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THE RELATION OF *PYTHIUM* SPECIES TO THE GROWTH OF A SUGARCANE VARIETY IN HAWAII

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BOTANY

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By

Charles Norman Adair

Dissertation Committee:

Gladys E. Baker, Chairman
Noel P. Kefford
Edison W. Putman
Ivan W. Buddenhagen
Eduardo E. Trujillo
Chester A. Wismer

ABSTRACT

A factor inhibiting the growth of sugarcane, Saccharum officinarum L., Hawaiian variety 37-1933, was evident in the soils of some fields of Ewa Plantation Company, Ltd., Hawaii, in which this variety had been cultivated continuously for 16 years. In a series of greenhouse pot tests involving two sugarcane varieties (37-1933 and 50-7209) this soil factor was characterized as biological, variety-specific, and pythiaceous. Pythium graminicola Subramaniam was associated with the inhibited growth of the sugarcane variety 37-1933, and was pathogenic toward this variety when evaluated according to Koch's Postulates. P. acanthicum Drechsler was associated with the field soils in which the growth of sugarcane variety 37-1933 was depressed, but not as a sugarcane root pathogen. P. acanthicum was very weakly pathogenic toward sugarcane when evaluated according to Koch's Postulates, but was strongly mycoparasitic in culture, attacking many soil fungi.

P. graminicola inhibited growth of the sugarcane variety 37-1933 when inoculated in fumigated or non-fumigated soil. Specificity of P. graminicola isolates toward the sugarcane variety from which they were isolated (37-1933 or 50-7209) was not significant in pathogenicity tests except when P. acanthicum was introduced into the fumigated soil with P. graminicola.

The levels of soil fungi and actinomycetes antagonistic toward P. graminicola and P. acanthicum in high-carbohydrate media were much lower in the Ewa soil than in soils from another plantation (Waialua Agricultural Company, Ltd.) in which the variety 37-1933 had never been grown. Nearly all of the antagonistic fungi noted in these studies,

including species of *Trichoderma*, *Penicillium*, and *Aspergillus*, were parasitized by *P. acanthicum* in low-carbohydrate media.

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CHAPTER I

HISTORICAL REVIEW

(a) Yield Decline

(i) General Growth Failures of Crops

Declining levels of yield with time have been associated with many orchard or plantation crops which are grown for prolonged periods in the same soils. Savory (1966) has reviewed aspects of this phenomenon in relation to several crops. Progressively poorer yields in some orchard crops have been referred to as replant problems or "specific replant diseases" (Savory 1966), indicating the failure of new trees to produce the yields of earlier plantings. The same phenomenon in some other crops has been termed decline, or yield decline. The lower yields in these crops were not attributed to poor climatic conditions, deficiency of essential elements, or incorrect cultural practices, although frequently low yields are associated with the particular area. The poor growth of the crops appears to be caused by inhibiting factors present in the soil in these areas. The growth-inhibiting factors in the soil in which the crops have been grown for a number of years may show a specificity toward the genus or species of crop plant (Savory 1966).

In many cases, the only above-ground symptoms associated with such replant or decline problems is a general stunting. Root systems are often reduced, but for some crops no root pathogens are evident. Facultative root pathogens may contribute to the poor growth of some crops, or may attack plants weakened by some other factors associated with the soil (Savory 1966).

Pythiaceous pathogens have been associated with yield decline or "specific replant diseases" of several crops. Powell, Owen and Campbell (1965) and Hendrix, Powell and Owen (1966) observed Phytophthora cinnamomi Rands, Phytophthora parasitica Dastur, Phytophthora cactorum (Lebert and Cohn) Schroeter, Pythium irregulare Buisman, P. ultimum Trow, and P. vexans deBary in association with roots of declined peach trees. P. ultimum was also reported by Hine (1961) in connection with the peach replant problem, and DeVay et al. (1967) associated a reduced population of P. ultimum and nematodes with improved growth of peach following fumigation.

Pythium species were not considered a primary cause of pear decline, however (Nichols et al. 1964). Although the population of Pythium species increased in association with the decline of a pear orchard, they were not pathogenic to the pear varieties involved. Pathogenic Phytophthoras were at low levels (Nichols et al. 1964).

Loblolly pine has been reported declining in some areas of the southern United States, and pythiaceous fungi have been associated with this condition. Phytophthora cinnamomi, Pythium irregulare, P. vexans, and P. splendens Braun were reported on Loblolly pine in Louisiana by Lorio (1966).

Pythiaceous fungi were also associated with the papaya replant problem, in which Pythium aphanidermatum (Edson) Fitzpatrick and Phytophthora parasitica were found to increase in population in the papaya residues (Trujillo 1965).

The recovery of many fruit trees after transplanting to new soil indicates that systemic pathogens are not affecting growth in such cases

(Savory 1966).

Soil toxins affecting a crop may be produced by the crop itself or by microorganisms associated with the crop or its residues (Borner 1960; Savory 1966). Toxic materials have been reported which increased susceptibility of roots to disease (Graham and Greenberg 1939; Carley and Watson 1967; Toussoun et al. 1968), but a varietal specificity of such substances has not been indicated, and adequate nitrogen and aeration limit the influence of such toxins to a brief period (Savory 1966).

(ii) Yield Decline of Sugar Cane

The failure of a sugarcane variety to maintain its original yields has been described as yield decline, varietal yield decline or growth failure (Jensen et al. 1959; Martin 1938; Martin et al. 1960). The concept of yield decline includes a trend of progressively poorer yields than were obtained earlier in an area in which the variety has been grown for some time; the poor growth is associated only with the soil in which the variety has been grown, and is not apparent when other varieties are planted in the same area (Carpenter 1928; Stevenson 1947; Warner, in press).

Yield decline has occurred in plantations of the sugarcane Cheribon and Bourbon (equivalent to Lahaina in Hawaii) in Java, Mauritius, and the West Indies, and in Hawaii it has been reported for Lahaina, Yellow Caledonia, POJ 36, and the Hawaiian varieties 109, 32-8560, and 37-1933 (Carpenter 1920; Jensen et al. 1959; Martin et al. 1960; Warner, in press).

Genetic deterioration of sugarcane clones resulting in lower inherent vigor and sugar-yielding ability has been considered a cause of yield decline, as indicated by the terms "running-out" and aging of varieties used to describe the decline of some varieties, but deleterious effects of mutations on the yields from extensive plantings of a variety are considered unlikely (Carpenter 1920; Stevenson 1947; Mangelsdorf 1960; Trippi and Montaldi 1960).

Carpenter (1920, 1928) examined a number of potential causes of yield decline, with particular reference to the variety Lahaina which had declined or failed in Hawaii by 1910 (Martin _et al_. 1960). Attacks by parasites, senility (genetic deterioration), root rot, soil toxins, deletion of available elements, bacterial relations, and black alkali soils were considered. Carpenter (1920, 1928) concluded that root rot was the principal cause of the poor growth of this variety. He also noted the residual nature of the factors causing yield decline: the poor growth effect was apparent in Lahaina when it was replanted in an area from which it had been absent a number of years following yield decline (Carpenter 1928).

Recently, the role of cultural, environmental, biological and genetical factors in causing yield decline was reviewed by Martin _et al_. (1960). The cultural and environmental conditions which may depress yields were listed as poor drainage, herbicidal damage, inadequate water or sunlight, low temperatures, and mineral deficiencies. Biological factors were emphasized as responsible for yield decline, and the importance of genetic changes was considered in terms of more virulent genotypes of pathogenic organisms, rather than less resistant genotypes

of the sugarcane (Carpenter 1920, 1928; Stevenson 1947; Edgerton 1958; Martin et al. 1960).

Toxic materials produced during the growth of a sugarcane variety have been suggested as contributing to yield decline by increasing susceptibility to diseases (Lyon 1923). As discussed above, such materials are associated with residues of some crops; the possible role of toxins in the etiology of soil-borne diseases of sugarcane is discussed below.

Pathogens which have contributed to the decline of some varieties of sugarcane include Helminthosporium sacchari (van Breda de Haan) Butler (affecting the Hawaiian variety 109), Physalospora tucumanensis Spegazzini (affecting the Hawaiian variety 38-2915), and Pythium graminicola Subramaniam (affecting the variety Lahaina) (Martin et al. 1960). Other pathogens capable of causing decline of sugarcane varieties include the viruses causing the mosaic, chlorotic streak, and ratoon stunting diseases (Martin et al. 1960). The nematodes Meloidogyne incognita var. acrita Chitwood and Helicotylenchus nanus Steiner, have also been implicated (Jensen et al. 1959).

(b) Sugarcane Soil-Borne Diseases

(i) Non-pythiaceous Pathogens

Fungi other than Pythium which have been reported as pathogenic on sugarcane roots include Armillaria, Leucoporus, Dictyophora, Marasmius, Olpidium, and Rhizoctonia (Martin, Abbot and Hughes 1961; Hsu and Chu 1966). Marasmius sacchari Wakker was associated with root rot of sugarcane in many areas of the world, and at one time it was suggested

as the cause of the decline of Lahaina in Hawaii (Lewton-Brain 1905; Johnston 1916). Marasmius displayed very weak virulence toward sugarcane in later tests (Matz 1920), and has since then been considered unimportant as a root pathogen (Martin 1938; Martin, Abbot and Hughes 1961). Rhizoctonia displayed pathogenicity toward sugarcane in tests in Barbados (Bourne 1922), but was not pathogenic when tested by other workers (Carpenter 1920; Rands and Dopp 1938b). Other fungi which have been recovered from infected sugarcane roots include Fusarium herbarum (Corda) Fries, Fusarium oxysporum Schlechtendahl, Meliola, Trichoderma, and Mucor; tests of pathogenicity of the fusaria and Meliola produced negative results (Carpenter 1920; Rands and Dopp 1938b).

(ii) Pythiaceous Pathogens

Species of Pythium that have been reported to be pathogenic on sugarcane include: P. acanthicum Drechsler, P. aphanidermatum, P. arrhenomanes Drechsler, P. artotrogus (Montagne) de Bary, P. butleri Subramaniam, P. catenulatum Matthews, P. debaryanum Hesse, P. dissotocum Drechsler, P. graminicola, P. irregulare, P. Megalacanthum de Bary, P. monospermum Pringsheim, P. periilum Drechsler, P. rostratum Butler, P. splendens, P. ultimum, and P. vexans (Carpenter 1921; Rands 1930; Rands and Dopp 1938b; Rangaswami 1961; de Carvalho 1965; Adair 1968a). In Hawaii those species which have been reported pathogenic toward sugarcane include: P. acanthicum, P. aphanidermatum, P. arrhenomanes, P. artotrogus, P. debaryanum, P. graminicola, P. rostratum, and P. splendens (Sideris 1932; Parris 1940; Klemmer and Nakano 1964; Adair 1968a; Raabe, unpublished data).

Carpenter (1920, 1921) reported Pythium as pathogenic on sugarcane roots and first identified the causal organism as P. butleri. Carpenter (1928) later revised his identification to P. aphanidermatum, and later again to P. graminicola (Carpenter 1934). The species considered most important in sugarcane root diseases in Louisiana and in other areas is P. arrhenomanes (Rands 1930; Martin, Abbot and Hughes 1961). Although P. arrhenomanes appeared similar to P. graminicola (Carpenter 1938), the distinctiveness of the two species was pointed out by Drechsler (1936). The Hawaiian pathogen may represent a strain of P. arrhenomanes, which apparently is a variable species (Rands and Dopp 1934; Stevenson and Rands 1938). However, the identity of the Hawaiian pathogen recently has been reported to be P. graminicola (Apt and Koike 1962a, 1962b; Koike 1964, Warner, in press).

Carpenter (1930a) suggested that organic residues in plantation soils may predispose sugarcane to attack by Pythium; nitrogenous residues were principally considered in this report. In later reports by Carpenter (1930b, 1932, 1934) fertilizer imbalance was emphasized as predisposing certain varieties to root rots, especially as it involved excessive nitrogen.

Salicylic aldehyde accumulating in the soil from decomposition of debris under certain conditions has been reported to increase susceptibility of sugarcane to root infection (Rands and Dopp 1938a). Organic acids and phenolic compounds in soil have been found by Tung, Yang, and Wang to inhibit sugarcane growth under some conditions as summarized by Wang, Cheng and Tung (1967), but no relationship to pathogenesis was indicated.

(iii) Soil Microflora Influencing Pathogenicity of *Pythium* toward Sugarcane

Actinomycetes, fungi, and bacteria have been reported antagonistic toward *Pythium* species which are pathogenic on sugarcane (Cooper and Chilton 1950; Connell 1952; Luke 1952). An extensive program has been conducted in Louisiana to characterize antagonistic populations affecting *Pythium* in sugarcane plantation soils. Cooper and Chilton (1950), Johnson (1952, 1954) and Hadden (1965) have all reported a negative correlation between populations of antagonistic actinomycetes and severity of *Pythium* root rot. Connell (1952) and Luke and Connell (1954) found a similar negative correlation between populations of antagonistic bacteria and *Pythium* root rot severity. On the other hand, fungi antagonistic toward *Pythium* were associated with areas of root rot severity, indicating an absence of a controlling effect by this group of organisms (Luke 1952). This finding was in contrast with the effect reported by LeBeau (1938) in sterile soil of a control of *Pythium* damage to corn when *Trichoderma* was introduced with the *Pythium* inoculum.

Srinivasan (in press) reported an influence of the sugarcane rhizosphere microflora on the sugarcane pathogen *P. graminicola*. Bacteria, actinomycetes, and the fungi *Trichoderma* and *Penicillium* were present in the rhizosphere of plants resistant to *Pythium* root rot, and were antagonistic toward *P. graminicola*. Lower populations of organisms antagonistic toward *P. graminicola* were present in the rhizosphere of susceptible plants.

(c) Pythium

(i) Genetic Potential for Varietal Specificity

One of the assumptions made in relating Pythium to yield decline of sugarcane is that the pathogenic Pythium population in the soil is capable of acquiring the varietal specificity which is one of the characteristics of yield decline (sensu Warner, in press). It may be that a display of such specificity by the soil population is a result of the increase in the level of strains inherently better adapted to parasitism of the variety in question (Rands and Dopp 1934), in which case a genetic change in a given strain would not be a necessary prerequisite for the display of specificity.

The possibility of a genetic change in a strain of Pythium relates to the frequency of mutations and the nature of the life cycle. The occurrence of mutations in Phytophthora has been reported (Buddenhagen 1958) but there are no such reports for Pythium. Apparently all Pythium species, with the exception of P. sylvaticum Campbell and Hendrix, are homothallic (Vanterpool 1939; Middleton 1943; Papa, Campbell and Hendrix 1967). P. graminicola typically produces monoclinous antheridia (Matthews 1931; Middleton 1943) which reflects the homothallic nature of this species, and which reduces the probability of crossing between different strains of this species.

The mycelium of Pythium has been considered to be haploid, with meiosis occurring in the oospore (Miyake 1901; Matthews 1931). Recently meiosis has been reported to occur in the oogonium and antheridium prior to fertilization, which indicates that the mycelium of Pythium is diploid (Sansome 1961, 1963, 1966). If the gamete nuclei

in the oogonium and monoclinous antheridium were reduced from a diploid condition, there is the possibility of the production of a zygote homozygous for a gene affecting pathogenicity from a parent strain heterozygous for such a gene. If the gamete nuclei fusing to produce the zygote represented the same genotype as present in the haploid mycelium from which they originated, new recombinations are unlikely at fertilization. A heterocaryotic condition could provide for the production of new genetic combinations from a haploid mycelium, but this condition has not been suggested for Pythium (Fincham and Day 1963).

The status of the mycelium in terms of haploidy apparently requires genetic evidence for its confirmation, as there is difficulty in interpreting division figures in the Phycomycetes (Fincham and Day 1963). The variability of colonies arising from single zoospores of mutated Phytophthora isolates may be an indication of a diploid condition of the asexual zoospores (Buddenhagen 1958; Sansome 1966).

Strain variation and host specificity in P. arrhenomanes and P. graminicola have been reported. Rands and Dopp (1934) found that strains of P. arrhenomanes displayed specificity toward the host (sugarcane or corn) from which they were isolated, and suggested that strains with higher virulence toward a host had increased in population in the soil where the host was planted. A similar specificity of P. graminicola isolates toward the grain plant from which they were isolated was reported by Hampton and Buchholtz (1962), and was suggested for isolates of this species toward the sugarcane variety from which they were obtained in Hawaii (Anon. 1963). Koike (1964) reported that different levels of sensitivity to antibiotics were associated with

isolates of P. graminicola from particular varieties, indicating correlation of sensitivity and strains associated with varieties.

(ii) Relationship of Pythium with Soil Factors

Antagonism toward Pythium species by soil organisms has been investigated in relation to Pythium-caused damage of several crops in addition to sugarcane. Lytic strains of Arthrobacter from rhizospheres of tomato and rice protected tomato seedlings from damping-off caused by P. debaryanum in nonsterile soil when seeds were inoculated with these bacteria (Mitchell and Hurwitz 1965). Damping-off of mustard by Pythium in nonsterile soil was controlled by dusting the seed with spores of Trichoderma viride Pers. ex Fr. and Penicillium species (Wright 1956).

Singh (1964) found that Trichoderma viride inhibited P. ultimum in sterile soil, but not in nonsterile soil. The culture filtrate from a Penicillium species controlled damping-off by P. debaryanum when added to sterile soil (Van Luijk 1938). Inhibition of P. debaryanum in vitro was caused by the culture and culture filtrate of a Penicillium species, and by cultures of Streptomyces, Gliocladium, Trichoderma, Cylindrocarpon, Suillus, Armillaria, and Pythium periplocum Drechsler (Van Luijk 1938, Vaartaja and Salisbury 1965).

The relationship between organisms colonizing substrates in the soil was suggested by Barton (1960) to involve physical crowding and production of waste materials rather than depletion of nutrients or antibiotic production. Although Pythium species colonized new substrates in the soil very rapidly, certain other fungi could limit colonization by Pythium if they were established in the substrate

previously. Fungi causing this exclusion of P. mamillatum included Trichoderma, Penicillium, Dicoccum, Mucor, Rhizopus, Cephalosporium, and Fusarium (Barton 1960).

Mycostasis of Pythium species in nonsterile soil has been reported (Harper and Buchholtz 1962; Vaartaja and Agnihotri 1966). Glucose and sucrose amendments partially counteracted the mycostatic effect of field soil toward P. graminicola and P. debaryanum, and several carbohydrates and organic nitrogen amendments overcame a similar effect toward P. ultimum (Harper and Buchholtz 1962; Agnihotri and Vaartaja 1967). Similar amendments have overcome mycostasis of several other genera of fungi, as summarized by Jackson (1965). Sporangia of P. ultimum were stimulated to produce germ tubes by such amendments, but the germ tubes often lysed, indicating a continued effect of mycostasis under these conditions (Agnihotri and Vaartaja 1967).

Mycoparasitic behavior has been reported for a fungus which is probably P. acanthicum (Haskins 1963). This species of Pythium was recently reported for the first time in Hawaii (Adair 1968a), and is one of a group of Pythiums which were reported to parasitize other Pythiums (Drechsler 1943). Among the soil fungi which this species parasitized were Mucor sp., Penicillium funiculosum Thom, P. spinulosum Thom, P. thomii Maire, Trichoderma viride, Aspergillus flavus Link, Rhizoctonia solani Kühn, Pythium mamillatum, and P. spinosum Sawada (Haskins 1963). Some of the fungi which this species did not parasitize were Streptomyces aureofaciens Duggar, S. caeruleus (Baldacci) Waksman, Aspergillus niger van Tieghem, and Pythium acanthicum. Those soil fungi which inhibited this Pythium were Mortierella renispora Dixon-Stewert,

Acremonium sp., Aspergillus sclerotiorum Huber, Metarrhizium anisopliae (Meschinelli) Sorokin, Penicillium chrysogenum Thom, P. herquei Bainer and Sartory, and Sporotrichum epigaeum Brunard (Haskins 1963).

The type of mycoparasitism displayed by the Pythium species discussed above appears to fall into the category of necrotophic mycoparasitism (Barnett 1963). There is some question as to the significance of mycoparasitism detected in vitro in terms of the situation in the soil (Boosalis 1964; Boosalis and Mankan 1965). Much of the interest in phytopathology in mycoparasitism is in relation to potential means of control of pathogens, but the mycoparasitism by the Pythium species discussed above is expressed toward several soil fungi which may be expected to antagonize phytopathogenic pythiums (Haskins 1963; Boosalis 1964; Vaartaja and Salisbury 1965).

The relationship between nutrition and mycoparasitism among Pythium species reported by Haskins (1963) was not investigated. All of his tests of parasitism were carried out on one medium. A marked influence of medium on mycoparasitism was reported for a Penicillium species parasitic on Rhizoctonia solani: in potato dextrose agar made with 20 gm/l of dextrose, parasitism was severe; in the same medium with 10 gm/l of dextrose there was negligible parasitism (Boosalis 1954). An influence of medium has also been reported for mycoparasitism by other fungi (Barnett 1963; Boosalis 1964).

(iii) Approaches to the Study of Pythium

The populations of Pythium and Phytophthora have been estimated in soil by methods of baiting, dilution end-point, and plating on selective media, as reviewed by Menzies (1963). Most baiting methods

have been reported in terms of isolating phytophthoras, although corn seed, sugarcane leaf segments, and pineapple roots have been utilized as baits for the isolation of Pythium from soil (Goth, DeVay and Schick 1967; Koike, personal communication; Srinivasan, in press). A dilution end-point method utilizing pineapple roots as bait has been developed for estimating Pythium populations, based on a modification of the "most probable number" method reported by Tsao (1960) (Buchanan and Fulmer 1928; Maloy and Alexander 1958; Koike, personal communication).

The estimation of populations by the "most probable number" method is valid statistically (Cochran 1950), but the data obtained in the dilution end-point methods developed by Tsao (1960) and Koike (personal communication) were not interpreted in terms of organisms per unit volume. Such an interpretation of the data in the latter method is possible with density estimation procedures described by Fisher and Yates (1948), the validity of which has been established (Fisher 1922).

Selective media have been developed for the isolation of pythiaceous fungi, based on certain physiological differences between this group and many other fungi (Eckert and Tsao 1962; Hine 1963). Such media have been utilized in isolations from diseased roots (Eckert and Tsao 1962), colonized baits (Klemmer and Nakano 1962), and soil particles (Schmitthenner 1962); and spores recovered from soil by screening have also been detected by their growth on such media (McCain, Holtzmann and Trujillo 1967).

Various reactions of pythiaceous fungi to antibiotics, fungicides, and other substances indicate somewhat distinctive physiological characteristics of this group of organisms (Tolmsoff 1962, Hine 1963;

Sietsma and Haskins 1967). The relative sensitivity to antibiotics has been utilized in the selective media discussed above, and also provides means of comparing species and strains of Pythium (Hine 1963; Koike 1964).

The specific effect of the fungicide "Dexon" (Bayer-22555, p-dimethylaminobenzenediazo sodium sulfonate) on pythiaceous fungi has been utilized in their selective control by soil treatments (Tolmsoff 1962; Mitchell and Hagedorn 1966; Zentmyer 1966; Alconero and Hagedorn 1968). As reviewed by Kreutzer (1963), "Dexon" has been effective against Pythium, Phytophthora, and Aphanomyces. Apparently no studies have been made of the taxonomic implications of the fact that "Dexon" affects one member of the Saprolegniales and two members of the Peronosporales.

The nature of exogenous sporogenic substances required by various members of the Pythiaceae has been investigated for a number of years (Leonian and Lilly 1937; Haskins and Tulloch 1964; Hendrix 1964; Klemmer and Lenney 1965; Yang and Mitchell 1965). An undefined exogenous factor was present in carrot fragments which stimulated oospore production (Johann 1928). Materials which are apparently required by many species, including P. graminicola and P. acanthicum, are sterols (Haskins, Tulloch and Mircetich 1964; Hendrix 1964, 1965; Lenney and Klemmer 1966). The stimulatory materials in the carrot amendment utilized by Johann (1928), and in various lipids tested by Klemmer and Lenney (1965), were apparently sterols. Calcium ions were also reported to be essential for oospore production in several Pythium species by Yang and Mitchell (1965).

The requirement for sporulation by the species of Pythium studied by Haskins (1963) was apparently satisfied by several of the fungi which it parasitized.

CHAPTER II

THE RELATION OF PYTHIUM SPECIES TO THE GROWTH OF A SUGARCANE VARIETY IN HAWAII*

Charles N. Adair†

Department of Botany, University of Hawaii

Honolulu, Hawaii

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† Present Address: Division of Biosciences and Marine Studies, University of Guam, Agana, Territory of Guam, U. S. A.

SUMMARY

The diminishing yields of certain crops grown for prolonged periods on the same soil are typical of the yield decline phenomenon. Many sugarcane varieties have suffered yield decline, in Hawaii and other areas of the world, and contributing factors in many cases have been plant pathogens. Root damage caused by *Pythium* species has been associated with the yield decline of certain sugarcane varieties, such as Lahaina in Hawaii. Such root damage has been suggested as an influence on the variety 37-1933 which is thought to have declined in Ewa Plantation in leeward Oahu.

Previous work has demonstrated the adverse effect of certain *Pythium* species on sugarcane growth, and has also suggested the operation of various modifying factors in the soil, such as antagonistic organisms and fungistasis. One assumption has been the development of variety-specific strains of *Pythium* in the soil which progressively inhibit the growth of the variety to which they are adapted, as they increase in population as a result of their successful invasion of the host root tissue.

A series of experiments were conducted to characterize the growth depressing factor affecting the variety 37-1933 in a typical field in Ewa Plantation, and to reveal the biological interactions present in the soil in this field as they may influence this factor. Greenhouse experiments demonstrated that there was a variety-specific pythiaceous factor in the soil affecting the variety 37-1933, and pathogenicity tests showed that the causal factor was *P. graminicola*.

An inverse relationship was found between the level of this pathogenic *Pythium* species and the level of soil organisms antagonistic toward this species, using a Waialua Plantation soil for comparison. A second *Pythium* species--*P. acanthicum*--was discovered in the Ewa soil and was parasitic toward most of the antagonistic soil organisms in either soil.

It was concluded that the variety 37-1933 in the Ewa Plantation field was affected by yield decline, and that *P. graminicola* was a major causal factor. *P. acanthicum* apparently was influencing the soil microflora, but the overall controlling factors were not made evident in these investigations.

I. INTRODUCTION

A feature of plantation cultivation of sugarcane, *Saccharum officinarum* L., in Hawaii and elsewhere has been the periodic replacement of the variety grown in a given area with a variety new to the area. In some cases, the different variety has been introduced because of its inherent superiority to the older variety in terms of yield. In other cases, a new variety, without necessarily greater yield potential, has been required because the older variety was failing to maintain yields. The reduction of vigor in the older variety has been known as yield decline (*sensu* Warner, in press).

The yield decline of some sugarcane varieties is similar to the "specific replant diseases" of orchard crops, reviewed by Savory (1966), in which progressively poorer yields are obtained from continued plantings of a crop in a given soil. The factors contributing to "specific replant diseases" are crop-specific and persistently associated with the soil (Savory 1966). Similar characteristics are associated with the yield decline of certain sugarcane varieties, although the decline of others has been attributed to their susceptibility to leaf or stem diseases, which were not soil-borne (Martin *et al*. 1960).

Fungal pathogens which have been implicated in the decline of sugarcane varieties during the history of sugarcane cultivation in Hawaii include: *Helminthosporium sacchari* (van Breda de Haan) Butler, causative agent of the leaf disease "eye spot", resulting in the decline of the Hawaiian variety 109; *Physalospora tucumanensis* Spegazzini

causing the stem disease "red rot" which resulted in the decline of the Hawaiian variety 38-2915; and *Pythium graminicola* Subramaniam causing root rot which resulted in the decline of the variety Lahaina (Martin *et al*. 1960).

It is suspected that another Hawaiian sugarcane variety, designated 37-1933, recently suffered yield decline in certain areas of Ewa Plantation Co., Ltd., on leeward Oahu (Koike and Warner, 1965; Warner, in press). This variety was grown on the major part of Ewa Plantation from 1949 to 1963. Following a period of drought conditions imposed by a labor strike in 1958 yields from 37-1933 remained depressed below the levels obtained during the years preceding the strike. Sugarcane fields in Hawaii are harvested on a two year cycle. The yield records for two fields, No. 13 harvested in even numbered years and No. 53 harvested in odd numbered years, are presented in Fig. 1 to provide examples of this trend toward decline in comparison with the average yields for the entire plantation (Ewa Plantation Yield Records, unpublished data). Environmental and cultural conditions were not considered primarily responsible for the characteristics of the depressed yields in this area, although they may modify the influence of other factors (Lyon 1923; Carpenter 1928; Martin *et al*. 1960; Warner, in press).

Biological factors which could contribute to these depressed yields include: genetic deterioration of the sugarcane variety, which would limit potential level of yield; accumulation in the soil of self-produced, variety-specific toxins and consequent inhibition of the growth of the variety which produced them; increasing severity of

soil-borne diseases resulting from increasing populations or increasing virulence of pathogens for the variety; and decreasing populations of antagonists in the soil and consequent reduction in their effectiveness in limiting damage by soil-borne pathogens.

Genetic change has been proposed as a cause of yield decline of sugarcane varieties. It is often identified as the "running-out" or aging of a clone after vegetative propagation for a number of generations (Stevenson 1947; Trippi and Montaldi 1960). Although occasional mutations of sugarcane are evident, significant alteration of an entire field of a given variety by mutation appears unlikely (Mangelsdorf 1960; Martin _et al_. 1960).

The absence of above-ground symptoms on the variety 37-1933 in association with the reduced yields in Ewa Plantation fields indicated that the cause of the reduced yields was associated with the soil. Known toxins affecting sugarcane growth and increasing susceptibility to root disease may be part of the cause, although according to some reports these toxins appear to be non-specific in activity (Lyon 1923; Rands and Dopp 1938; Wang, Cheng and Tung 1967). Sugarcane roots have displayed differential sensitivity to toxic root exudates from the same sugarcane variety, however, suggesting the possibility of variety-specific materials of this type in soil (Coleman, in press).

Although nematodes may contribute to yield decline of sugarcane in some areas of Hawaii, root damage by these organisms has not generally been associated with irrigated areas such as Ewa Plantation, and has not been reported for 37-1933 in the field soil investigated in this study (Jensen et al. 1959; Martin _et al_. 1960; Apt and Koike 1962_a_, 1962_b_;

Warner, in press).

Of several genera of soil fungi isolated from sugarcane roots in Hawaii, only Pythium species are recognized currently as pathogenic when inoculated onto sugarcane roots (Carpenter 1920, 1928; Koike and Warner 1965). A recent investigation (Warner, in press) showed that the growth of the variety 37-1933 in certain Ewa Plantation soils was adversely affected by pythiaceous pathogens.

The levels of actinomycetes, bacteria, and fungi antagonistic toward Pythium have been reported negatively correlated with severity of Pythium root rot in Louisiana plantation fields and in studies of rhizosphere influence (Cooper and Chilton 1950; Connell 1952; Johnson 1952; Luke 1952; Johnson 1954; Luke and Connell 1954; Srinivasan, in press). There have been no reports of such correlation in Hawaiian plantation soils.

Because a soil-borne factor has been implicated in yield decline of sugarcane variety 37-1933, this investigation was focused on the relationship of this variety and an Ewa Plantation field soil. By this approach it was hoped that the reason for the depressed yields of the variety 37-1933 might be elucidated and its status in terms of yield decline clarified. A series of greenhouse experiments was conducted to characterize the growth inhibiting factor in the field soil. Studies were made of the soil microflora to determine their interactions mutually and with sugarcane specifically.

The nature of the growth inhibiting factor was defined to some extent in several preliminary experiments. Dilution of the Ewa Plantation field soil by sterile soil affected the factor very slightly,

but steam sterilization nullified the factor (Adair 1969). Since the influence of a biological factor may be expected to be comparable following any adequate level of introduction into a sterile medium, and since steam could remove biological factors, these results were interpreted as evidence of the biological nature of the growth inhibiting factor. "Dexon" (Bayer-22555, p-dimethylaminodiazobenzene sodium sulfonate) controlled the growth-inhibiting factor to some extent when applied to the field soil in pots (Adair 1969). This fungicide is specific for pythiaceous fungi (Kreutzer 1963), and its effect suggests the possible role of pythiaceous pathogens in the growth inhibition of sugarcane. Of the three genera of fungi which have been reportedly controlled by "Dexon"--*Pythium*, *Phytophthora*, and *Aphanomyces* (Mitchell and Hagedorn 1966; Zentmyer 1966; Alconero and Hagedorn 1968)--only *Pythium* is known to be associated with infected sugarcane roots (Martin, Abbott and Hughes 1961). Varietal specificity of the growth-inhibiting factor was not significant in a factorial comparison of growth of varieties exposed to different field soils, but an interaction might have been detected with additional replication (Adair 1969).

Additional greenhouse experiments, involving exposure of sugarcane varieties to field soil, were designed to test the varietal specificity of the growth inhibiting factor in an Ewa field soil, and to test the effects of dilution, sterilization, and "Dexon" application on the factor. The identity of *Pythium* species in the soil, and their population levels, were investigated by several culturing and isolation methods. The effect of these species on sugarcane was determined by direct inoculation experiments.

To test the hypothesis that the *Pythium* population may become adapted to a variety of sugarcane present in an area for a prolonged period of time, the genetic characteristics of such a population were compared with those of a second population which originated in an area free from the influence of the variety. Earlier studies have suggested an association of varietal specificity with physiological strains of *Pythium* (Anon. 1963; Koike 1964). The sensitivity to antibiotics, the requirements for sporulation, and the virulence toward sugarcane roots were evaluated for a series of isolates of *P. graminicola* to obtain information on their genetic makeup.

The influence of antagonistic members of the soil microflora on *Pythium* species was examined *in vitro* and inferred from results of sugarcane inoculation experiments.

A species of *Pythium* recently reported in an Ewa Plantation field soil--*P. acanthicum* Drechsler (Adair 1968*a*)--displays mycoparasitic behavior *in vitro* (Drechsler 1943; Haskins 1963). The interactions between *P. acanthicum* and other soil fungi in relation to sugarcane were investigated to determine what influence this species may have on the variety 37-1933 in the Ewa Plantation field.

II. METHODS

(a) Soils and Sugarcane Varieties Used

Soil samples from Ewa Plantation Company, Ltd., field 53 (Ewa 53) and Waialua Agricultural Company, Ltd., Opaeula section field 17 (Waialua 17) were selected for these investigations. Ewa 53, an irrigated field in leeward Oahu with a gray hydromorphic soil, is an area in which the Hawaiian sugarcane variety 37-1933 had been present for 16 years. Samples were collected from Ewa 53 in the vicinity of plantings of RA 37-1933 (a variety propagated from irradiated planting material of the variety 37-1933). Collections were made in both poor (Ewa 53-A) and better (Ewa 53-B) sugarcane growth areas of this field, based on Ewa Plantation Co. Yield Records (unpublished data). Waialua 17, an irrigated field in leeward Oahu with a low humic latosol soil, is an area in which the variety 37-1933 has never been present. Samples were collected from Waialua 17 in the vicinity of the Hawaiian sugarcane variety 50-7209. Potting soils utilized in greenhouse experiments were a composted mixture which had been exposed to many sugarcane varieties, and a red Kunia (leeward Oahu) subsoil which had not been exposed to sugarcane.

Sugarcane variety 37-1933 was utilized in all of the experiments involving the sugarcane plant, and the sugarcane variety 50-7209 was included in several tests as an example of a variety which had not displayed depressed growth in Ewa Plantation.

(b) Characterization of Growth Depressing Factors

Depression of growth in Ewa 53-A was characterized in two greenhouse experiments in which sugarcane plants were grown in Mitscherlich pots according to a standardized procedure established at the Experiment Station of the Hawaiian Sugar Planters' Association (Koike and Warner 1965; Koike 1967a; Warner, in press). Two plants of each sugarcane variety were grown in five liters of soil in each pot for 10 to 12 weeks. The growth responses of 37-1933 and 50-7209 to various field soil amendments and treatments were measured as shoot dry weight, number of tillers, root dry weight, or severity of root rot damage.

In the first greenhouse experiment, the two varieties were grown in 4.5 liters of methyl bromide fumigated composted soil to which 500 ml of screened soil from Ewa 53-A or Waialua 17 were added. The four combinations of variety x soil were replicated five times.

In the second greenhouse experiment, the two varieties were grown in fumigated composted soil to which screened soil from Ewa 53-A was added in volumes of 50, 200, and 800 ml, with appropriate adjustment to give a total volume of five liters. The treatments with these volumes of Ewa 53-A soil were repeated for each variety in three series, two using the non-fumigated field soil, the other using the field soil after fumigation with methyl bromide. One of the series involving the non-fumigated field soil amendments was treated with "Dexon" at 0.25 gm per pot at planting and at four-week intervals, following Warner's methods. The two varieties were also grown in control pots containing fumigated composted soil without field soil amendments. The controls were repeated in two series: untreated and treated with "Dexon". Each

variety x treatment combination was replicated five times.

(c) Investigations of Soil Microflora

(i) Pythium Studies. - Pythium species were isolated from soil samples by a soil plating procedure (McCain, Holtzmann and Trujillo 1967) and from several plant materials obtained by host plant infection and baiting methods.

Soil particles ranging in size from 25 μ to 43 μ were plated on antibiotic supplemented V-8 agar (4% agar containing 1% "V-8 Juice" (Campbell Soup Co., Camden, New Jersey) cleared by centrifugation, 50 μg/ml "Mycostatin" ("nystatin"), 10 μg/ml "Terrachlor" (pentachloronitrobenzene), 100 μg/ml "Vancomycin hydrochloride", and 100 μg/ml "Pimaricin") (McCain, Holtzmann and Trujillo 1967; Ooka, personal communication). The soil particles in this size range were obtained by passing a soil sample through a graded series of screens ending with a mesh count of 325, and washing the filtrate from the 325 mesh screen to a 500 mesh screen to remove the clay particles. The residue on the 500 mesh screen was resuspended and plated on the antibiotic supplemented V-8 agar. Pythium colonies originating from an aliquot of the suspension were counted to provide an estimate of the population in six soil samples from two Ewa 53 locations (A and B) and Waialua 17.

Isolates of Pythium were recovered from lesioned segments of sugarcane roots, lesioned segments of pineapple roots, Ananas comosus (L.) Merr., (Klemmer and Nakano 1962), and sugarcane leaf baits (pieces of sugarcane leaf lamina, 2 cm x 0.5 cm) recommended by

Srinivasan (in press). All plant materials were plated on 2% water agar. The lesioned root segments were surface sterilized with 0.25% sodium hypochlorite for 10 min prior to plating.

Segments of sugarcane roots bearing lesions, plated out for Pythium recovery, were from the varieties 37-1933 and 50-7209 exposed to field soil from Ewa 53-A and Waialua 17 in the greenhouse experiments, and from these varieties inoculated with pure cultures of Pythium species.

Roots developing from pineapple crowns were utilized as a convenient baiting material in a dilution end-point estimation of the population of Pythium species in soil samples from Ewa 53-A and Waialua 17 (Tsao 1960; Klemmer and Nakano 1962; Koike, personal communication). Pineapple roots which were infected when exposed to two-fold dilutions of sample soil in water (using sterile soil as the diluent) were plated for recovery of the pathogen.

The leaf baiting method utilized in studies of sugarcane root infecting Pythium species by Srinivasan (in press) was employed to estimate populations of Pythium in soil samples from Ewa 53-A, Ewa 53-B, Waialua 17, and pots in one greenhouse experiment. Five leaf baits were exposed to ten-fold water dilutions of the soil samples, then plated for recovery of colonizing organisms. The proportion of colonization of the five leaf baits at each dilution was utilized in a "most probable number" estimation of the Pythium population under these conditions (Buchanan and Fulmer 1928; Maloy and Alexander 1958).

Final isolation for pure culture was accomplished by transferring unbranched hyphal tips (approximately 1 mm long) of colonies developing on water agar or antibiotic supplemented V-8 agar. Stock cultures were

maintained on potato dextrose agar (Difco 0013).

Colony characteristics on potato dextrose agar and spore morphology on 2% water agar with and without fresh carrot fragments (Johann 1928) served as the basis for species identification. Based on descriptions presented by Drechsler (1930), Matthews (1931), and Middleton (1943), two species of Pythium were identified: P. graminicola and P. acanthicum.

P. graminicola isolates from sugarcane and pineapple roots were exposed to antibiotics incorporated into the medium (Koike 1964). The sensitivity of P. graminicola to streptomycin, aureomycin, and actidione (cyclohexamide) was compared for these isolates. Colony growth with each antibiotic in comparison with growth without antibiotic was used as a measure of sensitivity. Each isolate was exposed to antibiotics in three replicates.

Sixteen P. graminicola isolates from the variety 37-1933 exposed to Ewa 53-A soil and from the variety 50-7209 exposed to Waialua 17 soil were cultured in the water agar with carrot fragments for a comparison of sporulation under these conditions. Oospores and sporangia were counted in measured sectors of duplicate petri dish cultures after 5 days to measure the level of sporulation.

(ii) Antagonism Studies. - Soil organisms antagonistic toward P. graminicola were detected by an agar layer method (Koike 1967b). Composite samples from Ewa 53-A and Waialua 17 were assayed for populations of antagonists in Cooke rose bengal agar (Difco 0703) at a dilution of 10^{-4} and in sodium albuminate agar (Waksman and Fred 1922) at a dilution of 10^{-5}. Four samples from Ewa 53-A and Waialua 17, and

three each from Ewa 53-B and from greenhouse pots inoculated with *P. acanthicum* or non-inoculated, were assayed for number and kinds of antagonistic fungi, using rose bengal agar. Non-fungal antagonists were characterized as bacteria or actinomycetes, and fungal antagonists were identified to genus.

(d) Interactions Between Organisms

(i) Pathogenicity Studies. -- The pathogenicity of *P. graminicola* and *P. acanthicum* toward sugarcane was tested by exposing roots to inocula in water: a procedure similar to that reported by Zentmyer and Mircetich (1965). Sections of sugarcane stalk were prepared so that roots developing from a node extended into a beaker of water, while the cut ends of the stalk remained dry and out of the water. Inoculation of the roots was accomplished by the introduction of sections of cultures of the *Pythium* species grown on water agar with carrot fragments. Selected isolates from among those tested for sporulation were compared for virulence toward roots of 37-1933 and 50-7209 by this method. Similar tests were made utilizing rooted side shoots of sugarcane, as reported by Adair (1968*b*; 1969).

Pathogenicity of the two species of *Pythium* toward sugarcane was also tested by introduction of inocula into soil in three inoculation experiments. These experiments followed the procedure used in the characterization of the growth depressing factors in the field soil.

In the first of the inoculation experiments, two groups of *P. graminicola* isolates recovered from 37-1933 roots exposed to Ewa 53-A soil (differentiated according to their growth rate in culture as "fast"

and "slow") and one group recovered from 50-7209 roots exposed to Waialua 17 soil were tested against the two varieties. The inoculum consisted of mycelial mats of the isolates grown 10 days in 100 ml of potato dextrose broth in 500 ml flasks; these mats were washed, placed in water for a total volume of 100 ml/mat, and fragmented for five seconds at high speed in a Waring Blendor. The suspensions of isolates in each group were pooled. Into each pot of fumigated composted soil for each sugarcane variety, 25 ml of the mycelial fragment suspension was introduced. For comparison in an additional treatment, the inoculum was introduced into an additional pot for each variety in the case of the "fast" Ewa 53-A and the Waialua 17 isolates. Each variety was also grown in two pots of uninoculated soil. The additional treatment involved the addition of "Dexon" to one pot inoculated with the "fast" Ewa 53-A isolate group, one with the Waialua 17 isolate group, and one uninoculated pot, for each variety. The "Dexon" was added at 0.25 gm per pot at planting and at four-week intervals. The treatments were replicated five times.

The second inoculation experiment used two isolates of P. graminicola which had displayed specificity toward 37-1933 and 50-7209 in the water inoculation procedure, corresponding to the variety from which they had been isolated. An isolate of P. acanthicum from Ewa 53-A was also used in this experiment. The inocula of the three isolates were prepared as described for the first inoculation experiment. Each of the three isolates was introduced separately to the two sugarcane varieties grown in the red Kunia subsoil. Also, each P. graminicola isolate was introduced into pots simultaneously with P. acanthicum. Uninoculated

pots of fumigated Kunia soil were prepared for each variety as controls. The treatments were replicated five times.

In the third inoculation experiment, one isolate each of P. graminicola and P. acanthicum from Ewa 53-A were tested against 37-1933 in non-fumigated red Kunia subsoil. The inocula, which contained numerous oospores, were prepared by fragmenting in a "Waring Blendor" cultures of the isolates on water agar with carrot fragments. The treatment combinations consisted of each species introduced separately, both species introduced together, and both species absent (uninoculated). The treatments were replicated six times, rather than five as in the above experiments, in an attempt to determine with greater precision the presence of any interaction.

(ii) Soil Microflora Studies. - Eighteen isolates of fungal antagonists detected in the individual samples from the field and from the second inoculation experiment were paired with P. graminicola and P. acanthicum on 2% water agar and potato dextrose agar. The macroscopic responses of the colonies, including amount of sporulation and zones of inhibition, and the microscopic behavior of the hyphae, including reaction to antibiotics and occurrence of parasitism, were noted.

III. RESULTS

(a) Characterization of Growth Depressing Factors

A significant interaction between variety and field soil was observable in the first greenhouse experiment, according to shoot dry weight and tiller number (Table 1).

The addition of non-fumigated field soil to potting soil in the second greenhouse experiment resulted in poorer shoot growth of the sugarcane than was obtained in the other treatments (fumigation of field soil amendment, non-fumigated amendment followed by soil treatment with Dexon, and non-amended control) (Table 2). There were no differences in shoot growth among the three levels of soil amendment in the non-fumigated series. There were positive correlations between shoot and root dry weights, and between shoot dry weight and tiller production. Severity of root rot was inversely correlated with plant growth measured by these parameters (Table 3).

(b) Characteristics of Soil Microflora

(i) *Pythium species*. - *P. graminicola* was isolated consistently from lesioned segments of roots of both sugarcane varieties exposed to Ewa 53-A and Waialua 17 soils and also from pineapple roots infected in *Pythium* population assays of these soils. The *Pythium* species consistently isolated from the leaf baits exposed to Ewa 53-A, Ewa 53-B, and Waialua 17 soils in water was identified as *P. acanthicum*, as confirmed by Vaartaja (personal communication). *P. graminicola* and *P. acanthicum* were both isolated by the direct screening and plating

method. The morphological characteristics used in the identification of these species are illustrated in Plate 1.

The population of P. graminicola estimated by the pineapple root baiting and the direct plating methods (Table 4) was much higher in Ewa 53-A soil than in Waialua 17 soil. The population in Ewa 53-B soil was intermediate between those in the other two soils. The population of P. acanthicum, estimated by the leaf baiting and direct plating methods, was also high in Ewa 53-A soil, low in Waialua 17 soil, and intermediate in Ewa 53-B soil.

The levels of P. acanthicum in potting soils in the second greenhouse experiment were correlated with the presence or absence of soil amendments and reflected the effect of fumigation and "Dexon" application (Table 5), but were not correlated with the volume of soil amendment.

The sensitivity of P. graminicola isolates to antibiotics varied somewhat (Table 6), but given levels of sensitivity were not associated with any particular source of isolate.

Although the absolute numbers of oospores and sporangia produced showed variation for the different isolates, the presence or absence of either of these spore types, and the relative numbers of those present, were fairly consistent (Fig. 2). Isolates from Ewa 53-A soil obtained from the sugarcane variety 37-1933 produced fewer oospores and more sporangia than isolates from Waialua 17 soil obtained from the variety 50-7209.

(ii) Antagonists. - Higher numbers of actinomycetes and fungi antagonistic toward P. graminicola were present in Waialua 17 soil than in

Ewa 53-A soil. The numbers of propagules per g, based on dilution plate counts, are listed in Table 7.

More fungi antagonistic toward P. graminicola were present in soil without inoculum than in soil to which P. acanthicum had been introduced in the second inoculation experiment (Table 8). The genera of fungi displaying antagonism toward Pythium species are indicated in Table 9.

(c) Interactions Between Organisms

(i) Pathogenicity of Pythium Species Toward Sugarcane. - P. graminicola and P. acanthicum were reisolated from sugarcane roots inoculated with these organisms in water, but very low virulence toward sugarcane was displayed by the latter species under these conditions. Varietal specificity was not displayed by all of the P. graminicola isolates tested, but there were several which were more virulent toward the variety from which they had been originally isolated than toward another variety (Fig. 3).

Inoculation of either sugarcane variety with P. graminicola resulted in a marked reduction in growth in the first inoculation experiment, as measured by shoot dry weight and tiller number (Table 10). There were no significant differences among the three groups of isolates in their effect on the growth of a given variety. Treatment of inoculated soil with "Dexon" resulted in a level of growth comparable with that in uninoculated soil.

Shoot dry weight, tiller number, and ratoon production of both sugarcane varieties were inhibited by inoculation with either type of isolate of P. graminicola in the second inoculation experiment, but

neither variety was affected significantly by P. acanthicum alone (Table 11). A significant interaction between variety and treatment was detected in this experiment which reflected a differential response of the varieties to the isolates. This was especially evident in the presence of P. acanthicum, when the dry weights of each variety were relatively lower in the presence of the P. graminicola isolate obtained from that variety, in comparison with that in the presence of the other isolate of P. graminicola. However, there was no significant interaction between inoculations with P. acanthicum and with isolates of P. graminicola.

The shoot dry weight of the variety 37-1933 was reduced in the presence of P. graminicola in the non-fumigated field soil in the third inoculation experiment. There was no inhibition caused by P. acanthicum, and there was no interaction between inoculations with the two species (Table 12).

(ii) Relationships Between Soil Organisms. - Most of the 18 isolates of antagonistic organisms inhibited P. graminicola and P. acanthicum in potato dextrose agar, but the effects were not often evident in water agar (Table 9; Plate 2).

P. acanthicum was parasitic toward most of the antagonistic fungi in water agar, making a necrotrophic invasion of the hyphae of the host fungi (Plate 3). The aerial mycelium of the host fungus was usually restricted, and sporulation was sparse. P. acanthicum was also mycoparasitic toward P. graminicola, but little inhibition of the latter species resulted from the parasitism in water agar or potato dextrose agar. Sporulation of P. acanthicum was profuse following parasitism of

a non-pythiaceous host.

IV. DISCUSSION

(a) Characterization of Growth Depressing Factors

A varietal specificity of the factor in Ewa 53 soil, suggested in preliminary experiments, was demonstrated in the first greenhouse experiment by the significant interaction between variety and soil. The growth depressing factor in soil from Ewa 53-A was more effective against the variety 37-1933 than against the variety 50-7209. Such a differential depression of growth of 37-1933 was not evident in soil from Waialua 17.

The effect of the factor in Ewa 53-A soil was also shown in a second greenhouse experiment by poorer growth of 37-1933 in the presence of non-fumigated soil amendments compared with the growth obtained in their absence. There was also inhibition of growth of 50-7209 exposed to Ewa 53-A soil in comparison with growth in non-amended soil, suggesting that the growth-depressing factor may affect other varieties to some extent.

The absence of an effect of amendment concentration shows that the inhibiting factor may be developing to comparable levels of effectiveness in the amended soil from any volume introduced. Such behavior may be interpreted as characteristic of a living entity such as a plant pathogen, which is proliferating in the soil to comparable levels from each volume of soil amendment introduced, and displaying comparable pathogenicity and growth inhibition at all levels of introduction.

The sterilization of field soil by methyl bromide fumigation nullified the growth inhibiting effect of the soil amendments toward 37-1933 and 50-7209. A similar effect of steam sterilization was evident in preliminary experiments, but could have been related to destruction of thermolabile toxins, as well as to killing of pathogens. Fumigation could be expected to affect only living organisms such as pathogens, the removal of which would allow disease-free growth of the sugarcane, which could be evident as more vigorous growth.

The severe root rot damage evident as low root dry weights and high root rot ratings (Table 3) for plants in soil amended with non-fumigated field soil demonstrates that the inhibited growth of these plants is due to root infection. The positive response to "Dexon" applied with the non-fumigated field soil amendments indicates that a pythiaceous organism is inhibiting the growth of sugarcane in this soil. In most cases, the growth in the presence of "Dexon" was comparable with that in sterile or non-amended soil, although for some parameters the growth was lower for higher field soil amendment volumes plus "Dexon" than for fumigated amendments. This could mean that there was incomplete control of infection by pythiaceous organisms by "Dexon", or that there was some inhibition of growth by non-pythiaceous organisms.

In the investigations reported by Warner (in press), relative responses to sterilization and "Dexon" have also demonstrated an influence on sugarcane of a biological factor which was pythiaceous in nature.

(b) Characteristics of Soil Microflora

The frequent association of Pythium with young lesions on sugarcane roots recovered from soils in which sugarcane grew poorly suggested an association of this genus with the poor growth. The Pythium species recovered from infected sugarcane roots was P. graminicola, which has been reported as the principal pythiaceous pathogen of sugarcane in Hawaii (Carpenter 1934; Koike 1964).

The species recovered by means of the leaf-bait technique, P. acanthicum, was not reported from Hawaii prior to this investigation (Adair 1968a). A rough-spored species mentioned by Carpenter in Hawaii (1928) may have been P. acanthicum. P. artotrogus (Montagne) deBary, reported pathogenic in Hawaii on the sugarcane variety Lahaina by Sideris (1932), may also have been P. acanthicum growing under conditions not conducive to sporangial formation; the two species are quite similar except for the failure of P. artotrogus to produce sporangia (Middleton 1943). Of the other spinyspored Pythium species reported from Hawaii--P. acanthophoron Sideris and P. oligandrum Drechsler (Sideris 1932; Parris 1940; Klemmer and Nakano 1964; Raabe, unpublished data)--neither resembles the isolate obtained by the leaf baiting technique. P. acanthophoron has been described as having a pulvinate appearance in culture, while the leaf bait isolate has a cumulous appearance; P. oligandrum infrequently produces antheridia, whereas the leaf bait isolate typically has one to three per cogonium (Middleton 1943). One species not previously reported from Hawaii appearing similar to the leaf bait isolate is P. periplocum Drechsler, but this species produces diclinous antheridia and aplerotic oospores. The leaf bait isolate

produces monoclinous antheridia and plerotic oospores, although the oospores are usually detached from the oogonial wall (Plate 1) (Middleton 1943).

Populations of Pythium species were at much higher levels in the poor-growth area of Ewa (53-A) than in the better growth area (53-B) of this field or in Waialua 17. Although Ewa 53 and Waialua 17 are different soil types, both areas are irrigated, so that they may be considered comparable in terms of water relationships. Preliminary studies on another field in Ewa Plantation--field 13, which is a low humic latosol similar to Waialua 17 soil--also revealed high levels of both Pythium species (Adair 1969). Thus soil type alone is not responsible for the population differences between Ewa 53 and Waialua 17.

Antibiotic sensitivities showed the possibility of variation among the P. graminicola isolates tested. There was little correlation between the source and the levels of sensitivity, in contrast to the results of Koike (1964), but there were significant differences among some isolates which could be related to genetic differences.

Requirements for sporulation were satisfied by the standard medium for isolates from some sources, but not for certain other groups of isolates. The failure of many isolates from sugarcane roots to produce oospores in the standard medium was apparently a characteristic of the group from a given source, rather than a condition due to age in culture. Cultures obtained in several programs of isolation, and thus of varying age, had the same range of sporulation requirements.

Varietal specificity of isolates expressed by infection of sugarcane roots in water suggested a wide range of potential for pathogenicity,

but the relative virulence toward varieties did not necessarily reflect the source of the isolate (Fig. 3). However, the presence of several isolates expressing varietal specificity corresponding to their source demonstrated the potential for development of such a population in the soil. A similar specificity of isolates toward their original host has been suggested previously (Anon. 1963).

The low population of organisms antagonistic toward P. graminicola in Ewa 53 soil may permit the development of the high population of P. graminicola in this soil. Obversely, the high population of antagonists in Waialua 17 soil may be responsible for the low levels of P. graminicola in that soil (compare Tables 4 and 7). A similar relationship between Pythium and antagonists was proposed for some soils in Louisiana (Cooper and Chilton 1950; Connell 1952; Luke 1952; Johnson 1954; Hadden 1965).

Fields of Ewa Plantation have received large amounts of "Atrazine" (Jones, personal communication), which is known to influence growth and sporulation of some fungi in soil. Curl, Rodriguez-Kabana and Funderburk (1968) found the effect of "atrazine" on Trichoderma to be stimulatory. This genus has been among the antagonistic genera present at low levels in "Atrazine"-treated Ewa 53 soil. It may be that the levels of "Atrazine" remaining in the soil are not adequate to influence Trichoderma, and the low level of the fungus is due to some inhibitory factor.

(c) Interactions Between Organisms

The association of *P. graminicola* with infected roots of sugarcane plants affected by the growth inhibiting factor in Ewa 53-A soil satisfied the first of Koch's Postulates for the pathogenicity of this species. *P. graminicola* was isolated in pure culture by transfer of hyphal tips, and identified. Inoculation of sugarcane roots in water and in sterile soil resulted in root lesions which were similar to those observed in the original disease, and growth depression was produced in the inoculation experiments as further duplication of the symptoms of the original disease. *P. graminicola* was reisolated from the root lesions developing in water and in soil, as final proof of pathogenicity.

Since *P. acanthicum* was not isolated from sugarcane roots infected in the field, the first of Koch's Postulates was not established for pathogenicity of this species. Consequently, the ability of *P. acanthicum* to infect sugarcane roots reflects only potential pathogenicity of this species toward sugarcane.

There was no interaction between the two *Pythium* species when inoculated on sugarcane roots in water or in soil, although the varietal specificity of *P. graminicola* isolates toward sugarcane in soil was most strongly evident in the presence of *P. acanthicum*. The role of *P. acanthicum* in increasing the specificity of *P. graminicola* in soil could be related to an interaction among the *Pythium* species and the soil antagonists, but there is no direct evidence for this.

The inhibition of *P. graminicola* by antagonists on potato dextrose agar, or in the agar layer method used for the initial detection of antagonists, may have been related to the nutritional status of the

antagonistic organisms. The absence of an antibiotic effect by several antagonists toward P. graminicola in water agar suggests that production of inhibitory levels of antibiotics depends on a rich nutrient base for the antagonist. A similar influence of the substrate on fungal interactions has been suggested by Barton (1960). Certain antagonists inhibited growth of P. graminicola in water agar, but mycelial growth of the two organisms in such cases was present in the same areas of the agar medium, with the reproduction potential of the Pythium colonies apparently reduced because of repression of sporangial production.

The importance of Pythium antagonists detected in potato dextrose agar in the suppression of P. graminicola in the soil may be questionable. The inhibition of zoosporangial production by P. graminicola in water agar in the presence of certain antagonists, however, indicates that these organisms may limit production of zoospores of P. graminicola at the low nutrient levels present in the soil.

The active mycoparasitism of most of the fungal antagonists by P. acanthicum may explain the low levels of antagonistic fungi in soils containing high levels of P. acanthicum. However, observations of mycoparasitism were only made on cultures in vitro, so that the effect of P. acanthicum on antagonistic fungi in vivo can only be inferred from the levels of such organisms present in soil with P. acanthicum. The assay of antagonistic fungi in soil to which P. acanthicum was added in the second inoculation experiment showed a lower level of antagonists than in soil without P. acanthicum (Table 8). The total numbers of fungi in these soils were approximately the same however, which shows

that the presence of P. acanthicum had not resulted in low populations of fungi in genera.

In water agar, parasitism by P. acanthicum sometimes involved invasion of conidiophores of the antagonist. A reduction in conidial production due to invasion of the conidiophores may be the cause of the reduced sporulation of the antagonist observed macroscopically. If such an effect occurred in the soil, the population of the profusely sporulating genera detected on dilution plates could be depressed.

The occurrence of one Trichoderma species that is not parasitized by P. acanthicum, and which appears to be inhibitory to P. acanthicum in water agar and potato dextrose agar (Table 9), demonstrates that the fungal host range of P. acanthicum does not include all species antagonistic toward P. graminicola. The fungal host range of the parasitic Pythium reported by Haskins (1963) included Trichoderma viride, and some Aspergillus and Penicillium species, but there were other Aspergilli and Penicillia which were not parasitized. The inhibitory Trichoderma, identified as T. lignorum, was present at low levels in Ewa 53 soil, which may explain the high levels of P. acanthicum in that soil. If the low level of this Trichoderma species cannot be explained by the parasitic activity of P. acanthicum, then its low population is caused by some other factor, possibly one that is affecting the actinomycetes, which were also at low levels in this soil.

The sporulation response of P. acanthicum following parasitism of the non-pythiaceous hosts is apparently related to the availability in the host of sterols required for the sexual stage, as reported by Haskins (1963) and Haskins, Tulloch and Mircetich (1964).

TABLE 1

EFFECT OF TWO DIFFERENT FIELD SOIL AMENDMENTS

ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

First greenhouse experiment:

means of parameters of growth in pots

Sugarcane Variety	Field Soil*	Growth Parameter	
		Shoot Dry Weight (g)	Tillers (No.)
37-1933	Ewa 53-A	24.8	0.6
	Waialua 17	47.3	1.8
50-7209	Ewa 53-A	53.3	2.0
	Waialua 17	49.7	1.2

*Mixed with methyl bromide fumigated potting soil at 500 ml/5 l. total volume.

TABLE 2

EFFECT OF TWO TREATMENTS AND FOUR VOLUMES OF EWA 53-A SOIL AMENDMENTS ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

Second greenhouse experiment:

Means of parameters of growth in pots

Amendment Treatment*	Amendment Volume+ (ml)	Culture Condition‡	Growth Parameters			
			37-1933 Shoot Dry Wt. (g)	50-7209 Shoot Dry Wt. (g)	37-1933 Tillers (No.)	50-7209 Tillers (No.)
--	0	- Dexon	127.6	154.4	4.2	4.4
--	0	+ Dexon	125.6	185.4	3.2	5.0
Fumigated	50	- Dexon	119.0	165.6	4.4	4.2
Non-fumigated	50	- Dexon	25.0	92.6	0.8	2.6
Non-fumigated	50	+ Dexon	111.0	168.4	3.0	4.6
Fumigated	200	- Dexon	99.2	163.2	3.8	4.0
Non-fumigated	200	- Dexon	21.4	102.8	0.8	3.2
Non-fumigated	200	+ Dexon	98.2	157.0	3.0	4.8
Fumigated	800	- Dexon	126.2	165.4	3.6	4.6
Non-fumigated	800	- Dexon	33.4	90.6	1.0	3.2
Non-fumigated	800	+ Dexon	97.2	149.4	3.2	3.8

* "Fumigated" and "Non-fumigated" refer to field soil amendment, thus do not apply to zero amendment level.

TABLE 2 (Continued)

EFFECT OF TWO TREATMENTS AND FOUR VOLUMES OF EWA 53-A SOIL AMENDMENTS ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

Second greenhouse experiment:

Means of parameters of growth in pots

+ Total volume adjusted to 500 ml with fumigated potting soil to compensate for different amendment volumes.

‡ Presence or absence of Dexon in soil (added at 0.25 g/pot in two applications after planting of sugarcane).

TABLE 3

EFFECT OF TWO TREATMENTS AND FOUR VOLUMES OF EWA 53-A SOIL AMENDMENTS ON ROOT GROWTH AND ROOT DISEASE OF TWO SUGARCANE VARIETIES

Second greenhouse experiment:

means of parameters of disease and growth in pots

Amendment Treatment*	Amendment Volume+ (ml)	Culture Condition‡	Growth and Disease Parameters			
			37-1933 Root Dry Wt. (g)	50-7209 Root Dry Wt. (g)	37-1933 Root Disease** (Rating)	50-7209 Root Disease (Rating)
--	0	- Dexon	17.42	40.74	0.8	0.8
--	0	+ Dexon	26.98	45.12	0.8	0.4
Fumigated	50	- Dexon	19.72	36.50	1.6	1.0
Non-fumigated	50	- Dexon	3.10	12.70	4.8	3.0
Non-fumigated	50	+ Dexon	17.50	36.00	1.0	0.6
Fumigated	200	- Dexon	14.64	29.28	2.0	1.0
Non-fumigated	200	- Dexon	2.74	14.50	4.0	3.2
Non-fumigated	200	+ Dexon	16.82	29.44	1.0	1.0
Fumigated	800	- Dexon	22.94	37.92	1.4	0.6
Non-fumigated	800	- Dexon	4.24	13.90	4.0	2.8
Non-fumigated	800	+ Dexon	15.24	26.34	1.6	0.4

* Defined in Table 2.

+ Defined in Table 2.

TABLE 3 (Continued)

EFFECT OF TWO TREATMENTS AND FOUR VOLUMES OF EWA 53-A SOIL AMENDMENTS ON ROOT GROWTH AND ROOT DISEASE OF TWO SUGARCANE VARIETIES

Second greenhouse experiment:

means of parameters of disease and growth in pots

+ Defined in Table 2.

**Relative severity of root infection, on a scale from 0 = healthy to 10 = complete decay.

TABLE 4

COMPARISONS OF POPULATIONS OF TWO *PYTHIUM* SPECIES IN THREE PLANTATION SOILS ESTIMATED BY THREE METHODS OF MEASUREMENT

Means of measurements as propagules per gram

Pythium Species	Plantation Soil	Method of Measurement		
		Leaf Bait	Pineapple Root Bait	Soil Plating
P. graminicola	Ewa 53-A	--	0.13	0.23
	Ewa 53-B	--	--	0.13
	Waialua 17	--	0.02	0.01
P. acanthicum	Ewa 53-A	16.00	--	0.25
	Ewa 53-B	3.21	--	0.07
	Waialua 17	0.45	--	0.00

TABLE 5

EFFECT OF SUGARCANE VARIETY AND GREENHOUSE SOIL AMENDMENTS AND CULTURE CONDITION ON POPULATION OF *P. ACANTHICUM* IN GREENHOUSE SOIL

Leaf bait method of population estimation in soils from second greenhouse experiment

Sugarcane Variety in Soil	Amendment Treatment*	Amendment Volume+ (ml)	Culture Condition‡	*P. acanthicum* Population (Propagules/g)**
37-1933	--	0	- Dexon	0.02
"	Non-fumigated	50	- Dexon	6.04
"	Non-fumigated	200	- Dexon	4.06
"	Fumigated	800	- Dexon	0.59
"	Non-fumigated	800	- Dexon	1.86
"	Non-fumigated	800	+ Dexon	0.59
50-7209	--	0	- Dexon	0.07
"	Non-fumigated	50	- Dexon	9.04
"	Non-fumigated	200	- Dexon	0.94
"	Fumigated	800	- Dexon	0.21
"	Non-fumigated	800	- Dexon	4.86
"	Non-fumigated	800	+ Dexon	0.42

* Defined in Table 2.

+ Defined in Table 2.

‡ Defined in Table 2.

**Means of 3 replicates.

TABLE 6

RELATIVE SENSITIVITY TO ANTIBIOTICS IN VITRO OF *P. GRAMINICOLA* ISOLATED FROM THREE HOSTS EXPOSED TO TWO SOILS

Mean ratios of linear growth in the absence of added antibiotics to linear growth in the presence of added antibiotics

P. graminicola Isolate		Antibiotic Sensitivity		
Host Plant	Soil	Streptomycin*	Aureomycin+	Actidione++
Pineapple	Ewa 53-A	1.42	2.23	4.76
	Waialua 17	--	1.79	3.94
Sugarcane variety 37-1933	Ewa 53-A	1.53	2.36	3.29
	Waialua 17	--	2.07	4.18
Sugarcane variety 50-7209	Ewa 53-A	--	2.56	2.60
	Waialua 17	--	1.92	3.38

* 100 μg/ml in potato dextrose agar.

\+ 5 μg/ml in potato dextrose agar.

++ 5 μg/ml in potato dextrose agar.

TABLE 7

COMPARISON OF POPULATIONS OF ORGANISMS ANTAGONISTIC TOWARD *P. GRAMINICOLA* IN THREE FIELD SOILS

Mean propagules x 10^4/g detected by the "agar layer method"*

Field Soil Sampled	Fungal Antagonists	Actinomycete Antagonists
Ewa 53-A	0.3	3.0
	1.4+	
Ewa 53-B	2.2+	
Waialua 17	5.7	30.0
	4.4+	

* Koike 1967*b*

+ Sampled April 1968; all others sampled June 1966.

TABLE 8

EFFECT OF *P. ACANTHICUM* INOCULATION IN GREENHOUSE SOIL ON POPULATIONS OF ORGANISMS ANTAGONISTIC TOWARD *P. GRAMINICOLA*

Mean propagules x 10^4/g detected by the "agar layer method"* in second inoculation experiment

Greenhouse Soil+	Total Fungi	Antagonistic Fungi	Antagonists/Total
Non-inoculated control	25.2	1.5	0.060
P. acanthicum inoculated	24.2	0.2	0.008

* Koike 1967*b*

+ Samples taken from pots in which 37-1933 was grown.

TABLE 9

EFFECT OF CULTURE MEDIUM ON INTERACTIONS BETWEEN TWO *PYTHIUM* SPECIES AND ISOLATES OF SOIL FUNGI IN THREE GENERA

Numbers of pairings showing given reactions*

Medium	*Pythium* Species	Soil Fungus	Reaction+ Negative	Neutral	Positive
PDA‡	*P. graminicola*	*Aspergillus*	4	0	0
		Penicillium	8	0	0
		Trichoderma	5	1	0
	P. acanthicum	*Aspergillus*	4	0	0
		Penicillium	5	3	0
		Trichoderma	2	4	0
WA**	*P. graminicola*	*Aspergillus*	4	0	0
		Penicillium	5	3	0
		Trichoderma	2	4	0
	P. acanthicum	*Aspergillus*	0	0	4
		Penicillium	0	0	8
		Trichoderma	2#	0	4

* Following simultaneous inoculations of *Pythium* species and soil fungus.

\+ Negative = *Pythium* species inhibited by soil fungus; Neutral = no reaction between two fungi; Positive = soil fungus parasitized by *Pythium* species.

TABLE 9 (Continued)

EFFECT OF CULTURE MEDIUM ON INTERACTIONS BETWEEN TWO PYTHIUM SPECIES AND ISOLATES OF SOIL FUNGI IN THREE GENERA

Numbers of pairings showing given reactions*

+
+ potato dextrose agar

**water agar

The two Trichoderma isolates not parasitized by P. acanthicum were identified as T. lignorum (Tode) Harz; the other isolates were identified as T. viride Persoon.

TABLE 10

EFFECT OF TWO GREENHOUSE CULTURE CONDITIONS AND INOCULATION WITH THREE *P. GRAMINICOLA* STRAINS ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

First inoculation experiment: means of parameters of growth in pots

P. graminicola Strain*	Culture Condition+	37-1933 Shoot Dry Wt. (g)	50-7209 Shoot Dry Wt. (g)	37-1933 Tillers (No.)	50-7209 Tillers (No.)
--‡	- Dexon	103.0	97.2	5.0	3.7
--	+ Dexon	107.7	118.7	5.3	3.8
Ewa "fast"	- Dexon	15.7	16.7	0.5	0.2
"	+ Dexon	112.5	127.7	4.2	5.0
Ewa "slow"	- Dexon	16.8	15.0	0.6	0.0
Waialua	- Dexon	17.5	23.0	0.2	0.3
"	+ Dexon	113.7	125.7	4.3	4.5

* Related groups of isolates obtained from plantations as indicated;

Ewa "fast" = rapid-growing group of isolates recovered from 37-1933 exposed to Ewa 53-A soil;

Ewa "slow" = slow-growing group of isolates recovered from 37-1933 exposed to Ewa 53-A soil;

Waialua = group of isolates recovered from 50-7209 exposed to Waialua 17 soil.

\+ Presence or absence of Dexon in soil (added at 0.25 g/pot in two applications after planting of sugarcane).

TABLE 10 (Continued)

EFFECT OF TWO GREENHOUSE CULTURE CONDITIONS AND INOCULATION WITH THREE *P. GRAMINICOLA* STRAINS ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

First inoculation experiment: means of parameters of growth in pots

‡ Non-inoculated control.

TABLE 10 (Continued)

EFFECT OF TWO GREENHOUSE CULTURE CONDITIONS AND INOCULATION WITH THREE *P. GRAMINICOLA* STRAINS ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

First inoculation experiment: means of parameters of growth in pots

‡ Non-inoculated control.

TABLE 11

EFFECT OF INOCULATION WITH *P. ACANTHICUM* AND TWO STRAINS OF *P. GRAMINICOLA* ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

Second inoculation experiment: means of parameters of growth in pots

Inoculum*	37-1933 Shoot Dry Wt. (g)	50-7209 Shoot Dry Wt. (g)	37-1933 Tillers (No.)	50-7209 Tillers (No.)	37-1933 Ratoons (No.)	50-7209 Ratoons (No.)
--+	36.2	66.4	3.4	5.8	4.8	11.8
37-1933 *P. graminicola*	10.0	18.4	1.0	1.6	2.8	5.0
50-7209 *P. graminicola*	11.0	15.2	0.6	2.2	4.0	3.6
P. acanthicum	37.0	77.4	3.6	5.2	4.4	8.2
P. acanthicum + 37-1933 *P. graminicola*	7.0	19.2	0.8	2.2	2.2	4.2
P. acanthicum + 50-7209 *P. graminicola*	13.6	12.0	0.8	0.6	2.2	3.2

* *P. graminicola* strains designated according to the variety from which they were isolated, and toward which they displayed specificity in water culture (37-1933 isolates originated in Ewa 53-A soil; 50-7209 isolates originated in Waialua 17 soil).

TABLE 11 (Continued)

EFFECT OF INOCULATION WITH *P. ACANTHICUM* AND TWO STRAINS OF *P. GRAMINICOLA* ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

Second inoculation experiment: means of parameters of growth in pots

+ Non-inoculated control.

‡ *P. acanthicum* isolated from Ewa 53-A soil.

TABLE 12

EFFECT OF INOCULATION WITH TWO SPECIES OF *PYTHIUM* ON SHOOT GROWTH OF THE SUGARCANE VARIETY 37-1933 IN NON-FUMIGATED SOIL

Third inoculation experiment: means of shoot dry weights in pots

Inoculum*	37-1933 Shoot Dry Wt. (g)
--+	9.27
P. graminicola	4.60
P. acanthicum	7.65
P. graminicola + *P. acanthicum*	4.03

* Both species originating from Ewa 53-A soil; *P. graminicola* isolated from roots of 37-1933.

\+ Non-inoculated control.

FIGURE 1

History of yields from sugarcane variety 37-1933 in Ewa Plantation fields 13 (0———0) and 53 (Δ———Δ) in comparison with average plantation yields (□———□). The biennial harvests from each field are plotted with the contemporary plantation harvests as tons of cane per acre. Data for the years 1958 and 1959 are omitted because of atypical conditions (drought imposed by labor strike).

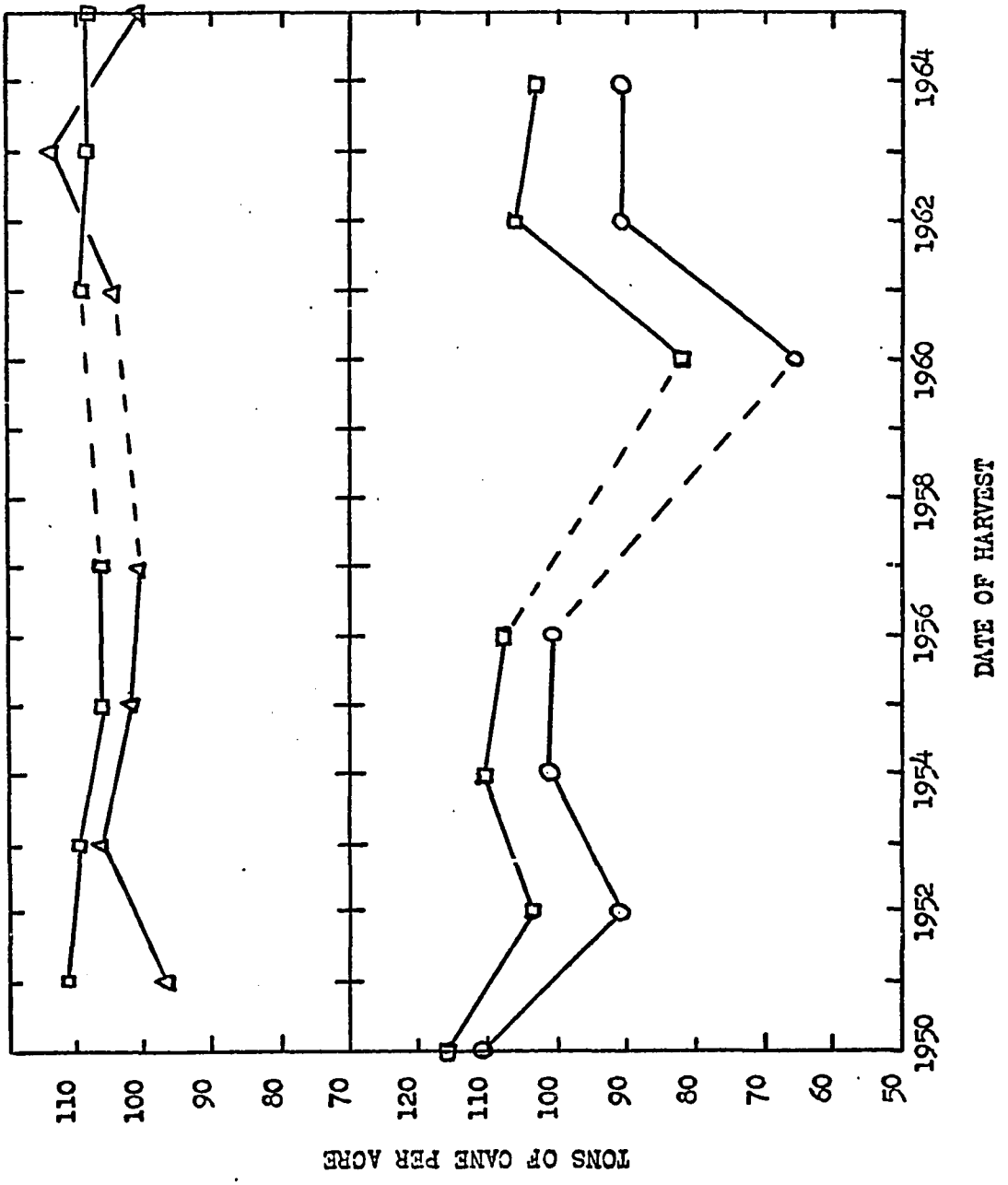
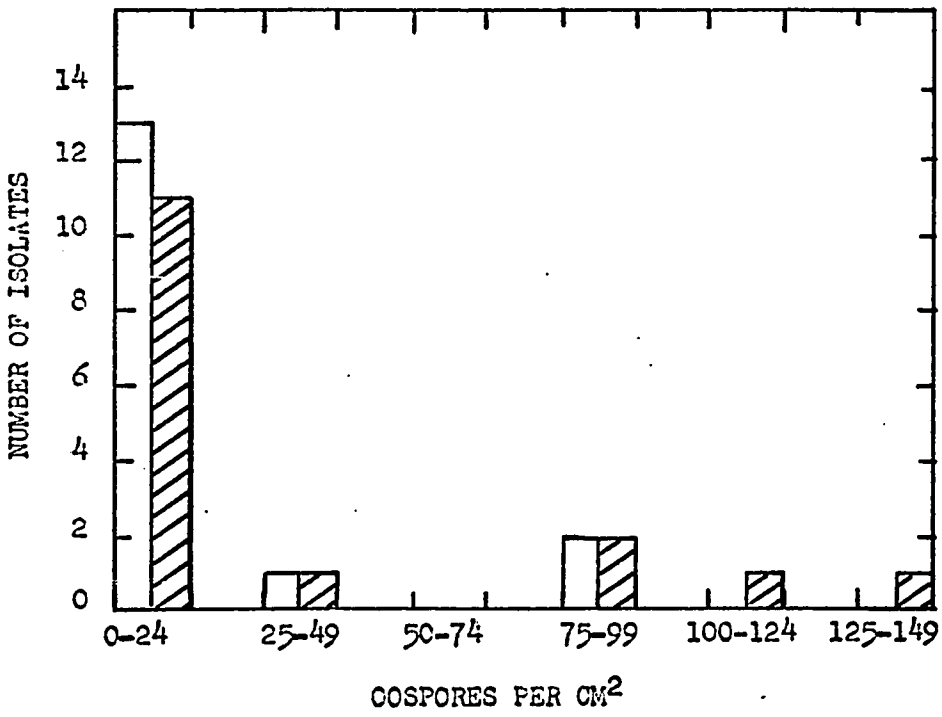

TONS OF CANE PER ACRE
110
100
90
80
70
120
110
100
90
80
70
60
50
1950
1952
1954
1956
1958
1960
1962
1964
DATE OF HARVEST

FIGURE 1

FIGURE 2

Comparison of sporulation of *P. graminicola* isolates from 37-1933 exposed to Ewa 53-A soil (open rectangles) and from 50-7209 exposed to Waialua 17 soil (cross-hatched rectangles) in plate cultures on water agar with carrot fragments. Frequency of sporulation level among isolates indicated on vertical axis.


NUMBER OF ISOLATES
14
12
10
8
6
4
2
0
0-24
25-49
50-74
75-99
100-124
125-149
COSPORES PER CM2

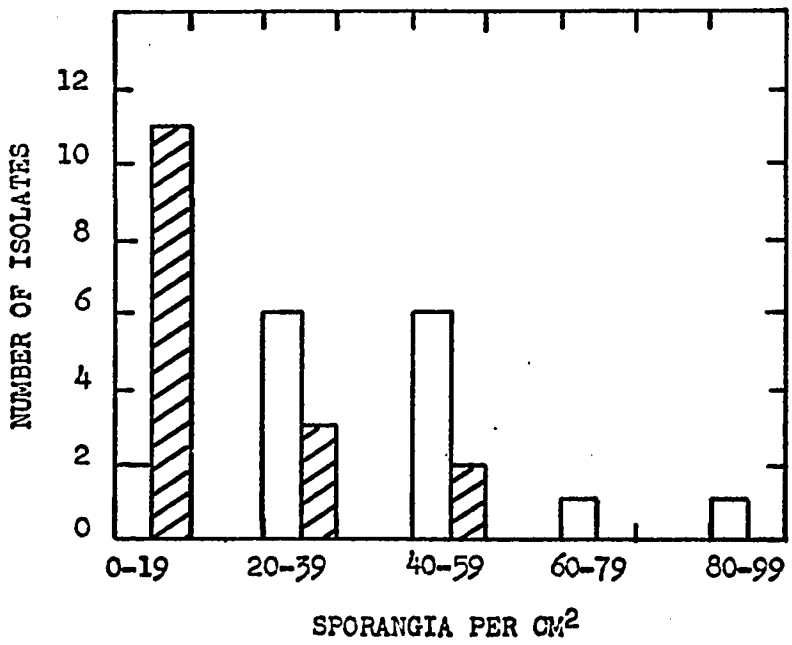

NUMBER OF ISOLATES
12
10
8
6
4
2
0
0-19
20-39
40-59
60-79
80-99
SPORANGIA PER CM2

FIGURE 2

FIGURE 3

Relative virulence of *P. graminicola* isolates from 37-1933 exposed to Ewa 53-A soil (open rectangles) and from 50-7209 exposed to Waialua 17 soil (cross-hatched rectangles) toward 37-1933 and 50-7209 in water culture. Frequency of root infection severity among isolates indicated on vertical axis. Root infection rated on a scale from 0 = healthy to 10 = complete decay.

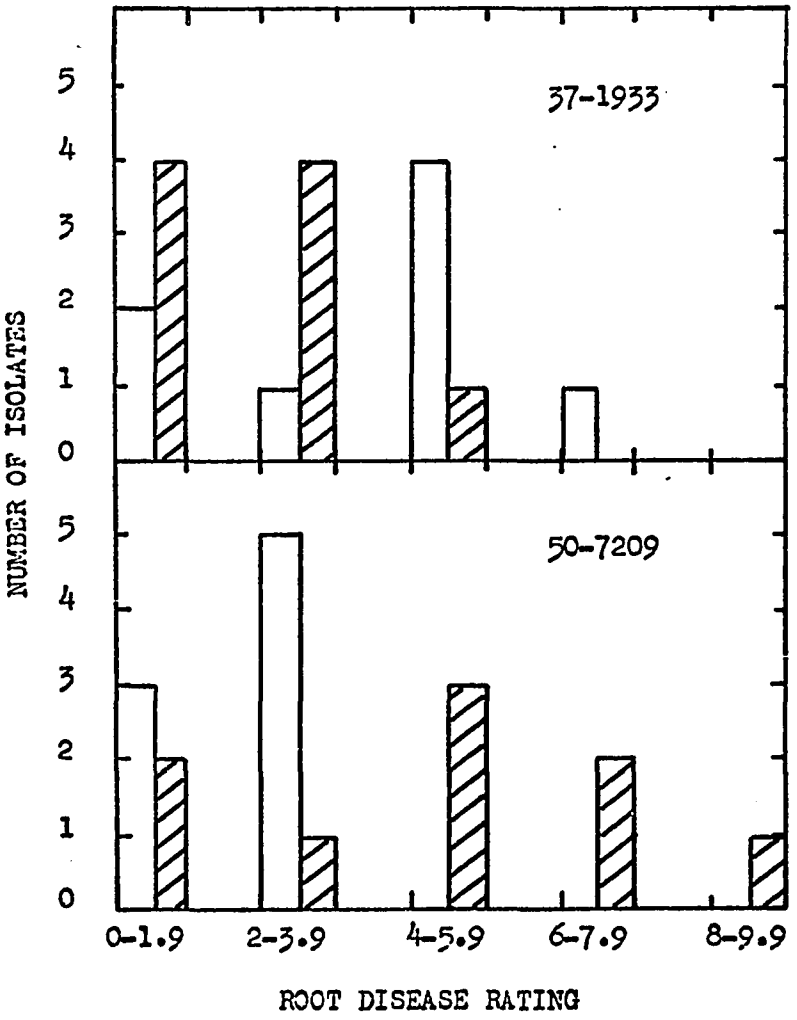

37-1933
50-7209
NUMBER OF ISOLATES
0
1
2
3
4
5
0-1.9
2-3.9
4-5.9
6-7.9
8-9.9
ROOT DISEASE RATING

FIGURE 3

PLATE 1

Fig. 1. - *Pythium acanthicum* oospore with antheridium. X 750.

Fig. 2. - *P. acanthicum* oospore with antheridium. X 750.

Fig. 3. - *P. acanthicum* oospore with sporangium associated with it. X 1000.

Fig. 4. - *P. acanthicum* sporangium. X 750.

Fig. 5. - *Pythium graminicola* oospore. X 1000.

Fig. 6. - *P. graminicola* sporangium. X 500.

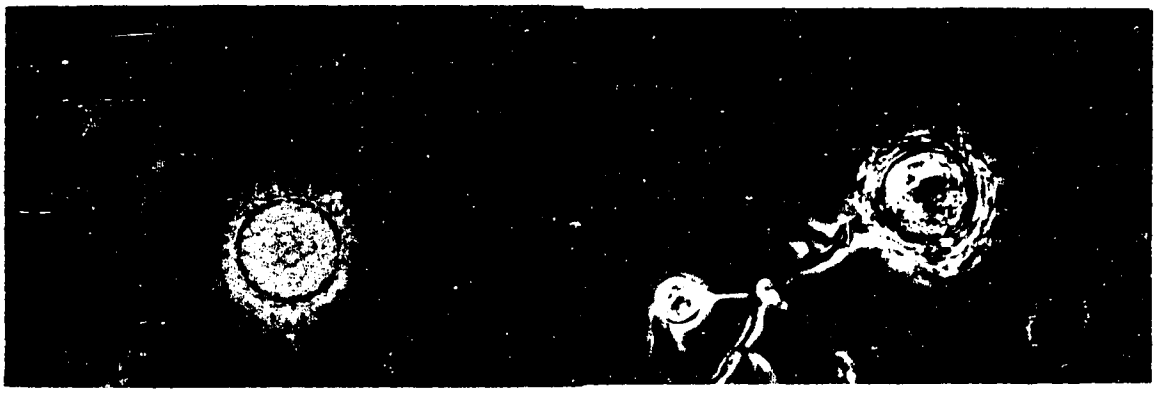


Fig. 1.

Fig. 2.

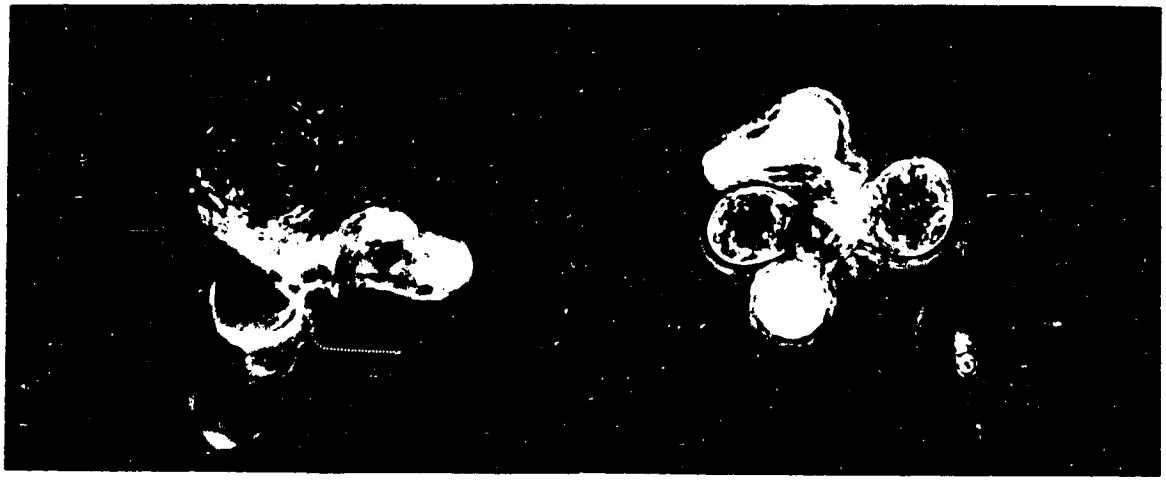


Fig. 3.

Fig. 4.

Fig. 5.

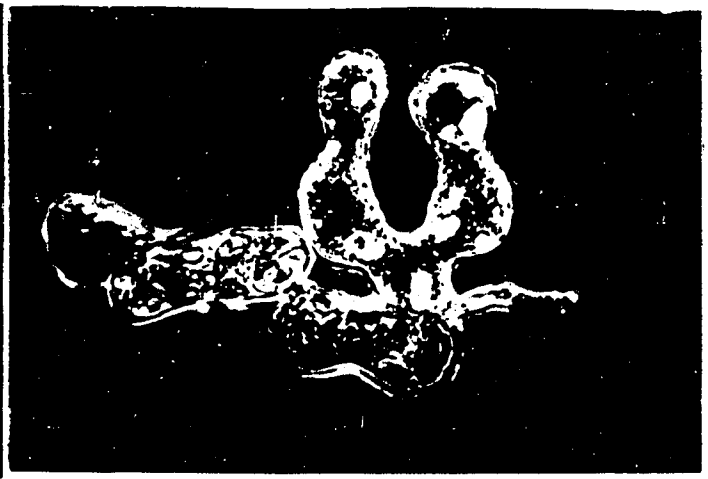


Fig. 6.

PLATE 2

Fig. 1. - *Pythium graminicola* hyphae showing curling response in vicinity of colony of *Penicillium* sp. in potato dextrose agar. X 500.

Fig. 2. - *P. graminicola* hypha growing into a colony of *Penicillium* sp. in water agar. The *Pythium* hypha is entering the field in the lower left corner, with a branch leading off to the right. X 500.

Fig. 1.

Fig. 2.

PLATE 3

Fig. 1. - *Pythium acanthicum* parasitizing *Trichoderma* sp. X 750.

Fig. 2. - *P. acanthicum* parasitizing *Trichoderma* sp. Note constriction of *Pythium* hypha passing through septum. X 750.

Fig. 3. - *P. acanthicum* parasitizing *Trichoderma* sp. Note short hyphal peg pushing out through wall of host hypha. X 1000.

Fig. 4. - *P. acanthicum* parasitizing *Penicillium* sp. X 1000.

Fig. 1.

Fig. 2.

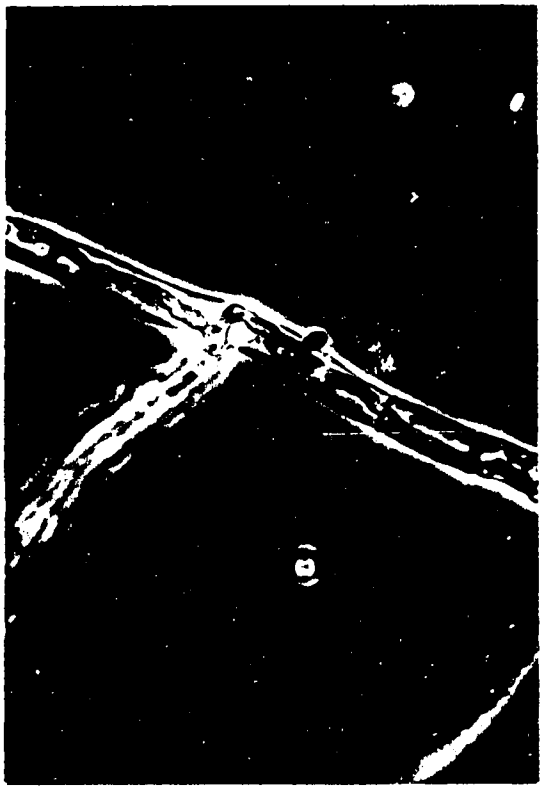


Fig. 3.

Fig. 4.

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CHAPTER III

CONCLUSIONS

The inhibition of the sugarcane variety 37-1933 by a specific, soil-associated factor from Ewa 53 is a condition similar to those recognized as "specific replant diseases." Such a condition in sugarcane cultivation is considered yield decline.

The higher population of *P. graminicola*, and the greater damage resulting from root rot of the sugarcane variety 37-1933 by this *Pythium* species in Ewa 53-A (poor growth area) soil than in Waialua 17 soil (in which 37-1933 has never been grown) suggests that *P. graminicola* can be identified with the growth inhibiting factor. The factor can be removed by fumigation and controlled by "Dexon" application, as shown in greenhouse experiments involving amendments of Ewa 53-A soil. The physiological variability of *P. graminicola* isolates, and the varietal specificity displayed by some, demonstrates that there are physiological strains of this species in the soil. There was no evidence for the preponderance in the soil population of a physiological strain having high specificity toward a given variety.

The lower population of organisms antagonistic toward *P. graminicola* in Ewa 53-A soil than in Waialua 17 soil may have permitted the development of the higher population of *P. graminicola* in the former soil than in the latter. Although the antagonists may not be completely inhibitory to *P. graminicola* in the soil environment, there may be inhibition of sporulation of the *Pythium* species in the presence of colonies of some antagonists.

The higher population of P. acanthicum in Ewa 53-A soil than in Waialua 17 soil may have reduced the population of certain other soil fungi in the former soil in comparison with the latter, by parasitic destruction of mycelium and sporophores. Fungi antagonistic toward P. graminicola could be included among those affected.

The nature of the soil microflora in Ewa 53 may have evolved under the influence of 17 years of cultivation of the sugarcane variety 37-1933, or may have been characteristic of this field at the first planting of this variety. Although the relative populations of the Pythium species and Pythium antagonists may account for the damage to roots by Pythium invasion, they do not account for the varietal specificity of the soil factor. The two varieties compared in this study may influence the soil microflora differently, so that the populations in Ewa 53 soil respond in a manner favorable or unfavorable for the growth of a given variety, or a truly variety specific component of the P. graminicola population may be present which attacks sugarcane variety 37-1933 more severely than sugarcane variety 50-7209.

APPENDIX I

SUPPLEMENTARY EXPERIMENTS

(a) Preliminary Characterization of Growth Inhibiting Factor in Ewa 53 Soil

(i) Varietal Reaction to Treatments of Soil Amendments

The sugarcane varieties 37-1933 and 50-7209 were grown in fumigated composted soil in Mitscherlich pots according to the procedure established at the Hawaiian Sugar Planters' Association Experiment Station (Koike and Warner 1965; Koike 1967a; Warner, in press). Field soils from Ewa 53-A and Waialua 17 were added in 500 ml volumes to 4.5 l. of potting soil in three treatments, one involving steam sterilized field soil and two involving non-sterilized field soil. Two pots of non-amended soil were also prepared for each variety. "Dexon" was applied to one pot of non-amended soil and to one treatment with non-sterilized field soil amendment for each variety. The treatments were replicated three times.

Few of the differences between treatment means in this experiment were significant, and the relative effects that appeared to occur were not consistent (Table I). The presence of non-sterilized Ewa 53-A soil without "Dexon" application seemed to cause lower shoot dry weight of 37-1933 than occurred in the absence of this soil, or in its presence with "Dexon". There were significant differences in tiller production between treatments which reflected these same effects. The shoot dry weight of the variety 50-7209 was significantly reduced in the presence of Waialua 17 and Ewa 53-A soils, indicating its sensitivity to factors

in both of these soils.

Although the results from this experiment were not consistent, an occurrence of growth inhibition by a factor in the soil was indicated by those differences which were significant. The conditions of the experiment may have obscured treatment effects, since growth of several plants was impaired by drought injury. The more vigorous plants, such as those in fumigated soil without amendments, tended to transpire more water than less vigorous plants, so that the potting soil dried out faster, and wilting developed more rapidly. The small number of replicates tended to make only large differences detectable significantly, even when they were consistent.

(ii) Response of 37-1933 to Levels of Field Soil Amendments

The variety 37-1933 was grown in fumigated composted soil to which non-sterilized Ewa 53-A soil was added in volumes of 50, 100, 200, 300, 400, and 500 ml. This variety was also grown in non-amended soil. The experiment was replicated three times.

There were no significant differences between treatments, but there appeared to be an equivalent reduction in growth caused by all amendment volumes above 50 ml (Table II). The small number of replicates tended to make significant effects of treatment less detectable than could have been possible with greater replication.

(b) Pythium Studies

(i) Pythium Population in Ewa Field 13

Soil from Ewa field 13 was assayed for Pythium population by the leaf bait method in conjunction with population studies of Ewa 53-A and Waialua 17. An average of 22.5 propagules of P. acanthicum per gram were present in this soil according to the "most probable number" determination.

(ii) Sensitivity of Pythium Isolates to Antibiotics

P. graminicola isolates from sugarcane and pineapple roots were exposed to antibiotics applied to filter paper discs placed on the surface of the medium (Sherwood, Falco and DeBeer 1944). The relative sensitivities to aureomycin and actidione were rated according to diameters of zones of clearing around the discs in the growth of Pythium from a layer of 2% water agar into a layer of potato dextrose agar (Koike 1967b).

There were variations in sensitivity of isolates to antibiotics as detected by this method (Table III), but few differences were statistically significant. The sensitivities to certain antibiotics were in general agreement with those displayed in the direct incorporation method (described under "Experimental Methods"), but the paper disc method appeared to give more variable results than were obtained by direct incorporation. Inconsistencies in agar thickness and in absorptive capacity of the paper discs may account for this variability.

(iii) P. graminicola Requirements for Sporulation

Bermuda grass, Cynodon dactylon Pers., segments were utilized for induction of P. graminicola sporulation by Koike (personal communication), and in preliminary tests were effective for several isolates. However, the failure of many isolates to sporulate with Bermuda grass indicated that the nature or level of sterols in this material were not adequate for all isolates (Klemmer and Lenney 1965) (Table IV). This was especially true for those isolates recovered from root lesions of 37-1933 exposed to Ewa 53-A soil.

The carrot fragments recommended by Johann (1928) satisfied the requirements for oospore production for many isolates, although several from Ewa 53-A and Waialua 17 failed to respond to this amendment also. There were several isolates which had failed to produce oospores with Bermuda grass, but which did produce oospores with the carrot fragments. The sporangial morphology of such isolates was similar to that of isolates which produced oospores with both types of plant material, and was also similar to that of isolates which failed to produce oospores with either sterol source.

Wheat germ oil was introduced into the water agar medium in which isolates were cultured to determine the suitability of this material for induction of oospores, based on such an effect reported by Klemmer and Lenney (1965). A uniform emulsion of this material in the aqueous medium was difficult to maintain, and consistent responses of the Pythium cultures to the oil were not obtained. Further development of the technique for successful incorporation of this material into the medium was not attempted.

Calcium chloride was introduced into water agar media, with and without the wheat germ oil amendment described above, to determine the effectiveness of the calcium ion in promoting sporulation (Yang and Mitchell 1964). A concentration of Ca^{++} of 27 μg/ml failed to modify the response of P. graminicola in the presence or absence of wheat germ oil.

(c) Pathogenicity Studies

A preliminary study of susceptibility of sugarcane roots to Pythium infection was conducted utilizing rooted "lalas" (side shoots) in aerated water, as described by Adair (1968b). Infection of roots occurred when pure cultures of Pythium were introduced into the water in weighted gauze bags. An assortment of recent and older sugarcane varieties displayed varying degrees of resistance to P. graminicola isolates from Ewa 53-A and Waialua 17 and to one P. acanthicum isolate from Ewa 53-A (Table V). An interaction between the two Pythium species was also tested for, in terms of pathogenicity toward sugarcane roots, but no significant effect was noted.

There were practical difficulties in this method which required the utilization of the rooted stalk cuttings described under "Experimental Methods." The principal problems involved in the use of rooted "lalas" were the limited supply of such material ("lalas" are produced spontaneously by some varieties, but often develop only after topping or tasseling of the main stalk of other varieties), the susceptibility of the "lalas" to drought injury and leaf infection during the rooting period, and the danger of introduced contaminants on the

leaf sheaths. Treatment of the stumps with phenyl-mercuric acetate prevented contamination, but also prevented infection by Pythium.

(d) Mycoparasitism by P. acanthicum on Non-antagonists

In preliminary studies of the effect of P. acanthicum on other fungi, parasitism against Helminthosporium and Fusarium was observed in water agar. An invasion of the host hyphae was made by P. acanthicum, with death of the conidiophores of Helminthosporium often evident. Infection of a Trichoderma species was also observed, but more extensive studies of this relationship were made in the primary investigation.

TABLE I. EFFECT OF TWO DIFFERENT FIELD SOIL AMENDMENTS, TWO AMENDMENT TREATMENTS, AND TWO GREENHOUSE CULTURE CONDITIONS ON SHOOT GROWTH OF TWO SUGARCANE VARIETIES

First preliminary experiment: means of parameters of growth in pots

Field Soil*	Amendment Treatment+	Culture Condition‡ + or - Dexon	Growth Parameters			
			37-1933 Shoot Dry Wt. (g)	50-7209 Shoot Dry Wt. (g)	37-1933 Tillers (No.)	50-7209 Tillers (No.)
--**	--	-	203	278	8.3	8.3
--	--	+	201	255	8.3	7.0
Ewa 53-A	Sterilized	-	185	261	7.7	6.7
Ewa 53-A	Nonsterilized	-	190	222	5.3	7.7
Ewa 53-A	Nonsterilized	+	255	255	8.0	7.7
Waialua 17	Sterilized	-	202	273	8.3	9.7
Waialua 17	Nonsterilized	-	209	233	5.7	8.0
Waialua 17	Nonsterilized	+	188	237	9.0	8.3

* Mixed with methyl bromide fumigated potting soil at 500 ml/5 l. total volume.
+ "Sterilized" and "Nonsterilized" refer to field soil amendment, thus do not apply to nonamended controls.
‡ Presence or absence of Dexon in soil (added to 0.25 g/pot in two applications after planting of sugarcane.
**Nonamended controls.

TABLE II. EFFECT OF SEVEN EWA 53-A SOIL AMENDMENT VOLUMES ON SHOOT GROWTH OF THE SUGARCANE VARIETY 37-1933

Second preliminary experiment: means of parameters of growth in pots

Amendment Volume* (ml)	Growth Parameters	
	Shoot Dry Wt. (g)	Tillers (No.)
0	167	8.0
50	103	2.7
100	90	4.3
200	91	3.3
300	94	3.3
400	79	2.3
500	84	5.0

* Total volume adjusted to 500 ml with fumigated potting soil to compensate for different amendment volumes.

TABLE III. RELATIVE SENSITIVITY TO ANTIBIOTICS *IN VITRO* OF *P. GRAMINICOLA* ISOLATED FROM THREE HOSTS EXPOSED TO TWO SOILS

Paper disc method: means of diameters of zones of clearing for two replicates with the isolates from each host exposed to each soil, measured in mm

P. graminicola Isolate			Antibiotic Concentration			
Host Plant	Soil	Number Tested	Aureomycin 1 mg/ml	Aureomycin 5 mg/ml	Actidione 1 mg/ml	Actidione 5 mg/ml
Pineapple	Ewa 53-A	2	23.0	33.0	35.5	40.5
	Waialua 17	2	23.0	29.0	35.0	40.0
Sugarcane variety 37-1933	Ewa 53-A	5	26.8	34.4	32.6	38.2
	Waialua 17	2	26.0	33.0	29.0	36.5
Sugarcane variety 50-7209	Ewa 53-A	3	27.7	35.3	32.7	39.0
	Waialua 17	5	31.4	38.4	32.4	38.4

TABLE IV. RESPONSE TO TWO SPOROGENIC MATERIALS OF *P. GRAMINICOLA* ISOLATED FROM THREE HOSTS EXPOSED TO TWO SOILS

Percentage of isolates producing oospores

P. graminicola Isolates		Sporogenic Material	
Host Plant	Soil	Bermuda Grass*	Carrot Fragments+
Pineapple	Ewa 53-A	50	--
	Waialua 17	40	67
Sugarcane variety 37-1933	Ewa 53-A	0	40
	Waialua 17	25	--
Sugarcane variety 50-7209	Ewa 53-A	33	--
	Waialua 17	0	50

* Boiled segments placed on surface of water agar culture.

\+ Sterilized with water agar medium used as substrate.

TABLE V. RELATIVE VIRULENCE OF TWO *P. GRAMINICOLA* ISOLATES AND ONE *P. ACANTHICUM* ISOLATE TOWARD THIRTEEN SUGARCANE VARIETIES IN WATER CULTURE

Root rot ratings*

Sugarcane Variety	Inoculum			
	None+	Waialua 17 *P. graminicola*	Ewa 53-A *P. graminicola*	Ewa 53-A *P. acanthicum*
54-5882	1	6	6	4
56-192	0	6	6	6
56-5840	1	0	2	6
57-1444	1	8	10	6
57-6466	0	5	8	0
57-7118	0	3	2	2
58-1543	1	2	8	2
58-1564	1	4	4	2
58-1921	1	2	4	2
58-5513	0	2	7	2
59-3182	1	4	4	2
60-2315	0	2	6	6
61-3054	0	6	8	6

* Relative severity of root infection, on a scale from 0 = healthy to 10 = complete decay.

+ Control.

APPENDIX II

DISCUSSION OF TECHNIQUES

(a) Greenhouse Experiments

(i) Soil Samples

Although the variety 37-1933 was not growing in the Ewa 53 soil at the time of sampling, residual factors affecting this variety were expected to be present, based on Carpenter's experience with the variety Lahaina on the island of Hawaii (Carpenter 1928). The collection of soil samples in the vicinity of RA 37-1933 was intended to provide samples of soil microflora that were still under an influence similar to that of 37-1933. The RA 37-1933 is distinguishable from 37-1933 morphologically (Chi, personal communication), and physiological differences may also be present. These differences might make erroneous the assumption of a similar influence on soil microflora described above. However, the varietal specificity of the soil factors toward 37-1933 evident in the second greenhouse experiment indicated that a residual effect toward this variety was still present in this soil in any case.

(ii) Addition of Amendments and Inocula

The procedure utilized in the incorporation of amendments and inocula to potting soil were intended to allow distribution of the material throughout the soil in each given pot. The mechanical process of mixing field soil into the potting soil was judged adequate to accomplish this, but inocula were added to the surface layer of soil, which could be questioned as a means of providing distribution of the

introduced organism. However, the striking inhibition of growth and extensive root damage of sugarcane plants in pots treated in this way indicated that such a method of inoculation was adequate. It was assumed that the mechanical forces of the percolation of water through the soil would carry zoospores of *Pythium* to lower levels in the pot, allowing colonization of the entire soil mass by this organism.

(iii) Application of "Dexon"

The application of "Dexon" was accomplished in large volumes of water, as recommended by Raabe and Hurlimann (1965), to provide through penetration of the soil in pots treated with this fungicide.

(iv) Recording Results of Greenhouse Experiments

The choice of parameters for measurements of experimental results was based on characteristics that could be related to potential yield in the field, and on aspects of particular interest in a given test. The dry weight of shoot growth was measured in all experiments as a direct indication of yield. Tiller production was also measured in all experiments, although this was to some extent proportional to shoot dry weight. Production of sugarcane in the field depends to some extent on the production of tillers, since the final harvest includes a number of stalks (the "stool") which have developed from the original planting of a seed piece. Although the tillers counted in the greenhouse experiments were fairly small, their presence indicated the potential size of the stool that could develop from the sugarcane plant growing under the conditions present in the pot.

Root dry weight and root rot were observed in one test because of the interest in the mechanism of action of the inhibitory factor that was being investigated. The high correlation between root dry weight and shoot dry weight (r = 0.83 **) indicated the interrelationship between the growth of these two portions of the plant, although the question of which portion affected the other was not apparent from this information alone. The high root rot rating associated with low root dry weight indicated that the root development was poor because of loss of tissue caused by pathogenesis; thus shoot development was poor because of impaired root function.

(b) Pythium Studies

(i) Pure Culture Isolation

The method of hyphal tip isolation of *Pythium* cultures obtained from the various soils and plant materials was chosen because mycelial growth of *Pythium* in water agar was suitable for this type of procedure, and oospores and zoospores were unsuitable for isolation. The wide-ranging, sparse habit of growth on water agar allowed the selection of single hyphal tips without interference with other hyphal branches that could have represented different colonies. Oospore isolation was actually accomplished by the screening and plating method, but the recovery and "purification" of colonies arising from this source were also accomplished by hyphal tip isolation. The leaf and pineapple root baits were presumably colonized by zoospores, but selection of colonies arising from invaded baits was again accomplished by hyphal tip isolation. Zoospores, presumably suspended in the water in which soil

was placed, could have been planted out on a selective medium, but the probability of bacterial contamination would have required further selection by hyphal tip isolation in this case also.

(ii) Soil Plating Counts

The estimates of *Pythium* populations by the direct screening and plating method were based on samples of the colonies present on the original plates. Samples from small numbers of colonies were considered to be representative, so that the estimation of total numbers of each species on the original plate was fairly efficient. However, when large numbers of colonies were present, many of which appeared to be non-pythiaceous, the sample tended to be biased, in that colonies appearing to be pythiaceous were selected. The percentage identified as *Pythium* was higher among the selected colonies than among the total number of colonies on the plate. Thus, a calculation based on the proportion selected resulted in a higher count of pythiaceous fungi for the plate than actually existed. For this reason, figures based on the actual numbers of colonies identified as *Pythium* were considered along with the upward biased estimates for soil samples giving rise to the large numbers of non-pythiaceous fungi.

(iii) Analysis of Pineapple Root Infection Data

The occurrence of infection of pineapple roots exposed to dilutions of soil was recorded in the dilution end-point estimation of *Pythium* populations in soil samples. Similar results were expressed by Tsao (1960) and Koike (personal communication) in relative terms, such as "disease potential index," which was the reciprocal of the average

highest dilution at which Pythium infection was evident. An attempt was made to interpret the data from the pineapple root baiting method in terms of propagules per gram. The "most probable number" method (Buchanan and Fulmer 1928) was not suitable for data based on the two-fold dilutions utilized in this method, but these data could be evaluated by Stevens' density estimation method, as described by Fisher and Yates (1948). The data obtained in a population assay of Ewa 53-A and Waialua 17 soils were analyzed by both methods. The "disease potential index" for each soil was approximately 40 times the number of propagules per gram (Table I).

(iv) Leaf-Bait Population Assay

The leaf-bait procedure for isolating Pythium described by Srinivasan (in press) involved exposure of five strips of boiled sugarcane leaf to one gram of soil in 100 ml of water. The proportion of the baits colonized by Pythium was considered related to the level of Pythium in the soil, but apparently no estimation of propagules per gram was made by this method. This leaf-bait method and a pineapple root bait method utilized by Koike (personal communication) involved exposure of baiting material to relatively undisturbed soil in water; apparently these assays were based on colonization of bait by the motile zoospores of Pythium.

Preliminary tests were made using the leaf bait procedure to test its suitability for a "most probable number" type of population assay. Comparisons were made of water and sterile soil as diluents, and efficiency of two-fold and ten-fold dilutions were compared. The ability of P. graminicola and P. acanthicum to colonize the boiled leaf

substrate in water was tested by exposing the leaf baits to pure cultures of the Pythium species.

A test involving exposure of leaf baits to soil diluted at ten-fold levels with water resulted in a "diluting-out" of the Pythium (Table II) indicating the possibility of a dilution end-point estimation of population as described by Tsao (1960).

The dilution of soil samples in water involved dispensing suspensions of the soil, which may have resulted in deposition of suspended oospores on the leaf strips. A test was conducted in which the same soil was diluted at the same levels with sterile soil and with sterile water. The water diluent apparently provided for greater consistency of dilution, and resulted in generally higher estimates of population (Table III). The higher counts with the water diluent may have been due to colonization by suspended oospores in addition to the zoospores that apparently are produced under these conditions, or due to greater accessibility of zoosporangia to the water when the soil aggregates were disintegrated in the dilution procedure.

A test of the effect of dilution ratios on estimated population was made using water as the diluent. Two-fold and ten-fold dilutions resulted in approximately the same apparent population, as indicated in Table IV. Because of the large amount of material required in two-fold dilutions, and the difficulty in interpreting the results according to the "most probable number" method, ten-fold dilutions were considered acceptable for population assays by the leaf bait method.

The failure to recover P. graminicola from the leaf baits made this method unsuitable for estimation of the population of this species.

A water agar culture of a sporulating strain of P. graminicola introduced into water colonized the leaf baits rapidly, but a non-sporulating strain was not recovered under similar conditions. P. acanthicum rapidly colonized the leaf baits under these conditions. The failure of P. graminicola in soil to colonize the leaf baits may relate to competition between the Pythium species.

(v) Sensitivity of Pythium to Antibiotics

The determination of sensitivity of P. graminicola isolates to antibiotics incorporated into the medium required a separate petri dish for each isolate antibiotic combination, limiting the number of combinations possible at one time. This method did allow for a measurable control growth rate, however, which provided a basis of comparing the sensitivity of isolates.

The paper disc method allowed the exposure of one culture to several antibiotic treatments on the same agar disc, providing more direct comparison between treatments, but not providing a measurable control of growth without antibiotic. Appropriate concentrations of antibiotic solutions for application in paper discs were determined in preliminary tests utilizing antibiotics at several levels.

Streptomycin was applied in filter paper discs at 10,000, 5,000, 1,000, 500, and 100 μg/ml, and aureomycin was applied at 1,000, 500, 100, 50, and 10 μg/ml, in 0.1 ml volumes. None of the streptomycin applications caused a zone of clearing around the disc, but aureomycin at 1,000 and 500 μg/ml caused clear zones to five and three mm beyond the edge of the discs. The 0.1 ml volume was excessive for the absorptive capacity of the discs.

Streptomycin was tested in the filter paper discs again at 100,000 μg/ml in an 0.05 ml volume, and actidione was tested at 1,000, 500, 100, 50, 10, 5, and 1 μg/ml in 0.05 ml volumes. No inhibition of Pythium was caused by the streptomycin, but actidione caused zones of clearing at the 1,000 and 500 μg/ml levels.

(vi) Sporogenic Medium

The choice of the sporogenic medium was based on the need for an effective, reproducible, and readily available substrate which apparently satisfied the requirements for sporulation of a portion of P. graminicola isolates. As discussed in Appendix A, a wide range of natural products apparently contained the sterol required for sporogenesis, but the materials containing the highest levels (oils) were difficult to incorporate in the level and distribution desired. The carrot fragments were utilized in uniform batches of media, and were introduced in suspension so that uniform quantities were present in each container of medium.

(c) Antagonism Studies

(i) Population of Antagonists

The antagonistic fungi detected by the agar layer method represented genera which produce large numbers of spores, so that their population levels are probably only relative. This method probably favored only certain groups of soil fungi, so that the presence of other antagonistic organisms in the soil important as potential suppressors of Pythium may not have been detected. There is also some question as to the effectiveness in the soil of organisms appearing

antagonistic in plate cultures (Huber and Watson 1966). However, the importance of many of the genera isolated as aggressive colonizers in the soil suggests that their populations may be relevent in terms of the level of antagonists toward Pythium (Barton 1960).

The assays for actinomycetes were performed on fresh soil samples, although some soils assayed for antagonistic fungi had been stored for several weeks at a low temperature. It was assumed that few relative population changes had occurred under these conditions, although this possibility is evidently present (Agate and Bhat 1967).

(d) Pathogenicity Tests

(i) Inoculation in Soil

The utilization of fumigated soil as a medium when inoculating sugarcane with pure cultures of Pythium tended to reduce competition and antagonism by other soil organisms during the colonization of the soil by Pythium. The relevance of infection by Pythium under these conditions to the importance of this organism in unfumigated field soil may be questionable. One attempt to resolve this problem was the inoculation of sugarcane with Pythium species in unfumigated soil in the third inoculation experiment. The relative increase in development of lesions on roots inoculated in this manner, and the marked effect on shoot growth, indicated that pathogenesis by P. graminicola is expressed even in soil containing a native microflora. Another aspect which indicated that the expression of pathogenicity in fumigated soil may be related to potential conditions in field soil is the rapid recontamination of fumigated soil by air-borne spores of many of the fungi which

appear to be antagonistic toward P. graminicola. This phenomenon was apparent in studies on antagonists present in soil from the second inoculation experiment, and has been reported frequently, as reviewed by Kreutzer (1965).

A greater resistance of actinomycetes and certain bacteria than of fungi to methyl bromide fumigation has also been reported, as summarized by Kreutzer (1965). There is thus the possibility that the eradication by methyl bromide was not complete, although no fungi were recovered from fumigated soil which was plated out in preliminary tests. Pathogenicity expressed by P. graminicola introduced into the fumigated soil might have been reduced if antagonists present at the time of inoculation were inhibiting the Pythium. The ability of P. graminicola to attack sugarcane in the fumigated potting soils indicated that any surviving antagonists were not limiting pathogenesis by this species.

TABLE I. COMPARISON OF TWO METHODS OF ANALYSIS OF *PYTHIUM* POPULATION ASSAY DATA

Mean values based on infection of pineapple roots in soil dilutions

Field Soil Sampled	Method of Analysis	
	"Disease Potential Index"	Propagules/gm*
Ewa 53-A	5.0	0.132
Waialua 17	1.0	0.024

*Calculated according to Stevens' density estimation method.

TABLE II. EFFECT OF DILUTION ON RECOVERY OF *PYTHIUM* BY LEAF BAITS

Number of leaf baits colonized by *Pythium* out of five exposed to each dilution of Ewa 53-A soil in water

Replicate	Dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	5	2	0	0	0	0	0
2	5	4	2	0	0	0	0

TABLE III. EFFECT OF DILUENT ON RECOVERY OF *PYTHIUM* BY LEAF BAITS

Propagules/gm* detected in Ewa 53-A soil according to colonization of leaf baits exposed to ten-fold dilutions

Replicate	Diluent	
	Sterile Soil	Water
1	3.57	4.38
2	0.36	4.38

*Calculated according to Stevens' density estimation method.

TABLE IV. EFFECT OF DILUTION RATIO ON RECOVERY OF *PYTHIUM* BY LEAF BAITS

Mean propagules/gm* detected in Ewa 53-A soil according to colonization of leaf baits exposed to dilutions in water

Dilution Ratio	Mean Propagules/gm
2	6.80
10	4.38

*Calculated according to Stevens' density estimation method.

APPENDIX III

SUPPLEMENTARY TABLES

The supplementary tables include a tabular presentation of the data represented in Figs. 1, 2, and 3, and a detailed presentation of the data summarized in Table 6.

TABLE I. HISTORY OF YIELDS FROM SUGARCANE VARIETY 37-1933 IN TWO EWA PLANTATION FIELDS IN COMPARISON WITH AVERAGE PLANTATION YIELDS*

Tons of sugarcane per acre per harvest

Date	% 37-1933 in Total	Area Harvested		
		Total Plantation	Field 13	Field 53
1950	99.1	115.34*	110.77	
1951	99.5	110.67		97.46
1952	99.0	104.16	92.70	
1953	99.7	109.78		107.37
1954	99.0	111.41	102.88	
1955	99.4	107.03		103.28
1956	99.0	108.03	101.32	
1957	97.7	107.49		100.54
1958	--‡	--	--	
1959	--	--		--
1960	97.0	82.55	65.02	
1961	84.4	109.33		105.69
1962	85.9	106.53	92.50	
1963	70.0	109.75		113.74
1964	34.9	103.42	91.99	
1965	21.7	108.28		103.00

* Average for plantation calculated as total yield/total acreage.
+ Yearly figures represent harvest from approximately half the plantation, since each field is harvested biennially.
‡ Yields for 1958 and 1959 were atypical because of labor strike.

TABLE II. COMPARISON OF SPORULATION OF *P. GRAMINICOLA* ISOLATES FROM TWO HOSTS EXPOSED TO TWO SOILS

Mean spores/cm^2 in plate cultures on water agar with carrot fragments*

P. graminicola Isolates			
37-1933 Exposed to Ewa 53-A		50-7209 Exposed to Waialua 17	
Oospores	Sporangia	Oospores	Sporangia
0	12	0	0
0	17	0	0
0	21	0	0
0	22	0	0
0	23	0	8
0	30	0	14
0	36	0	56
0	39	0	59
0	40	3	4
0	57	5	4
0	58	20	38
0	59	49	4
0	84	82	32
44	63	95	25
83	50	100	6
83	51	152	6

* Triplicate counts of spores in measured sectors of petri dish; cultures grown in two replicates.

TABLE III. RELATIVE VIRULENCE OF *P. GRAMINICOLA* ISOLATES TOWARD TWO SUGARCANE VARIETIES IN WATER CULTURE

Root disease ratings* after exposure to inoculum

P. graminicola Isolate		Sugarcane Variety	
Host Plant	Soil	37-1933	50-7209
Sugarcane variety 37-1933	Ewa 53-A	6.0	2.5
		5.0	1.5
		5.0	2.5
		4.5	2.0
		4.5	3.0
		3.5	3.0
		0.5	0.0
		0.0	0.0
Sugarcane variety 50-7209	Waialua 17	5.0	6.0
		3.5	5.0
		2.5	4.5
		2.5	8.0
		2.0	4.5
		1.0	3.5
		1.0	6.0
		0.0	0.0
		0.0	0.0

* Relative severity of root infection, on a scale from 0 = healthy to 10 = complete decay.

TABLE IV. RELATIVE SENSITIVITY TO ANTIBIOTICS *IN VITRO* OF *P. RAMINICOLA* ISOLATED FROM THREE HOSTS EXPOSED TO TWO SOILS

Ratios of linear growth in the absence of added antibiotics to linear growth in the presence of added antibiotics*

P. graminicola Isolate		Antibiotic Sensitivity		
Host Plant	Soil	Streptomycin+	Aureomycin‡	Actidione**
Pineapple	Ewa 53-A	1.37	2.19	4.66
		1.47	2.28	4.85
	Waialua 17	--	1.73	4.06
		--	1.85	3.82
Sugarcane variety 37-1933	Ewa 53-A	1.53	2.29	3.36
		1.53	2.18	3.58
		1.49	2.42	3.21
		1.57	2.55	3.11
		--	2.38	3.24
	Waialua 17	--	2.00	4.30
		--	2.14	4.07
Sugarcane variety 50-7209	Ewa 53-A	--	2.48	2.68
		--	2.64	2.51
		--	1.69	3.91
		--	1.89	3.65
		--	2.18	2.90
		--	1.99	3.27

* Means of three replicates for the isolates from each host exposed to each soil.
+ 100 μg/ml in potato dextrose agar.
‡ 5 μg/ml in potato dextrose agar.
** 5 μg/ml in potato dextrose agar.

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