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Host-parasite interactions with reference to polyphenols in coconut roots

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Central Plantation Crops Research Institute
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8. (a) Objectives (Specify) briefly the AIMS & GOALS of the project in not more than 150 words.

The involvement of a pathogen in coconut root (wilt) disease is undisputable although the exact etiological agent is still unknown. Basic information relating to metabolic sequences in the coconut palm and their alterations subsequent to Pathogenesis in scanty. Alterations in phenol metabolism due to disease is a wide spread phenomenon. The consensus on the pattern of the phenolic compounds from their site of synthesis was yet another reason. The objective of the present investigations is to assess the rate of metabolism of phenolic compounds in relation to coconut root (wilt) disease.

The mode of inducing disease syndreme including the foliar and root symptoms can be attributed in many instances to enzymes and toxic principle elaborated by the pathogen. It was therefore one of the objectives to undertake investigations on these aspects relating to various microorganisms found associated with the root (wilt) affected palms.

(b) Practical utility

It is not uncommon to undertake such investigations on the unknown etiology. Altered metabolism of phenolic compounds often provide results with a practical bias. In the lethal yellowing diseas of coconut the quantitative determination of phenolic compounds and its oxidizing enzymes have been made use of for forcasting the disease before the onset of the visible symptoms. The ability of the potential pathogen to cause alterations in the cell leading to defined symptoms can be made use of in the process of elemination.

(c) Technical Programme

1. The estimation of total phenol in the healthy, apparently healthy and root (wilt) affected coconut palms and its characterisation.
2. Determination of peroxidase and polyphenol oxidase in the roots and its multiple form with particular reference to root (wilt) disease. Influence of the disease on the rate of biosynthesis of phenolic compounds.
3. In vitro and invico ability of the various microorganisms found associated with disease in causing alterations in the roots and leaves.

Host-Parasite Interations wit[illegible]ference to Polyphenols in coconut roots.

FINAL REPORT

Introduction

The implication of the phenolic compounds in the defence mechanism of the host is an acceptable factor, since its presence is noted at the infection and wounding sites (Hare, 1966). The infection of the host

generally associated with an increased synthesis of polyphenols and its oxidation which essentially constitute the mechanism of defense by the host (Parkas and Kiraly, 1962). The oxidation products are more toxic to the invading pathogen and the extent of toxicity depends on the stages of oxidation. (Farkas and Ledingham, 1959).

The coconut root (wilt) disease is one of unknown etiology and the involvement of a pathogen is not ruled out. The results are indicative that there is a deranged metabolism in the host tissue. In the past, no detailed studies have been carried out in relation to host-parasite interactions with reference to polyphenols in coconut palms. In view of the importance of the polyphenols in the defense mechanism a detailed study was undertaken and the result obtained are detailed below. The studies were restricted to the metabolism of the phenolic compounds viz. its general status and the levels of the oxidative and synthesizing enzymes. The investigations were mainly restricted to root samples except some initial studies where leaf material was also analysed.

The cultivars selected for the studies were West coast tall (WCT), and the hybrids Tall x Dwarf (TxD) and Dwarf x Tall (D x T). Among these cultivars WCT is cultivated since centuries and over 90% of the total coconut cultivation was also WCT, the detailed studies were restricted to WCT palms except some initial studies where other cultivars were also included.

MATERIALS AND METHODS

1. Selection of palms/samples

Palms were indexed for the incidence and increase in intensity of coconut root (wilt) disease using the method described by George and Radha (1973) based on the foliar symptoms consisting of flaccidity, necrosis and intermitant yellowing. Samples collected from coconut palms cultivated in the root (wilt) free tract at Kasaragod was classified as healthy (A). While samples from palms free of the symptoms of the disease from Kayangulam a disease affected tract was designated, apparently healthy (AH). Likewise applying the foliar indexing system of George and Radha (1973) samples were collected from palms in

the disease early (DE) and advanced stages (DA). Based on the obseravations made in the preliminary experiments bulk of the present investigations were restricted to the root samples in view of the comparitively better consistency.

2. Collection of samples

a) Roots

Root samples were collected from the basal part of the palms within a diameter of 1 M. The soil was removed from the basal part and the freshly enrovging roots were located. Roots showing no external injury were used for the entire studies. Samples were cut within 6 to 12" in length and the applaced in ice chests brought to the laboratory and stored appropriately. Details of the root samples collected for each studies are given under.

Table I

Details of the root samples collected for the study

Particulars of the study	WCT	TxD	DxT
Total phenol	142	115	85
O-dihydroxyphenol	58	-	-
Polyphenol Oxidase (PPO)	80	-	-
Peroxidase	80	-	-
Phenylalanine ammonia lyase	57	-	-

b) Leaves

The normal practise in vogue is the analysis of the leaflets from the 14th leaf of the coconut palms. (Fremond and Lamothe 1956). However, during the present studies difficulties were encountered to locate the 14th leaf in root (wilt) affected palms particularly in the advanced stage of the disease. Consequently sampling was restricted to the first dully opened leaves. Immediately after the collection the samples were transported to the laboratory and processed in the manner de with earlier.

3. Extraction of total phenols.

The total phenol fractions were extracted from the roots by the

method described by Biehn et al (1968). The root samples were washed first in tap water followed by distilled water. The washed roots were cut into small pieces, 5.0g of the sampled material was placed in 100 ml EM flask containi[illegible] ml of 80% methanol and boiled for 15 min. The boiled samples were decanted and the residue was disintigrated in another 25.0 ml of 80% elthanol and pooled with the decanted liquid heated for 5 min. and filtered while hot using methanol water washed Whatman No.I filer paper. The filterate was made up to 100 ml in a Volumetric flask. Suitable aliquotes were taken for the estimations.

4. Estimation of total phenol

Total phenol content was estimated by using Folin-Dennis reagent (Swain and Mills; 1959). 1-0 ml of the aliquote was mixed thoroughly with 5.0 ml of 2.8 N. sodium carbonate in a 25.0 ml volumetric flask. To the mixture 2.0 ml of diluted (1:2) Folin-Dennis reagent was added with shaking. The volume was made upto 25.0 ml with distilled water and the colour intensity was measured in a Klett Summerson Colorimeter by using the red filter (640-700 mm). Chlorogenic acid was used as the standard.

5. Characterisation of the [illegible]olic compounds

The characterisation of the phenolic compounds was achieved by the method Biehn et al (1968). The methanol extract was evaporated to near dryness, suspended in distilled water, acidified to P^H 4.5 with 0.1N. hydrochloric acid and extracted three times with 5.0 ml aliquote of ethylacetate. The ethylacetate fractions were pooled together and evaporated to near dryness. The residue was dissolved in ethylacetate (5.0 ml) and washed with acidified distilled water. Tracees of water was removed by the addition of 1.0g anhydrous sodium sulphate. The ethylacetate fraction was evaporated to dryness and the residue was dissolved in 1.0 ml of 90% ethanol. All the evaporations was done under reduced pressure at less than 40°C in a vaccum desicator.

Extractions from the leaf samples was similar to the procedure described above.

6. Chromatoographic separati[illegible]

Thin layer chromatography was used for the separation with silica gel G, polyamide and cellulose. (glass plates of size 20 x 20 cm).

Cellulose was found to be the most suitable and further studies was made with this material. In all the experiments the slurry was prepared in water except for polyamide for which chloroform was used. A known aliquots (0.1 ml) was applied on the plate with the help of a micropipette and was kept in a chromatographic chamber which contained the solvent system.

7. Selection of solvent system.

In all, 14 solvent systems suggested by Bickel (1964) were tried, among which dilute formic acid was found to give the best resolution and further studies were made with formic acid (2%). The different solvents systems used for the separation are given under.

Table-2

Solvent system used for TLC Seperations

Solvent system	Proporations
1. Acetic acid	2-5%
2. Acetic acid : Hydrochloric acid:Water (Forestal solvent)	30:3:10
3. Formic acid	2-5%
4. Isoamylalcohol:acetic ecid:water	4:1:5
5. Amylalcohol:Ammonia	10:3
6. Butanol:Acetic acid:water	4:1:5
7. Benzene:acetic acid:water	6:7:3
8. Butanol:Formic acid	10:3
9. Butanol:Ammonia:water	10:3:3
10. Butanol:Ethanol:water	5:2:1
11. Butanol:water	6:1
12. Chloroform:acetic acid:water	2:1:1
13. Ethanol:Ammonia:water	35:2:13
14. Ethylacetate:Formic acid:water	3:1:3

8. Determination of O-dihydrolyphenols

a. Preparation of the sample

The root samples were washed thoroughly and kept in an oven maintained at 80°C for drying. Oried samples were powdered in a dry grinder and was used for the estimation.

b. Quantitative determinations

Orthodihydroxyphenol content in the root was determined by the method of Dabek (1974) employed for its estimation in palms affected by lethal yellowing. The principle of the method was based on the histochemical test reported by Revee (1951) and similar to that used by Zucker and Ahrens (1958) in the estimation of chlorogenic acid.

1 gm sample was extrac twice with 50 ml quantities of boiling water each for a period of 5 min. afterwhich the residul polyphenol content was only negligible. The combined extracts were filtered through Whatman No.I filter paper and made up to 100 ml.

To 5.0 ml of the extract in a 25.0 ml volumetric flask 1.0 ml of 10% (w/v) sodium nitrate, 1.0 ml of 20% (w/v) urea and 1.0 ml of 10% (v/v) acetic acid were added in succession. The mixture was shaken well and after 3-4 min. 2.0 ml of 2.0 N Sodium hydroxide was added. The cherry red colour indicated the presence of O-dihydronyphenols. The colour intensity of the solution was measured in a Klett Summerson Colorimeter by using the Green filter (500-570 nm). Chloroginic acid was used as the standard.

9. Enzyme Studies

1. Removel of Tannins

Different methods were tried for the removal of tannins by the addition of extraneous protein like gelatin, Casein, egg albumin and the antioxidant 2-mercaptoethanol in the grinding medium (Panlson and Falton, 1968). The efficacy of individual protains and 2-mercaptoethanol alone and both in combination were also tried.

2. Methods

Ten gm. of freshly collected leaf samples were macerated in a waring blender with 100.0 ml of distilled water containing any of the following.

1. 1% gelatin
2. 1% egg albumin
3. 3% casein
4. 1% 2-mercaptoethanol
5. 1% gelatin + 1% 2-mercapt[illegible]hanol
6. 1% egg albumin + 1% 2-mercaptoethanol
7. 3% casein + 1% 2-mercaptoethanol.

The macerates were filtered through chese cloth and to this filterate 50.0 ml solvent ether was added to remove the chlorophyll. The tannin content in clear extract was estimated by permanganate titiation using indigo carmine as the indicator (Horwitz, 1955).

3. Acetone powder preparation

The root samples collected were stored in a deep freezer for 24 hours for freezing. About 15 g. of the frozen samples of the roots were sliced into small bits and transferred to a waring blender containing 150 ml of cold citrate buffer (0.1M) of P^H 7.0 incorporated with 1% 2-mercaptoethanol. The contents were mascerated for 1 min. and the slurry filtered through chees cloth. The residue was reextracted with 100 ml of the same buffer. The slurry was filtered similarly and both the filterate were pooled in a 2.0 liter beaker. To the combined filterate double the volume of chilled acetone was added with gentle stirring. The precipitate was allowed to settle for 5.0 min. and filtered with the aid of Buchner funnel using Whatman No.I filter paper. The precipitate was removed from the filter paper and kept in a vaccum desicator overnight. The acetone powder preparations were stored in cold in screw caped test tubes for further studies.

4. Assay of Polyphenol oxidase (PPO)

Polyphenol oxidase activity was measured using a method similar to that of Matta and Dimonds (1963). About 250 mg of dried acetone powder was extracted in 10.0 ml of citrate-phosphate buffer (0.1M) of P^H 7.0 was used as the crude enzyme. The reaction mixture consisted of 3.0 ml catechol (0.5%) as substrate, 0.75 ml citrate-phosphate buffer (0.1M) P^H 7.0 and 0.25 ml of the enzyme preparation in a cuvette. The

reaction mixture without the enzyme preparation was used as the control. The optical density of the reaction product was measured in a Beckman DU-2 model spectrophotometer at 400 nm after 5.0 min incubation. The specific activity was expressed as OD/mg of protein/5 min.

5. Assay of Peroxidase(PO)

Peroxidase was assayed using the pyrogallol test (Chance and Machly, 1955). 250 mg of dried acetone powder was extracted with 10.0 ml of citrate phosphate buffer (0.1M) PH 70 and the assay system consisted of 2.0 ml of pyrogallol solution (40 mM), 1.5 ml citrate phosphate buffer (0.1M) PH 7.0, 0.5 ml hydrogen peroxidase (3.0%) and 1.0 ml of enzyme preparation. The reaction mixture was incubated at 37°C in a water bath for 5 min. and checked by the addition of 5.0 ml of 5.0N sulphuric acid. The coloure purpurogallin formed was extracted twice with 5.0 ml aliquots of solvent ether. The combined extract was evaproated to dryness at room temperature and the residue was dissolved in 5.0 ml of ethanol. The optical density of the resultant product was measured at 430 nm in a Beckman DU-2 spectrophotometer. The control contained all the ingredients except the enzyme preparations. The specific activity was expressed as OD/mg of protein/5 min.

8. Determination of protein

Protein was determined by the method of Lowry et al 1951. The reaction mixture consisted of 1.0 ml of the sample solution containing approximately 5-100 mg protein and 5.0 ml of alkaline copper solution. The micture was kept for 10 min. and added 1.0 ml of Folin-Ciocalteau reagent. The volume was made upto 10.0 ml and the colour intensity was measured after 30 min. in a Klett Summerson Colorimeter using the red filter (640-700 nm). Bovine serum albumin was used as the standard.

10. Determinatation of the isoenzyme pattern of Polyphenol Oxidase (PPO)

1. Partial purification

The partial purification of the PPO was carried out by the method described by Kahn (1975, 1976) with some modification.

Step-1-Acetone powder preparation.

Acetone powder was prepared from the roots same way as described above.

Step-2- Ammonium sulphate precipitation

The acetone powder prepared was suspended in cold sodium phosphate buffer (0.05M) P^H 6.5.(5mg Powder/ml) and stirred continuously for 30 min. at 0-4°C. The mixture was centrifuged at 15000 x g for 20 min. in a refugerated centrifuge. The supernatent was used as the crude enzyme fraction.

To the supernatant, solid ammonium sulphate was added slowly with stirring untill 90% concentration of ammonium sulphate was reached. The solution was stirred further till all the ammonium sulphate added was dissolved completely and kept in a refrigerator overnight to complete the precipitation. The suspension was centrifuges at 15000 - x g for 15 min and the pallet was dissolved in cold sodium phosphete buffer (0.05M) of P^H 6.5 (about 60.0 ml).

Step-3-Dialysis

The solution was then transferred to dialysing tube, tied properly and kept in 500 ml of cold sodium phosphate buffer (0.05M) of P^H 6.5. in a beaker kept in a refrigerator overnight with changes of buffer 3 and 6 hr. intervals.

Step-4- Gil filteration

The dialysed enzyme preparation was concentrated by sprinkling 0.5 g. sephadex G 200 over the dialysing tube. Concentrated sample was applied to column containing sephadex G-200 gel which was equiliberated with sodium phosphate buffer (0.05M) of P^H 6.5 with aid of a pipette. The solution was allowed to drain off, but not completely rendering the gel to dry. After the application of the sample the column was filled with sodium phosphate buffer (0.05M) of the P^H 6.5 which was used as the eluent.

After allowing the first few ml of the eluent to drain off 5.0 ml fractions were collected at the 0.5 ml/min in test tubes. PPO activity was determined in each fraction by the method described above.

Step-5- Ammonium sulphate precipitation

The fractions containing PPO activity were pooled together in a 500 ml beaker. Solid ammonium sulphate was added slowly with stirring

till it reached 90% saturation. The stirring was continued for 30 min. till all the ammonium sulphate crystalls were dissolved. The solution was allowed to stay overnight and centrifuged at 15000 x g for 15 min. in a refrigerated centrifuge. The pellet was dissolved in 10 ml sodium phosphate buffer (0.05M) of p^H 6.5.

Step-6- Dialysis

The solution was transferred to a dialysing tube, tied properly and kept in 500 ml of cold sodium phosphate buffer (0.05M) of p^H 6.5. in a refrigerator at a temperature of 0-4°C overnight with a change of buffer 3 hour and 6 hour inntervals. After the dialysis the solution was concentrated by sprinkling dry sephadex G-200 over the dialysing tube.

All the operations wer carried out at temperature below 10°C. in a cold room. The partially purified enzyme was resolved into its isoenzyme pattern.

11. Determination of Isoenzymes of polyphenol coxidase (PPO).

Isoenzyme pattern of the PPO was carried out by polyacrylamide gel electrophoreses (Davis, 1964). Cleaned glass tubes of the size 12 cm x 5 mm were used for the gel polymerisation. To contain unpolymerized gel solutions, the tubes were stoppeered with polyethylene cups. The tubes, were placed in a rack veritically and kept between two fluorescent tube lights for enhancement of polymerisation. Stock solutions were prepared as described- Wrigley (1971).

Separating gel formulations

Stock solutions 10% acrylamide, 0.1% Bis. p^H8.9.

a) IN Hcl 48.0 ml
   Tris 36.3 g
   TEMED (Tetramethylethylenediamine) 0.23 ml. resulting P^H 8.8 to 9.0.
   Volume made up to 100 ml with glass distilled water.

b) Acrylamide 60.0 g
   Bis 0.6 g
   Water 100 ml

c) Ammonium persulphate 0.14 g, water 100 ml. Working solution was

prepared according to the following combinations.

1 part (a), 1.34 parts (b); 4 parts (c); 1.66 parts H_2O.

Stacking gel formulations

Stock solutions

a) Tris 5.98 g

TEMED 0.46 ml

IN Hcl about 48.0 ml to p^H 6.7 volume made up to 100 ml with glass distilled water.

b) Acrylamide 10 g

Bis 2.5 g

Water 100 ml

c) Riboflavin 4.0 mg

Water 100 ml

d) Sucrose 40 g

Water 100 ml

Working solution was prepared according to the following combination.

1 part (a), 1 part (b), 1 part H_2O, 1 part (c), 4 parts (d).

The working solution for the separating gel was transferred to the tubes with the help of a pipette. About 8 cm length of the tube was filled with the working solution. The top of the solution was layered with distilled water. The tubes were there kept for polymerisation in the fluorescent light for about 40 min.

The water layer of the above gel was removed with the aid of a filter paper strip. About 0.5 cm above the separated gel was filled with stacking solution and was layered with distilled water and again kept for polymerisation as mentioned above for about 30 min. After the polymerisation the tubes were removed from the rack and fixed in the perforation of the upper tank.

12. Application of the sample solution.

The water layer present in the top of the gel was removed with the help of filter paper strip. Solid sucrose was added to the partially purified enzyme to get 10% concentration and was used as a sample solution. About 0.1 ml of the solution which contains 50 to 100 μg of protein was transferred to the gel tubes and was then layered with 10% sucrose solution. Both the compartments of the electrophoretic

apparatus were filled with Tris glycine buffer (10 m M) of P^H 8.3. The electrophoresis was carried out at 2.5 mA per tubes. Bromophenol blue (0.1%) was used as tracking dye.

13. Staining.

The gel were removed from the tubes and stained for PPO according to the method described by Srivastara and Van Huystee (1973). The gels were incubated under vigorous areation in 0.01M DOPA (Dihydroxyphenylalamine) in phosphate buffer (0.05M) of P^H 7.0. After one and half hours incubation brown bands appeared at the site of PPO activity. The stained gels were kept in 7% acetic acid in test tubes with few drops of benzine.

14. Determination of Phenylelamine ammonia lyase (PAL) activity.

a) Collection of sample and preparation of acetone powder.

Sampling and acetone powder preparations were similar to those employed for PPO and PO.

b) Assay for PAL

250 mg of the acetone powder preparation was extracted in 10.0 ml of Tris-Hcl buffer (0.05M) of P^H 8.8 and the assay was carried out by the method described by Subba rao and Towers (1970) and used subsequently by Yamamoto et al 1977.

The assay system consisted of 2.0 ml of Tris.Hcl buffer (0.05M) of P^H 8.8, 2.0 ml of 0.01M L-phenylalamine and 2.0 ml of the enzyme solution. The mixture was incubated at 37°c for 4 hr. The reaction was stopped by the addition of 1.0 ml of 1.N. Hcl and the cinnamic acid formed was extracted with per ..de free ether. The ether layer was separated and evaporated to dyness under a jet of hot air.

The residue was dissolved in 3.0 ml of 0.05N sodium hydroxide and the amount of cinnamic acid was determined spectrophotometrically at 268 nm. using Beckman DU-2 model spectrophotometer. The reaction mixture with 1.0 ml of 1 N. Hcl was the blank, specific activity was expressed as µg of cinnamic acid/mg of protein/4 hour.

RESULTS

1. Concentration of total phenols in roots of WCT, T x D and D x T coconut palms.

The mean total phenolic content in the roots of WCT, TxD and DxT palms

was in the order of 128 mg, 126 mg and 88 mg per 100 g dry tissue respectively. (Fig 1). The rangeof phenolic content and number of samples analysed are indicated in table 3 and Fig. 1.

Table 3: Total phenol content in the roots of healthy WCT, TxD and DxT palms (mg/100 g dry wt.).

No.	Coconut cultivars	No. of samples analysed	Total phenol content (mg/100 g dry wt.	Mean
1.	WCT	36	85-205	128
2.	TxD	32	126 70-213	126
3.	DxT	33	88 20-210	88

The incidence of the root (wilt) disease, however, had an impact on the phenolic content with a decrease in the roots of the palm cultivated in root (wilt) affected tract compared to healthy. (Fig 2). Like wise with the increase in the intensity of the disease there was a fall in the total phenol content in the different cultivars studied. Fgs 3, 4, and 5) and decrease was more pronounced in the case of the WCT palms.

2. <u>Variation in the concentration of total phenols with the incidence and increase in the intensity of coconut root (wilt) disease.</u>

Percentage variations in the concentration of total phenols with the incidence and increase in the intensity of coconut root (wilt) disease is given in table No.4.

Table 4: Percentage variation in the concentration of total phenols with the incidence and increase in intensity of root (wilt) disease.

No.	Healthy	Apparently healthy	Disease early	Disease advanced
1.	100	- 22	- 45	- 49
2.	100	- 4	- 22	- 45
3.	100	+ 20	- 33	- 61

In the case of WCT and TxD there was gradual decrease in the concentration of total phenols from healthy to disease advanced while in DxT the

the apparently healthy palms were having the maximum concentration. From the apparently healthy (AH) the concentration of total phenols gradually decreased.

3. Chromatooraphic studies.

Inorder to standardize the optimal conditions required for the Chromatographic analysis of the qualitative nature of the total phenols in coconut root (wilt) disease different solvent systems were tried (Table 2. Materials & Methods). The Chromatographis pattern of the root extract showed three fluorescent spot under U.V. light irrespective of the disease conditions. The fluorescent sports were having the Rf values 0.28, 0.42 and 0.57 respectively. The diseased samples were also exhibiting the same U.V. fluo[illegible]cent spot but they were very faintly seen (Fig 6.).

The trend was not identical in coconut leaves. Healthy samples contained three fluorescent spots with Rf values 0.36, 0.50 and 0.57 (Fig 7). Preliminary observations in this direction indicated that one of the compounds (Rf value 0.36) was absent in the leaves of the coconut palms cultuvated in the diseased tract. This observation was since then investigated in detail with leaf samples collected from different parts of Keral, Hirehalli (Mysore) and Goa. The study was also extended to other cultivars (Table 5). The presence of this compound was noticed in 131 WCT palms. Out of 139, 18 TxD palms out of 20 and 18 DxT palms out of 20 samples analysed from disease free area (Kasaragod). The presence of the same compound was recorded in the sample out of 20 collected from Hirahalli and all the three samples collected from Goa. All the WCT samples collected from root (wilt) affected area showed its absence.

Table 5. Distribution of ether extractable phenolic compound in the coconut leaves.

Locality	Variety and source		Total No. of palms	Presence	Absence
Root (wilt)	Kasaragod	WCT	139	131	8
Tree tract	"	TxD	20	18	2
	"	DxT	20	18	2
	Hirahalli	WCT	20	16	4
	Goa	WCT	3	3	-

Root (wilt) affected tract	Kayangulam	WCT	50	-	50
	Chavara	"	7	-	7
	Purakad	"	15	-	15
	Thazava		10	-	10
	Muthukulam	"	8	-	8
	Chathannoor	"	12	-	12
	Thazava	DxT	9	7	2

The interesting observation made in this connection was the Chromatographis pattern observed in sample collection from DxT palms in the disease affected tracts. Contrary to the WCT palms where the phenolic compounds was completely absent 7 DxT palms out of 9 showed its presence.

4. Reaction of ethanolic extract with Erlich's (1946) Reagent, P-dimethyl aminobenzaldehyde (PDAS).

While characterising the phenolic constituents its was observed that an alcoholic extract of the root produced a red colour with Erlich's reagent consisting of an acidic solution of paradimethyl amino benzaldehyde (PDAB). This finding was fur[illegible] investigated since PDAB has been employed under varying conditions for the quantitative estimation of pyrogallol the result was a rapid method for the estimation of pepogallol at a range of 5-20/ ug

5. The Method

All the chemicals used were Analytical reagent grade.

Standard pyrogallol

50 mg pyrogallol was dissolved in 500 ml glacial acetic acid. Suitable dilutions of this stock solution were made with glacial acetic acid to give working standard of varying concentrations.

PDAB-reagent-10% PDAB in 99-100% glacial acetic acid.
Sulphuric acid - concentrated.

To 5.0 ml aliquots of sample containing 5-20/ug of pyrogellol was added to 1.0 ml of PDAB so[illegible]ion and the mixture was kept in an ice bath. This was followed by the addition of 1.0 ml of concentrated sulphuric acid and the colour intensity was measured in a Klett Summerson

photoelectric Colorimeter-using the green filter No.54 (520-580 nm). The absorption maxima of the ur complex was recorded in a Beckman DU-2 model spectrophotometer.

The absorption spectrum of the pyrogallol and PDAB in the presence of H_2SO_4 showed in absorption maximum at 405, 485 nm (F.g 8). When colorimeter filter No.42 (400-450 nm) used the readings were exhorbitantly high and its was not used further. The main basis of the procedure depends on the use of PDAB under controlled conditions. The intensity of the colour complex increases with the increase in the concentration of PDAB. Any increase beyond 10% was not significantly beneficial to the colour formation. PDAB is used for the chromatographic detection of a variety of phenolic compounds (Shaw and Trevarthan 1958) but has not been used for the quantitative estimation of pyrogellol. There is linearity between the concentration of pyrogallol and intensity of the colour in the range of 5-20/ug (Fig 9).

Amongst the compounds tested, rescreinol, phloroglucinol, m-cresol and catechol responded to the test in varying degrees (Table 6). Their interferences on the estimation of pyrogallol has also been indicated in table 6. Aromatic amines were known to interact with PDAB and the results revealed their possible interference on the chromogenic reaction at higher concentration. But the interference caused by any of these compounds that are likely to be present in a biological system can be avoided by chromatography prior to estimation.

Table 6: Specificity of the colour reaction and the effect of interfering substances on the development of the colour.

No.	Substances added	Klett reading using green filter: Colour reaction with individual compounds	Klett reading using green filter: Pyrogallor with * interfering compounds
1.	Pyrogallol (control)	160	160
2.	Catechol	15	175
3.	Resorcinol	133	220
4.	Phloroglucinol	50	210
5.	M-cresol	60	185

* A 5.0 ml solution containing 20 ug of pyrogallol and 20 /ug of each compound was used in the development of the colour. Control contained only 20/ug of pyrogallol.

Table 7: Effect of aromatic aminos on the reaction *

No.	Substance added	Klett reading using green filter No.54
1.	Pyrogallol (control)	160
2.	Anthranilic acid	176
3.	Aniline	184
4.	Napthylamine	192
5.	Sulphanilic acid	194
6.	O-Amino Phenol	176

* A 5.0 ml solution containing 20 /ug of pyrogallol and 60 /ug of each amine was used in the de velopment of colour. Control contained only 20 /ug of pyrogallol.

6. Concentration of Orthodihydroxyphenols.

The mean O-dihydroxoyphenol content in the roots of coconut palms cultivated in the healthy and disease affected tract is given in fig 10. It is clear from the figure the concentration of O-dihydronyphenol is higher in the diseased samples when companed to the healthy. It is observed that the apparently healthy samples showed the maximum concentration when compared to the other catogories studied. The concentration of O-dihydroxyphenol decrease with the advancement of the disease (Fig 11) In Fig 11 the mean concentration of the O-dihydroxyphenol as well as the range are presented. The apparently healthy samples showed a wider range in the concentration of O-dihydroxyphenol 15-95 with a mean value of 33.0. While in diseased early the range was 10-50 with a mean value of 20.0. The healthy samples showed a narrow range 11.30 when compared to other samples with mean value of 18.0. The values expressed mg/100 g dry tissue.

7. Studies on the enzyme related to the metabolism of Phenols.

While standardizing the assay of enzymes related to the metabolism of phenolic compounds in coconut palms with particular reference to root (wilt) disease an essential prerequisite was an appropriate procedure for the elimination of tannins which interfered with the assay. Consequently a method was standardized for the elimination of tannins using leaf samples. The method included the addition of extraneous protein, and

addition of an antioxidant and combination of both at different combinations percentage removal of tannin in terms of water extract with different treatment combination is given in table 8. Maximum eliminction in tannin was achieved with addition of 1% Gelation and 1% mercaptoethanol combination which removed 90% of tannins. Followed by this treatment 1% mercaptoethanol and 3% casein and 1% mercaptoethanol were found to remove 68% tannin. Since no extraneous proteins were used in 1% mescaptoethanol system and also it removed considerably good amount of tannins it was used for further studies.

Table 8: Percentage of tannins removed in terms of water extract

No.	Treatment	Percentage of tannin removed
1.	1% Gelatin	37.8
2.	1% Egg albumin	0.
3.	3% Casein	37.2
4.	1% mercaptoethanol	68.6
5.	1% Gelatin + 1% Mercaptoethanol	0.0
6.	1% Egg albumin + 1% Mercaptoethanol	60.8
7.	1% Casein) 1% Mercaptoethanol	68.6

8. Levels of Polyphenol oxidase activity (PPO).

Levels of polyphenol exidase (PPO) activity in healthy and diseased tract are given in Fig 12. Specific activity of PPO in the healthy sample was very low when compared to the palms in the diseased tract. Fig 13 showed the mean activity and range of PPO in the roots of coconut palms under varying disease conditions. With the increase of the disease intensity the activity was also increased. Highest activity was recorded in the diseased advanced palms while both healthy and apparently healthy palms showed negligible differences. There was no significant difference in the activities of PPO between disease early and disease advanced. The range varied from 0.61 to 8.0 in healthy, 1.3 to 8 in apparently healthy, 9.5 to 19.7 in diseased early and 10-20 in disease advanced. The total number of samples assayed, the mean value and range were given in table No.9.

Table 9 Details of the enzyme studies

No.	Enzymes studied	No. of samples ansayed H	AH	DE	DA	Activity of each enzyme H	AH	DE	DA
1.	Polyphnoloxidase*	24	30	18	10	4.4 (0.61-8.0)	5.4 (1.3-8.0)	[illegible].8 (9.[illegible]-19.7)	14.0* ([illegible]-20)
2.	Peroxidase *	24	30	[illegible]	10	4.4 (0.58-8.0)	4.8 (1.0-7.0)	[illegible].9 ([illegible].3)	12 (8.6-23.0)
3.	Phenylalanine ** ammonia lyase					0.90 (0.62-1.2)	20.3 (9.3-[illegible])	16.1 (8.1-48.3)	8.5 (6.1-15)

* Activity expressed in OD/mg protein/5 min.

** Activity expressed in /ug cinnamic acid produced/mg of protein/ 4 hour.

9. Peroxidase (PO) activity

The levels of peroxidase (PO) activity in healthy and diseased tract are given in Fig.14. The activity was very high in the diseased tract when compared to healthy. On Figure 15 is presented the mean activity and range of PO in the roots of coconut palms under varying disease conditions. With the increase in disease intensity the activity was also increased. It is cle[illegible] from the data that there was not much differences between the healthy and apperently healthy, disease early and diseased advanced samples. The range varied from 0.58 to 8.0 in healthy, 1.0 to 7.0 in apparently healthy, 8.[illegible] to 18.3 in disease early and 8.6 tp 23.0 in disease advanced. The total number of samples assayed, mean value and range were given in table 9.

10. Levels of phenylalanine Ammonia lyase (PAL)

Levels of phenylalanine ammonia lyase activity in healthy and diseased tract is given in Fig.16. The result indicated the activity of PAL in healthy samples was very low 1.0 while in samples from diseased tract it was very high. Figure 17 showed the mean PAL activity and range in the roots of coconut palms under varying disease conditions. From the figure it was clear that the PAL activity in apparently healthy palms was

very high when compared to other catogories studied. With the increase in intensity of the disease the activity was decreased. There was significant difference in the activity between each catagories of the samples studied. The range varied from 9.62 to 1.2 in healthy, 9.3 to 59.8 in apparently healthy, 8.1 to 48.3 in diseased early and 6.1 to 15.0 in disease advanced. The number of samples assayed, mean activity and the range are given in table 9.

11. Correlation between enzyme activities and disease index.

The correlation between phenol exidizing enzymes (PPO) and (PO) and disease index are given in Figures 18 and 19. With regard to these two enzyme activities, a perfect positive correlation was recorded as the disease index increased. The correlation coefficient worked out for PPO and PO were r = 0.51 and 0.613 respectively. Contrary to these two enzymes the PAL showed clean negative correlation with the increase of the disease index (Fig 20) and the correlation coefficient worked out for PAL was r = - 0.51.

12. Relation between the enzyme activities and total phenol content in the roots of healthy and root (wilt) affected palms.

, As the disease index increased the activities of the phenol oxidizing enzymes increased with a fall in the total phenol content. (Fig. 21 and 22). But in the case of PAL a perfect positive relation exist between PAL activity and concentration of total phenol content in the roots of coconut palms at different stages of the disease (Fig.23) PAL activity was increased from healthy to apparently healthy and then showed decreasing trend.

13. Relation between the enzyme activities and O-dihydroxyphenol content in the roots of healthy and root (wilt) affected palms.

The ralation between the phenol oxidizing enzymes and O-dihydroxyphenol content are given in figures 24 and 25. As the activity of the phenol oxidizing enzymes increased with the increase in the intensity of the disease the O-dihydroxyphenol content was decreasing. (Fgs 24 and 25). The relation between concentration of O-dihydroxyphenol and PAL activity is presented in figure 26. The highest activity of PAL and the highest concentration of O-dihydroxyphenol was observed in the case of apparently healthy palms. Both PAL activity and concentration of

O-dihydroxyphenol decreased with the advancement of the disease. It showed that a relationship exists between the PAL activity and the concentration of O-dihydroxyphenol.

14. Partial purification and determination of Isoenzyme pattern of polyphenoloxidase.

The activity of the c... e enzyme prepared (acetone powder) was very low to enable for the determination of the isoenzyme patter of PPO. It was therefore necessary to purify the enzyme further. PPO from the supernatent was precipitated at 90% ammonium sulphate concentration.

The precipitate was dissolved in sodium phosphate buffer (0.05M) of P^H 6.5. The protein content and specific activity of PPO were determined. The solution was then dialysed and the value was further reduced with sephadex G-200. The concentrated sample was subjected to gel filteration using sephadex G-200. The enzyme was eluted with sodium phosphate buffer (0.05M) of P^H 6.5 and 5.0 ml fractions were collected. The fractions showed PPO activity were pooled together (Fig 27) and the enzyme was against precipitated with 90% ammonium sulphate. The pellets were dissolved in sodium phosphate buffer C0-05M P^H 6.5, dialysed and concentrated. The Protein content and specific activity in each step was determined and is given in ta..e 10.

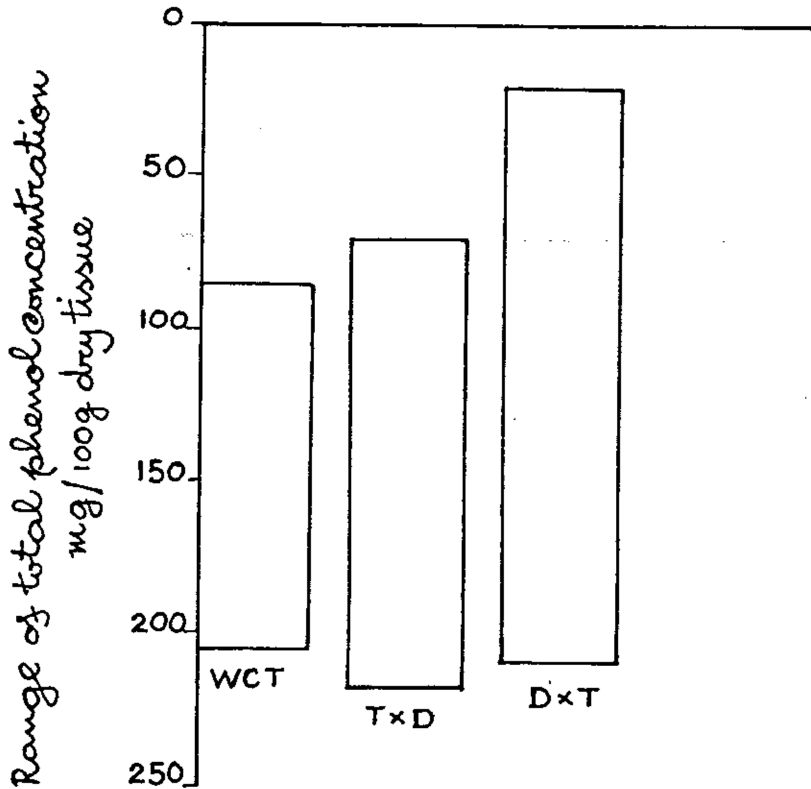
The partially purified enzyme was resolved for its isoenzyme pattern by acrylamide gel eletrophoresis. The electrophoresis was carried out with purified samples from healthy, apparently healthy and diseased. The isoenzyme pattern is given on figure 28. In case of healthy samples only one band, three in apparently healthy sample and five in diseases samples were obtained.

15. Phenol metabolism in relation to root (wilt) disease.

Figure 29 sums up the whole study in concerning phenol metabolism in relation to root (wilt) disease. There was a gradual fall in total phenol content from healthy to disease. Orthidihydiozyphenol concentration decreased with the increase in the intensity of the disease. Both PPO and PO activities were increased with the increase in the intensity of the disease. Apparently healthy samples had, the maximum PAL activity which was further decreased w... the increase in intensity of the disease.

FIG.1

Concentration of total phenol in the roots of healthy WCT, T×D and D×T palms.
(mg/100g dry tissue)



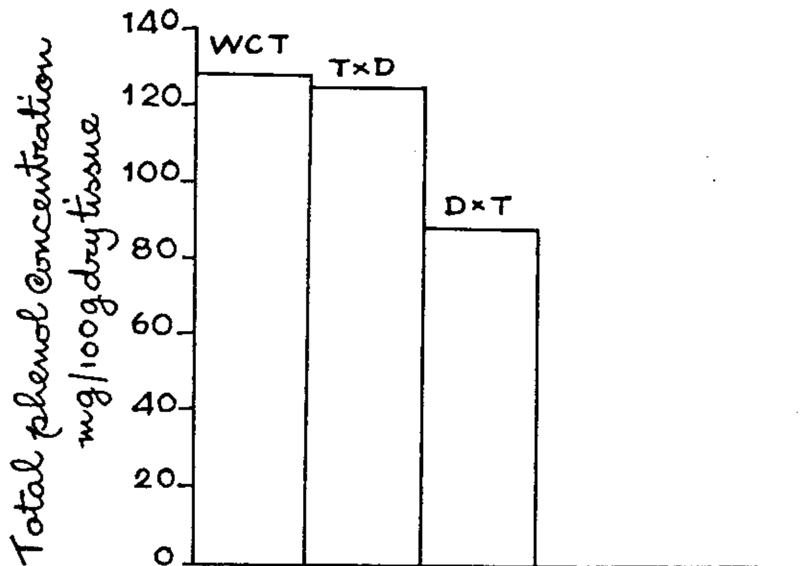


FIG. 2.

Concentration of total phenol in the roots of WCT, T×D and D×T coconut palms in healthy and disease affected tracts.

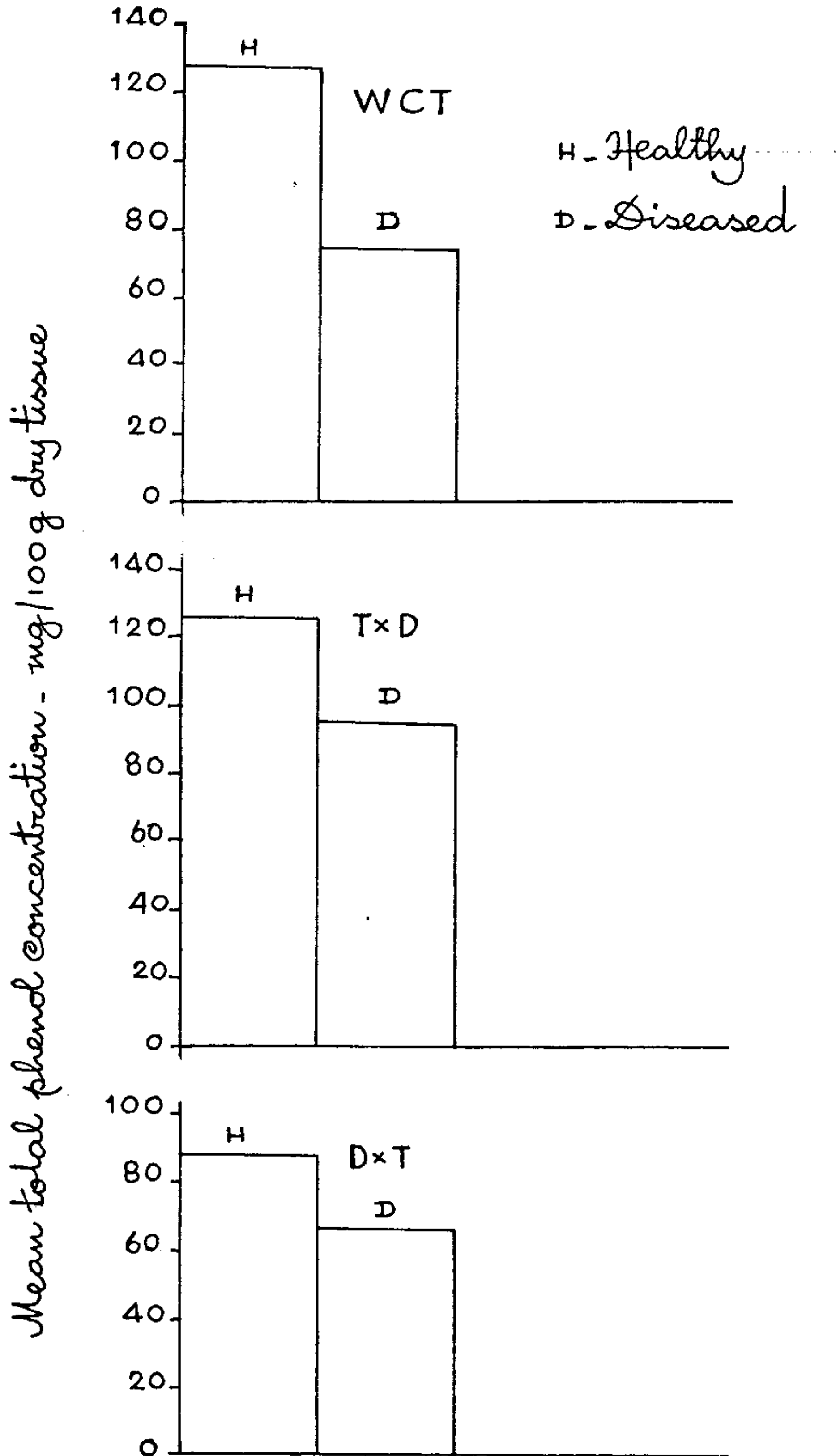


FIG. 3

Concentration of total phenol in the roots of VCT palms in different degrees of disease-intensity.

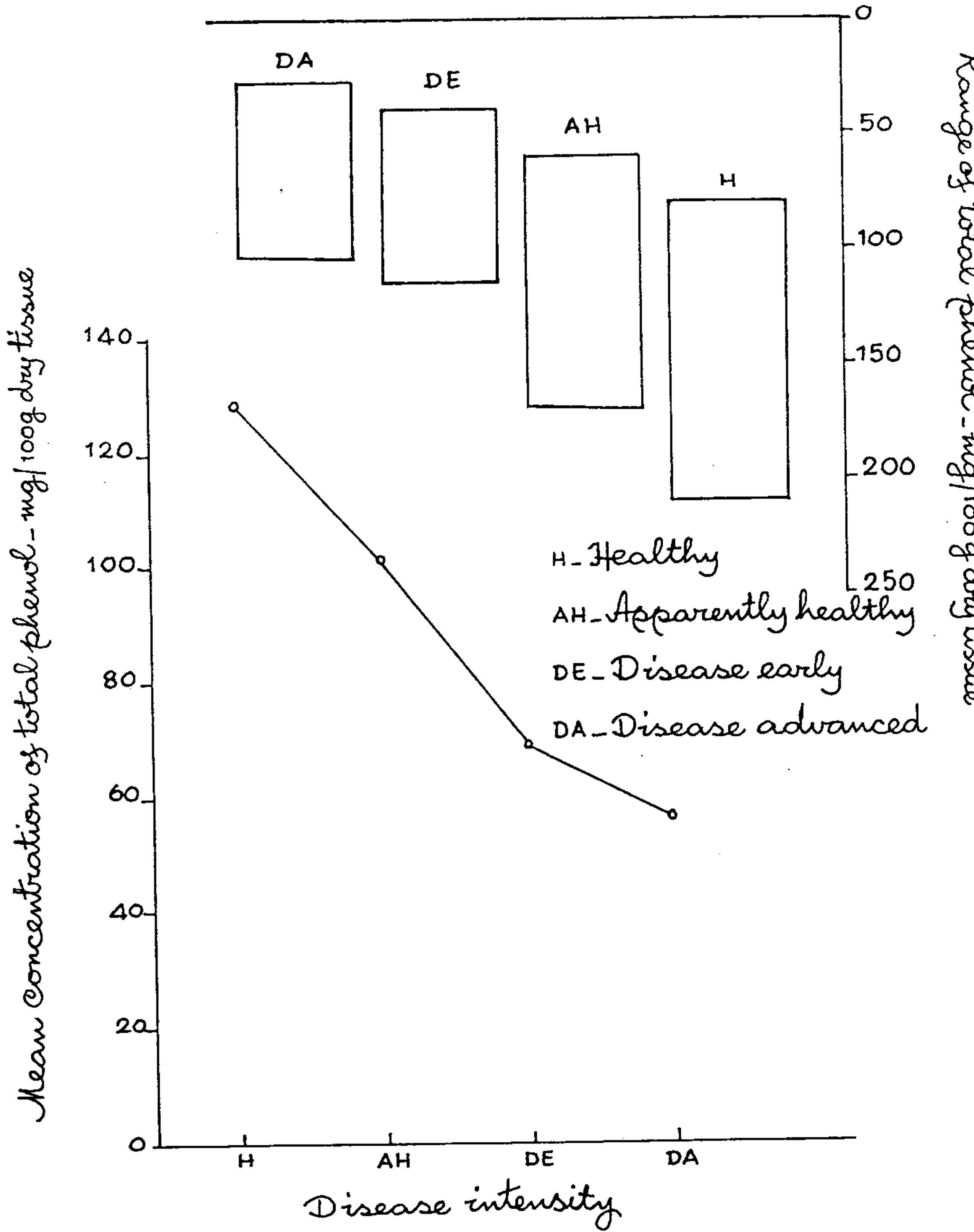


FIG. 4

Concentration of total phenol in the roots of T×D palms in different degrees of disease intensity.

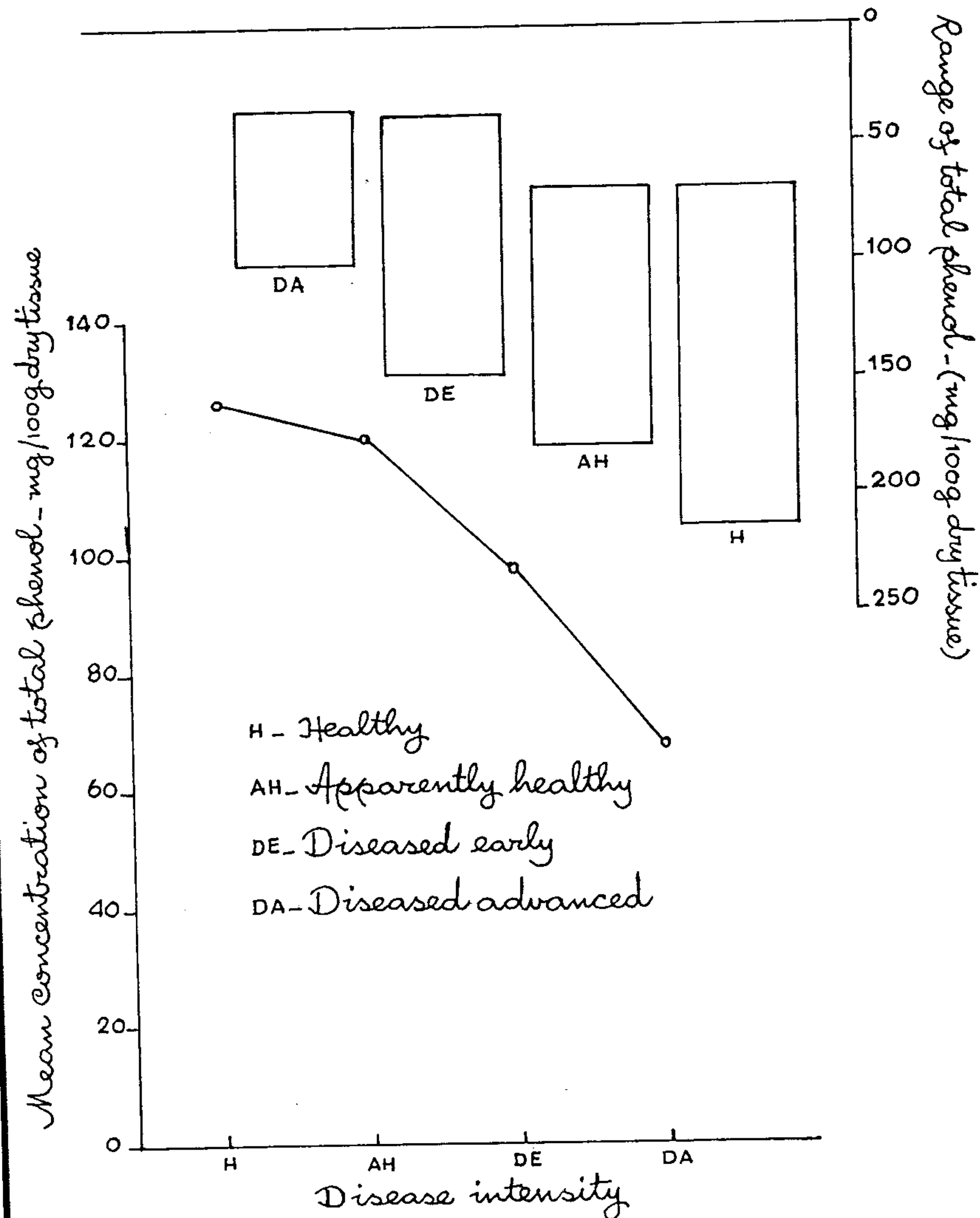


FIG. 5.

Concentration of total phenol in the roots of D×T palms in different degrees of disease intensity.

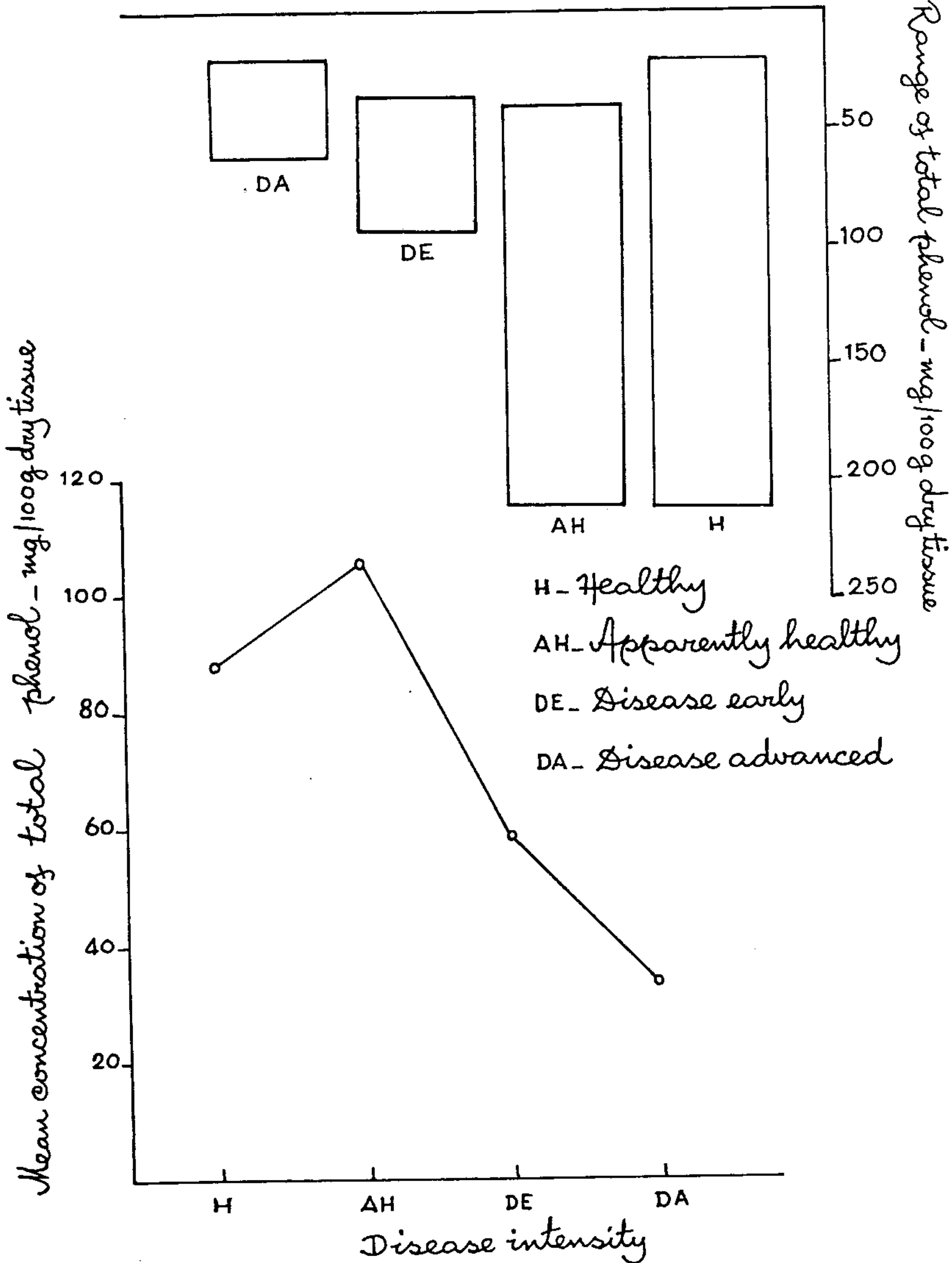


FIG. 6.

Chacterisation of phenolic compounds in the roots of WCT palms.

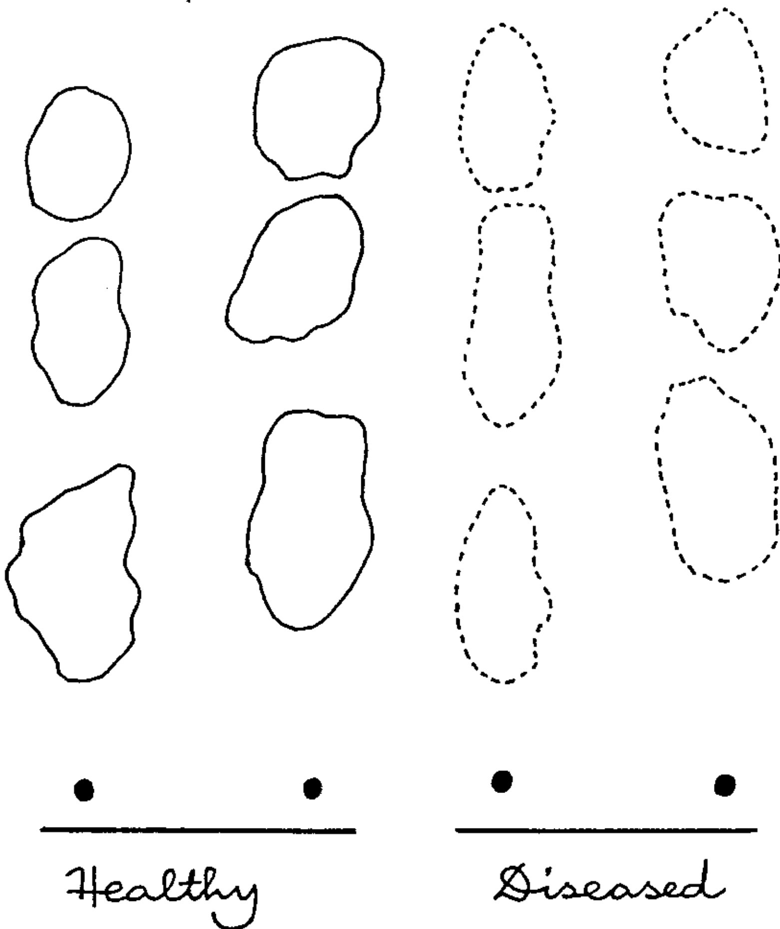


FIG. 7.

Chacterisation of the phenolic compounds in the leaves of WCT palms.

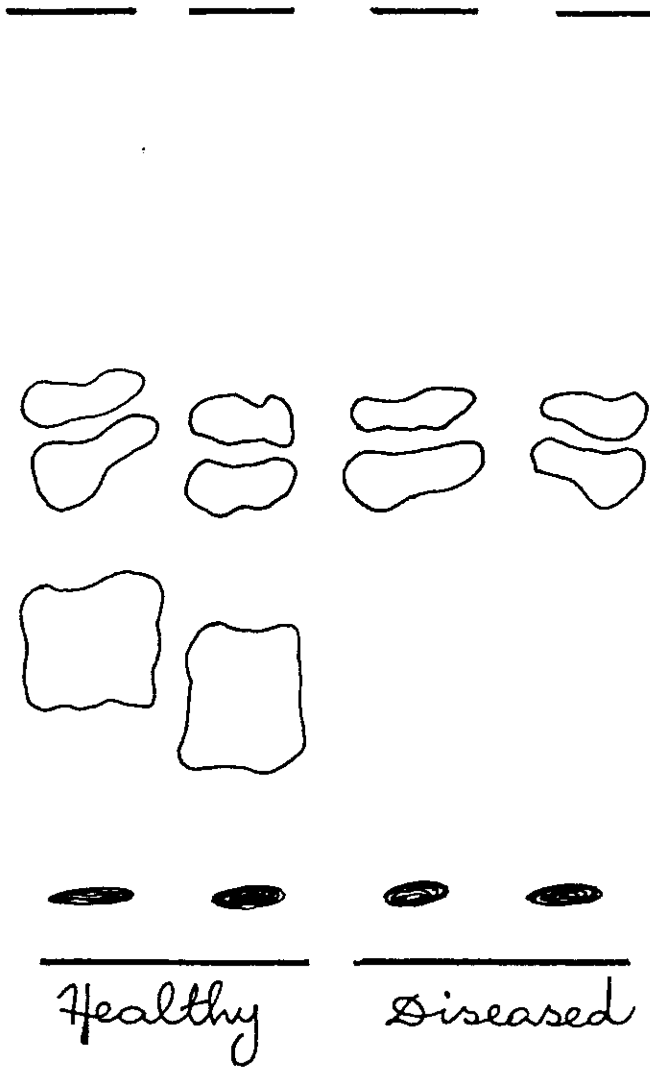


FIG. 8

Absorption spectrum of coloured complex formed by the reaction of pyrogallol with P-dimethylamino benzaldehyde (PDAB) in the presence of sulphuric acid.

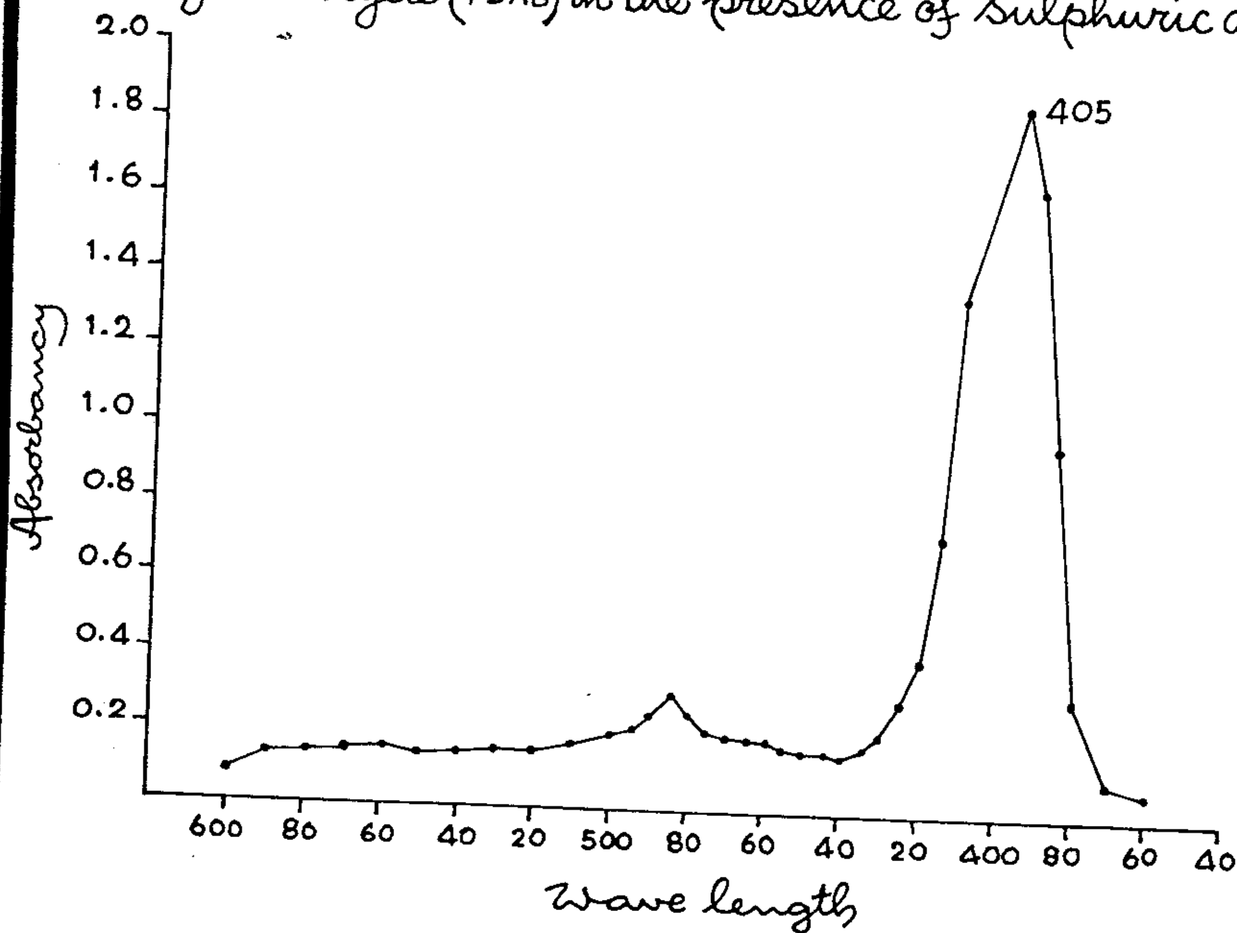


FIG. 9.

Linearity of the colour complex formed by the reaction of pyrogallol with PDAB

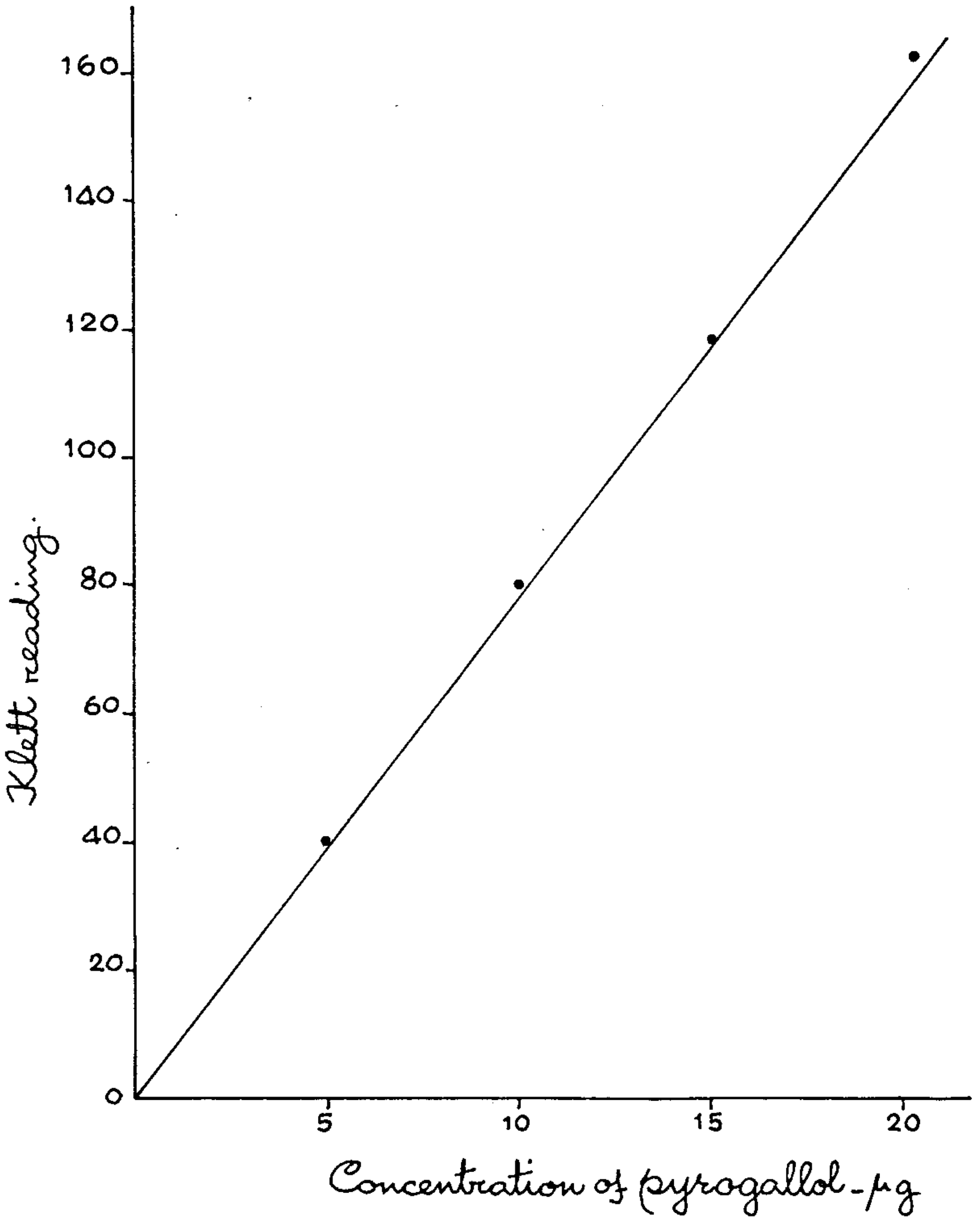


FIG. 10.

Concentration of O-Dihydroxy phenol in the roots of WCT palms cultivated in healthy and diseased tracts.

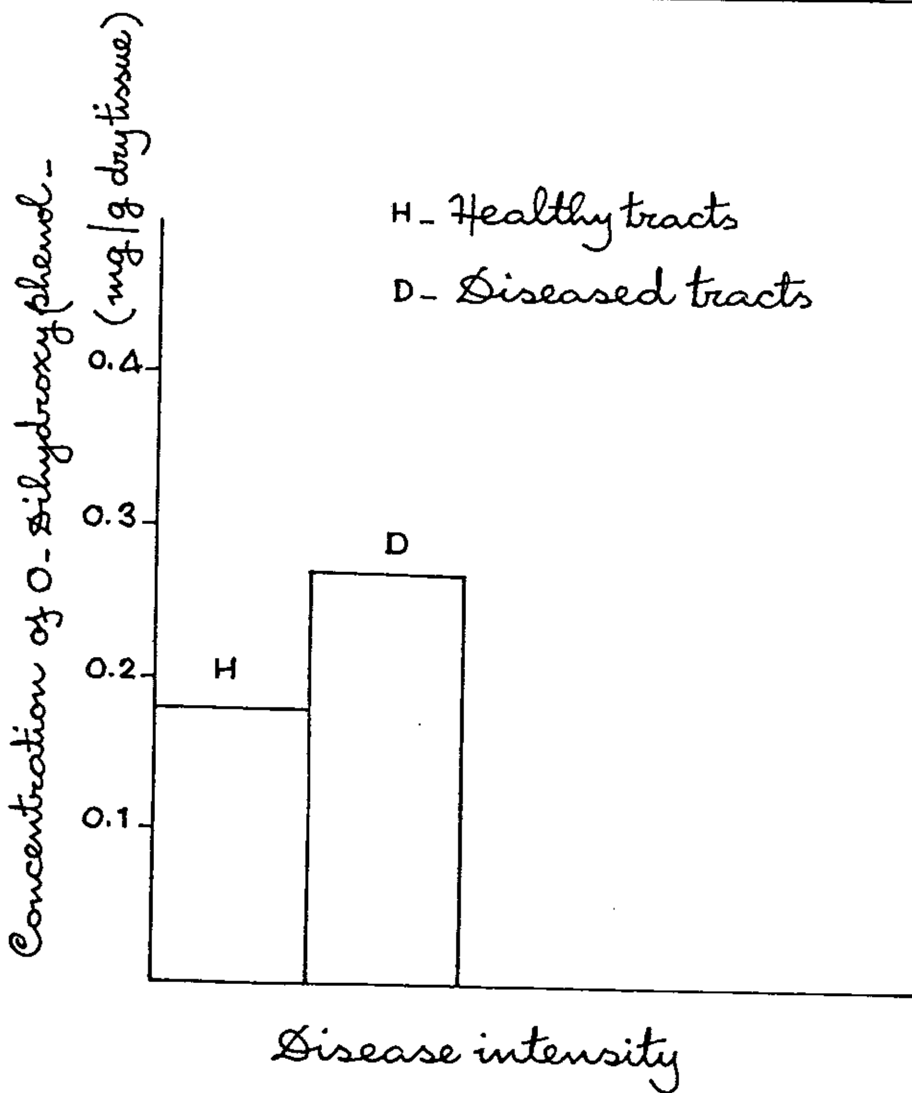


FIG. 11.

Concentration of O-Dihydroxyphenol in the roots of WCT palms in different degrees of disease intensity.

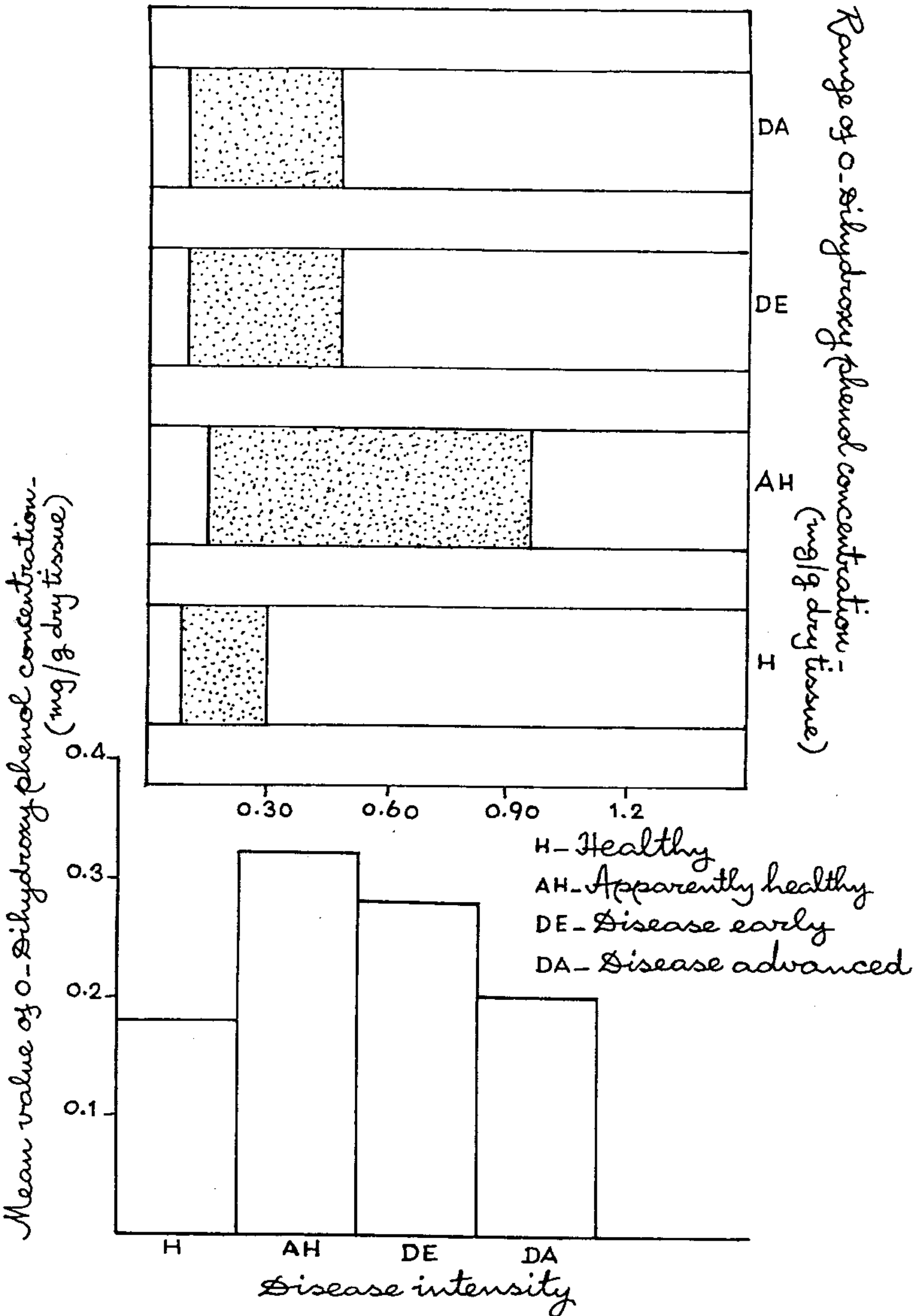


FIG. 12.

Level of Polyphenol oxidase (PPO) activity in the roots of WCT palms cultivated in the healthy and diseased tracts.

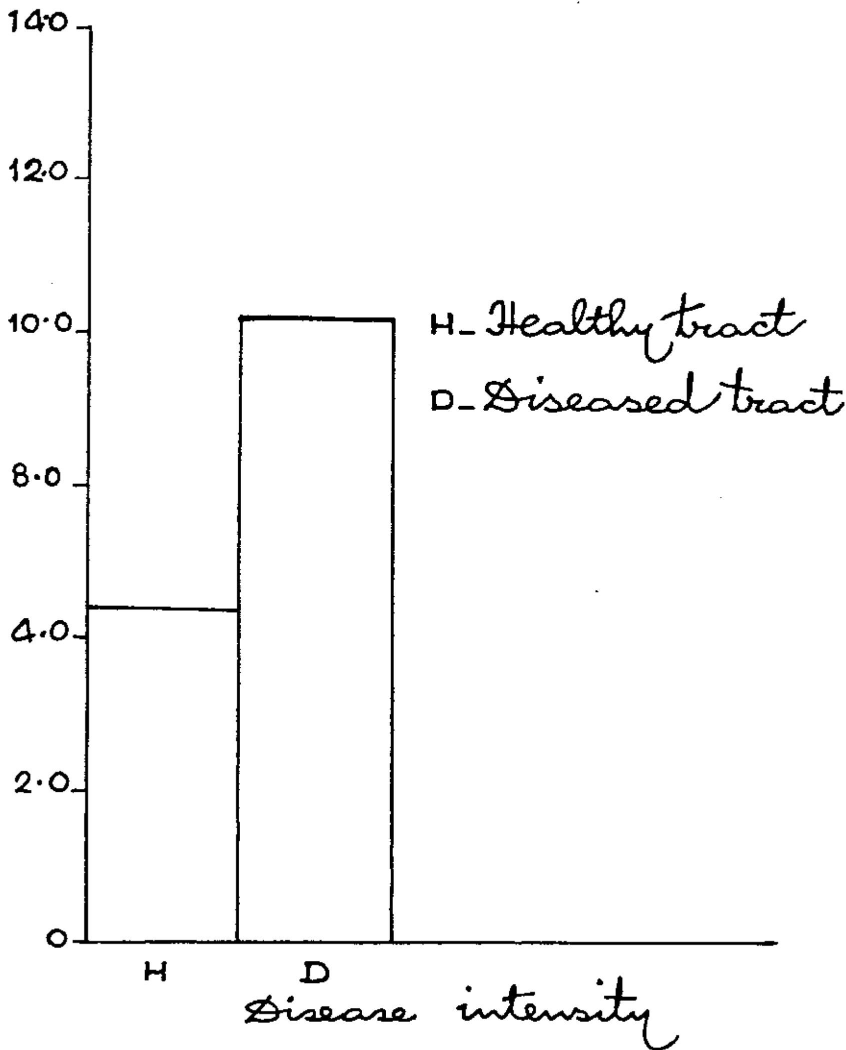


FIG. 13.

Level of Poly phenol oxidase (PPO) activity in the roots of WCT palms in different degrees of disease intensity.

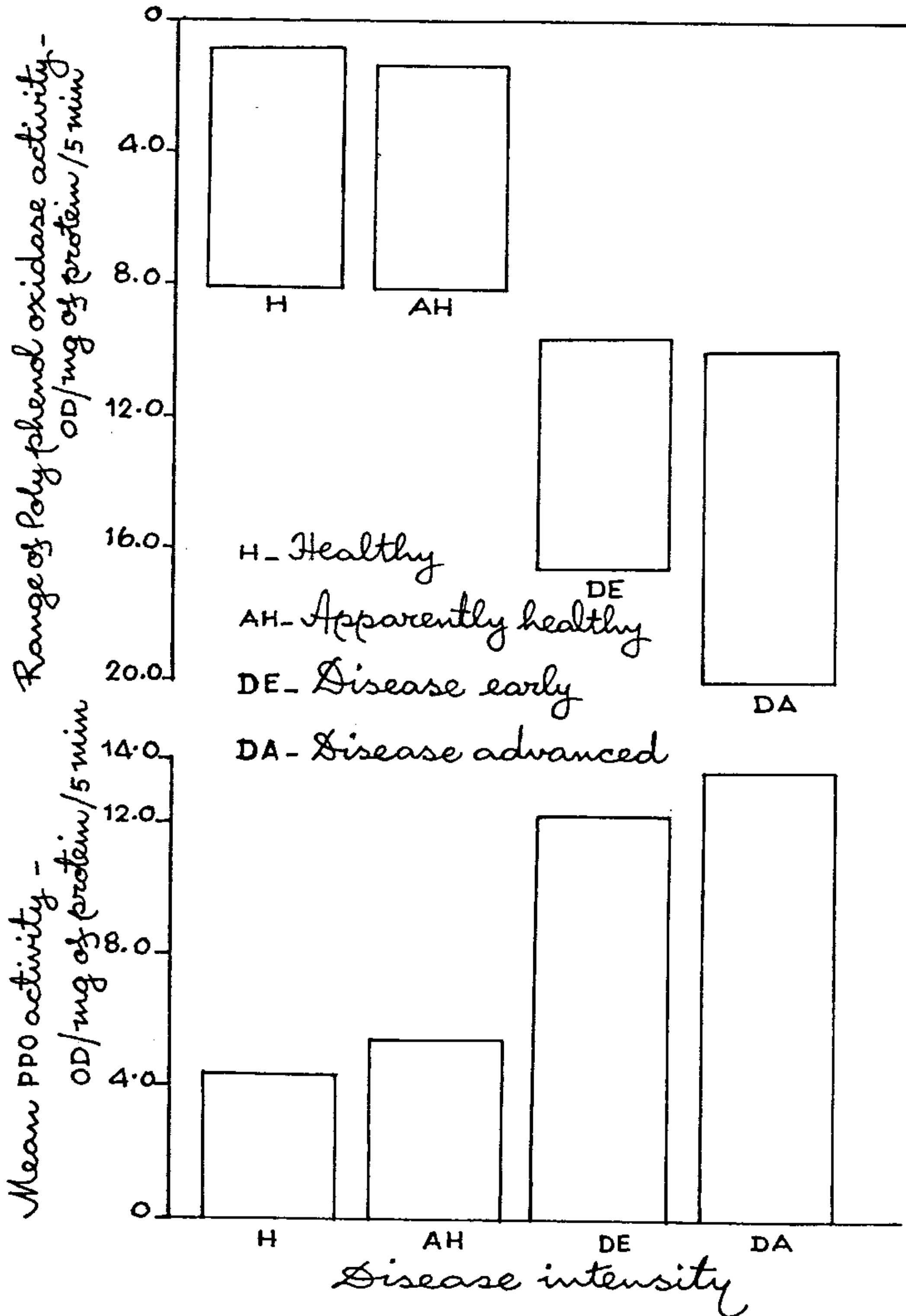


FIG. 14.

Level of Peroxidase (PO) activity in the roots of WCT palms cultivated in healthy and diseased tracts.

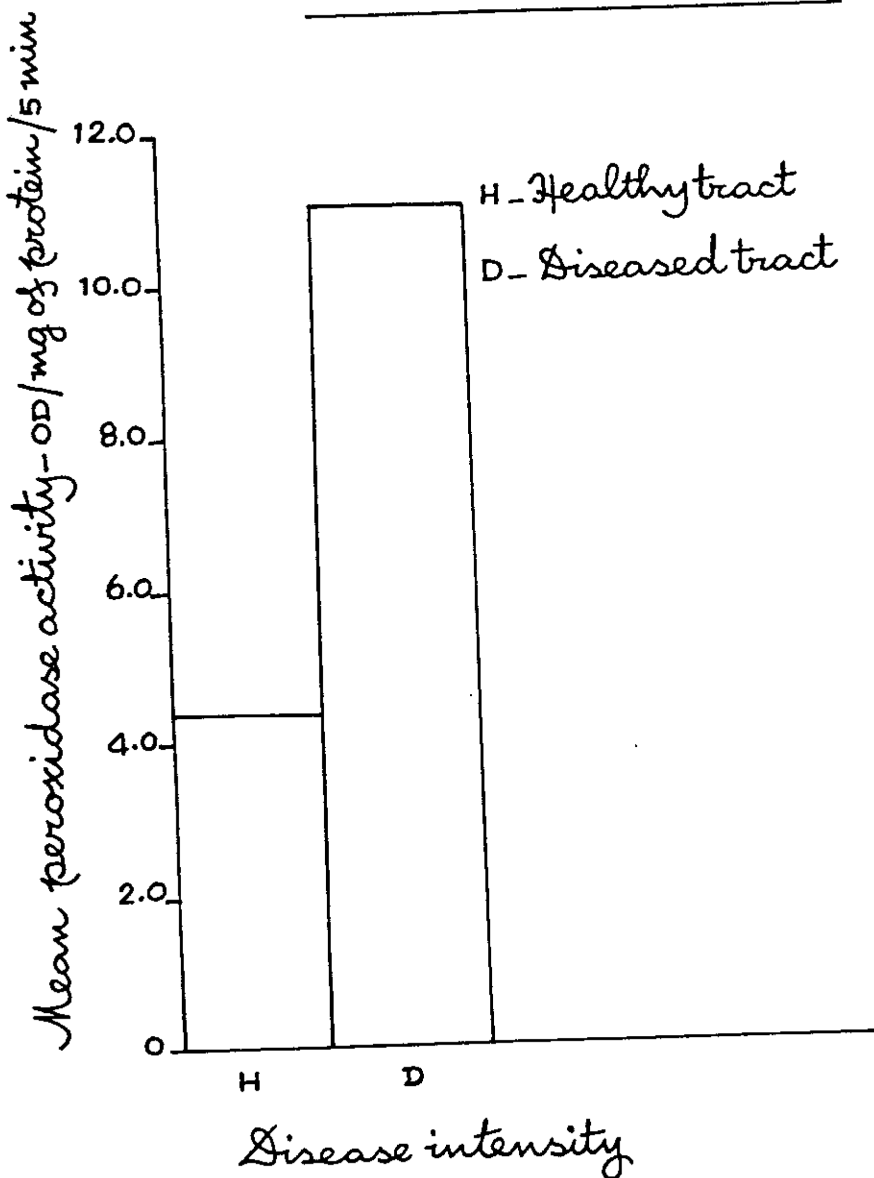


FIG. 15.

Level of Peroxidase activity in the roots of WCT-palms in different degrees of disease intensity.

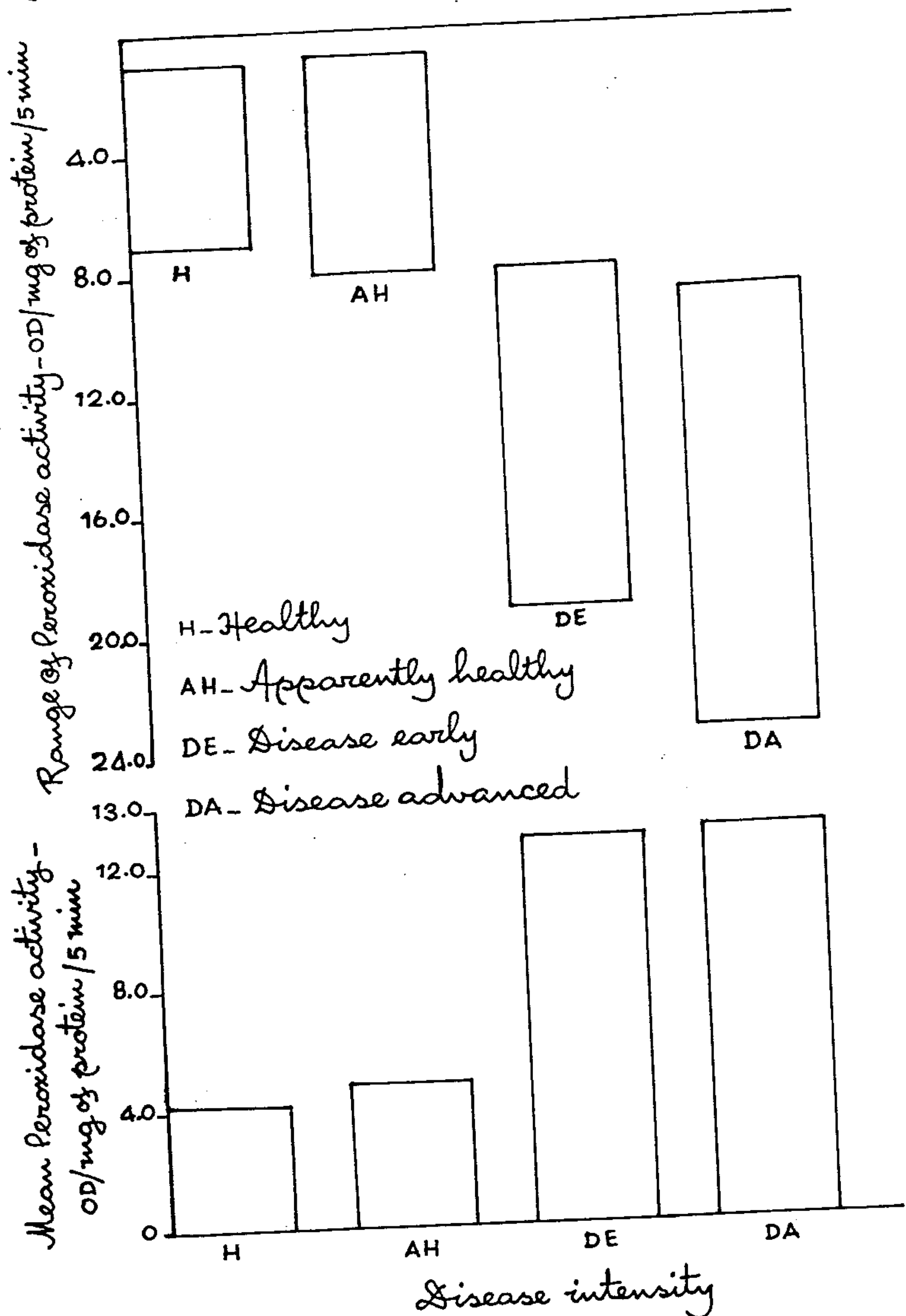


FIG. 16.

Level of Phenylalanine ammonia lyase (PAL) activity in the roots of WCT palms cultivated in healthy and diseased tracts.

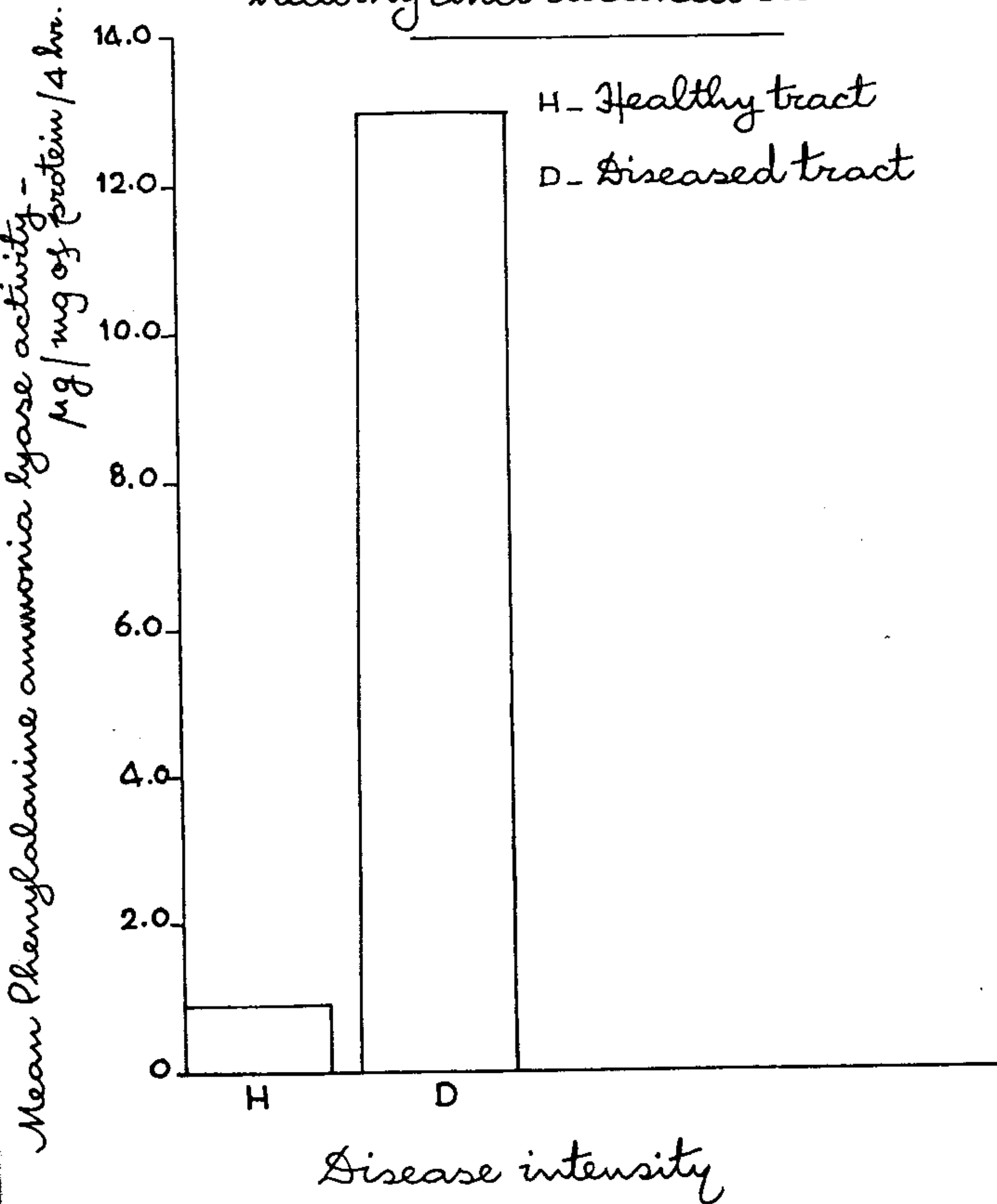


FIG. 17.

Level of Phenylalanine ammonia lyase (PAL) activity in the roots of WCT palms in different degrees of disease-intensity.

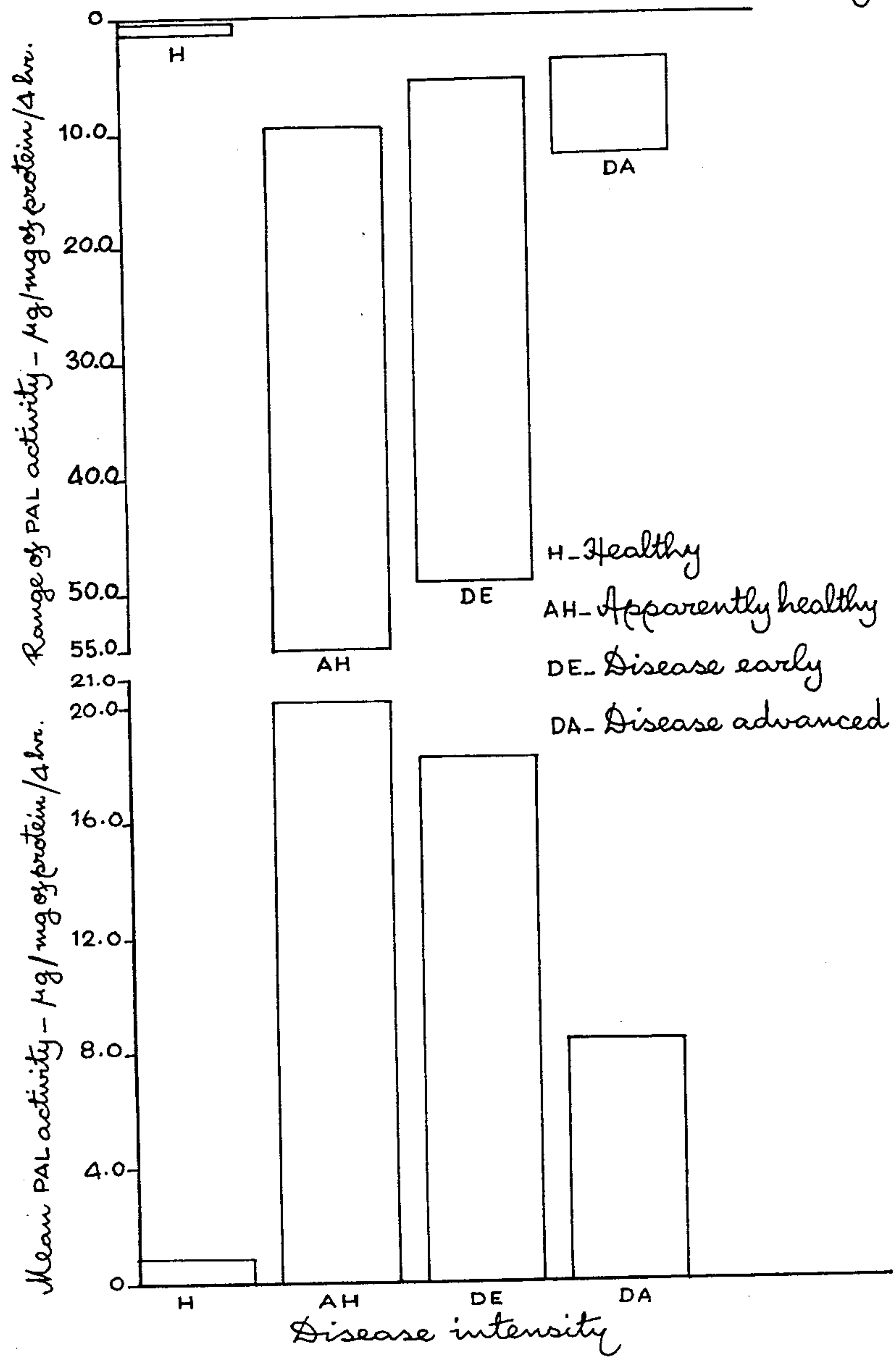


FIG. 18.

Correlation between Polyphenol oxidase (PPO) activity and disease index.

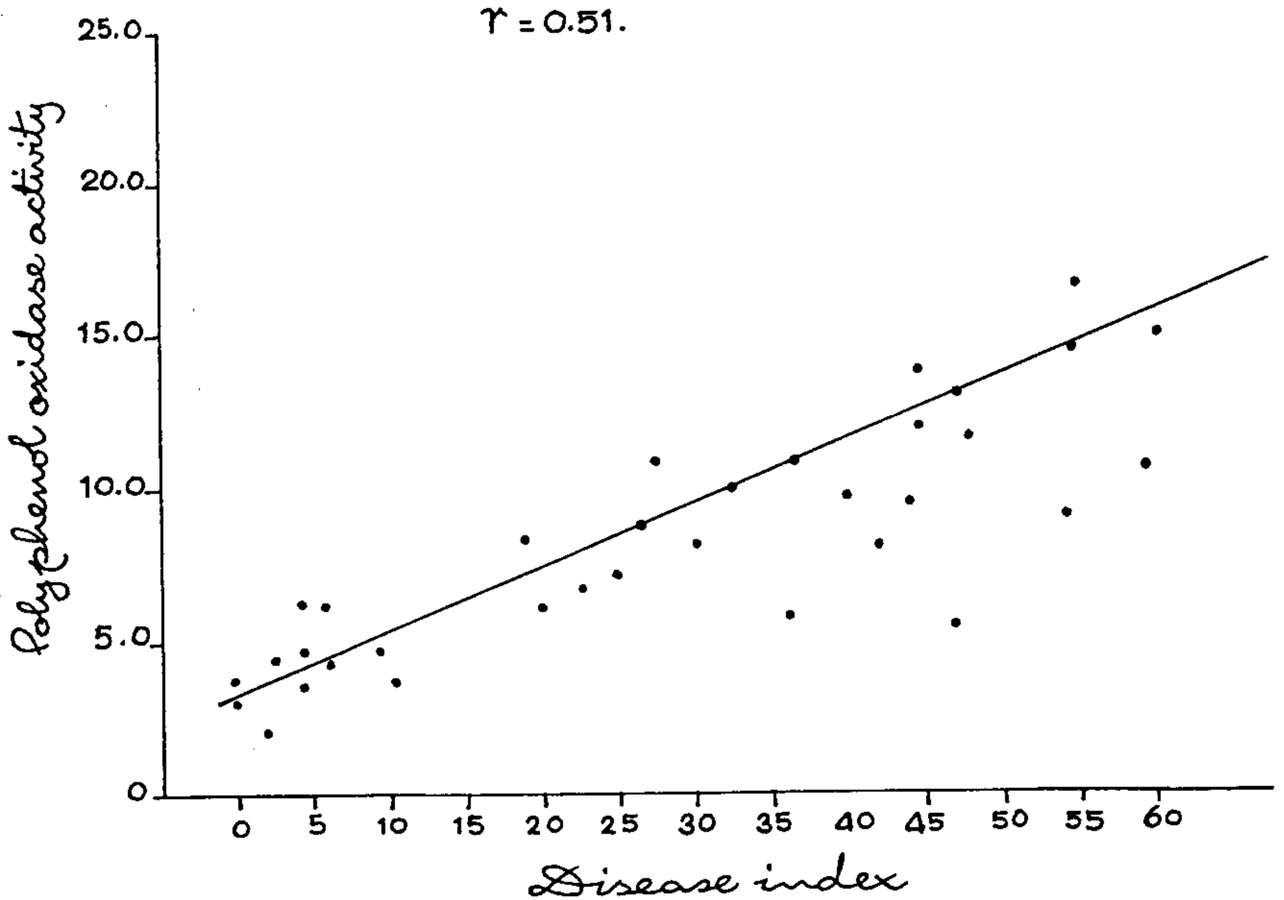


FIG. 19.

Correlation between Peroxidase activity and disease index

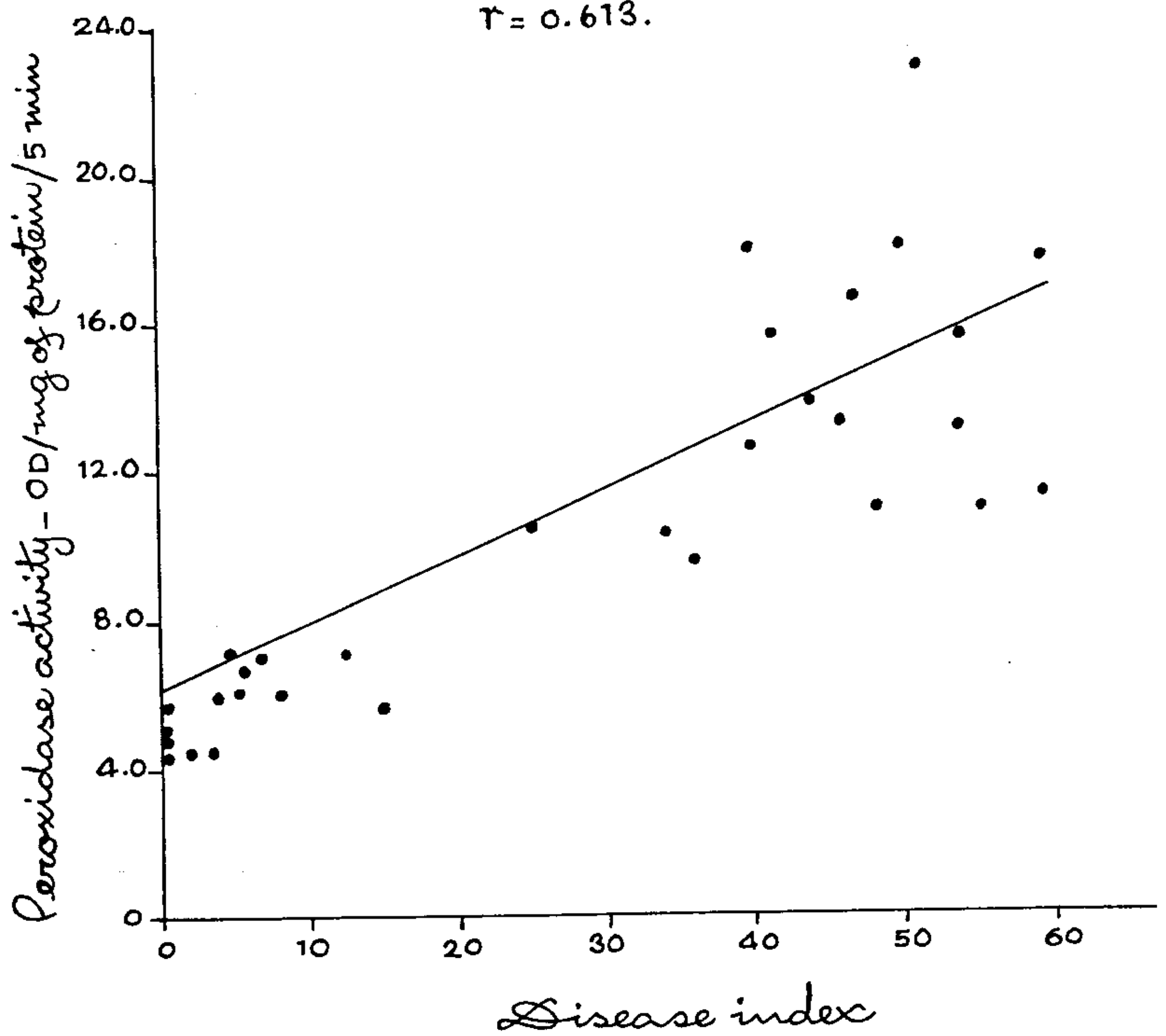


FIG. 20.

Correlation between Phenylalanine ammonia lyase (PAL) activity and disease index.

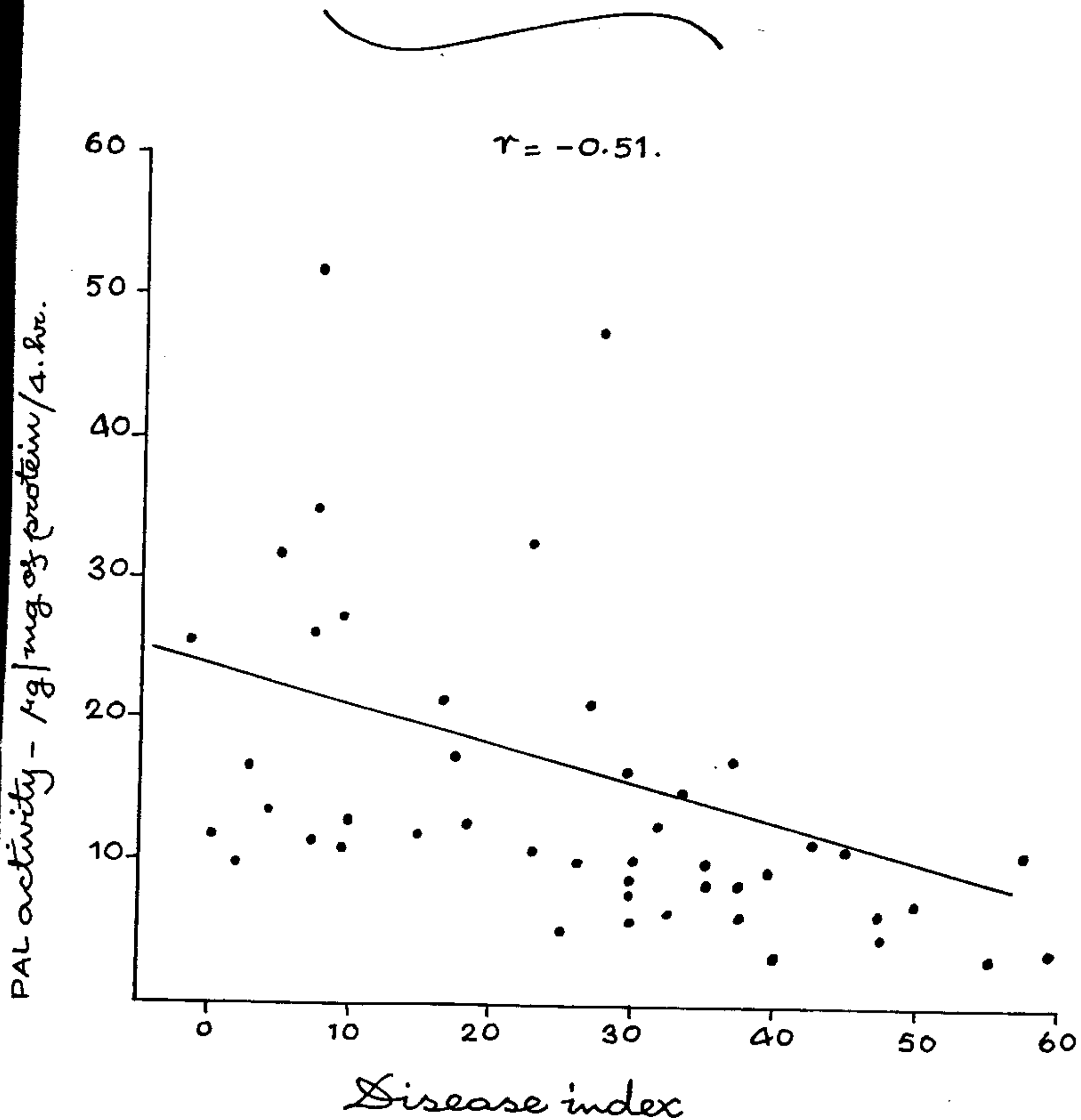


FIG. 21

Relation between Poly phenol oxidase (PPO) and Concentration of total phenol in the roots of WCT-palms in different degrees of disease intensity.

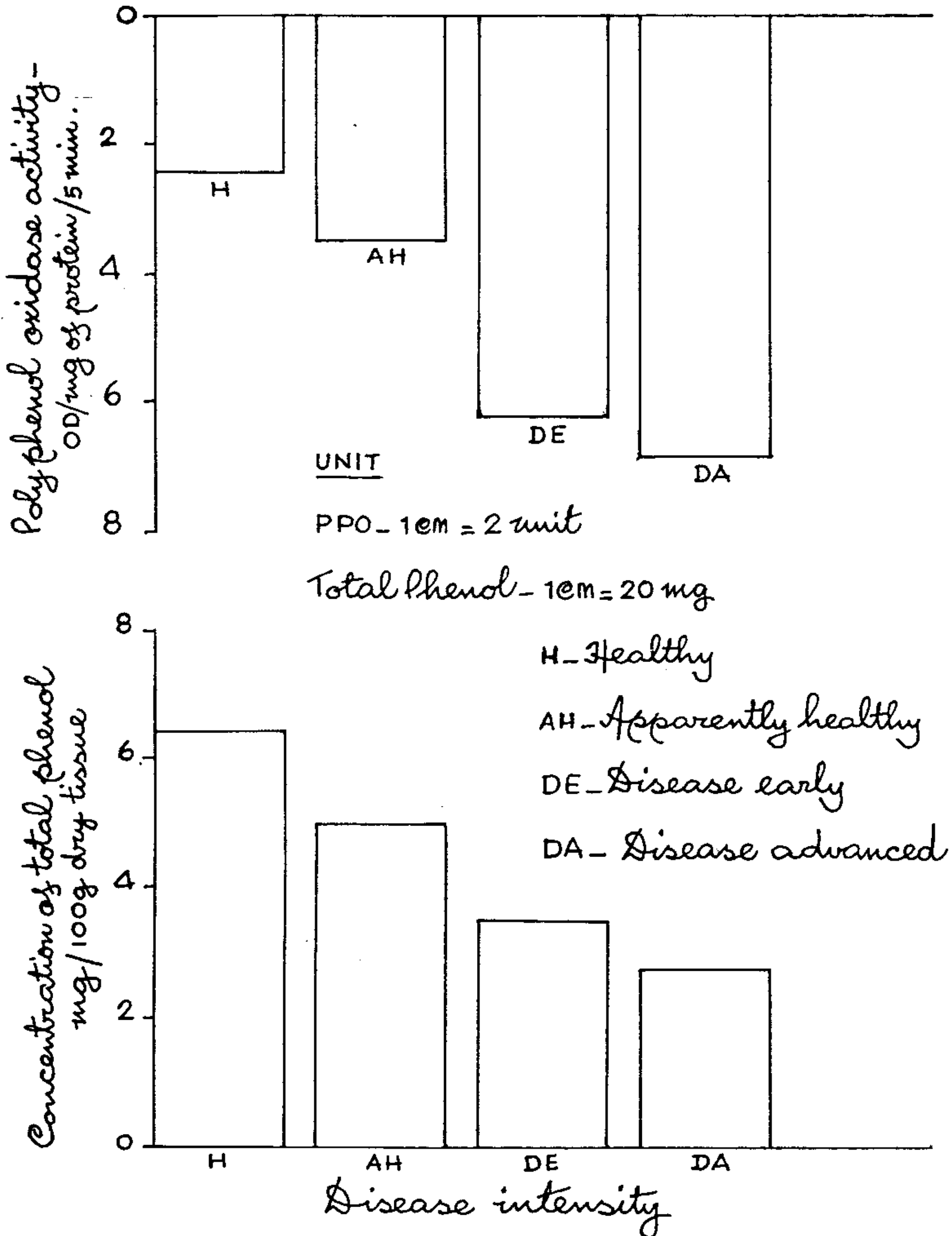
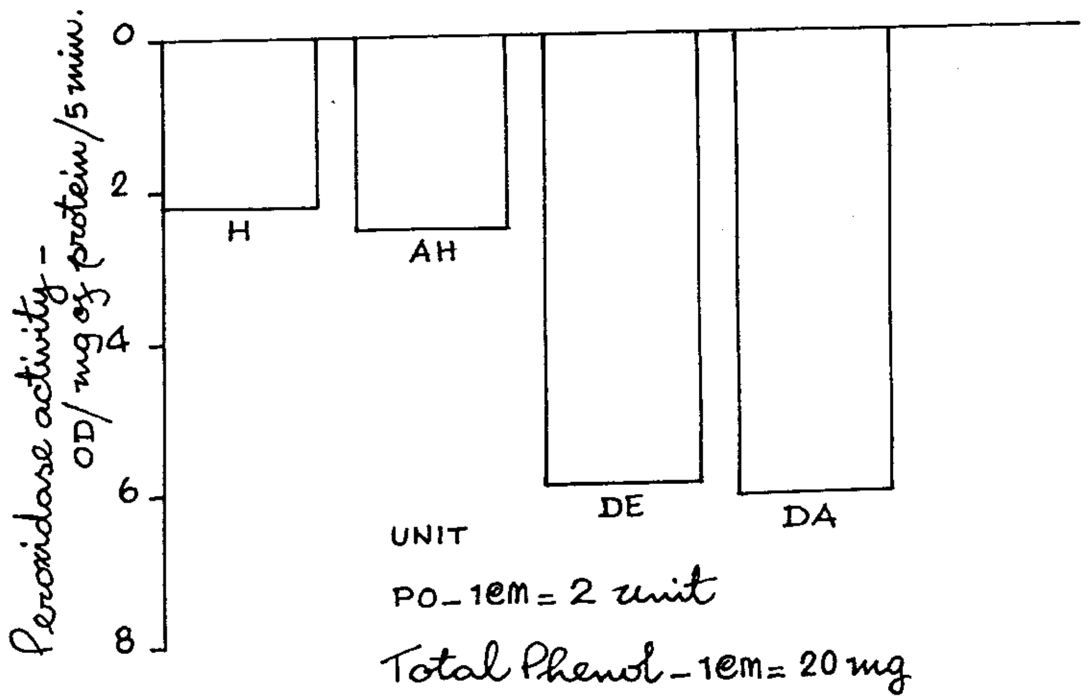


FIG. 22.

Relation between Peroxidase (PO) and concentration of total Phenol in the roots of WCT palms in different degrees of disease intensity.



Total Phenol - 1cm = 20 mg

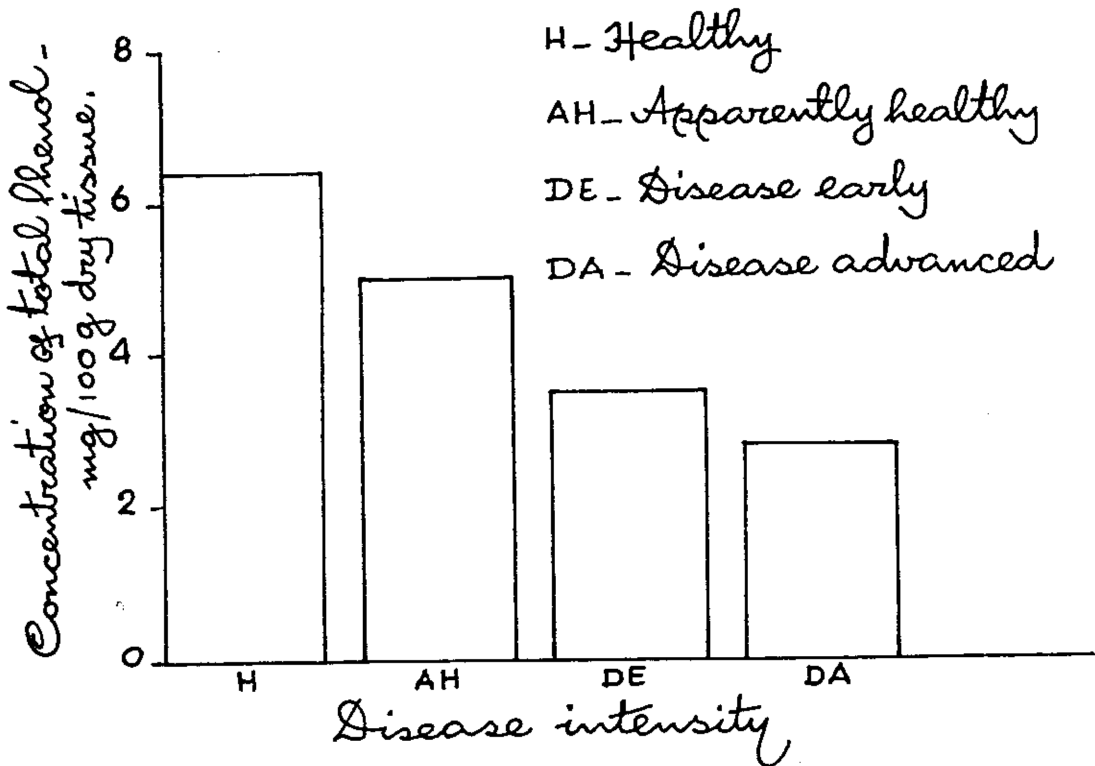


FIG. 23.

Relation between Phenylalanine Ammonia lyase (PAL) and concentration of total Phenol in the roots of WCT palms in different degrees of disease intensity

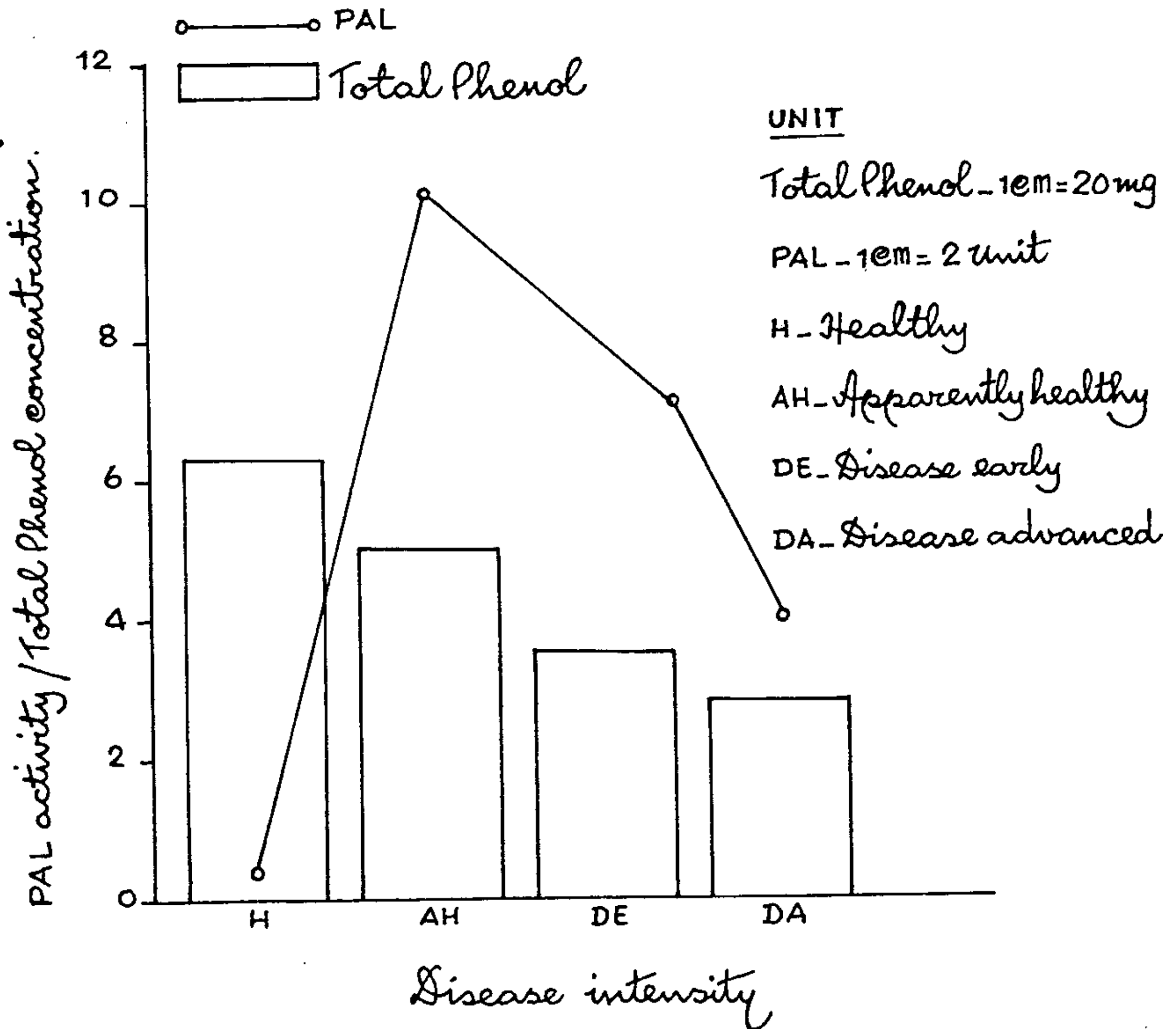


FIG. 2 4

Relation between Poly phenol oxidase (PPO) and concentration of O-Dihydroxy Phenol (OD Phenol) in the roots of WCT palms in different degrees of disease intensity.

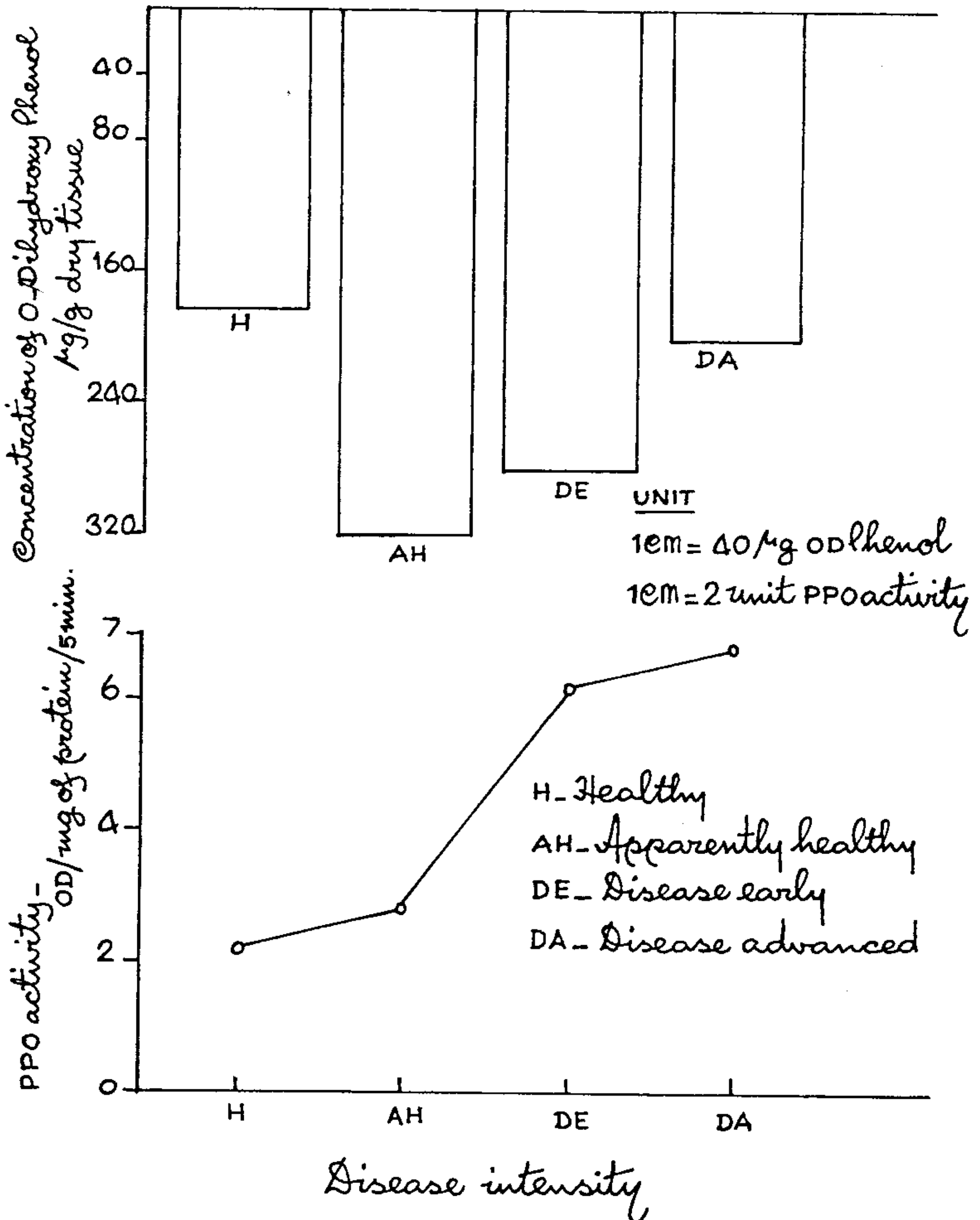


FIG. 25.

Relation between Peroxidase (PO) and concentration of O-Dihydroxy Phenol (OD Phenol) in the roots of WCT palms in different degrees of disease intensity.

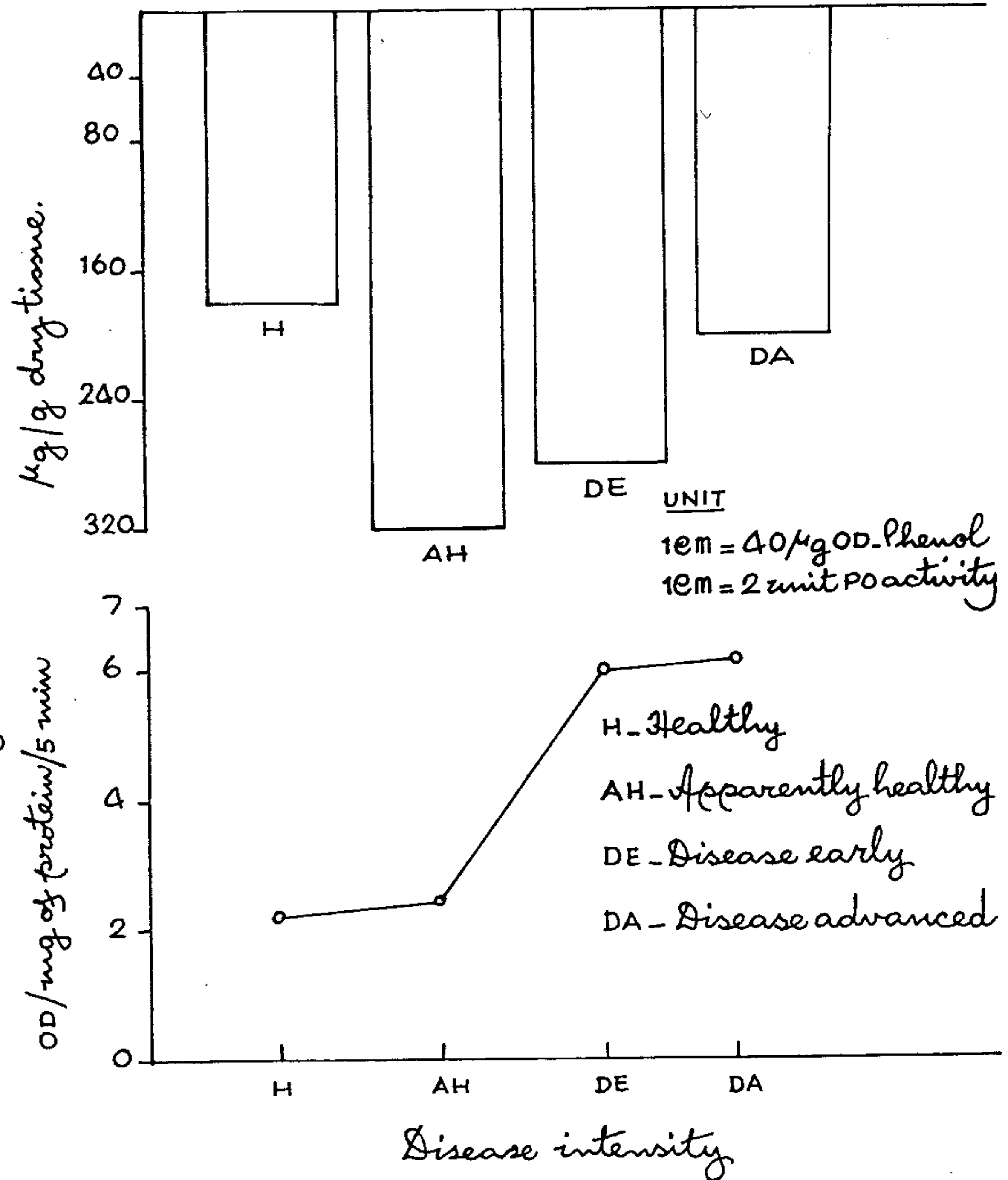


FIG. 26.

Relation between Phenylalanine Ammonia lyase (PAL) and concentration of O-Dihydroxy Phenol in the roots of WCT palms in different degrees of disease intensity.

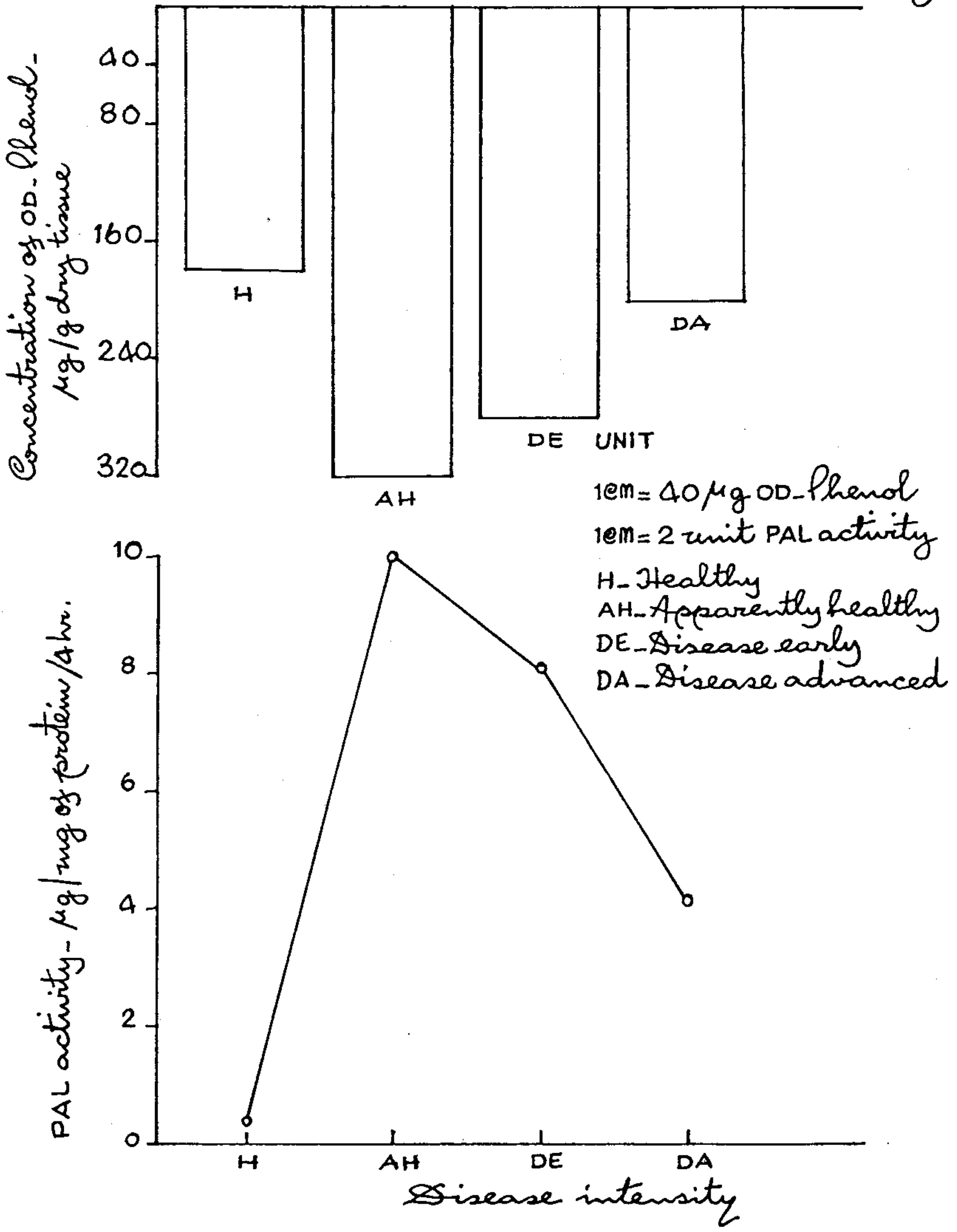


FIG. 27.

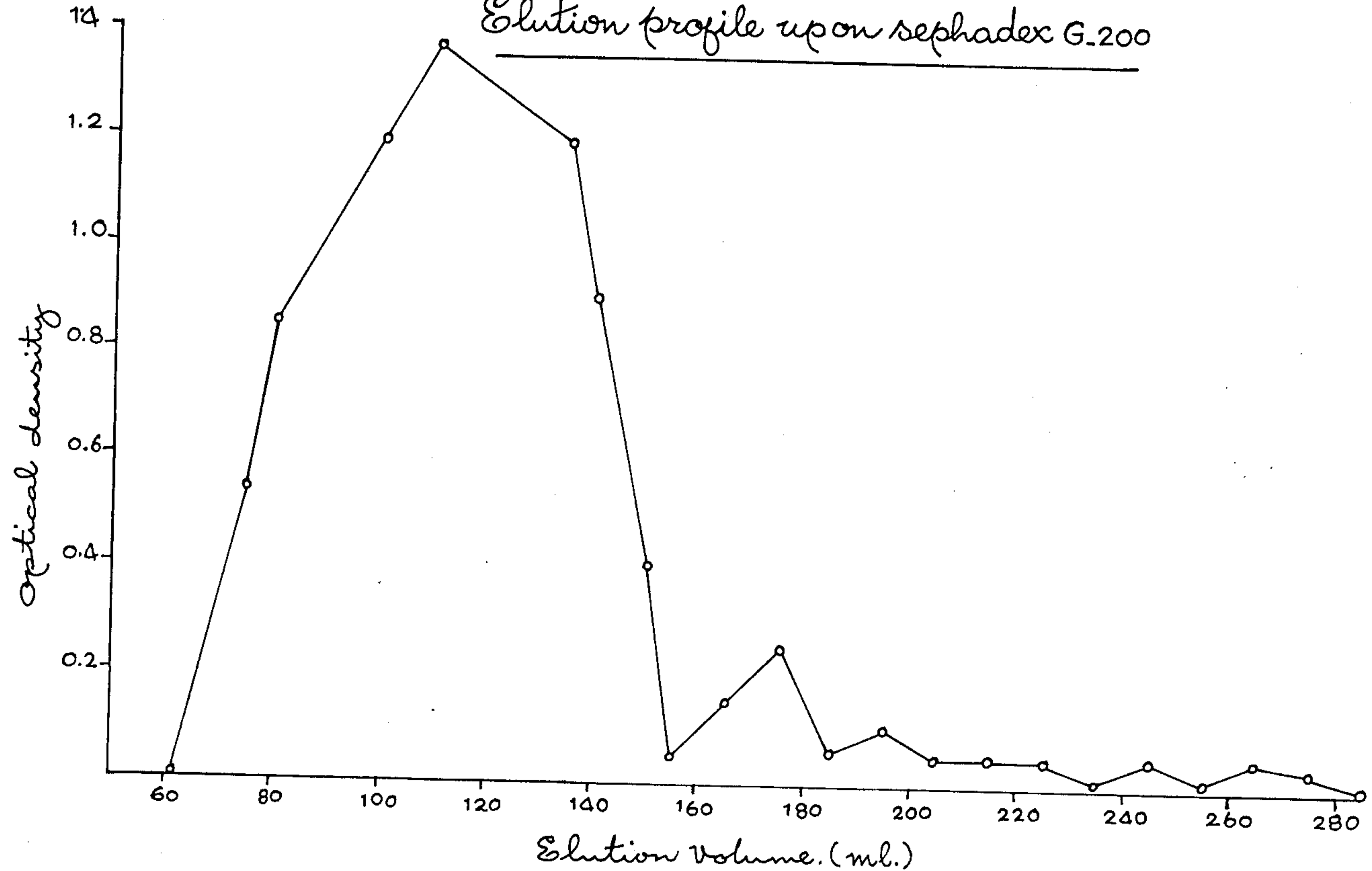


FIG. 28.

Isoenzyme pattern of PPO in the roots of healthy (H), apparently healthy (AH) and diseased (D) WCT palms.

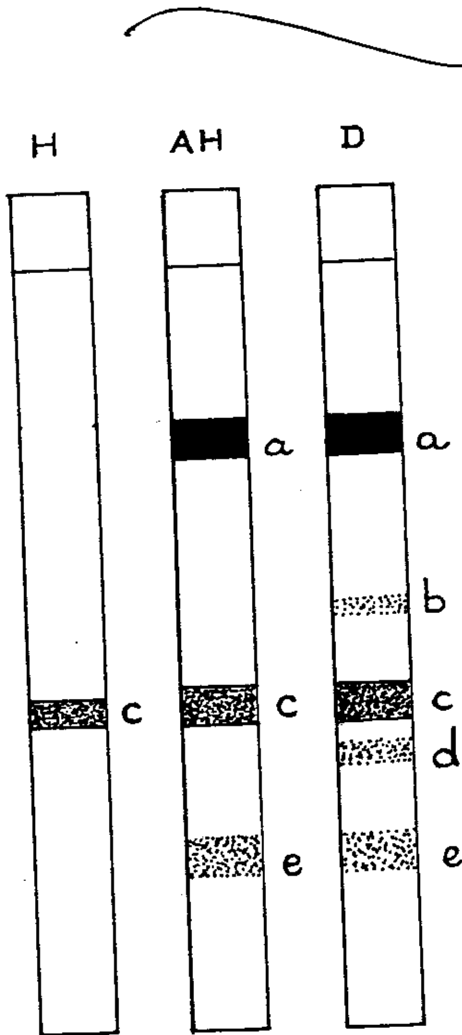
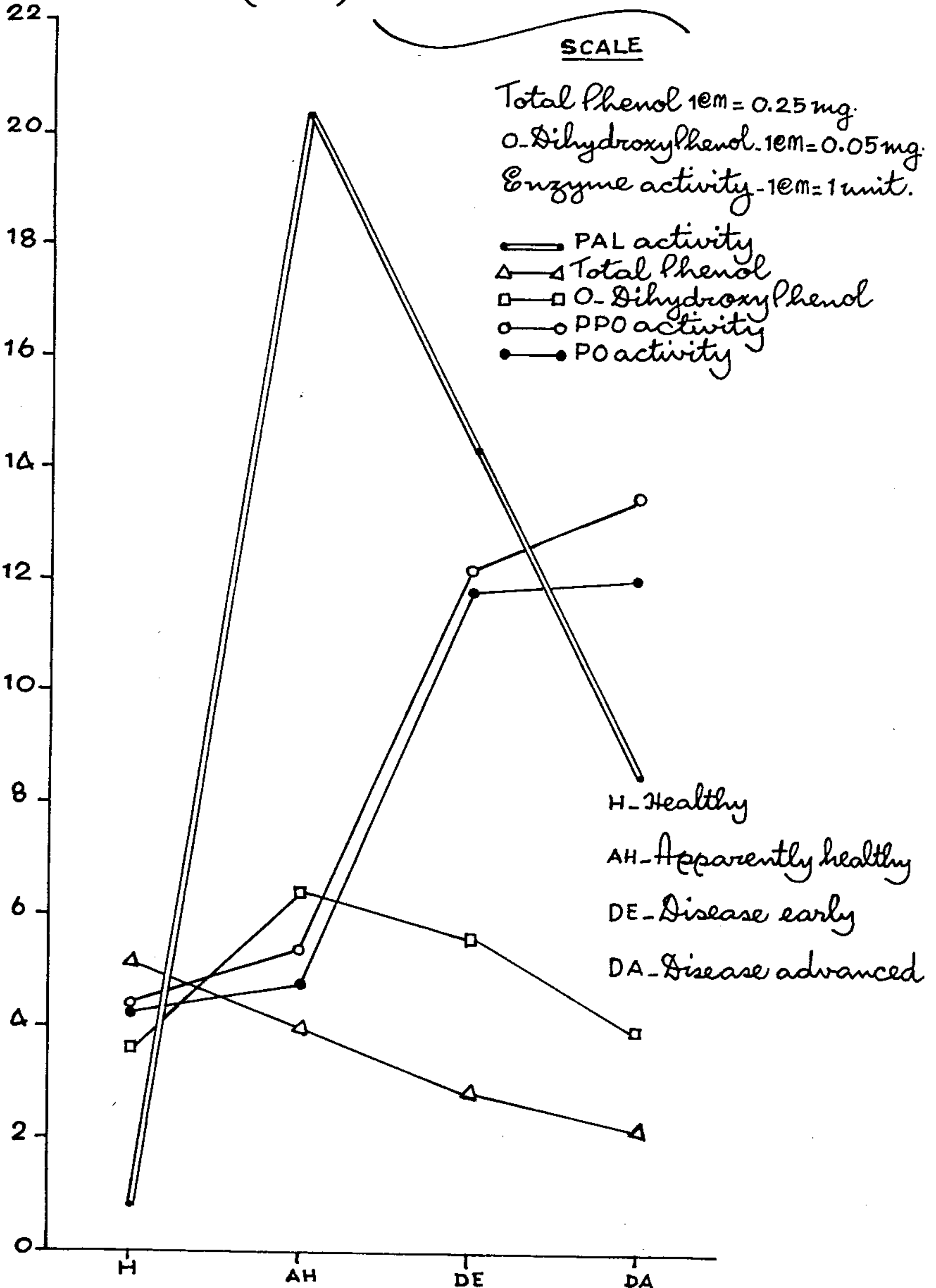


FIG. 29.

Phenol metabolism in Coconut palms in relation to incidence and increase in intensity of – Root (wilt) disease.



The healthy samples showed very low PAL activity.

TABLE 10: Purification of Polyphenol oxidase.

S.No.	Enzyme	Volume ml.	Total Protein mg.	PPO activity OD/mg of protein/5 min.	Percent purification
1.	Acetone powder	271	282	0.81	-o-
2.	$(NH_4)_2SO_4$ Precipitate	[illegible]	165	2.5	3.8
3.	After dialysis	53	129	2.7	4.0
4.	After concentration with Sephadex G 200	18	120	13.7	17.0
5.	$(NH_4)_2SO_4$ Precipitate after gel filteration	11	15	19.0	25.0
6.	After dialysis	11	12	23	28.0

II Involvement of associated fungi

1. Pectinolylic properties of Rhizoctonia solani Bipolaris halodes and Pestolotia palmarum the casual organisme of root rot, leaf rot and blight respectively were studied..

All the organisms could utilize pectin as sole source of carbon. The extend of degradation of pectin was studied by estimating the mycebial dry weights at fi[illegible] intervals. The organisms could eleborate pectin methyl esterace (PME), Polygalacturanase (PG) and Pectin trans eliminase (PTE). Maximum PME activity was in B: halodes PTE was more in P. Palmarum followed by B. halodes and R. coloni.

2. Studies on fungi isolated from apparently healthy roots.

Following studies were conducted with Cylindrocarpon effusum, Cylindrocarpon lucidum, Fusarium equisetti and two numbers of sterile isolates.

a) Studies on the ability of the above fungi to elaborate PPO activity in medium containing phenolic compounds were carried out.

Phenolic compounds of the coconut roots were extracted 80% alcohol after mascerating the tissue and filtered under vaccum, evaporated and the residue was in-corporated with Czepak's Dex's agar as well as liquid medium without agar. Organisms were grown in the medium. Radial spread of the mycelium was [illegible] in the case of the fungus grown in

agar medium. The optical density of the liquid medium was noted and as given in table 11. In solid agar medium a clear brown zine was noted around the mycelial growth and this isolate No.2. Which showed a spread of 0.4 cm radius from the margin as compared to other cultures having only 0.3 cm and 0.2 cm. The results were indicative for the elaboration of the phenol oxidizing enzymes.

Table 11: Optical density of the liquid medius after the inoculation of the fungi isolated from the roots.

No.	Fungal Isolation	Period Days	Optical Density of the liquid medium at 400 nm
1.	C. effusum	24	6.9
2.	F. equiseti	"	8.0
3.	C. Leucidum	"	6.0
4.	Sterile isolate No.2	"	7.45
5.	Sterile isolate No.1	"	4.45

b. Studies were also condic... in Czepak's Dox' agar and liquid medium in which 0.1% caffeic acid was incorporated.

A clear brown zone developed around the mycelial growth as in the case of previous experiment and this was prominent in isolate No.2 culture.

Culture filterate of the fungal organisms was taken after 14 days of growth. The optical density at 400 nm was noted in Spectronic 20. Colorimeter. Positive indications for the enzymic activities was obtained in (Table 12).

Table 12: Optical density of the culture filterate after Inoculation of the fungus isolated from the roots in Czepaks Dox's medium incorporated with Caffeic acid.

No.	Fungal isolates	Period days	Optical density at 400 nm
1.	C. effusum	14	0.21
2.	F. equiseti	"	1.05
3.	C. lucidum	"	0.75
4.	Sterile isolates No.2	"	1.00
5.	Sterile isolates No.4	"	0.8

C. In vive Studies

In vive study was conducted by inoculating the fungual isolates in the roots of potted seedlings. Seed nuts were brought from a disease free area (Kasaragod) germinated in plastic bags irrigated with sterile water and transplanted in cement pots. After the seedling got established, fungi like C. effusum, C. lucidum, F. equiseti and two number of sterile isolates were multiplied in sterilized coconut root bits and inoculated at root zone. 20 replicates were maintained keeping equal number of control seedlings, which was incorporated only with sterilized root bits. Total phenol content was estimated from the roots after one year and two year intervals. Total phenol content was found to reduce in the inoculated as compared to that of un-inoculated. It was also observed that reduction of total phenol content was increased as the infection period prolonged. This results were indicating the ability of the fungus to cause deplection of total phenol in diseased coconut roots.

Table 13: Total phenol content in the roots of seedlings inoculated with fungul isolates.

No.	Fungal isolates	1st uear mg/g dry tissue	2nd year mg/g dry tissue
1.	F. equiseti	6.9	2.2
2.	C. effusum	7.6	1.0
3.	C. lucidum	5.8	2.0
4.	Sterile isolate No.2	7.4	1.7
5.	Sterile isolate No.4	6.8	1.3
6.	Control	7.8	6.5

Conclusion

The emphasis of this investigation was to understand the mechanism on the alteration of the phenolic compounds and its relation, if any, in the root (wilt) syndrome of the coconut palm. Lack of adequate information on the host-pathogen interaction in the root (wilt) disease of coconut has been a limiting factor in restricting the studies on the role of phenolic. Studies relating to the metabolizm of the phenolic compounds confined to WCT palms only. Nonavailability or limited availability of equal number of samples both healthy and diseased palms at different

stages of the disease compelled to restrict the intestigations mainly on SCT palms. However, the available data on other varieties were incorporated into this investigations to the maximum extend. Detailed studies were carried out only with root samples as the root samples of the same length from the root tip assures uniformity in age and other physiological conditions.

The results obtained are indicative of an altered phenol emtabolism in the roots of coconut palm affected with coconut root (wilt) disease. Changes in total phenol and orthodihydroxyphenol concentration, increased activities of phenol odixizing enzymes (both PPO and PO) and a fall in the phenol synthesing enzymes(PAL) are characteristics of plant diseases. The variation is very high between the individual samples of the same catogory is little puzzling. It may due to the heterogenous nature of the palm. The most interesting result obtained are the activity of PAL and the isoenzyme pa tte rn of PPO. These two studies can be elaborated further and may be possible to arrive at a suitable test for the early diagnosis of the root (wilt) disease.

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