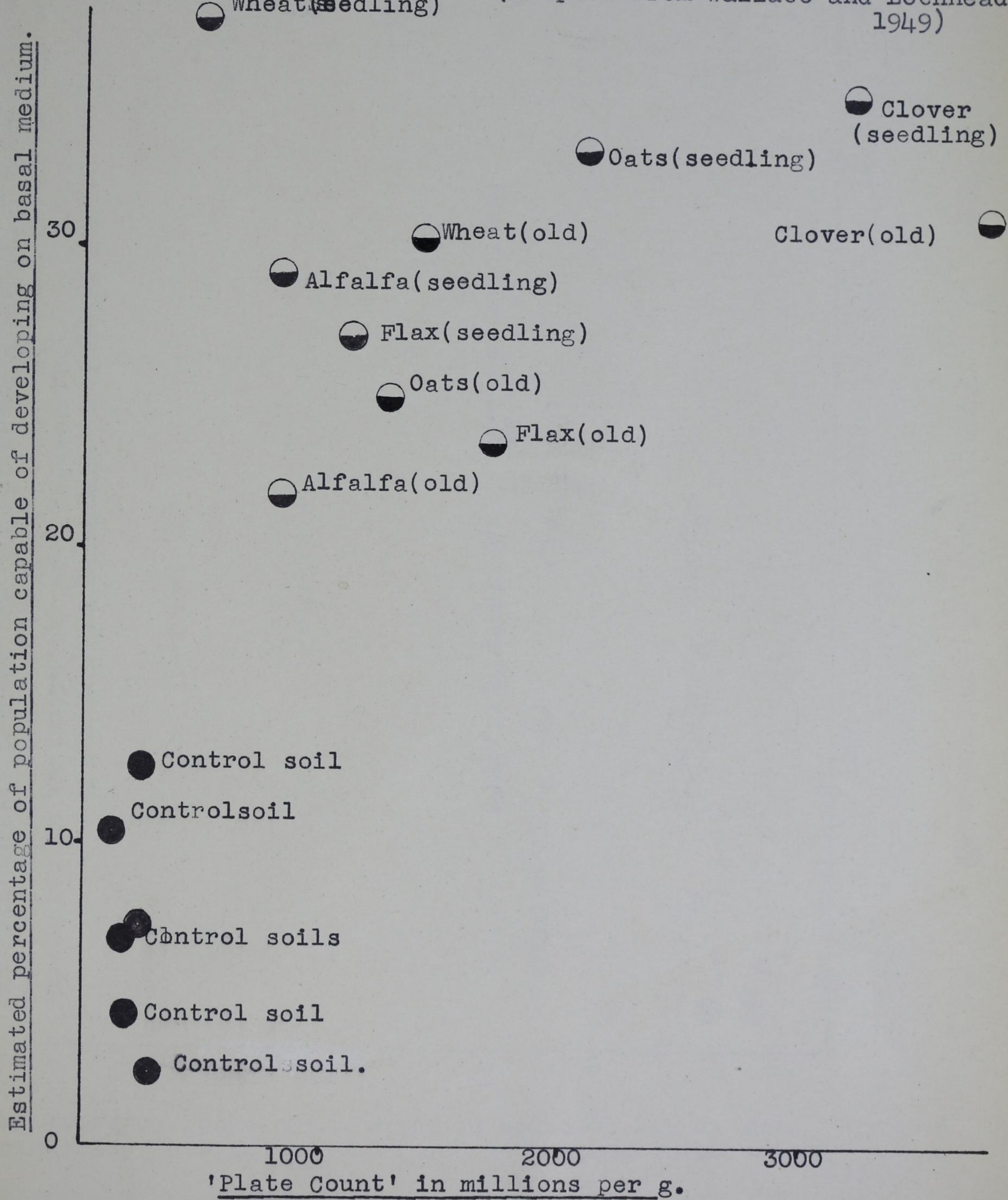
(Adapted from Timonin 1940)



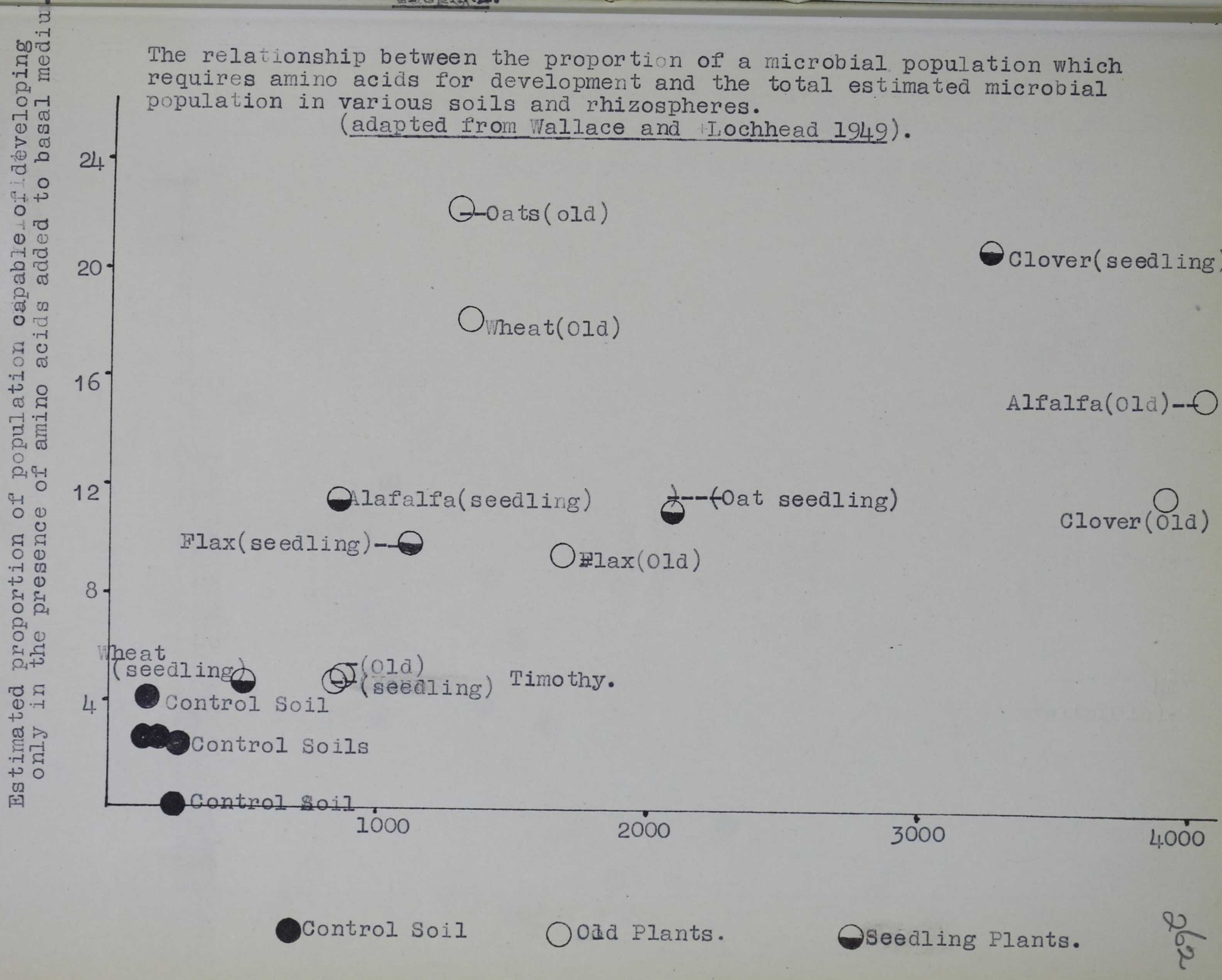
Graph XXXIX

The relationship between the proportion of a microbial population which is capable of growing on a glucose salts medium and the estimates of the total population of microbes in various soils and rhizospheres.

(Adapted from Wallace and Lochhead 1949)



The relationship between the proportion of a microbial population which requires amino acids for development and the total estimated microbial population in various soils and rhizospheres.
(adapted from Wallace and Lochhead 1949).

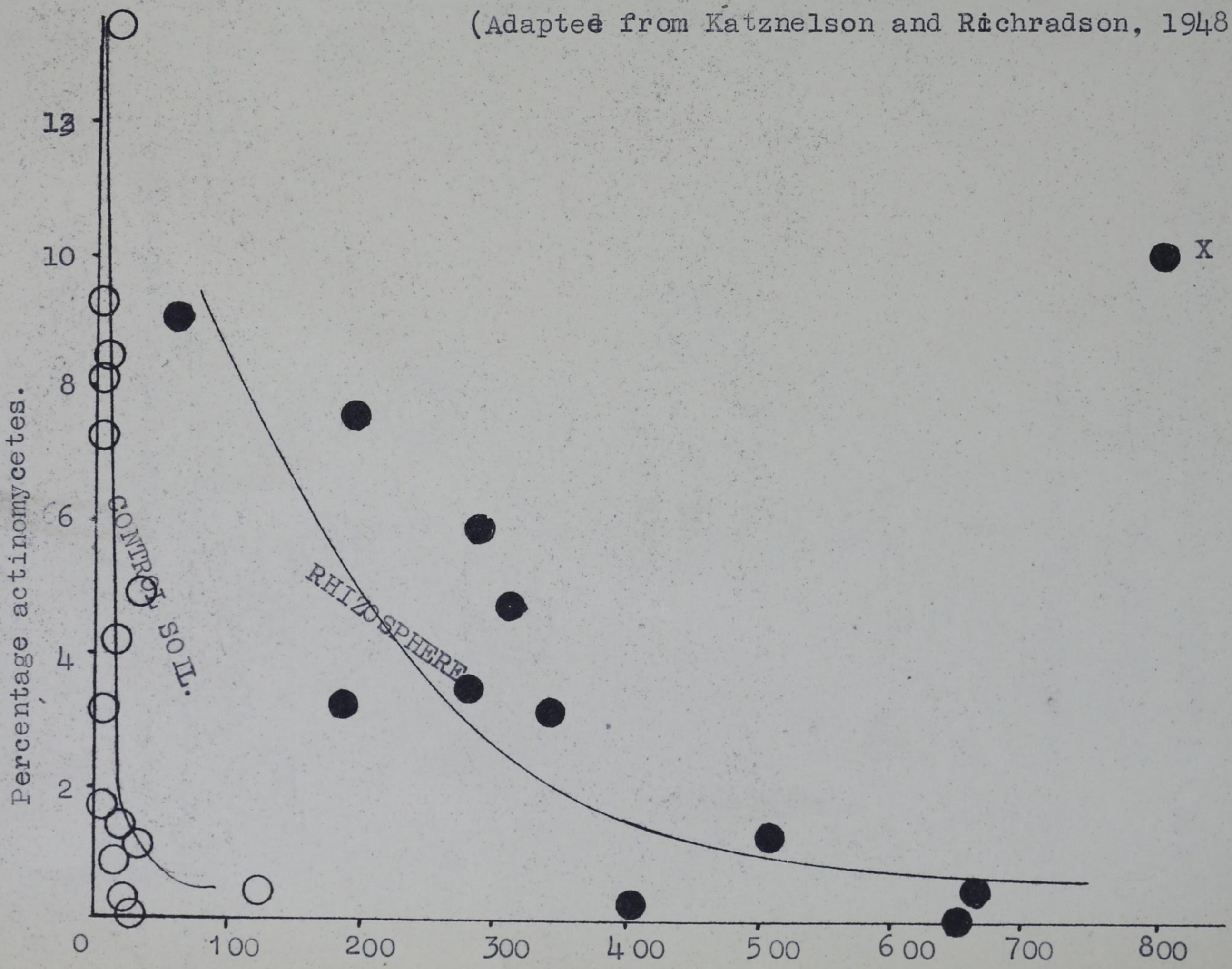


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Graph XLI.

The relationship between percentage actinomycetes estimated for a soil sample and the total estimates for the same rhizosphere or control soils.

(Adapted from Katznelson and Richradson, 1948).

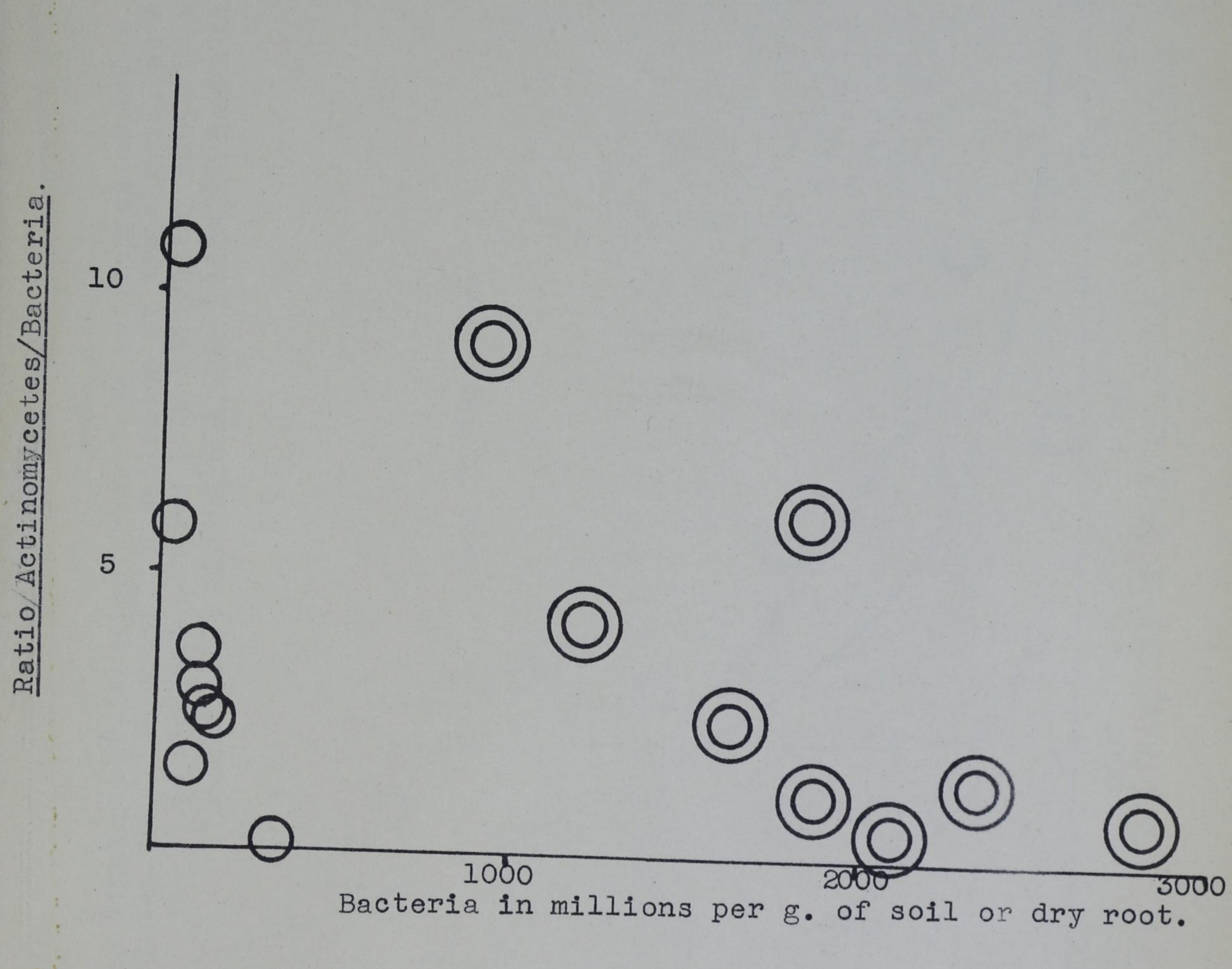


Bacteria plus actinomycetes in ten millions per g.

Graph XLII.

The relationship between the actinomycete/bacteria ratio obtained from soil and rhizosphere samples and the estimates of numbers of bacteria per g. of soil or root.

(Adapted from Katznelson and Richardson 1943)

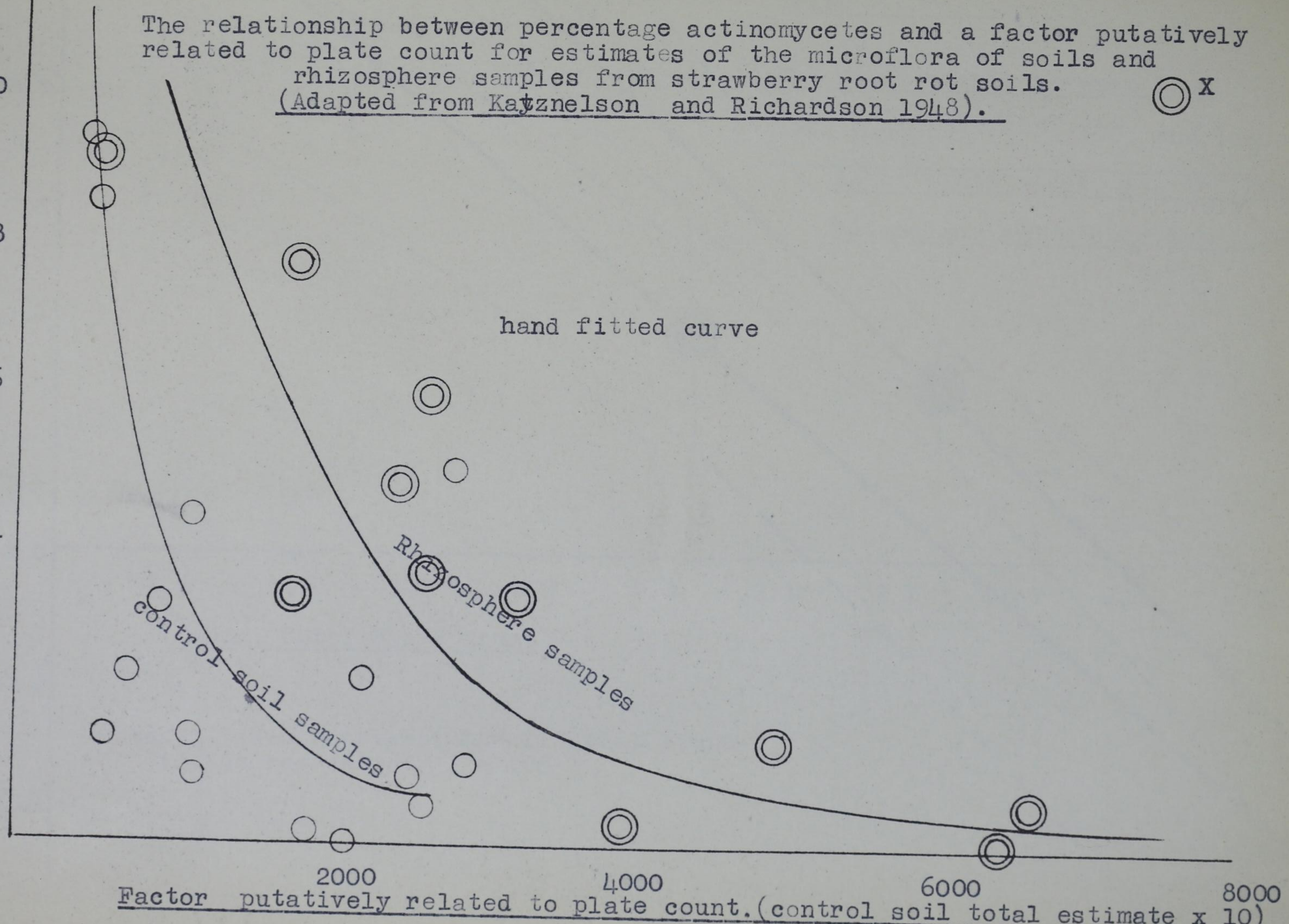


○ = Soil

◎ = Rhizosphere

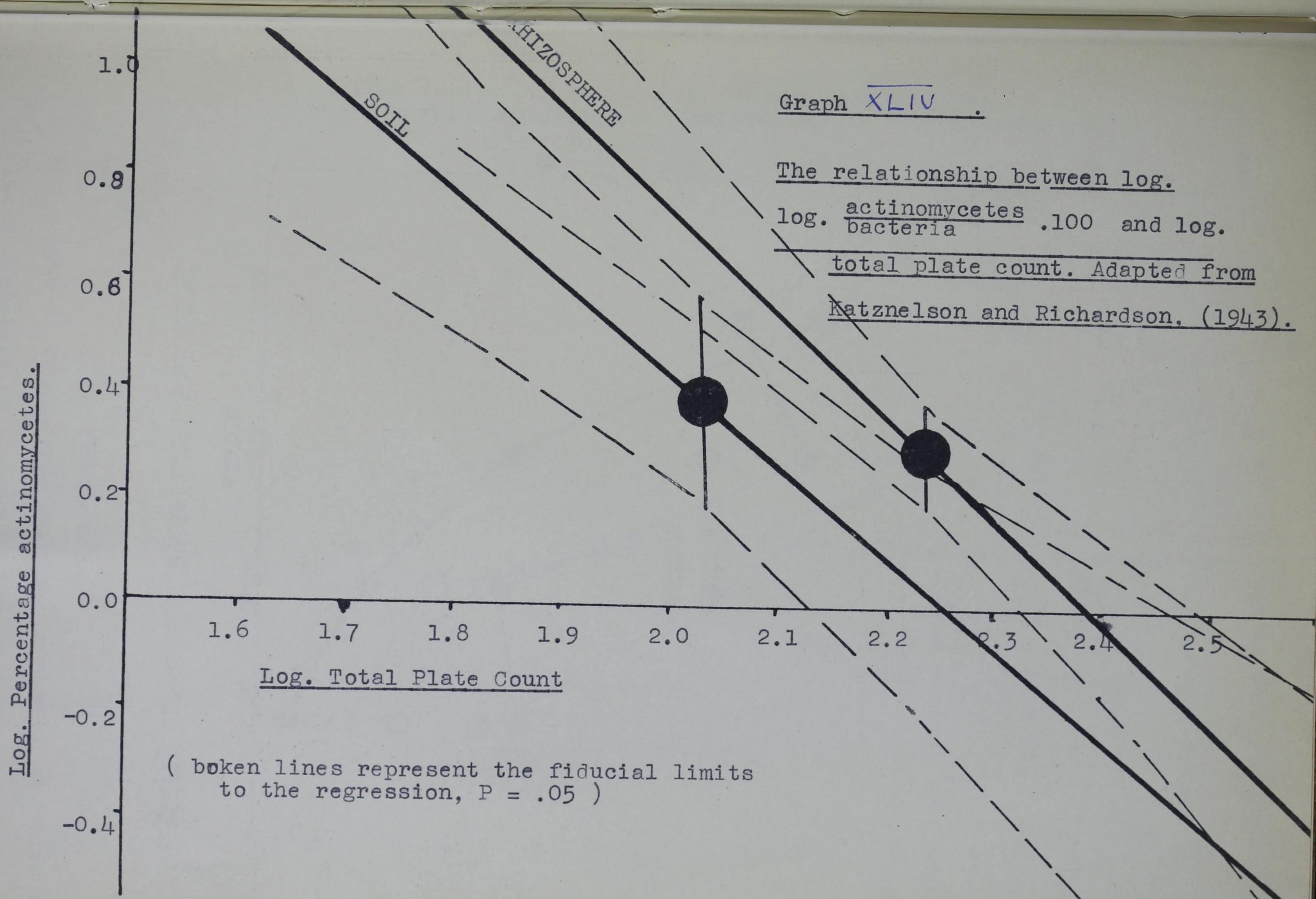
Graph XLIII.

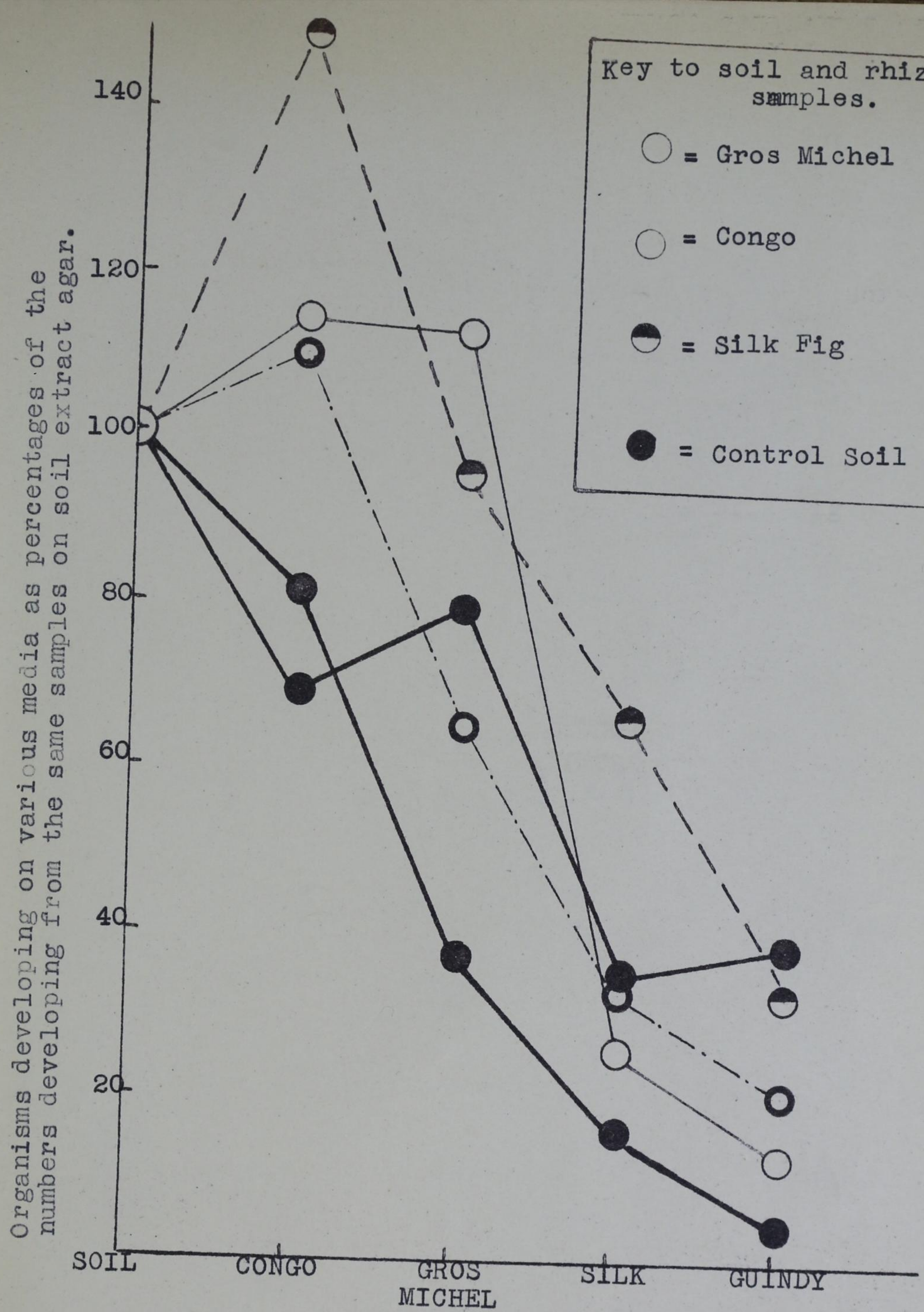
The relationship between percentage actinomycetes and a factor putatively related to plate count for estimates of the microflora of soils and rhizosphere samples from strawberry root rot soils. (Adapted from Katznelson and Richardson 1948).



Graph XLIV .

The relationship between log. log. $\frac{\text{actinomycetes}}{\text{bacteria}}$ .100 and log. total plate count. Adapted from Katznelson and Richardson. (1943).





Media used for plating rhizosphere samples.

Graph XLV.

The growth of banana rhizosphere organisms from various banana varieties on agar media prepared from various banana varieties, as percent

iii + 268 leaves