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**FINAL REPORT
OF
RESEARCH PROJECT**

Micro III (231)

P1 - 76/4 - ICI - H20/0311

ASSOCIATION OF BACTERIA IN THE
ETIOLOGY OF ROOT (WILT) DISEASE

1976 - 1983.



CENTRAL PLANTATION CROPS RESEARCH INSTITUTE

KERALA, INDIA.

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FINAL REPORT

1. Institute Code No. : Micro III (231)
2. I.C.A.R. Code No. : P1-76/4-ICI-H₂O/0311
3. Name and Address of Research Institute/Centre
Central Plantation Crops Research Institute,
Regional Station, Kayangulam
4. Project Title : Association of bacteria in the etiology of root
(wilt) disease.
5. Name and Designation of Project Leader : Mathew George,
Scientist S-1 (Microbiologist)
6. Name(s) and Designation(s) of Project Associates including
Project Leader and work to be done:

Sl. No.	Name and Designation	Time spent	Work done
1.	Mathew George, Scientist S-1 (Microbiology)	51 man months	Bacterial isolation and identification; Extraction, characterisation and purification of toxin from <u>Enterobacter</u> isolates. Serological studies with crude toxin. Serological comparison of coconut <u>Enterobacter</u> with standard <u>enterobacter</u> strains. Antibiotic assay against Coconut <u>Enterobacter</u> . Pathogenicity experiment in field tanks
2.	N.P. Jayasankar, Joint Director	6 man months	Control trials
3.	V.P. Potty, Scientist S-1 (Microbiology)	2 man months	Preliminary pathogenicity experiments

7. Location of Research Project with complete address (Division/Section/Sub-Centre)

Microbiology Section, CPCRI Regional Station, Kayangulam,
Kerala, PIN 690-533

8. Date of start : 1976
9. Date of termination : 1983
10. (a) Objectives (Not more than 150 words)

The symptom picture and nature of the disease suggests that the disease is of a complex nature. The main object of the project is to elucidate the role if any of bacteria in the etiology of the disease. The initial phase of the work will therefore consist of establishing the exclusive association of phytopathogenic bacteria in the root (wilt) affected palms. Samples will be drawn from outside the Institute farm to avoid the influence of soil treatments and consequent inequilibrium in microfauna/microflora. The associated bacterial isolates will be screened for their ability to produce toxic principles. Pathogenicity trials will be conducted on coconut seedlings in field tubs. Control trials with antibiotics both in vitro and in the field will also be initiated.

- (b) Practical Utility including background information (Not more than 150 words)

Root (wilt) disease extends at present to an area of 0.25 million hectares. The estimated reduction in yield due to this disease, is to the tune of 40 to 60 per cent causing an annual economic loss of Rs.300 million. Available information pose this malady to be of complex nature. The understanding of the role of bacteria, if any, in the disease complex will lead to the effective control measures for checking the incidence and spread of the disease.

11. Technical Programme:

1. Association of bacteria in wilt affected palms in the disease affected region and pockets of fresh occurrence of the disease in the transitional regions will be looked into. Surface sterilised root bits and aqueous extracts of the stelar region of freshly collected roots will be cultured in a range of media including enriched ones.

2. Pure cultures showing potential characteristics of pathogenicity will be cultivated and the ability of the culture filtrate and bacterial extracts will be assayed for the presence of any toxic principles.
3. Pathogenicity trials will be carried out by administering mass cultures of the bacteria and concentrates of toxic principles to potted seedlings.
4. The associated bacteria will be screened in vitro against chemicals followed by large scale field trials.

12. Final report on the project:

1. Introduction

A pioneering attempt in a bacteriological angle by Srivastava et al., (1969) on the root (wilt) disease of coconut revealed the vascular streaming movement of bacteria typical of a root pathogen. They have tentatively identified this bacterium as Pseudomonas sp. A probable involvement of a toxin with this disease was brought out by the development of epinasty and complete bending of tomato seedlings placed in the root sap collected from the diseased palm in contrast to those placed in healthy root sap and distilled water (Ramadasan, 1967). Prompted by these observations, the isolations made from stelar portions of roots for nearly three years have revealed the consistent association of Enterobacter cloacae with root (wilt) disease. Though this bacterium had not been considered a conventional phytopathogen, since then species of Enterobacter have been implicated in plant diseases (Mohrbach and Pfiffer, 1976; Hopkins and Elmstorm, 1977). Efforts to screen the bacterium for potential phytopathogenic properties by separation of toxin from cell-free culture filtrate, characterisation and antigenic nature of crude toxin, Purification and determination of molecular weight of toxin, Antibiotic assay against the bacterium, Field trials with oxytetracycline, and details of pathogenicity experiment are reported hereunder

2. Materials and Methods

2.1 Collection of samples:

Studies relating to the association of bacteria in the root (wilt) disease of coconut were conducted, mainly depending on the root samples collected from a disease-prevalent area of Kayangulam and a disease-free tract of Kasaragod situated 500 km apart in the state of Kerala. Coconut palms in the disease-prevalent area were categorised into apparently healthy and disease-affected palms on the basis of foliar symptoms (George and Radha, 1973). Samples of roots were collected from 75 palms of different disease intensities and 107 palms cultivated in the disease-free region. Subsequently samples of roots representing premonsoon and postmonsoon samples were also collected from palms cultivated in the areas bordering the contiguous strip of root (wilt) disease affected tract twice a year during May-June and October-November. Three palms were selected from each plot comprising apparently healthy, disease-early and disease-advanced categories. There were four plots randomly distributed in and around Irinjalakuda representing the northern border. These plots were located at Canal basin, Chelur, Pullur and Ala near Cranganoor. Two such plots selected in the southern border were located at Edagramam and Ottasekaramangalam. Similarly samples were also collected before the onset of rains and after the rains during May-June and October-November, respectively, from two mildly infected gardens in Mynagappally and Shertallai in the disease tract. Samples from plots at Thazhava, Sooranadu, Sooranadu north and Parakadavu represented Mynagappally area and collections from plots at Kavumkal, Kalavoor, Shertallai town and Porumpara comprised of samples from Shertallai area (Fig.2).

2.2 Method of sampling of coconut roots

Coconut roots were traced to the growing tip by opening 1/8 sector of the base of palm to a distance of 1 m and to a depth of 0.5 m. Apparently healthy roots, free of any external

injury were severed and transferred to polythene bags and stored in an ice box for transportation to the laboratory.

2.3 Bacterial isolation

Roots were thoroughly washed in a tap water and excess moisture adhering to the roots was removed by pressing in absorbant cotton. Individual root was surface-sterilised by flaming after immersion in alcohol. Routine precautions were observed for the maintenance of aseptic condition. Root length confined to 7-15 cm portion from the growing tips was dissected out in a sterile petri plate and bits of stelar portions were scooped off. Half of the scooped off stelar bits were transferred to another sterile petri plate and crushed in physiological saline. Loopful of this suspension was streaked on to three plates containing surface-dried nutrient agar, potato sucrose agar and coconut root extract agar medium respectively. The rest of the stelar bits was plated as such on these media. Plates were incubated at room temperature (30 to 35°C). Petri plates containing nutrient agar and potato sucrose agar were observed for bacterial growth after 24 to 48 h. Bacterial growth on coconut root extract agar medium was observed only after three to four days. The encountered bacterial colonies were streaked on nutrient agar twice to separate out individual colonies. Pure cultures of the isolates were maintained in nutrient agar slants for characterisation.

2.4 Identification of bacteria encountered

Based on morphological and physiological properties such as cultural characters, cell morphology and staining traits, spore test, Kovac's oxidase test, acid production from glucose, reduction of Nitrate and soft rot of potato tissue, the bacteria were classified up to generic level by a process of elimination at each step, according to the key of Bradbury (1970).

2.5 Standardisation of incubation period

Triplicate sets of 25 ml nutrient broth in 100 ml conical flasks were inoculated with uniform quantity of coconut Enterobacter

At 24 h interval, one set of flasks was withdrawn for assay over a period of 25 days. The broth was centrifuged to remove bacterial cells. Cell-free culture filtrate from each flask was treated with five times its volume of ethyl alcohol. The precipitate was sedimented overnight and dried in an incubator at 45°C. The average weight of dried precipitate on each day was plotted against the period of incubation.

2.6 Growth of *Enterobacter* in coconut root extract broth

Twenty five 250 ml conical flask, each containing 50 ml of coconut root extract broth were inoculated with one ml of 20 h bacterial broth culture diluted to 1:1000 with sterile coconut root extract broth. The inoculated flasks were shaken on a rotary shaker. Two flasks were removed on each day for enumeration, till the fifth day and then onwards on alternate days. The dilutions were plated on coconut root extract agar and counts were taken on fourth day of incubation.

2.7 Influence of coconut root extract on production of polysaccharide by *Enterobacter*

Triplicate sets of 25 ml nutrient broth containing different concentrations of coconut root extract in 100 ml conical flasks were inoculated with uniform quantity of coconut *Enterobacter*. On incubating for 13 days, the broth was centrifuged to remove bacterial cells. The culture fluid was treated with five times its volume of ethyl alcohol. The precipitate was sedimented overnight, dried in an incubator at 45°C and weighed.

2.8 Nutritional factors influencing the production of polysaccharide by *Enterobacter*

A series of experiments involving 0.2% broth of 10 sugars in basal salt solution, in 1% beef extract, in 50% coconut root extract and in 50% coconut root extract with 1% beef extract were conducted. Sugars tested were the monosaccharides, D-glucose, D-galactose, D-arabinose, dulcitol, D-ribose and D-xylose; the disaccharides, lactose, trehalose and sucrose; and the trisaccharide, Raffinose.

2.9 Production of polysaccharide by standard strains of
Enterobacter cloacae

Twenty four strains of Enterobacter cloacae from six culture collections were grown in triplicate sets of nutrient broth and CRE broth for 13 days. The broth was centrifuged to remove bacterial cells. Cell-free culture filtrate from each flask was treated with five times its volume of ethyl alcohol. The precipitate was sedimented overnight and dried in an incubator at 45°C. The average weight of dried precipitate elicited from each strain was calculated and compared with that of coconut Enterobacter. The accession numbers and the collection centres of tested strains of Enterobacter cloacae are the following:

I. Robert Koch Institute, Berlin, Germany

- | | | | |
|--------|---------|--------|---------|
| 1. Nr. | 978/70 | 2. Nr. | 921/70 |
| 3. Nr. | 1084/70 | 4. Nr. | 1100/70 |
| 5. Nr. | 1542/70 | 6. Nr. | 90/71 |

II. University College, Dublin, Ireland

IMD-116 (ATCC - 23355)

III. Ministry of Health, Ontario, Canada

1. 3401
2. Sc. 311
3. Sc. 312

IV. Pasteur Institute, Paris, France

1. 6022
2. 6085
3. 681

V. National Collection of Typecultures, London, U.K.

- | | | | |
|-----------|-------|-----------|------|
| 1. NCTC - | 10005 | 2. NCTC - | 5920 |
| 3. NCTC - | 6022 | 4. NCTC - | 8348 |
| 5. NCTC - | 9394 | 6. NCTC - | 9529 |
| 7. NCTC - | 9711 | 8. NCTC - | 9844 |

VI. U.S. Department of Agriculture, U.S.A.

1. NRRL-B-126
2. NRRL-B-169
3. NRRL-B-180

2.10 Preparation of toxin material

The crude toxin was extracted from 13-day-old culture of coconut Enterobacter grown in a broth containing 15 g glucose, 5 g calcium carbonate and 10 g yeast extract dialysate/litre (Spencer and Gorin, 1961). The culture fluid was centrifuged at 20,000 g for 30 min and the pellet was discarded. The supernatant liquid was concentrated to approximately one-fifth its original volume by freeze-drying. The preparation was treated with five to six volumes of 95% ethanol and allowed to precipitate overnight. The supernatant was decanted and the precipitate was dried over calcium chloride in a vacuum desiccator. The dried material was dispensed in distilled water to form approximately 2% suspension and centrifuged to discard the sediment. The clear supernatant was passed through a 20 cm x 2.5 cm column of Dowex-50 (200-400 mesh H⁺ form) and a 20 cm x 2.5 cm column of Dowex-1 (200-400 mesh, formate form) to remove the charged compounds. The effluent was collected and freeze dried in a lyophilizer. The crude toxin preparation was stored in a refrigerator.

2.11 Collection of root sap polysaccharide

Root sap was collected from disease-free and disease-affected palms according to the method of Davies (1964). A main root emanating from the bole of the coconut was cut obliquely about a metre from the bole. The distal end, severed from the tree was connected by a short rubber tube to a vertical glass tube of 0.5 cm diameter. The whole assembly was made water-tight and distilled water was injected through the rubber tube. The level of water was marked in the glass tube. The glass tube was plugged with cotton and covered with an inverted test tube. On the next day excess water was sucked out by a hypodermic syringe. On subsequent days root sap collected in the glass tube was drawn out from the rubber tube using a syringe. The root sap was treated with five to six volumes of 95% ethanol and allowed to precipitate. Aqueous solution of this precipitate also was passed through Dowex columns and the effluent was freeze dried to yield the toxin material.

2.12 Toxin effect on plant cuttings

Biological assay was carried out using 2,000 ppm aqueous solution of the crude toxin. Tomato plants having four or five leaves were cut at the base of the stem (above root zone) and placed in three ml of aqueous solution of the crude toxin in five ml test tubes for a period of 30 h. Three replications were maintained with control plants kept in distilled water. The biological activity of the crude toxin was assessed by the time required for the tomato cutting to begin wilting at the margin of the primary leaf (Strobel, 1967).

2.13 Revival of the wilted plants

Ten tomato cuttings were similarly treated with the crude toxin at 2,000 ppm concentration. After four h, five of the treated cuttings were transferred to distilled water. After 24 h the remaining five cuttings were also shifted to distilled water and observed for the revival of the wilted plants.

2.14 Effect of toxin on germination of seeds

To study the effect of the crude toxin on germination, seeds of seven different plant species viz. Crotalaria striata, DC., Crotalaria juncea, Linn., Nicotiana tabacum, Linn., Cyamopsis tetragonoloba, Taub., Lycopersicum esculentum, Miller., Amarantus caudatus, Linn., and Cleome viscosa, Linn. were thoroughly washed in tap water followed by sterile water. Fifty seeds of each species were soaked in 1% aqueous solution of the crude toxin overnight. Seeds soaked in distilled water served as the control. These soaked seeds were placed on sterile moist filter paper in petriplates and incubated for three to six days at room temperature (30-35°C). The rate of germination was observed.

2.15 Serological studies with coconut Enterobacter toxin Preparation of antiserum

Antiserum to Enterobacter crude toxin was prepared according to Hamilton and Ball (1966) and Rai and Strobel (1969 b). Normal sera were collected from rabbits that were selected for

immunisation. Ten mg crude toxin was dissolved in two ml of phosphate buffer saline (pH 7.0). This was emulsified with an equal volume of Freund's complete adjuvant. Two ml of emulsified toxin was injected into the thigh muscles of the animal. The same procedure was followed to inject the animals seven days later. A couple of weeks after the last injection the rabbits were bled by a small cut in the marginal vein of the ear. After clotting of the blood, the serum was collected and centrifuged at 3,000 rpm for 20 min and stored in five ml vials at -10°C for further tests.

Precipitin test

A drop of antiserum and a drop of 1% toxin solution were transferred to a clean micro slide and mixed up. The slide was observed for clump formation.

Titre determination

The titre values of the antiserum and antigen were determined by preparing a two-fold dilution series of both reagents and carefully bringing them into contact in a capillary tube. These capillary tubes were dipped in paraffin oil to have a column of one cm paraffin oil at the bottom, in turn fixed and arranged on a plastocine base. The dilutions of the antiserum were made in 10% glycerol and the dilutions of the antigen were prepared in physiological saline. Formation of precipitation bands at the interjunction was observed for different dilutions.

Preparation of the plant extracts for immunodiffusion

Coconut leaflets showing clear symptoms of flaccidity from the middle whorls of crown, leaflets from the spindle and stelar portions of roots 15 cm away from the growing tip were collected from disease affected palms. Five grams of these material were ground in five ml phosphate buffer saline (pH 7.0) in a pestle and mortar and filtered through absorbant cotton. The preparations were centrifuged at 3,000 rpm for 10 min and the clear supernatants were used for the test. Corresponding plant extracts from healthy palms served as controls.

Immunodiffusion tests

Immunodiffusion was carried out with Enterobacter toxin antiserum according to Ouchterlony (1958), Hamilton (1961, 1964) and Hamilton and Ball (1966). Plain agar was prepared with Bacto purified agar at a concentration of 0.75% in phosphate buffer saline solution (pH 7.0) and mixed with 0.2% sodium azide as preservative. The agar was poured on microscope slides to form a thin layer and wells were cut using five mm template. Antiserum was added to central well. Crude toxin, extract from leaf, extract from stelar portions of root and root sap were added to the peripheral wells. Normal serum, normal plant extracts and phosphate buffer saline (pH 7.0) were used as controls.

Screening of polysaccharide from standard cultures of Enterobacter cloacae.

Polysaccharides extracted from 24 standard cultures of Enterobacter cloacae (500 mg each) were dispensed in distilled water to form approximately 2% suspension and centrifuged to discard the sediment. The clear supernatant was passed through a 20 cm x 2.5 cm column of Dowex-50 (200-400 mesh, H⁺ form) and a 20 cm x 2.5 cm column of Dowex-1 (200-400 mesh, formate form) to remove charged compounds. The effluent was collected and dried in a stream of air. This preparation was dissolved in phosphate buffer saline (pH 7.0) to form a 1% solution and tried against coconut Enterobacter toxin antiserum by immunodiffusion.

2.16 Purification of Coconut Enterobacter toxin

Column chromatography

The crude toxin was further purified by passing through a column of Sephadex G-200. Twenty five mg of the crude toxin was dissolved into two ml distilled water and introduced dropwise on to the top of an 18x1 cm column of Sephadex G-200. The effluents were collected in three ml fractions. The percentage of transmittance of these fractions were read using a Spectronic-20 calorimeter at 540 m μ

2.17 Molecular weight determination

The molecular weight was determined by column chromatograph using sephadex G-200 with the aid of the following formula (Granath, 1965).

$$\frac{V_c}{V_t} = 3.20 - 0.58 \log MW$$

Where V_c = The volume at which the sample is eluted from the column - the void volume of the column (The void volume was 10.5 ml as determined using blue dextran)

V_t = Total bed volume of the column, which was 32 ml

MW = Molecular weight

Assuming that the elution volume as 'X' ml, the molecular weight of the toxin had been derived as:

$$\frac{X-10.5}{32} = 3.20 - 0.58 \log MW$$

$$3.20 - \left\{ \frac{X-10.5}{32} \right\} = 0.58 \log MW$$

$$\text{Antilog of } \frac{3.20}{0.58} - \left\{ \frac{X-10.5}{32 \times 0.58} \right\} = MW$$

2.18 Comparison of toxicity

The coconut Enterobacter crude toxin, its highly purified fraction and the toxin separated from sap of disease affected palms were assayed at three concentrations viz; 100 ppm, 1,000 ppm, 2,000 ppm in aqueous solution, for their ability to cause wilting tomato cuttings. Tomato plants having four or five leaves were cut at collar region and placed in three ml of the respective toxin preparation in five ml test tubes for a period of 20 to 56 h. Three replications were maintained in each case with control plants kept in distilled water. The time taken to cause wilt symptoms were recorded in each case.

2.19 Acid hydrolysis

The procedure of Strobel (1967) was followed for acid hydrolysis of the crude toxin. Thirty mg of the crude toxin was acid hydrolysed by refluxing in 10 ml of 1 N H_2SO_4 for five to six h. The hydrolysate was cooled to room temperature and neutralised with excess of Na_2CO_3 till the effervescence ceased. The supernatant was centrifuged at 3,000 rpm and the clear neutralised hydrolysate was used for fractionation.

2.20 Chromatography

The neutralised hydrolysate was passed through Dowex-1 (200-400 mesh, formate form) 10 x 2 cm column and Dowex-50 (500-400 mesh H^+ form) 10 x 2 cm column, to get neutral fraction. The anionic and cationic fractions were collected by adding 6 N formic acid and 6 N hydrochloric acid to Dowex-1 and Dowex-50 respectively followed by elution with about 10 ml distilled water. Different fractions were dried using a stream of air and kept in a vacuum desiccator overnight and weighed.

2.21 Amino acid analysis by thin layer chromatography

Cleaned and dried TLC plates were coated with 0.25 mm thick layer of silica gel-G. The silica gel plates after air drying were activated by storage at 105°C for two h. The cationic fraction was spotted on silica gel along with standards. The compounds were separated using the following solvent systems.

1. n. Butanol : Acetic acid : Water (3:1:1 V/V)
2. Methanol : Pyridine : Water (80:40:20 V/V)
3. n. Butanol : Acetic acid : Water (12:3:5 V/V)

Comparatively better resolutions were obtained with n. Butanol : Acetic acid : Water (12:3:5 V/V), system. The spots were developed by spraying 0.3% ninhydrin in ethanol (Kai and Strobel, 1969).

2.22 Sugar analysis by Paper chromatography

The neutral fraction of the crude toxin was spotted on Whatman No.1 filter paper along with standards. The chromatograms were developed by using the following solvent systems.

1. n. Butanol : Acetic acid : Water (4:1:5 V/V)
2. Ethyl acetate : Pyridine : Water (8:2:1 V/V)
3. Isopropanol : Pyridine : Water (7:7:2 V/V)

Isopropanol : Pyridine : Water (7:7:2 V/V) showed better resolution of component sugar and the sugar was detected by passing the dried developed chromatogram rapidly through a reagent solution prepared by diluting 0.1 ml saturated aqueous silver nitrate solution to 20ml with acetone and adding water dropwise, with shaking until the silver nitrate which separated on addition of acetone has dissolved. The dry paper was then sprayed with a 0.5 N solution of sodium hydroxide in ethanol. Excess brown silver oxide was dissolved by immersing the chromatogram strip for a few minutes in 6 N ammonium hydroxide. Then the paper was washed for one h. in running water and dried in an oven (Trevelyan et al., 1950). Black or dark brown spots were compared with standard sugars developed on the paper.

2.23 Sugar acid analysis

The anionic fraction of the crude toxin was also spotted with standards on Whatman No.1 filter paper. The chromatograms were developed with the following systems:

1. n. Butanol : Acetic Acid : Water (4:1:5 V/V)
2. Ethyl acetate : Pyridine : Water (8:2:1 V/V)
3. Isopropanol : Pyridine : Water : Acetic acid (8:8:4:1 V/V)

Comparatively better resolutions were obtained with Isopropanol : Pyridine : Water : Acetic acid (8:8:4:1 V/V) system. Here also the compounds were detected on chromatograms by following the procedure given by Trevelyan et al., (1950) as described earlier.

2.24 Competitive saprophytic ability of Enterobacter cloacae

The survival and mutual coexistence of Enterobacter cloacae with the two fungi Cylindrocarpon effusum and Fusarium equiseti in methyl bromide fumigated soil was studied by incorporating the bacterium alone and in different combination with the fungi in 500 g fumigated soil contained in pots. There were five treatments with three replications. Fifty ml of bacterial suspension at 10^6 cells/ml concentration and 50 ml of each fungal spore suspension at 10^5 spores/ml concentration were added to respective pots. The treatments were as follows:

T ₁	-	Control
T ₂	-	<u>Enterobacter cloacae</u>
T ₃	-	<u>Enterobacter cloacae</u> + <u>Fusarium equiseti</u>
T ₄	-	<u>Enterobacter cloacae</u> + <u>Cylindrocarpon effusum</u>
T ₅	-	<u>Enterobacter cloacae</u> + <u>Fusarium equiseti</u> + <u>Cylindrocarpon effusum</u>

Twenty g soil samples were collected first after 12 h of incubation and subsequently on 15th day, 30th day, 60th day and 90th day and enumerated for bacteria and fungi on coconut root extract agar with actidione 30 mg/l and Rose bengal agar respectively. Fifty ml sterile water was added to each pot at 15 days interval to maintain soil moisture.

2.25 Preparation of bacterial inoculum

The coconut Enterobacter cloacae was mass cultured in coconut root extract broth contained in 10 l glass jars. The broth was aerated continuously with sterile air flowing out of an improvised bacterial filter attached to a fish tank aerator. The culture was incubated for five days to attain the required cell concentration, before inoculating to field tanks

2.26 Control Trials

Antibiotic assay against coconut Enterobacter

Antibiotic discs of 6.5 mm diameter (Bharath Laboratories, Bombay) were used for the initial screening. The sensitivity discs were placed aseptically on nutrient agar plates seeded with Enterobacter cloacae. Observations were recorded after 18 h by measuring the diameter of the zone of inhibition. Those exceeding 12-15 mm were considered positive. Promising chemicals after the initial screening were selected for further assay by the tube dilution technique (Rangaswami and Soumini Rajagopalan, 1973). A series of test tubes, each with 5 ml of nutrient broth and containing respectively 0.2, 0.4, 0.6, 0.8 and 1 $\mu\text{g/ml}$ of oxytetracycline and 1, 2, 3, 4, 5, 6 and 7 $\mu\text{g/ml}$ of streptomycin was prepared. To each tube was added 0.5 ml of a 20 h bacterial broth culture diluted to 1:1000 with sterile nutrient broth. The series of test tubes was incubated for 18 h at room temperature (30-35°C) under static condition. The growth of the bacterium in each tube was determined using a Spectronic-20 colorimeter at 600 m μ . The minimum inhibitory concentration of the antibiotic for Enterobacter cloacae was recorded taking the lowest concentration of the antibiotic that prevents the growth of the bacterium.

Application of Terramycin tree formulation

Twenty coconut palms exhibiting the primary symptoms of root (wilt) disease of the age group of 10-20 yrs were selected and the intensity of disease was indexed. The terramycin tree formulation containing three active ingredients of oxytetracycline in one/^{litre} of distilled water was injected under pressure to each of the ten experimental palms for the first time in 1977 (January, March, June and September) followed by a subsequent dose in May, 1979. The remaining palms were left untreated which served as controls. The disease condition of the treated and untreated palms were indexed during the years 1977-1981.

3. EXPERIMENTAL RESULTS

3.1 The association of bacteria

The four groups of palms namely disease early, disease advanced, apparently healthy and healthy, from which root samples have been collected for bacterial isolations are presented in Table-3 on the basis of association of bacteria. Root samples from disease advanced palms showed highest incidence of bacteria, whereas root samples from healthy trees presented least incidence.

3.2 Characterisation of bacterial isolates

The isolation of bacteria was continued for a period of three years, from the various categories of palms already dealt with under materials and methods. The purified cultures were subjected to the cultural, morphological and physiological tests required for classification employing the key of Bradbury. Such a process of elimination has resulted in obtaining 82 gram negative bacterial isolates, which fell in to four groups such as Enterobacter, Azotomonas/Acromonas, Erwinia and Pseudomonas. The detailed characteristics of these isolates are furnished in Table.4 and Fig.3. Of the 82 gram negative isolates 54 were classified as Enterobacter sp. 21 fell under Azotomonas/Acromonas group, five were Erwinia sp. and two Pseudomonas Sp. The percentage of incidence of each group of bacterium is shown in Fig.5.

3.3 Distribution in disease-free and disease-affected tract

Isolations were made from representative roots of 117 palms in the disease affected tract and 107 palms from the disease free tract. Out of the 69 gram negative isolates from disease affected tract, 52 were classified as Enterobacter sp., 11 as Azotomonas/Acromonas group, five as Erwinia and one as Pseudomonas. In the disease free tract out of the 13 isolates 10 belonged to Azotomonas/Acromonas group, two belong to Enterobacter and one to Pseudomonas.

3.4 Occurrence of Enterobacter

The preponderance of Enterobacter was evident as it amounted to 65.85% of the total gram negative bacterial isolates. Its association with root (wilt) disease was revealed as 96% of Enterobacter isolates were derived from the disease tract. Competitive occurrence of Enterobacter in roots of palms of different stages of root (wilt) disease is expressed in Table-6.

3.5 Confirmation of identity of coconut Enterobacter

Six representative strains of coconut Enterobacter were sent to Commonwealth Mycological Institute, Kew Surrey, England for identification. These isolates were classified as Enterobacter cloacae (Jordan) Hornaeche and Edwards and the strains were accessioned in to the International collection of phytopathogenic bacteria, Davis, California, as ICBP 3661, ICBP 3662, ICBP 3663, ICBP 3664, ICBP 3665, ICBP 3666 strains and Commonwealth Mycological Institute as B.6250, B.6253, B.6254, B.6255 and B.6256 strains respectively.

3.6 Strainal variation of coconut Enterobacter

The colonies of the bacterium were off white in nutrient agar and light yellow in yeast extract (1%) glucose (2%) calcium carbonate (2%) agar. The isolates were gram negative and motile and utilised glucose fermentatively. Other cultural characteristics were a negative response to methyl red, urease and indole tests and positive one to Voges Proskauer, production of hydrogen sulphide and nitrate reductase tests. Among the biochemical characteristics the bacterium did not attack starch, polygalacturonic acid or pectin, but hydrolysed gelatin. Arabinose, sucrose, mannitol and salicin were utilised with the production of acid and gas. Arginine was decarboxylated, but γ -amino valeramide was not produced from lysine. However, variations among the strains were noticed in the case of a few morphological and biochemical characteristics viz., cell morphology and

encapsulation, ornithine decarboxylase activity and fermentation of rhamnose, lactose, glycerol and dulcitol. These differences of traits are presented in Table-7.

3.7 Production of polysaccharide in culture filtrate

The rate of production of polysaccharide in nutrient broth by coconut Enterobacter is indicated in Fig.6 which showed a gradual increase up to 17th day of incubation. The trend was static subsequently. The maximal production was 4-5 g of alcohol precipitable polysaccharide per litre of broth (13 days after incubation) and the extent of decrease was in the order of 1-2 g/l (13-17 days after incubation).

3.8 Enumeration of Enterobacter

The colony counts taken by pour plate method showed that the bacterium could attain a concentration of 10^{12} cells/ml within 8 to 9 days in coconut root extract broth. The viable counts decreased to 10^3 cells/ml by the 16th day. The \log_{10} of colony counts was plotted against days of incubation to obtain the growth curve (Fig.7).

3.9 Effect of coconut root extract on production of polysaccharide by Enterobacter

The effect of incorporating coconut root extract in nutrient media on the extent of production of polysaccharide by the coconut Enterobacter isolates (Fig.8) has brought out a linear increase of 8 to 9 g alcohol precipitated material per litre of the broth upto 50% concentration. Further increase of the coconut root extract did not have any appreciable effect on polysaccharide production during a period of 13 days.

3.10 Effect of nutritional factors on the yield of polysaccharide

In order to assess the response of carbohydrates to the extent of production of polysaccharide by coconut Enterobacter

10 different sugars were incorporated into four media such as basal salt solution, beef extract 1%, coconut root extract 50% and coconut root extract 50% + beef extract 1%. Sugars tested in presence of coconut root extract (50%) and beef extract (1%) facilitated yield of polysaccharide by coconut Enterobacter in the range of 6.20 g to 9.40 g/l culture broth, in which lactose and dulcitol recorded above 9 g/l while D.arabinose, trehalose, raffinose, D.galactose, D.glucose, and sucrose followed with above 8 g/l yield. Sugars with coconut root extract (50%) ranked as the next medium with an yield of 1.88 to 4.28 g/l broth. Here also dulcitol and D. galactose topped the list with 3-4 g/l production of polysaccharide. Sugars with beef extract (1%) yielded 0.640 g to 1.004 g followed by sugars in basal salt solution, aiding production of 0.320 to 1.120 g polysaccharide per litre culture broth. Overall superiority of lactose, D-galactose and dulcitol in all the combinations tested is evident from Fig.9.

3.11 Screening of standard strains of Enterobacter cloacae for their ability to produce polysaccharide.

Twenty four strains of Enterobacter cloacae from six international culture collections elaborated alcohol precipitable materials in the range of 1.0 to 5.2 g/l when cultivated in nutrient media, whereas in growing coconut root extract broth, the range of production of alcohol precipitable material varied from 1.28 to 4.64 g/l broth. The coconut root extract facilitated production of polysaccharide above 4.5 g/l broth in culture designates E₂₂, E₁₆, E₁₂ and E₁₀ and below 1.5 g/l in E₂, E₃, E₅ and E₆. The culture designate E₁ yielded 5.2 g/l and E₄, E₅ and E₆ produced above 3.5 g/l polysaccharide whereas E₂₁ and E₂₂ produced only 1.5 g/l poly saccharide when cultured in nutrient broth. Capability of individual strains of Enterobacter cloacae in production of polysaccharide in nutrient media and coconut root extract broth is presented in Table 9. The comparative efficiency of these isolates on elaboration of polysaccharide in two media are depicted in Fig.10 alongwith coconut Enterobacter culture No.CB₂.

3.12 Extraction of toxin material

On growing coconut Enterobacter in yeast extract dialysate broth for 13 days, the extracellular polysaccharide was harvested as described in materials and methods. Seven lots of two l culture gave 1,600 ml cell free broth on centrifuging, individually. The alcohol precipitated polysaccharide (17.546 g) derived from 10,800 ml cell free broth, yielded 2.070 g crude toxin on purification. In other words it is evident from Table 8.

The recovery of alcohol precipitated material from root sap of root (wilt) affected coconut palms is detailed in Table 11. Four hundred and forty eight mg. of alcohol precipitated material derived from 2,473 ml root sap collected at four stretch over a period of 62 days yielded 159 mg purified material on passing through ion exchange resins, i.e. an average of 181 mg alcohol-precipitated material per litre of root sap yielded 64 mg substance on freeze drying the effluent from Dowex columns. As the alcohol precipitated material obtained from root sap of healthy palms was negligible, attempts were not initiated on its purification.

3.13 Gaumann's test

Biological assay of the crude toxin employing Gaumann's test revealed the effect of crude toxin at three different concentrations viz., 100 ppm, 1,000 ppm and 2,000 ppm on tomato cuttings. The observation of tomato cutting was continued at serial intervals up to 30 h. Slight bending at the leaflets was rated as initial symptom. While tomato cuttings dipped in toxin at 100 ppm took 22 h to show the initial symptoms, 1,000 ppm took four and tomato cuttings at 2,000 ppm developed the initial symptom within 30 min. The symptoms progressed to the highest level observed, showing drooped stem and withered leaves within a period of 30 h at 2,000 ppm concentration of toxin. The index of wilting tomato cuttings in different concentrations of toxin at serial intervals of time is given in Table 12.

Likewise, highly purified toxin fraction was also tested for biological activity at three different concentrations for a period of 48 h. Within the stipulated period this toxin at 100 ppm concentration could not induce wilting; whereas 1,000 ppm took six h to show initial symptom and 2,000 ppm took three h. The index of wilting at serial intervals of time at different concentrations of highly purified toxin is shown in Table 13.

Toxin separated from root sap of root (wilt) affected coconut palms also were bioassayed with tomato cuttings. Toxin at a concentration of 100 ppm did not show any effect on tomato cuttings for a period of 56 h, while, 1,000 ppm concentration of toxin effected initial symptom within 10 h and 2,000 ppm concentration of toxin showed initial symptoms within eight h. The symptoms expressed by tomato cuttings at 100 ppm, 1,000 ppm and 2,000 ppm concentration of root sap toxin at serial intervals of time for 56 h is detailed in Table 14.

3.14 Revival of wilted plants

Tomato cuttings placed in 2,000 ppm toxin solution for four h, when transferred to distilled water revived wilting; whereas cuttings retained in toxin solution for 24 h could not regain turgidity.

3.15 Effect of toxin on germination of seeds

As it is evident from Table 15, 1% aqueous solution of crude toxin did not have any effect on the germination of seeds of seven species of plants tested.

3.16 Serological studies

The coconut Enterobacter crude toxin was antigenic. The titre value of antiserum was 1/512 and of antigen 1/128. Inimuno-diffusion tests crude toxin formed one clear precipitation band near the antiserum well. Similarly extracts from disease affected palms also formed a corresponding band. The buffer saline and normal plant extract used as control did not show any band.

None of the alcohol-precipitated polysaccharide from standard cultures of Enterobacter cloacae formed precipitation band in immunodiffusion when tested against coconut Enterobacter toxin antiserum.

3.17. Purification of toxin

On applying crude toxin to Sephadex G-200 column, the effluent was collected in three ml fractions and read colorimetrically, as described in materials and methods. The percentage transmittance of each fraction was plotted against elution volume as indicated in Fig.11. There was a lone peak at the elution volume of 21 and the peak fraction was dried under a stream of air and weighed to calculate the percentage of recovery. Seventy six percent by weight of the crude toxin applied was recovered as purified toxin, in a single peak.

3.18 Characteristics of toxin

Molecular weight

Molecular weight of the peak fraction was determined following the formula given in materials and methods. The elution volume of peak fraction being 21 ml, its molecular weight was determined as 89,130.

3.19 Fractionation

On passing through Dowex columns the neutralized acid hydrolysate of the crude toxin was separated into anionic, cationic and neutral fractions with a total recovery of 86.66% in a proportion of 13:8:5 respectively.

3.20 Amino acid profiles

The cationic fraction of crude toxin was resolved by thin layer chromatography for detection of amino acids. Three ninhydrin positive spots were located of which two were identified as glutamic acid and lysine. The unidentified spot had an R_f value of 0.12.

The Enterobacter crude toxin showed 16 amino acids when analysed by Amino acid analyser. Aspartic acid and glutamic acid were in greater proportions each showing 2.02 mg/100 mg sample. Lysine, valine, proline, alanine and leucine were in the range of 1 to 2 mg/100 mg sample. The rest of the nine amino acids represented each less than 1 mg/100 mg samples (Tables 16 & 12)

3.21 Sugar content

The neutral fraction of the crude toxin showed the presence of only one sugar compound. This was identified as glucose on comparison with standard sugar.

3.22 Sugar acid distribution

Paper chromatographic analysis of anionic fraction of crude toxin presented five spots none of which with Rf values 0.87, 0.58, 0.42, 0.21 and 0.05 resembled the known sugar acids available.

3.23 Competitive Saprophytic ability of Enterobacter cloacae

The dilution plate counts taken at periodical intervals of bacteria and fungi incorporated into the methyl bromide fumigated soil revealed that these microbes (Enterobacter cloacae, Cylindrocarpum effusum and Fusarium equiseti) can co-exist and proliferate together without any deleterious effect to each other (Table 15 and Fig.13).

3.24 Screening of antibiotics

Initial screening of antibiotic sensitivity discs against coconut Enterobacter revealed that the bacterium was sensitive to Chloramphenicol, Mandelamine, Kanamycin, Tetracycline, Oxytetracycline, and Streptomycin whereas it was resistant to Sulphadiazine, Erythromycin, Bacitracin and Penicillin. The minimum inhibitory concentration of Oxytetracycline and

Streptomycin was judged by tube dilution technique. Oxytetracycline prevented growth at 0.8 $\mu\text{g}/\text{ml}$ concentration and Streptomycin at 5 $\mu\text{g}/\text{ml}$ concentration. Growth of the bacterium as observed by per cent transmittance in various concentrations of the antibiotics is plotted in Fig.14.

3.25 Effect of oxytetracycline on coconut root (wilt) disease.

The percentage incidence of disease as indexed by the method of George and Radha (1973) of treated and control palms over a period of four years is given in Table 18. The change in disease index of individual palms of treated and control lots from 1977 to 1981 is calculated and is compared by 't' test. A mean reduction of 3.31% in the disease index of treated palms and a mean increase of 8.54% in the disease index of untreated control palms are observed indicating the effect of oxytetracycline in ameliorating the disease condition (Table 19).

Table 2. Technical details of antibiotic sensitivity discs

Code No.	Name of sensitivity discs	Concentration per disc	Solvent
H ₃	Chloramphenicol	30 µg	Water
H ₈	Mandelamine	30 µg	Dilute alkali
H ₆	Kanamycin	30 µg	Water
H ₁₉	Tetracycline	30 µg	Water
H ₁₆	Streptomycin	10 µg	Alcohol
H ₅	Erythromycin	10 µg	Alcohol
H ₁₇	Sulphadiazine	1 mg	Alkali hydroxide and Carbonates
H ₂	Bacitracin	10 µg	Water
H ₁₄	Penicillin	10 units	Water

Table 3. Extent of occurrence of bacteria in coconut palms in relation to root (wilt) disease.

Condition of palms	Number of palms	Percentage of palms yielding bacteria
Disease advanced	51	56.49
Disease early	52	43.20
Apparently healthy	14	30.95
Healthy	107	12.15

Table 5. Percentage occurrence of gram negative bacteria and their identity in disease-free and disease-affected tracts.

Identity	Disease-free tract	Disease-affected tract	Total
<u>Enterobacter</u>	2.44	63.41	65.85
<u>Azotomonas/</u> <u>Acromonas</u>	12.20	13.41	25.61
<u>Erwinia</u>	0	6.1	6.1
<u>Pseudomonas</u>	1.22	1.22	2.44

Table 6. Extent of occurrence of Enterobacter in relation to coconut root (wilt) disease

Categories of palms	Total number of palms	Percentage of palms yielding bacteria	Percentage occurrence of <u>Enterobacter</u>
Disease-advanced	51	56.49	42.34
Disease-early	52	43.20	73.75
Apparently healthy		30.95	16.67
Healthy	107	12.15	8.0

Table 7. Variation in the morphological and biochemical characteristics among different strains of coconut Enterobacter cloacae

Characteristics	Strain designations					
	6 ₂ N	6B ₄	C ₁₁ ^A	5 ₂ B	C ₇	CB ₂
Cell morphology	Medium rod	Medium rod	Short rod	Short rod	Medium rod	Medium rod
Encapsulation	+	-	-	-	-	-
Ornithine decarboxylase	-	-	+	+	+	+
Rhamnose fermentation	+	+	-	-	-	-
Lactose fermentation	+	+	-	+	-	+
Glycerol fermentation	-	-	+	+	+	+
Dulcitol fermentation	+	+	-	-	-	-

Table 8. Influence of different carbohydrates on the production of Polysaccharide by coconut *Enterobacter cloacae* (culture CB₂)

Sl. No.	Sugar*	Media			
		Basal salt solution	Beef extract 1%	** CRE 50%	CRE 50% + Beef extract 1%
1.	D-Xylose	0.320	0.840	1.88	6.32
2.	D-Ribose	0.346	1.040	1.92	6.20
3.	D-Arabinose	0.620	0.760	2.32	8.48
4.	Trehalose	0.747	0.840	2.36	8.08
5.	Dulcitol	0.600	0.720	4.28	9.04
6.	Raffinose	0.720	0.840	2.40	8.32
7.	D-Galactose	0.893	0.840	3.32	8.96
8.	Lactose	1.120	1.040	2.20	9.48
9.	D-Glucose	0.880	0.760	2.16	8.88
10.	Sucrose	0.840	0.640	2.04	8.96

* Sugars were incorporated at a concentration of 0.2%

** Coconut root extract

Table 9. Extent of production of Polysaccharide by different standard cultures of Enterobacter cloacae.

Culture designation and its source	Media	
	CR3 broth Polysaccharide in g/l broth	Nutrient broth Polysaccharide in g/l broth
ROBERT KOCH INSTITUTE, GERMANY		
E ₁ (Nr.978/70)	1.80	5.2
E ₂ (Nr.921/70)	1.28	2.8
E ₃ (Nr.1084/70)	1.36	3.0
E ₄ (Nr.1100/70)	2.52	4.2
E ₅ (Nr.1542/70)	1.60	3.8
E ₆ (Nr.90/70)	1.48	2.4
UNIVERSITY COLLEGE, IRELAND		
E ₇ (IMD 116)	1.88	2.2
MINISTRY OF HEALTH, CANADA		
E ₈ (3401)	3.44	1.4
E ₉ (SC-311)	2.40	3.2
E ₁₀ (SC-312)	4.04	1.8
PASTEUR INSTITUTE, FRANCE		
E ₁₁ (6022)	3.16	2.8
E ₁₂ (6085)	4.72	2.2
E ₁₃ (681)	1.48	2.8

Contd.....

Media

Culture designation and its source

CRE broth Polysaccharide in g/l broth

Nutrient broth Polysaccharide in g/l broth

NCTC, London

E₁₄ (NCTC-10005)

40.4

3.24

(318-02) 3.20

E₁₅ (NCTC-5920)

3.16

E₁₆ (NCTC-6022)

81.8

4.64

(8808) 3.81

E₁₇ (NCTC-8348)

87.4

1.88

(3908) 2.23

E₁₈ (NCTC-9394)

34.1

3.56

(288) 2.48

E₁₉ (NCTC-9529)

1.52

1.4

E₂₀ (NCTC-9711)

3.28

1.6

E₂₁ (NCTC-9844)

81.8

3.88

1.0

USDA

E₂₂ (NRRL-B-126)

5.36

1.2

E₂₃ (NRRL-B-169)

2.36

2.20

E₂₄ (NRRL-B-180)

40.4

3.2

(318-02) 1.61

CB₂ (Kayankulam culture)

8.5

8.8

81.8

(8808) 1.1

8.8

87.4

(3908) 3.1

8.8

34.1

(188) 81 E

.....

(18-02) E

Table 10. Extent of production of toxin by coconut Enterobacter from cell free culture broth in varying lots.

Lot No.	Volume of cell free culture broth	Weight of alcohol precipitated polysaccharide	Yield of crude toxin
1.	1200 ml	2.315 g (1.93 g/l)	246 mg (205 mg/l)
2.	1500 ml	2.331 g (1.46 g/l)	273 mg (171 mg/l)
3.	1600 ml	2.754 g (1.72 g/l)	331 mg (207 mg/l)
4.	1600 ml	2.425 g (1.52 g/l)	286 mg (179 mg/l)
5.	1600 ml	2.651 g (1.66 g/l)	328 mg (205 mg/l)
6.	1600 ml	2.438 g (1.52 g/l)	287 mg (179 mg/l)
7.	1600 ml	2.632 g (1.65 g/l)	319 mg (199 mg/l)
Total	10800 ml	17.546 (1.625 g/l)	2.070 g (192 mg/l)

Table 11. Recovery of purified material from root sap of disease-affected coconut palm in varying lots.

Lot No.	Volume of root sap collected	Period of collection in days	Alcohol precipitated material	Purified material
1.	184 ml	18	17 mg (92.39 mg/l)	2 mg (10.87 mg/l)
2.	137 ml	14	16 mg (116.77 mg/l)	4 mg (2920 mg/l)
3.	432 ml	9	93 mg (215.28 mg/l)	26 mg (10.19 mg/l)
4.	1720 ml	21	322 mg (187.21 mg/l)	127 mg (73.84 mg/l)
Total	2473 ml	62	448 mg (181 mg/l)	159 mg (43.53 mg/l)

Table 12. Gaumann's test with coconut Enterobacter toxin

Observation time/ Concentration of toxin	Observation time									
	15 min	30 min	1 h	2 h	3 h	4 h	5 h	6 h	22 h	30 h
100 ppm	-	-	-	-	-	-	-	-	+	+
1000 ppm	-	-	-	-	-	+	+	++	++	+++
2000 ppm	-	+	+	+	++	++	+++	+++	+++	+++

+ Slight bending at the tip of leaflets
 ++ Visible bending of leaflets
 +++ Petiole started bending with stem tip.
 ++++ Stem dropped with leaves withered.

Table 13. Gaumann's test with highly purified coconut Enterobacter toxin.

Concentration of toxin.	Observation time									
	1 h	2 h	3 h	4 h	5 h	6 h	22 h	30 h	48 h	
100 ppm	-	-	-	-	-	-	-	-	-	
1000 ppm	-	-	-	-	-	+	++	++	+++	
2000 ppm	-	-	+	+	+	++	+++	+++	+++	

~~+ Slight bending at the tip of leaflets~~
 ++ Visible bending on leaf lots.
 +++ Petiole started bending with stem tip

Table 14. Gaumann's test with Root sap toxin

Concentration of toxin	Observation time						
	6h	8th	10h	23h	30h	48h	56h
100 ppm	-	-	-	-	-	-	-
1000 ppm	-	-	+	++	++	++	++
2000 ppm	-	+	+	+++	+++	+++	+++

- + Slight bending at the tip of leaflets
- ++ Visible bending of leaflets
- +++ Petiole started bending with stem tip.

Table 15. Effect of coconut Enterobacter toxin on the germination of seeds

Seeds tested	Percentage of germination	
	Toxin treated	Control
<u>Crotolaria Striata</u>	98	100
<u>Crotolaria juncea</u>	100	100
<u>Nicotiana tabacum</u>	100	98
<u>Cyamopsis tetragonoloba</u>	100	100
<u>Lycopersicum esculentum</u>	100	100
<u>Amarantus caudatus</u>	100	98
<u>Cleome viscosa</u>	100	100

Table 16. Amino acid analysis of Enterobacter toxin

Sl. No.	Amino acids	mg/100 mg sample
1.	Lysine	1.15
2.	Histidine	0.20
3.	Arginine	0.66
4.	Aspartic acid	2.02
5.	Threonine	0.88
6.	Serine	0.62
7.	Glutamic acid	2.02
8.	Proline	1.03
9.	Glycine	0.84
10.	Alanine	1.10
11.	Valine	1.11
12.	Methionine	0.27
13.	Isoleucine	0.86
14.	Leucine	1.18
15.	Tyrosine	0.58
16.	Phenyl alanine	0.68

Table 17. Competitive Saprophytic ability of *Enterobacter cloacae*.

Bacterial counts $\times 10^7$ /g of soil

Period	Treatments				
	T ₂	T ₃	T ₄	T ₅	T ₁
12h	20	20	20	17	0.006
15th day	200	5.5	11.5	55	-
30th day	12	3.5	2	30	-
60th day	0.625	0.09	0.05	0.15	-
90th day	0.039	0.003	0.006	0.004	-

Fungal counts $\times 10^5$ /g of soil

12 h	20	20	20	0.4
15th day	4.5	-	3.7	-
30th day	3.3	4	3.4	0.85
60th day	-	0.1	0.3	1.20
90th day	75	0.5	0.6	1.00

- T₁ - Control
- T₂ - Enterobacter
- T₃ - Enterobacter + Fusarium equiseti
- T₄ - Enterobacter + Cylindrocarpum effusum
- T₅ - Enterobacter + Fusarium equiseti + Cylindrocarpum effusum

Table 18. Influence of Oxytetracycline on root (wilt) disease

Palm No.	Percentage of disease incidence*					
	Jan. 1977	Jan. 1978	Jan. 1979	Jan. 1980	Jan. 1981	
Treated Palms	1.	27.1	28.5	27.2	28.8	25.9
	2.	20.5	20.6	17.1	23.7	21.0
	3.	25.6	33.7	32.2	35.6	24.2
	10.	12.3	15.2	9.7	11.1	6.5
	11.	18.0	11.5	4.8	5.6	3.3
	13.	28.3	25.1	29.1	35.3	31.4
	15.	31.9	32.8	33.7	35.2	30.0
	17.	26.4	20.3	11.5	20.7	24.3
23.	26.3	25.4	22.4	25.8	20.0	
Untreated control palms	3.	23.2	31.9	26.3	34.8	29.3
	4.	30.0	37.0	33.3	41.9	37.4
	7.	24.1	33.0	26.1	32.0	28.5
	8.	19.0	27.8	25.2	30.5	21.3
	9.	31.1	37.0	36.7	36.5	42.1
	12.	14.0	16.3	13.7	16.3	18.0
	14.	11.3	23.7	40.1	39.3	39.6
	18.	26.0	31.9	34.3	37.3	36.1
	21.	36.6	45.6	48.1	50.4	48.4
24.	21.7	23.0	27.1	26.6	21.7	

* Indexed by the method of George and Radhn, 1973.

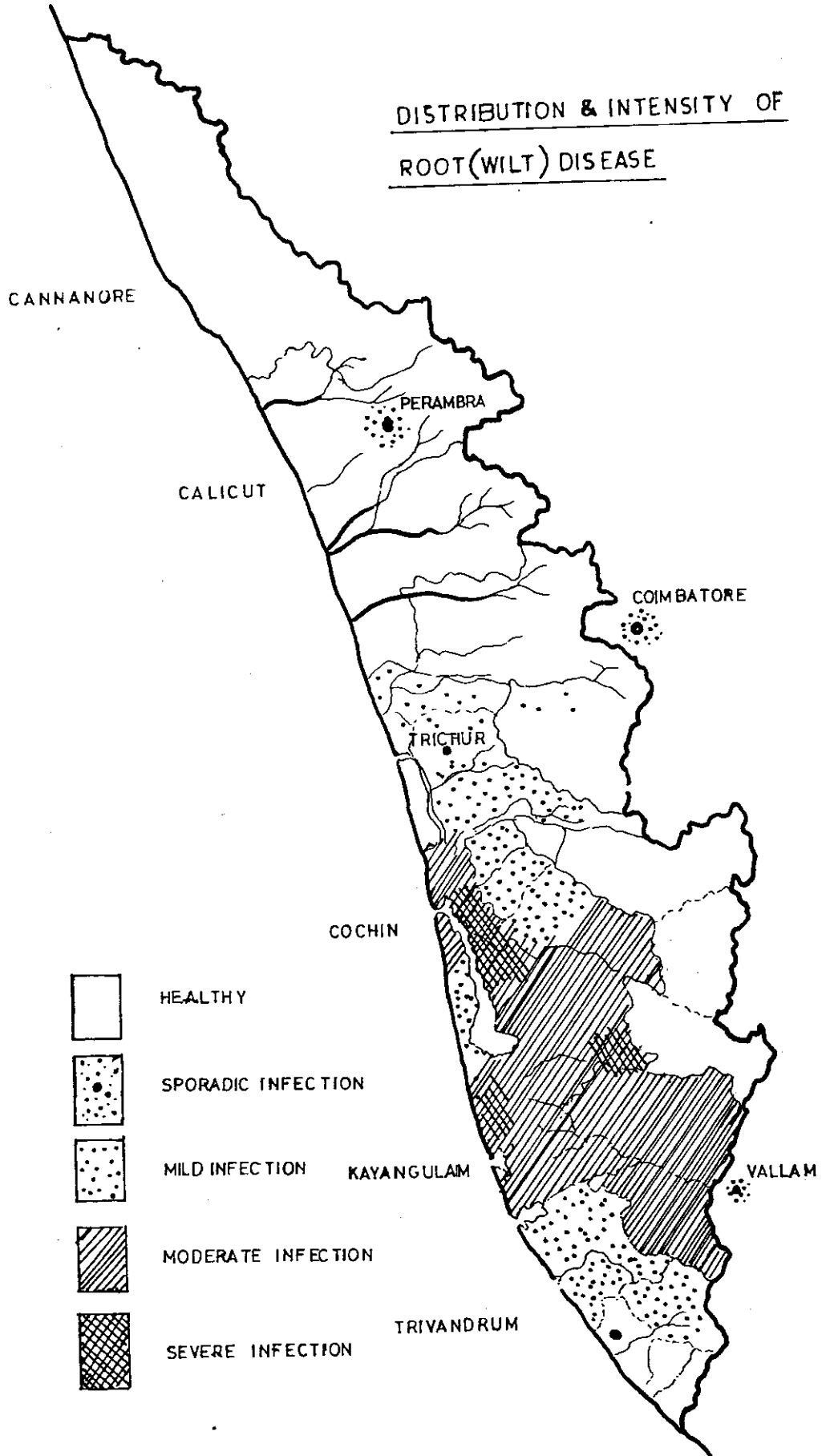
Table 19. Change in disease index over a period of four years (1977-1981)

OTC Treated Palms	Control Palms
1.2	- 6.1
- 0.5	- 7.4
1.4	- 4.4
5.8	- 2.3
14.7	-11.0
- 3.1	- 4.0
1.9	-28.3
2.1	-10.1
6.3	-11.8
	0.0
Mean 8.31	- 8.54

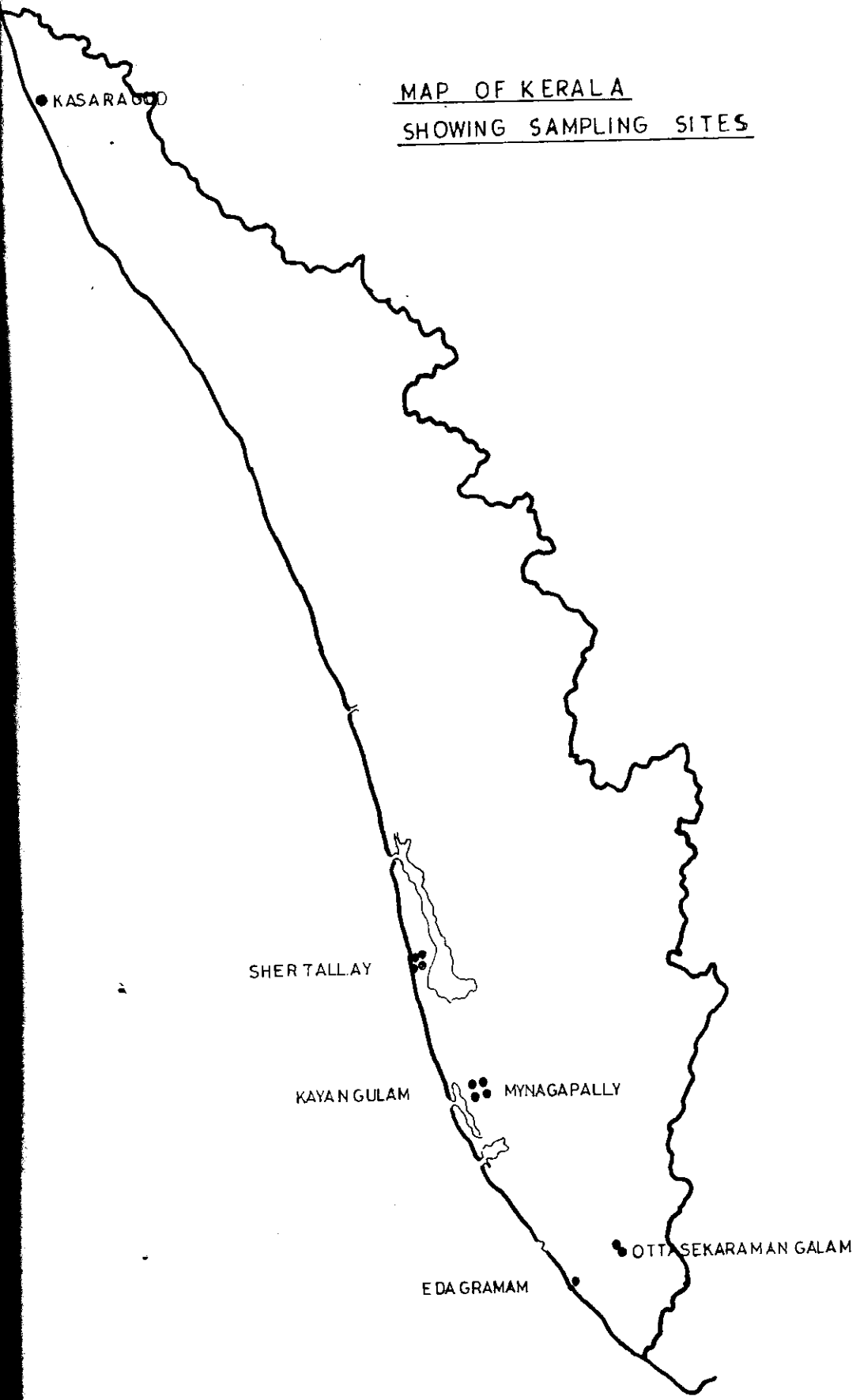
't' value 3.81 **
 ** significant at 1% level
 * OTC - Oxytetracycline

MAP OF KERALA

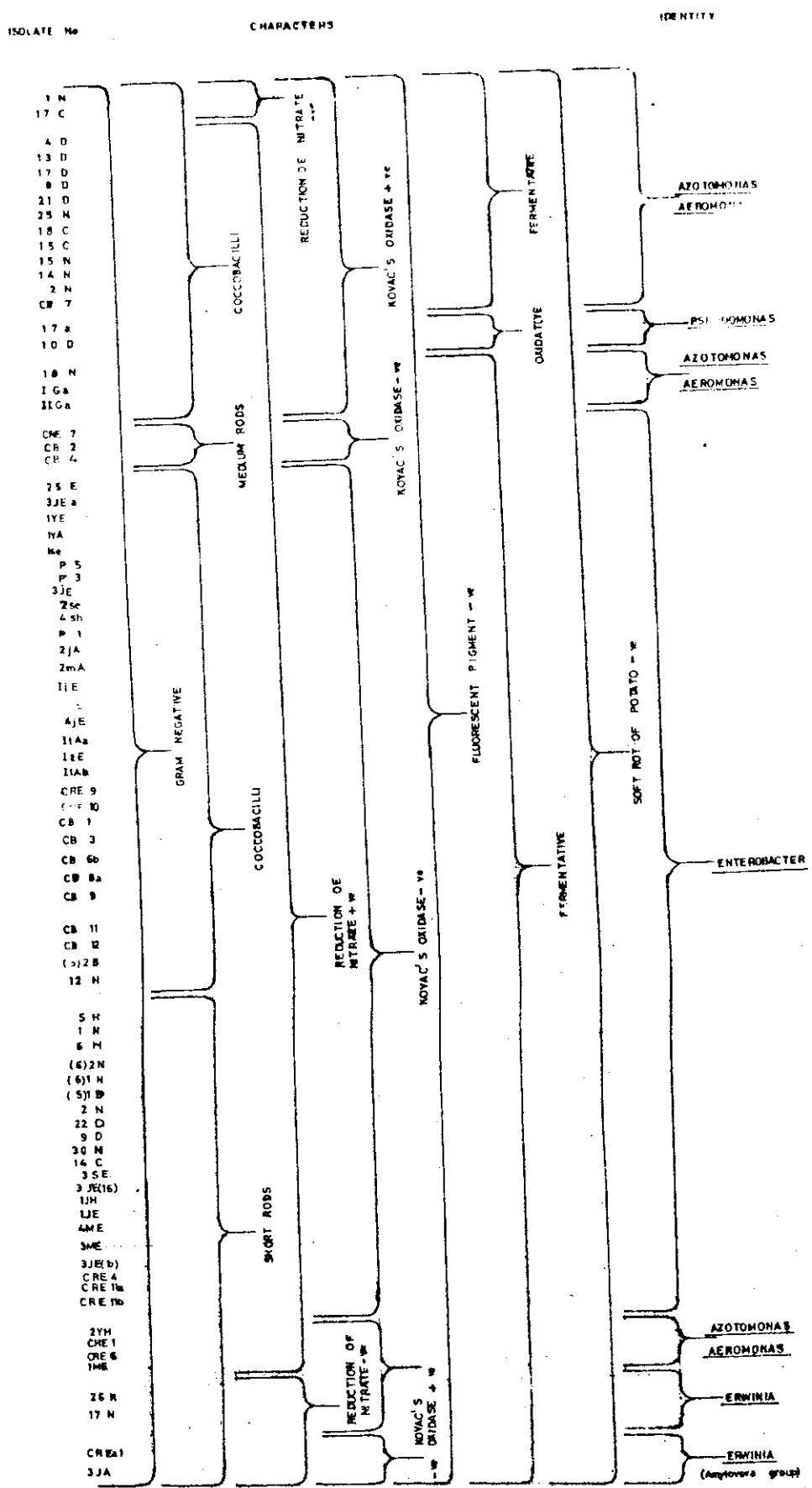
DISTRIBUTION & INTENSITY OF
ROOT(WILT) DISEASE



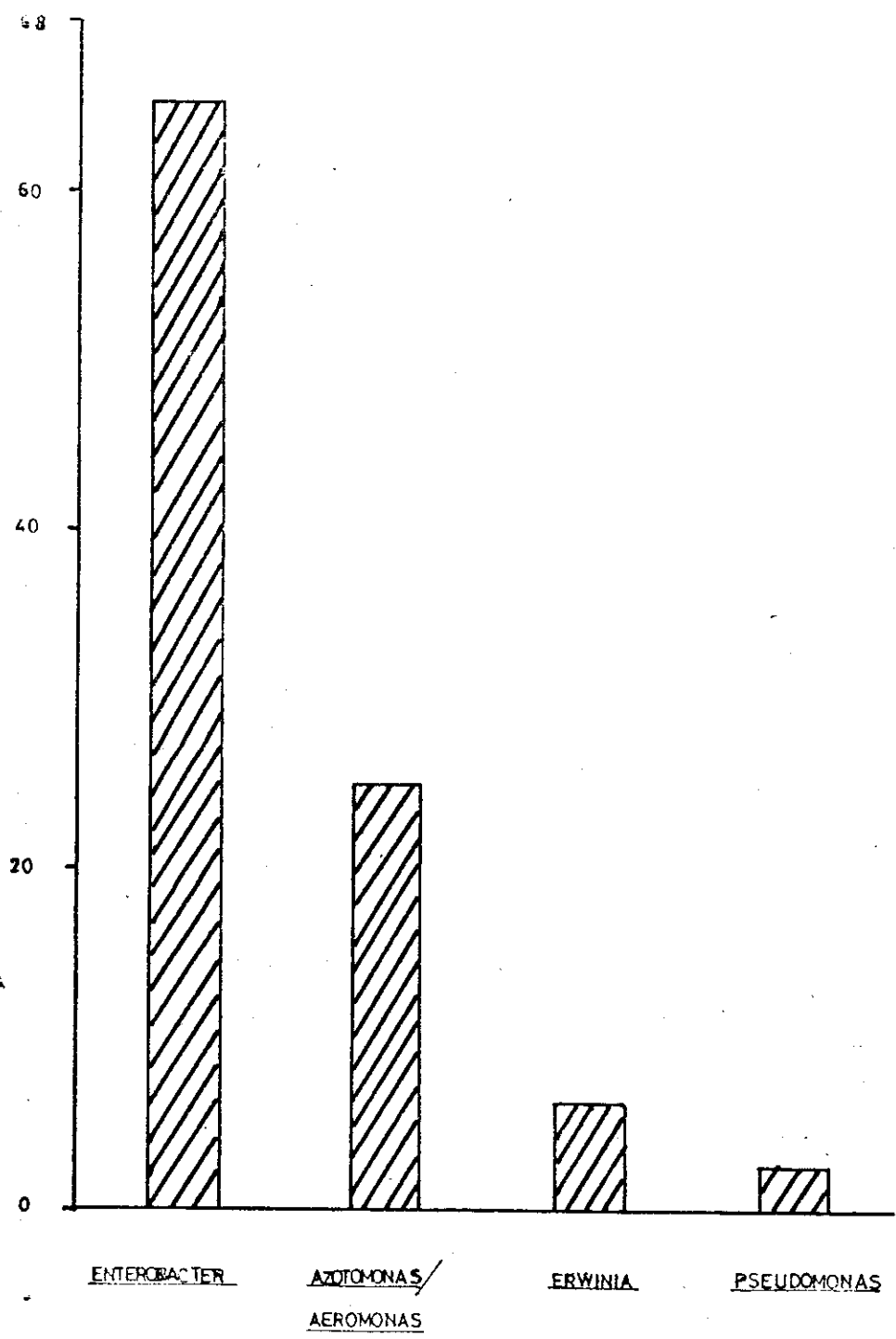
MAP OF KERALA
SHOWING SAMPLING SITES



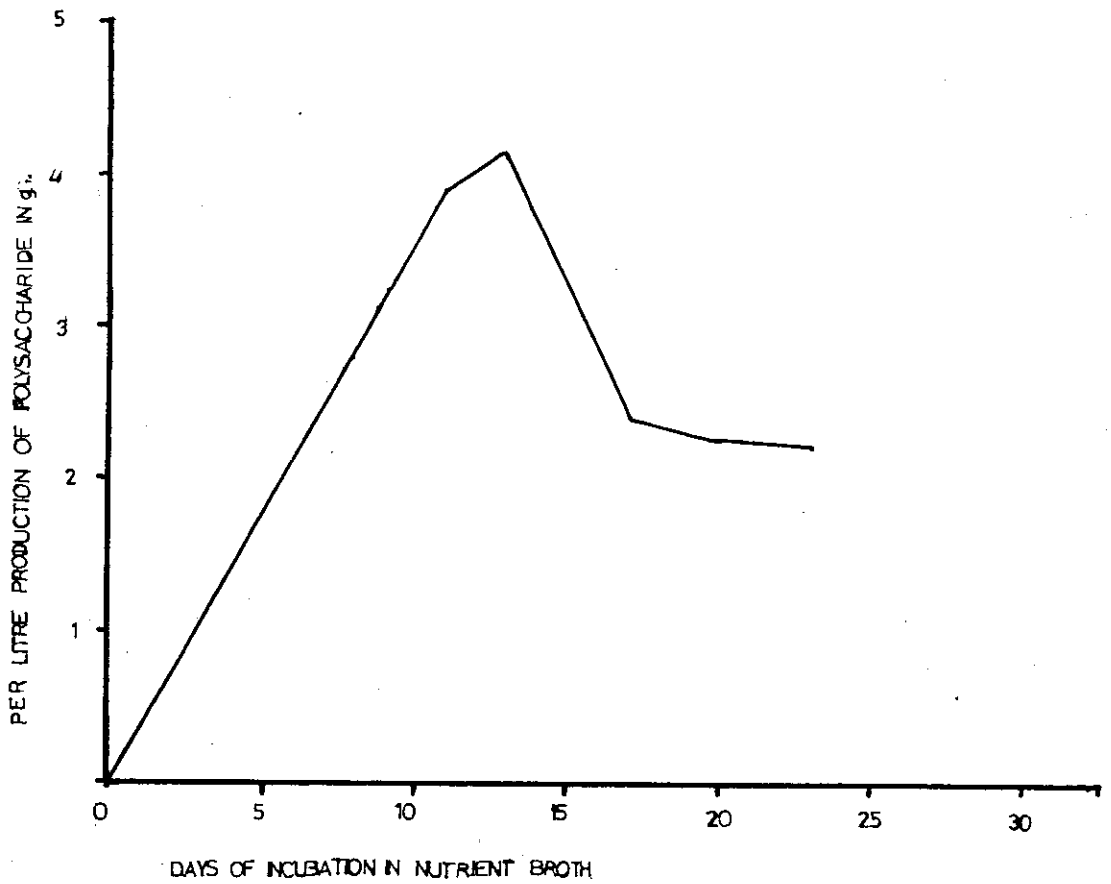
SCHEMATIC PROFILE ON THE TRAITS OF GRAM NEGATIVE BACTERIAL ISOLATES



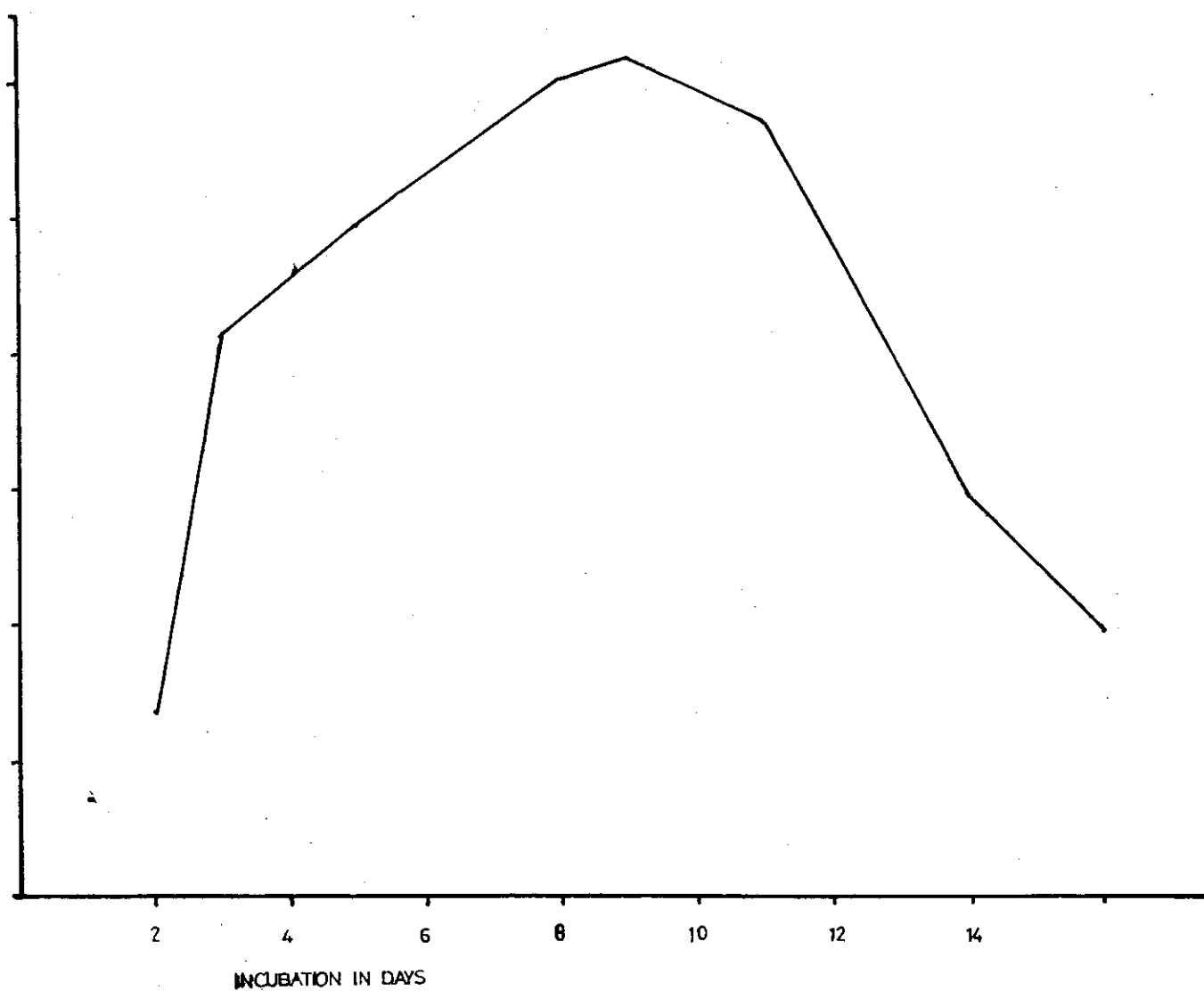
PERCENTAGE OF INCIDENCE OF BACTERIAL ISOLATES



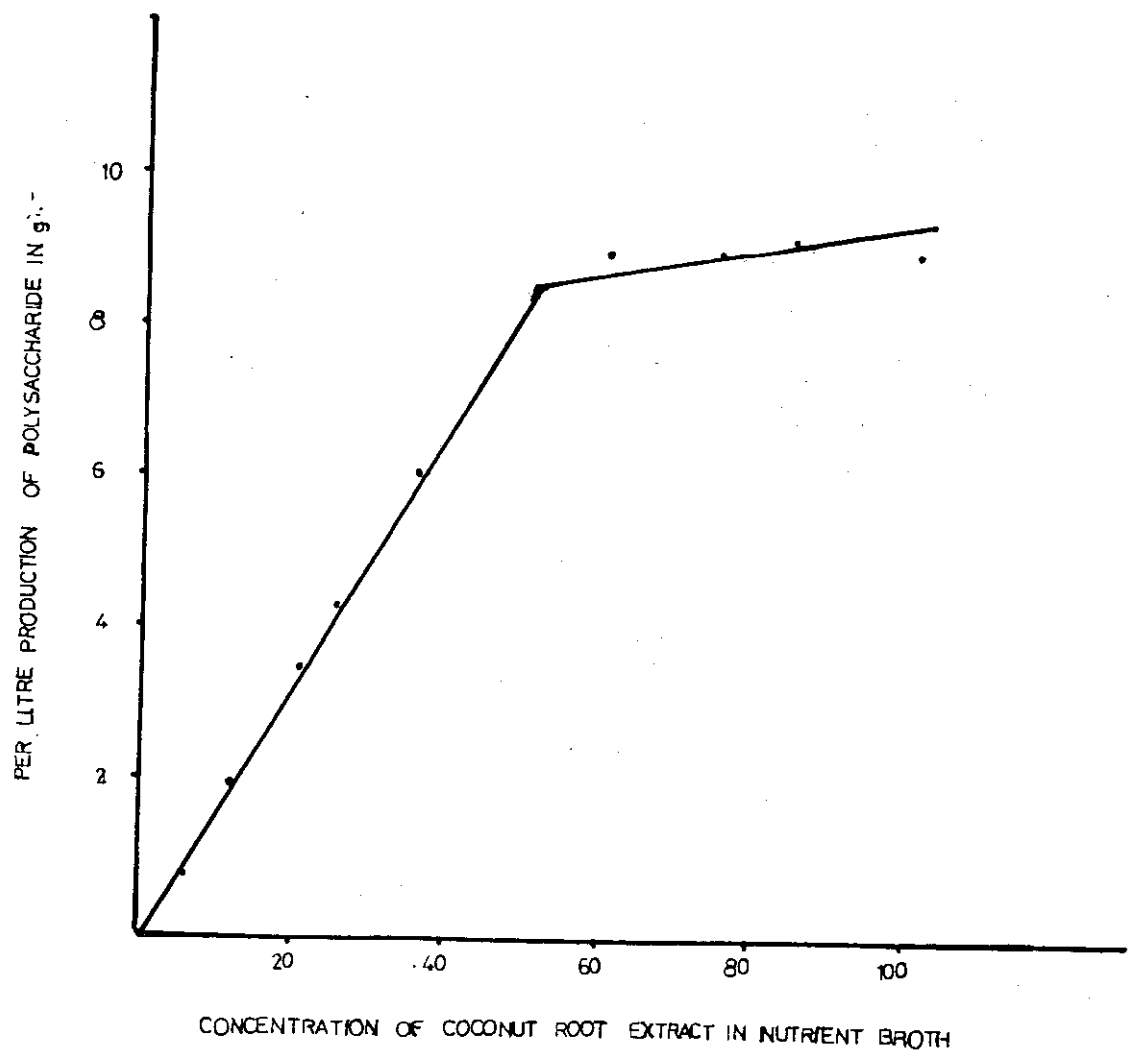
PRODUCTION OF POLYSACCHARIDE



GROWTH CURVE OF ENTEROBACTER CLOACAE



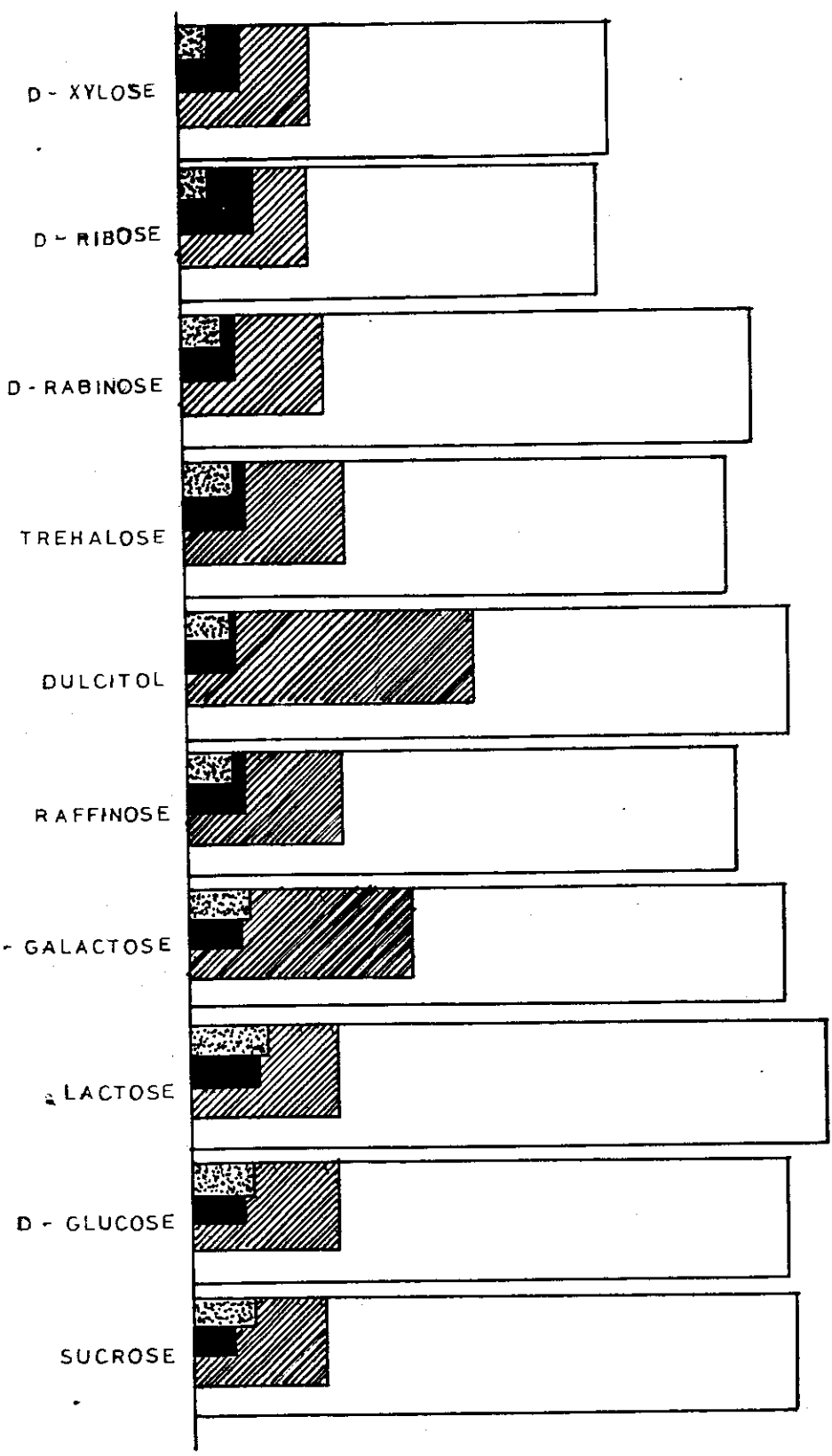
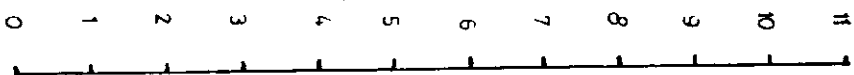
INFLUENCE OF COCONUT ROOT EXTRACT ON PRODUCTION OF POLYSACCHARIDE







NUTRITIONAL FACTORS AND YIELD OF POLYSACCHARIDE

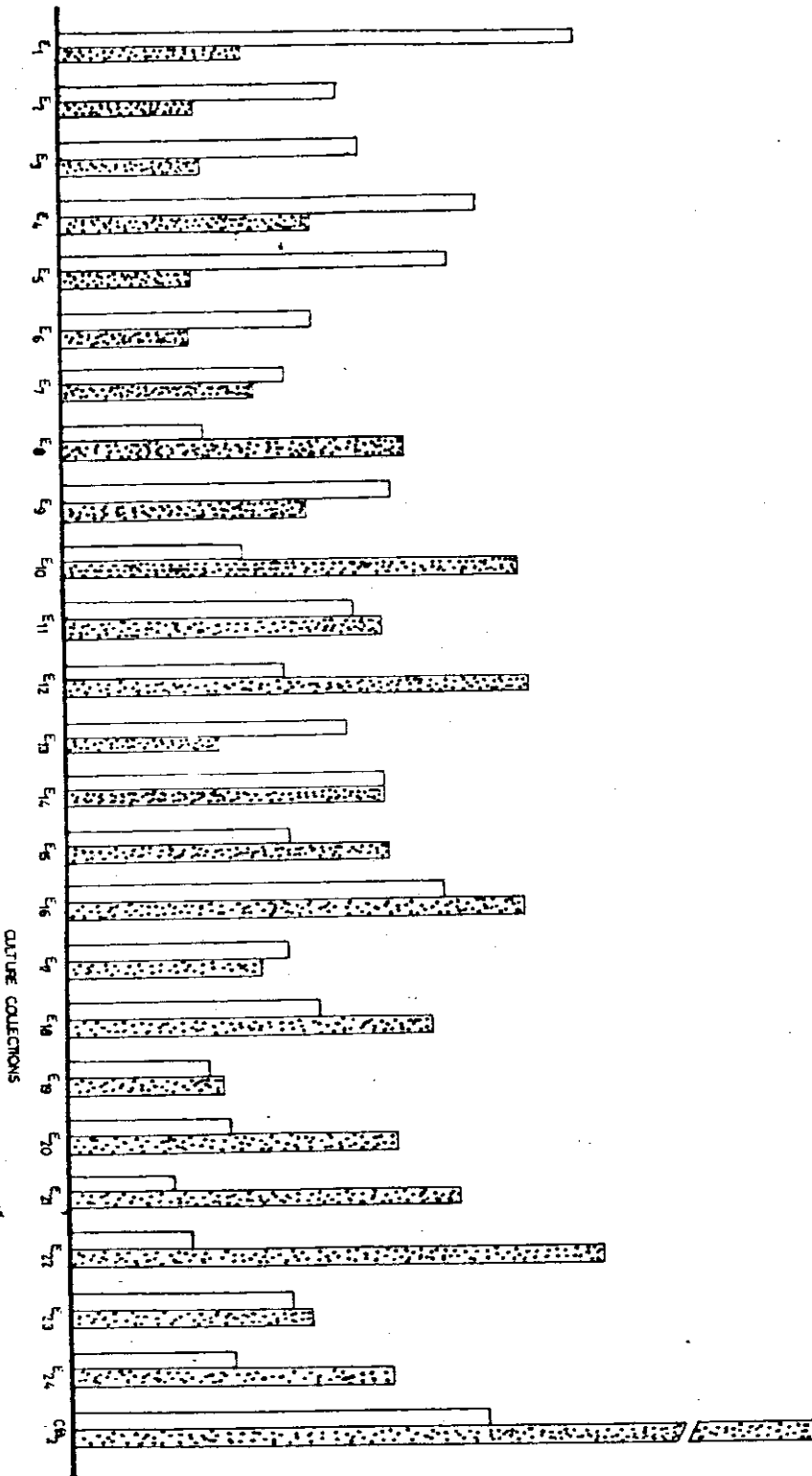
Fig-9

POLYSACCHARIDE YIELD IN g.



-  Coconut root extract 50% + Beef extract 1%.
-  Coconut root extract 50%.
-  Beef extract 1%.
-  Basal salt medium.

PER LITRE PRODUCTION OF POLYSACCHARIDE IN g



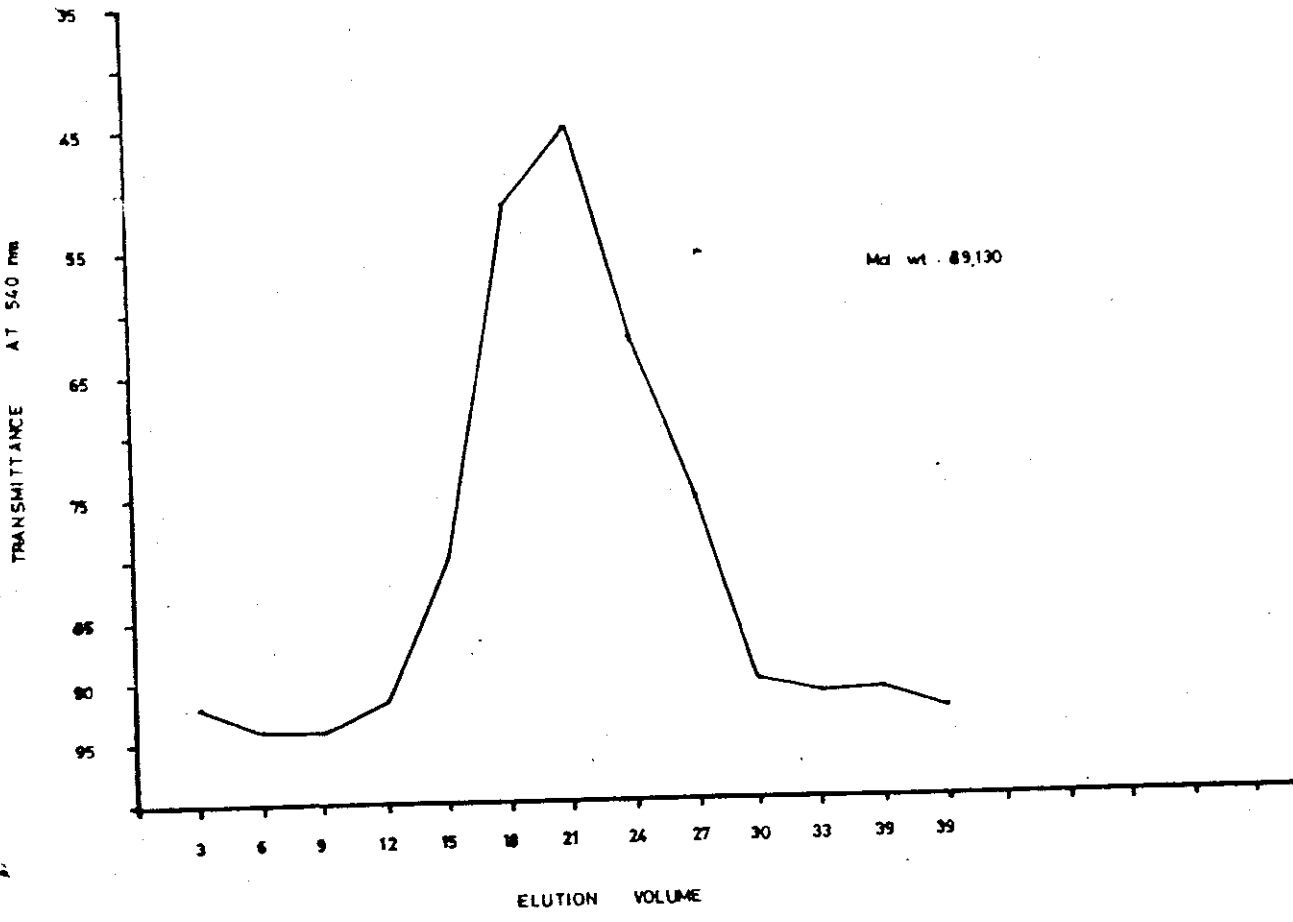
COMPARATIVE EFFICACY OF STANDARD ENTEROBACTER CULTURES IN ELABORATING POLYSACCHARIDE

E1-E6 ROBERT KOCH INSTT
 E7 DUBLIN
 E8-E10 CANADA
 E11-E13 PASTEUR
 E14-E21 NCIC
 E22-E24 USDA

[White Box] NUTRIENT BROTH
 [Stippled Box] CASE BROTH

CULTURE COLLECTIONS

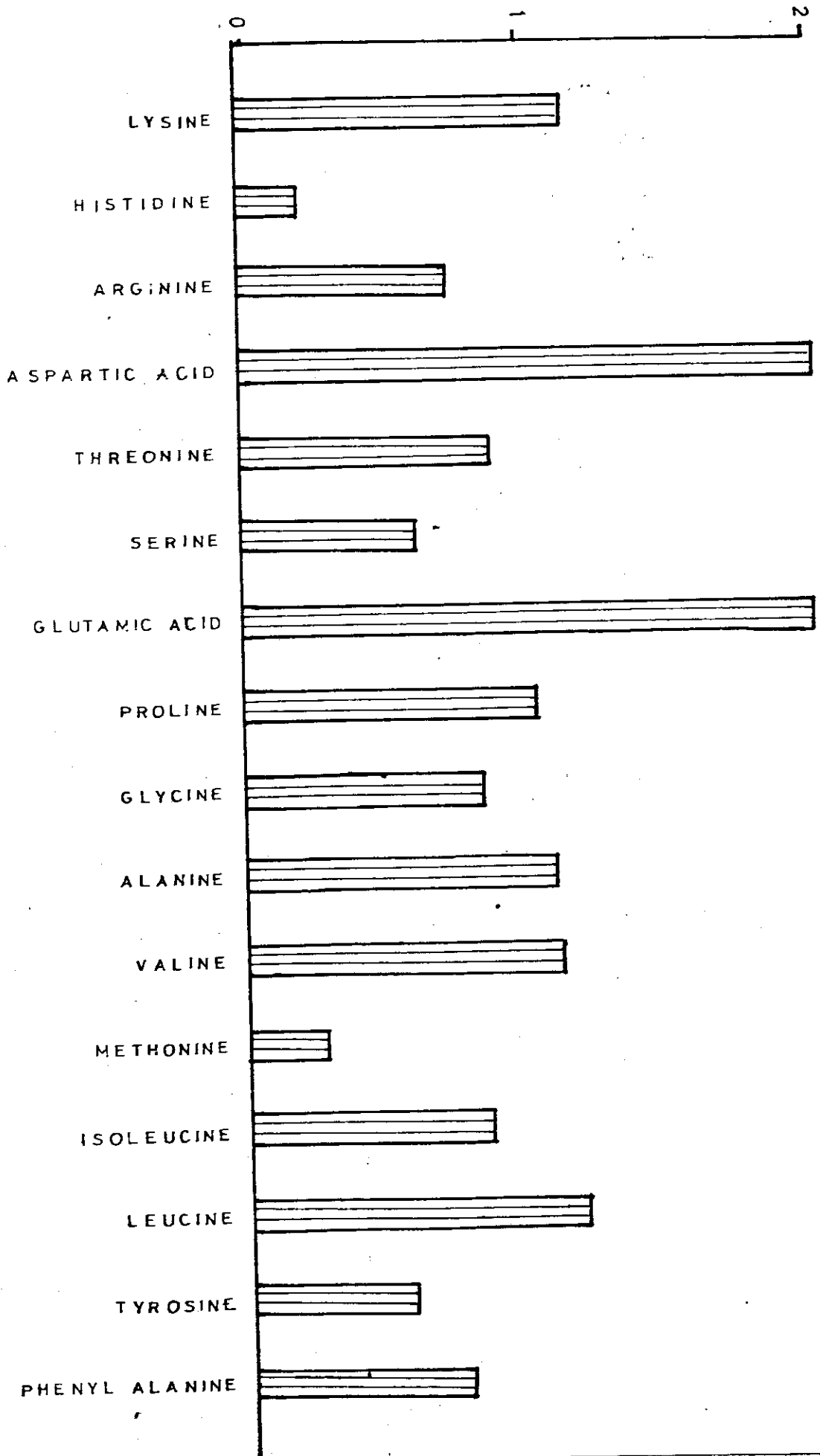
PURIFICATION OF COCONUT ENTEROBACTER TOXIN BY SEPHADEX G-200 COLUMN CHROMATOGRAPHY



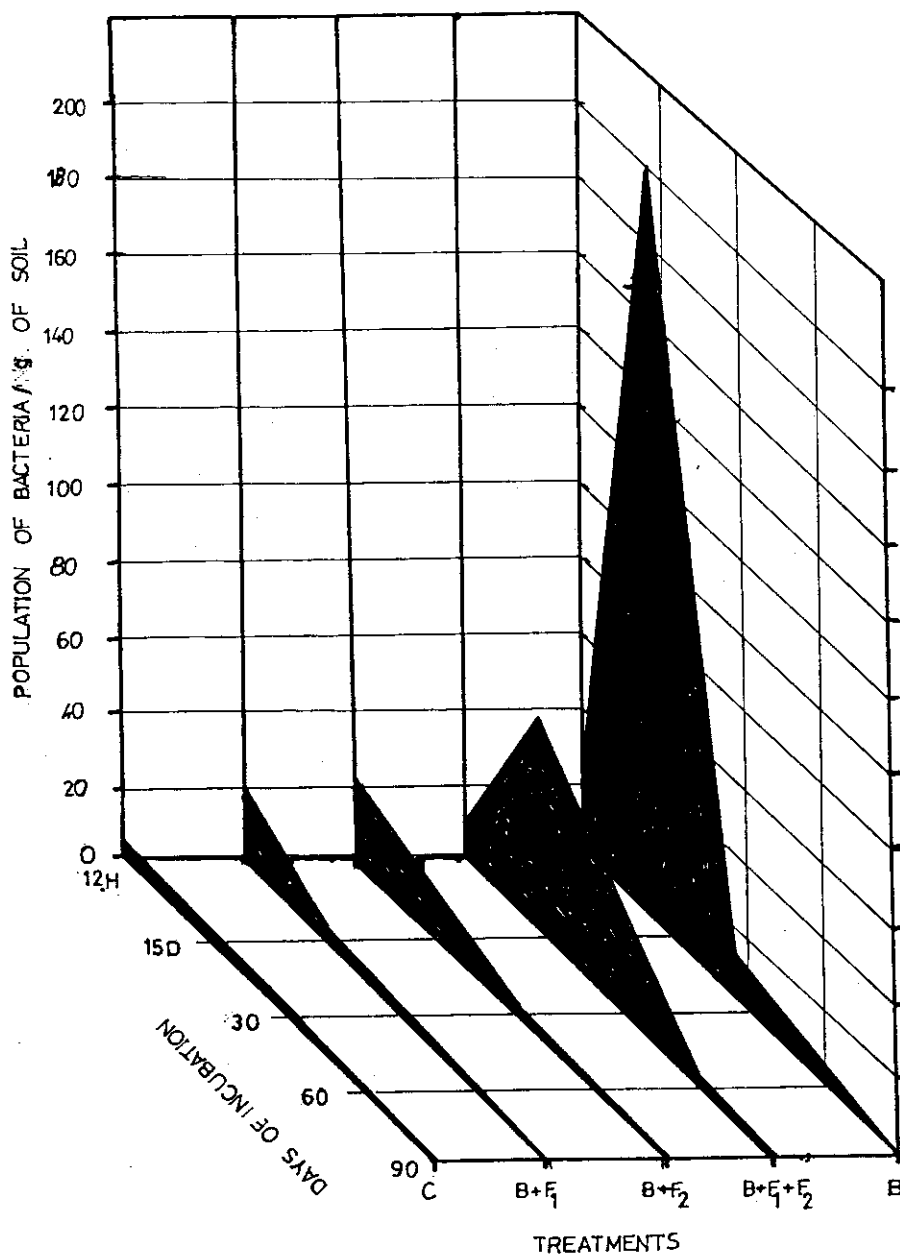
AMINOACID ANALYSIS OF ENTEROBACTER TOXIN

RESULTS EXPRESSED AS mg/100mg SAMPLE

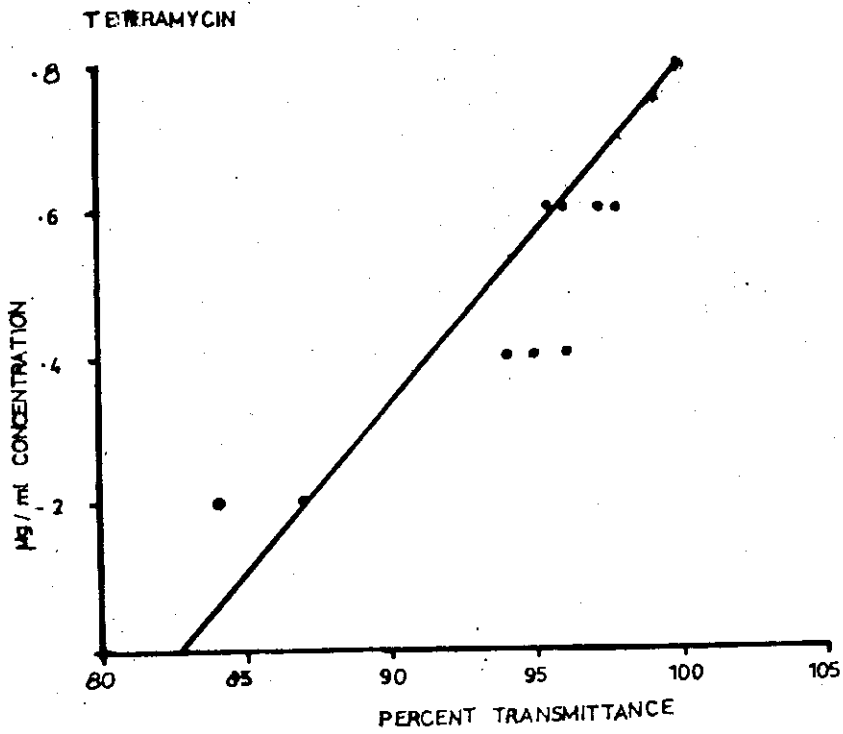
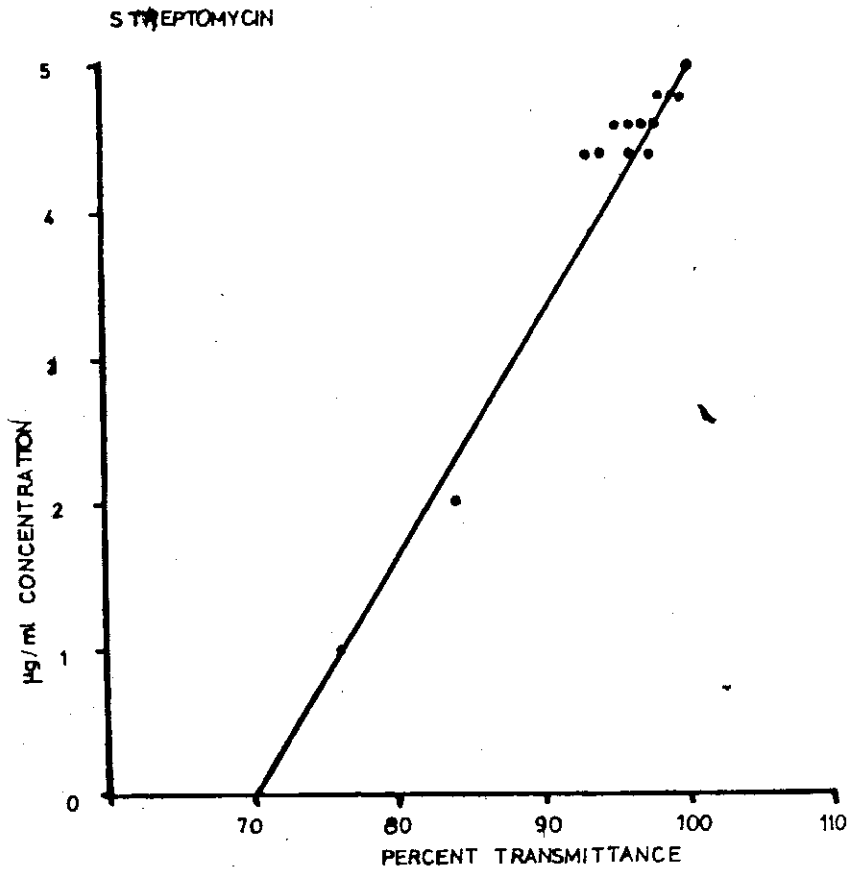
Fig-12



COMPETITIVE SAPROPHYTIC ABILITY OF ENTEROBACTER CLOACAE.



ANTIBIOTIC ASSAY AGAINST COCONUT ENTEROBACTER



5. SUMMARY

A detailed and systematic investigation on the association of bacteria with the root (wilt) disease of coconut was carried out. Culturing and isolation of bacteria from stelar bits of roots of palms showed the preponderance of Enterobacter cloacae and its affinity to palms in the diseased tracts with the comparative negligible incidence in the healthy tract. Relevant in this context is the formulation of a medium simulating the conditions akin to the natural habitat, with the incorporation of coconut root extract solidified with agar. These coconut Enterobacter cloacae isolates produced alcohol precipitable polysaccharide-like materials that could wilt test plants in vitro. The influence of different carbohydrates on the extent of production of extracellular polysaccharide has been tested in different media, wherein the profitability of coconut root extract was observed. Maximal production of polysaccharide was noticed on incubation of coconut Enterobacter isolates for a period of 13 days. The ability of coconut Enterobacter isolates to produce polysaccharide was compared with 24 standard cultures of Enterobacter cloacae from six international culture collections in two media namely coconut root extract broth and nutrient broth. The crude toxin extracted from the extracellular polysaccharide of coconut Enterobacter isolates on bioassay in vitro at different concentrations showed that the toxin at 2,000 ppm could wilt plant cuttings within a period of 30 min and the time required to wilt was inversely proportional to the concentration. The crude toxin has been observed to be antigenic. The antiserum formed clear precipitation band with crude toxin and extracts from diseased materials showed a serological relation; whereas no such relationship was observed with the polysaccharide preparations of standard Enterobacter cloacae cultures, thereby bestowing the coconut Enterobacter isolates with an individual attribute.

The coconut Enterobacter toxin subsequent to purification using sephadex G.200 has been eluted only to a single fraction with an estimated molecular weight of 89,130. On passing through Dowex columns the neutral acid hydrolysate of the crude toxin was separated into anionic, cationic and neutral fractions with a total recovery of 86.66% in a proportion of 13:8:5 respectively indicating the dominance of sugar acids and amino-acids attributing the possibility of the toxin being a glycopeptide. The cationic fraction of coconut Enterobacter toxin was resolved into three ninhydrin positive spots on Thin layer chromatographic plates, two of which have been identified as glutamic acid and lysine. On the other hand, fractionation with an Amino acid analyser has indicated the presence of 16 amino acids in the toxin. The neutral fraction of coconut Enterobacter crude toxin showed the presence of only glucose whereas the anionic fraction was resolved into five spots, none of which was identified.

On screening different antibiotics against the coconut Enterobacter isolates, the sensitivity of the bacterium to streptomycin and tetracycline groups of antibiotics was revealed which prompted investigations in this direction, that have provided rich dividends on the possibility of checking the disease to an extent using a research formulation of oxytetracycline. In the present investigation attempt has also been made to arrive at a precise conclusion on the exact role of the coconut Enterobacter cloacae strain in root (wilt) disease, by initiating a large scale pathogenicity experiment, by inoculating the bacterium alone and in combination with the other biotic agents implicated, to coconut seedlings planted in microplots containing methyl bromide fumigated soil, the results of which are awaited.

REFERENCES

- BRADBURY, J.F. 1970. Isolation and preliminary study of bacteria from plants. Rev. Pl. Path. 49: 213-218.
- DAVIS, T.A. 1964. Contribution to the physiology of the coconut palm. Proc. 2nd Sessn. FAO Tech. Wkg. Pty. Cocon. Prod. Fret. and Processg. Colombo pp. 150-174.
- GRANATH, K.A. 1965. Gel filtration, fractionation of dextran. pp. 20-28. In Methods in Carbohydrate Chemistry. Vol.V. ed. Whistler, R., Academic Press, Inc., New York.
- HAMILTON, R.I. 1961. Properties of bromo mosaic virus and its related antigens. Virology 15: 452-464.
- HAMILTON, R.I. 1964. Serodiagnosis of barley stripe mosaic facilitated by detergent. Phytopathology 54: 1290-1291.
- HAMILTON, R.I. and BALL, E.M. 1966. Antigenic analysis of extracts from barley infected with barley stripe mosaic virus. Virology 30: 661-672.
- HOPKINS, D.L. and ELMSTROM, G.M. 1977. Etiology of watermelon rind necrosis. Phytopathology 67: 961-964.
- OUCEPERLONY, O. 1958. Diffusion in gels, methods of immunological analysis. pp 1-78. In Progress of Allergy, V. ed. Kallos, D., B.S.Karger, Basel and New York.
- RAI, P.V. and STROBEL, G.A. 1969. Phytotoxic glycopeptides produced by Corynebacterium michiganense. II Biological properties. Phytopathology 59: 53-57.
- RAMADASAN, A. 1967. On the nature of wilt in the root (wilt) disease of coconut palm. Proc. 1st Internat. Symp. Plant Path. New Delhi. pp.670-675.
- RANGASWAMI, G. and SOUMINI RAJAGOPALAN. 1973. Bacterial Plant Pathology. Tamil Nadu Agricultural University, Coimbatore, India, pp.291.

- BOHRBACH, K.G. and PFIFFER, J.B. 1976. The interaction of four bacteria causing Pink disease of pineapple with several pineapple cultivars. Phytopathology 66: 396-399.
- SRIVASTAVA, D.N., SHEKHAWAT, G.S. and RAO, Y.P. 1969. Association of bacteria with root (wilt) of coconut in Kerala. Indian J. Agric. Sci. 39: 395-396.
- STROBEL, G.A. 1967. Purification and properties of phytotoxic polysaccharide-produced by Corynebacterium scopodonicum. Plant Physiol. 42: 1443-1441.
- TREVELYAN, W.E., PROCTOR, D.P. and HARRISON, J.S. 1950. Detection of sugars on paper chromatograms. Nature 166: 444.

13. Approximate expenditure incurred in the Project: (Give reasons for variation, if any, from original estimated cost)

Rs. 90,000/-

The additional expenditure was due to extension of the project for three more years subsequently.

14. Publications and material (one copy each to be supplied with this proforma)

a) Research papers:

1. Mathew George. 1983. Investigation on the association of bacteria in the root (wilt) disease of coconut (Cocos nucifera L. Ph.D. thesis submitted to University of Kerala, India.
2. Mathew George, Potty, V.P. and Jayasankar, N.P. 1976. Association of Enterobacter with coconut root (wilt) disease. Curr. Sci. 45 (18): 677-678.

b) Popular articles:

3. Mathew George. 1979. A comprehensive scheme to contain root (wilt) disease. Published in Malayala Manorama dated 27-7-1979.
4. Jayasankar, N.P., Joseph, K.V. and Mathew George. 1981. Microbiological aspects. In Review of research on coconut root (wilt) disease (Mimco.). CPCRI Regional Station, Kayangula India.

* c) Radio talks:

5. Mathew George. 1981. Certain assorted thoughts on "Kattuvcezhcha". Science broadcast on 30-1-1982, duration 10 min.

d) Seminars and workshops (Relevant to the project) in which the Scientists have participated:

Annual Research Council, CPCRI - 1976 to 1982.

e) Materials developed such as new varieties of crops or breeds of farm animals, implements, products, etc.)

15. Details (Nos. etc.) of Field/Laboratory Note books and final material and their location.

Laboratory Note Book - 5

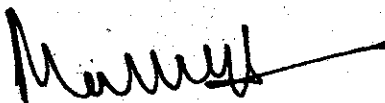
Project file - 1

CPCRI Regional Station, Kayangulam, Kerala - 690 533

16. Comments/suggestions of Project leader regarding possible future line of work that may be taken up arising of this Project:


Although Project Micro III(231) is closed, the large scale pathogenicity trials in field tanks are under observation and reported under Path 1.3 (231). The results of the experiment are awaited to suggest any future line of study.

17. Signatures with name of Project Leader and Associates:

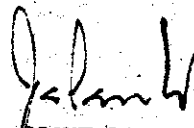


Mathew George
Project Leader.

18. Signature (with comments, if any) of Head of Division/Section/Station:



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JOINT DIRECTOR
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- KRISHNAPURAM-P.O. 690533.

19. Signature (with comments, if any) of Director:

