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Vascular wilt of cocoa (Theobroma cacao L.) caused by Verticillium dahliae Kleb.: Studies on pathogenicity and resistance

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VASCULAR WILT OF COCOA (*THEOBROMA CACAO* L.) CAUSED BY *VERTICILLIUM DAHLIAE* KLEB.: STUDIES ON PATHOGENICITY AND RESISTANCE.

A thesis submitted for the degree of PhD
of the University of Bath

by

Mario Lucio Vilela de Resende, M.Sc.

1994

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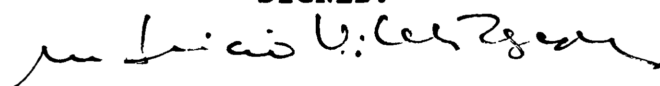

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FOREWORD.

Two papers (listed below) arising from this work have been accepted for publication and include results in chapters 3 and 5 of this thesis. Reprint of the paper already published is included at the back of this thesis.

Resende, M.L.V., Flood, J. & Cooper, R.M. (1994). Host specialization of *Verticillium dahliae*, with emphasis on isolates from cocoa (*Theobroma cacao*). Plant Pathology, 43, 104-111.

Resende, M.L.V., Flood, J. & Cooper, R.M. (1994?) Effect of method of inoculation, inoculum density and seedling age at inoculation on the expression of resistance of cocoa (*Theobroma cacao L.*) to *Verticillium dahliae* Kleb. Plant Pathology (In Press).

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LIST OF ABBREVIATIONS.

CEPLAC Comissao Executiva do Plano da Lavoura Cacaueira, here referred to as The Cocoa Research Centre in Brazil.

$CDCl_3$ Deuterochloroform.

CFU's Colony Forming Units.

cv. Cultivar.

E.I. Electron Impact; **e.V.** Electron Volts (related to mass spectrometry).

IEMS Electron Impact Mass Spectrometry.

GC-MS Gas Chromatography combined with Mass Spectrometry.

HPLC High Pressure Liquid Chromatography.

M^+ Molecular ion.

MeOH Methanol; **MeOD** Deuteromethanol.

MeTMSi Trimethylsilyl ethers.

MM Minimal Medium.

MS Mass Spectrometry.

nm Manometer (wavelength unit).

NMR Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR= Hydrogen NMR and ^{13}C-NMR= carbon NMR).

OFR Off Resonance Decoupled (^{13}C-NMR).

PCR Polymerase Chain Reaction.

Rf Reference value of a certain substance on TLC plates (in relation to solvent front).

RFLP Restriction Fragment Length Polymorphism.

STS Silver thiosulphate.

TLC Thin Layer Chromatography:
- **PTLC** Preparative Silica Gel Thin Layer Chromatography.
- **ATLC** Analytical Silica Gel Thin Layer Chromatography.

TMS Tetramethyl silane.

UV Ultra Violet light.

VCG Vegetative Compatibility Group.

WAA West African Amelonado.

To...

'The almighty God, creator and sustainer of heaven, earth and all the great and marvellous wonders therein to be discovered; Who, as sovereign and having exercised intrinsic power, saw that His work was very good'.

Genesis 1:31

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SUMMARY.

Verticillium wilt of cocoa is an increasingly serious problem in Brazil, the world's second largest producer of this commodity. Host range, isolate variation, physiology of infected plants, in addition to methods for selection and mechanisms of host resistance to the disease, constituted the intrinsic objectives of this thesis.

Verticillium dahliae isolates from cocoa were pathogenic, albeit to different degrees, to other crops such as aubergine, tomato, pepper and cotton and to native weeds from cocoa plantations. Some species were colonized, but did not show symptoms. Symptomless carriers of the disease are likely to be important in the multiplication and survival of the fungus in the field.

A rapid decrease in total transpiration, stomatal conductance and midday leaf water potential of cocoa seedlings was closely associated with the onset of foliar symptoms, indicating that water stress is a major cause of symptom development. Water stress was most pronounced when plants were inoculated with a 'non-defoliating' isolate. By contrast, a 'defoliating' isolate induced accumulation of ethylene in newly developed leaves, where the first symptoms generally appeared. This plant hormone was responsible for the accelerated senescence and defoliation, as demonstrated by the application of the ethylene inhibitor, silver thiosulphate.

Inoculation by soil drench and stem puncture were

compared as methods for selecting resistant cocoa cultivars. Disease progress was more rapid and symptoms were more severe following stem puncture and, under glasshouse conditions, differences between cultivars were detected by 15 days after inoculation. Moreover, using stem puncture, inoculum densities as low as 10^4 conidia/ ml differentiated between resistant and susceptible cultivars, whereas with the soil drench method, 10^7 conidia/ ml were necessary. Although a substantially higher proportion of plants were infected by stem puncture inoculation, the resistance of cultivar Pound-7 remained effective, even with very high inoculum levels (up to 10^8 conidia/ ml).

With either method, older seedlings were more susceptible to *V. dahliae* than younger ones. However, with stem puncture even young seedlings (15 days) became sufficiently infected to enable a valid disease assessment; in contrast, with soil inoculation, 60-day-old seedlings were required. In a nursery trial with 15-day-old seedlings, seven cocoa genotypes previously selected as resistant, moderately resistant or susceptible based on root inoculation, were ranked in the same order when stem punctured. Stem puncture inoculation of young seedlings is cost effective in terms of time and space, and is therefore recommended for screening of cocoa for wilt resistance, especially in large scale breeding programmes.

Variation of *V. dahliae* isolates from different countries and regions was studied in terms of pathogenicity and genetic relatedness as judged by

vegetative compatibility groups (VCG) using nitrate non-utilizing mutants. Isolates originating from cocoa in Colombia (VCG-4) and from cotton in the U.S.A. (VCG-1), overcame the resistance and caused severe defoliation on Pound-7. Cocoa isolates from different states in Brazil, although all in VCG-2, differed in their pathogenicity towards cocoa cultivars, which will guide the choice of isolates for selection trials.

Based on previous evidence on mechanisms of resistance to other cocoa pathogens, the level of phenolic compounds, including tannins, was initially investigated in susceptible and resistant cultivars, before and after inoculation. Pre-formed condensed tannins present in stems were toxic to conidia of *V. dahliae*, but only at high concentrations (ED_{50} conidial germination= 383μg/ml). No significant differences in tannin contents were detected between susceptible and resistant cultivars; nevertheless increased levels of these compounds were found in all three cultivars tested, 60 days post-inoculation.

These results led to the consideration of untested methods to extract antifungal compounds from cocoa. From four organic solvents with increasing polarities, diethyl ether was the most effective, extracting four inhibitory substances, here designated C-1, C-2, C-3 and C-4. Separation was achieved by flash chromatography combined with silica gel thin layer chromatography (TLC). These compounds were detected under UV light or by spraying the TLC plates with *V. dahliae* conidia. Mean Rf values with diethyl-ether: petroleum ether: methanol (6:3:1) were

respectively: C-1= 0.77, C-2= 0.46, C-3=0.38 and C-4= 0.25. Their structure was investigated using NMR and GC-MS spectroscopy. Compound-4 was identified as a triterpenoid (arjunolic acid), compounds 3 and 2 are phenolics (3,4 dihydroxyacetophenone and 4-hydroxyacetophenone, respectively). The least polar substance (Compound-1) seems to be a sulphur based compound. Their respective toxicities to *V. dahliae* (ED_{50} for conidial germination in μg/ml) were: C-1=4.6, C-2=7.8, C-3=118.7, C-4=10.3. These substances, not present at detectable levels in intact uninoculated plants, can be considered as novel phytoalexins. Bioassays indicated that they accumulate to maximum inhibitory levels in stems of Pound-7 by fifteen days after inoculation and seem to be present in much larger amounts in this cultivar than in the susceptible ICS-1. Compounds 1 and 4 were detected in cocoa stems in amounts well above their toxic level to *V. dahliae* conidia *in vitro*. The possible use of these compounds as tools in breeding for resistance remains to be investigated.

1. GENERAL INTRODUCTION.

1.1. The host *Theobroma cacao* L.

1.1.1. Origin, environment and vegetative growth of cocoa.

The cocoa tree belongs to the genus *Theobroma*, a group of small, wild trees which occurs mainly in the vast Amazon basin in South America. There are over twenty species in this genus, but *T. cacao* is the only one cultivated extensively. The headwaters of the Amazon basin is a region where great variation in morphological and physiological characteristics is found, thus, it has been considered the centre of origin or the primary centre of diversification of the cocoa tree (Wood, 1985). From there, it has spread into two directions, originating two distinct populations: The 'forastero' or amelonado cultivars originated in the eastern part of the region and spread along the Amazon Valley, whereas the 'Criollo' cultivars originated in the northen and western basin and spread through the Andes mountains, Central America and Southern Mexico (CEPLAC, 1982; Luz, 1989). Much later, in the seventeenth century, the Portuguese and Spanish introduced the crop into West Africa and the Dutch and Spanish brought cocoa to South-east Asia (Urquhart, 1961; CEPLAC, 1982).

Nowadays, cocoa is cultivated in almost every country in the lowland humid tropics, and the four major producers are, respectively, Ivory Coast, Brazil, Ghana and Malaysia (Figure 1.1.); all these countries produced more than 200,000 tonnes of dry beans in the 1990-1991

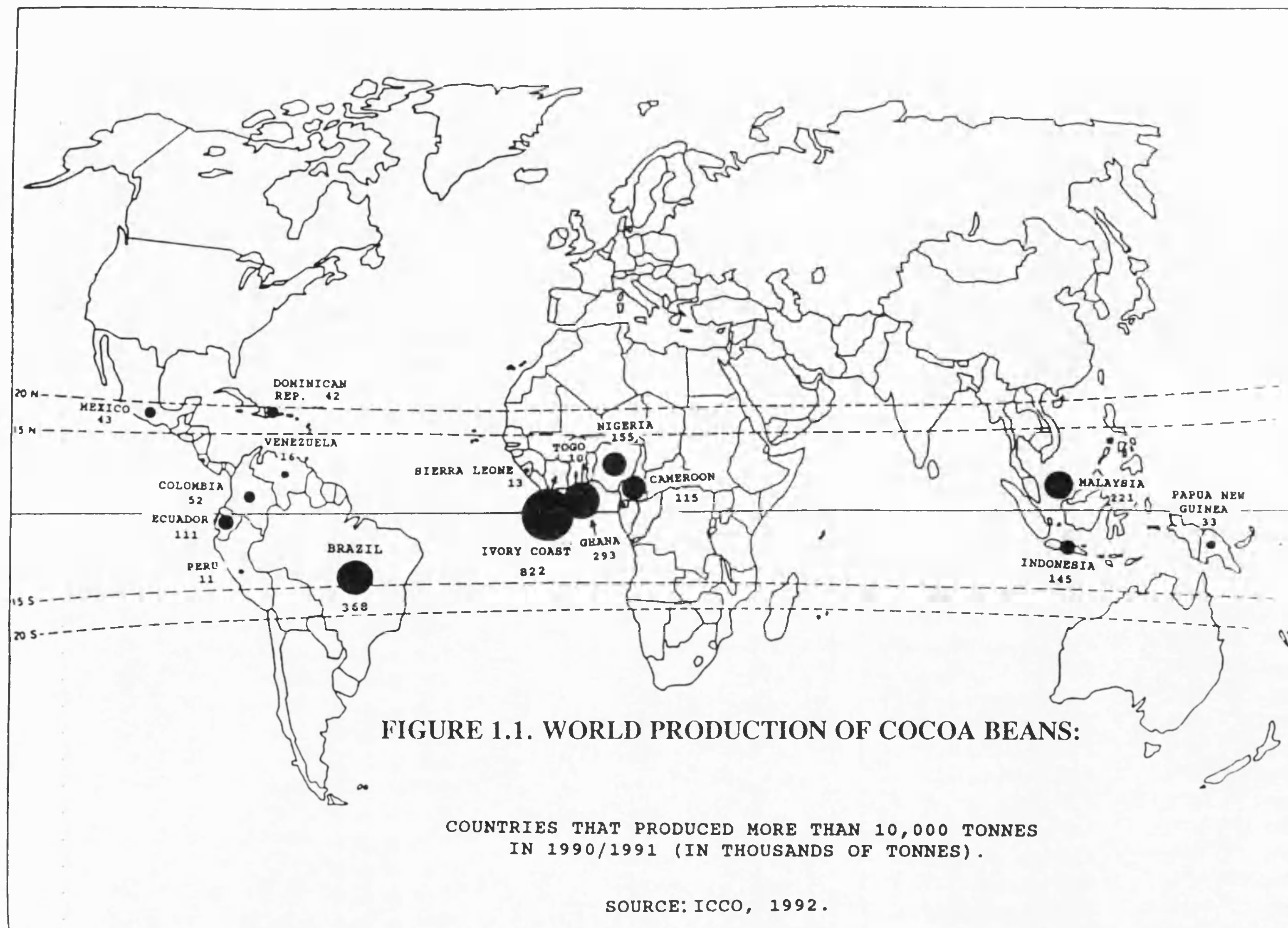


FIGURE 1.1. WORLD PRODUCTION OF COCOA BEANS:

COUNTRIES THAT PRODUCED MORE THAN 10,000 TONNES
IN 1990/1991 (IN THOUSANDS OF TONNES).

SOURCE: ICCO, 1992.

harvesting season (ICCO, 1992). In most areas where cocoa is grown, the annual mean air temperature is approximately 25°C and the total annual rainfall varies from 1250 to 2800 mm (Wood, 1985). Cocoa trees are usually grown under shade and the amount of light admitted to a plantation is mainly controlled by the level of shading utilized (Urquhart, 1961). In young plantations, the shade is not only needed to reduce light intensity, but also to buffer the micro-environment so that excessive moisture stress is avoided (Wessel, 1985).

Cocoa is a small tree, attaining an average height of 5 or 6 metres and having an uncommon and characteristic mode of branching. The seedlings form a straight orthotropic main stem 1-1.5m height, which forks into three to five plagiotropic branches, forming the so called fan or 'jorquette'. After considerable growth of the fan branches, an orthotropic shoot or 'chupon' develops just below the jorquette. This shoot will eventually reach above the tree's canopy and form a new fan (Urquhart, 1961; Greathouse & Laetsch, 1969). Leaf production on the fan branches is by a series of 'flushes', during which, the terminal bud grows out rapidly, producing three to six pair of leaves. After the flush has expanded, the terminal bud remains dormant for a determined period, and then produces another flush of growth (Toxopeus, 1985). Like shoot growth, root growth in cocoa seedlings in rhythmical, with maximum growth rate occurring between successive flush cycles of shoot growth (Hardwick, Sleigh & Collin, 1982). In mature cocoa seedlings, the root

system consists of a 50-120 cm long tap root combined with an extensive mat of lateral feeder roots, most of which, lie in the top 20 cm of soil (Toxopeus, 1985).

1.1.2. Diseases of cocoa.

Disease losses on cocoa are devastating; estimates of the total world production lost to diseases range from 20-30%, which is equivalent to 480-720,000 tonnes of dry beans per annum (Holderness, 1993). A range of pathogens can attack cocoa, including fungi and viruses. *Phytophthora* spp. are the most widespread pathogens in cocoa plantations throughout the tropical world and cause more losses than any other pathogen (Lass, 1985; Luz, 1989; Holderness, 1993). Others, although inducing more severe damage in certain regions, are generally of restricted occurrence. In South America, native fungal species as *Crinipellis perniciosa* and *Moniliophthora roreri* are very destructive. *Oncobasidium theobromae* in South-east Asia and cocoa swollen shoot virus (CSSV) in West Africa are other serious threats to cocoa production (Lass, 1985; Holderness, 1993). Attack by diseases may result in a direct loss of the crop, as with Phytophthora pod rot, or the tree may be debilitated, as with vascular-streak dieback caused by *O. theobromae*. The tree may even be killed, as with *Ceratocystis* (Lass, 1985) or Verticillium wilt (Oliveira, 1982).

A number of cocoa diseases can be controlled at a certain level, by simple field sanitation of pods, leaves

and shoots; however on certain occasions, chemical control may be necessary or complementary. The successful development of appropriate management strategies for diseases of this perennial crop should also include the selection and cultivation of resistant plant materials as a mean of obtaining durable and economical control (Lass, 1985; Holderness, 1993).

1.2. The pathogen *Verticillium*.

1.2.1. *Verticillium* diseases in general.

Verticillium species are responsible for some of the world's major diseases affecting vegetable, field, tree and ornamental crops. Most of the crop losses are caused by *V. albo-atrum* and *V. dahliae*. *V. dahliae* is more widely involved as a pathogen (Schnathorst, 1981). *V. dahliae* is more destructive in warmer climates, whereas *V. albo-atrum* is more apt to cause problems in crops in northern latitudes with humid climates (Snyder & Smith, 1981). These two species differ slightly in their optimal temperature requirements for growth in culture. For *V. albo-atrum*, this is usually 20-25°C, while for *V. dahliae*, it is 25-28°C. Taxonomically, *V. dahliae* is separated from *V. albo-atrum* mainly by the presence of microsclerotia and the absence of dark mycelium (Isaac, 1949; Schnathorst, 1973).

Fungal wilt pathogens, such as *V. dahliae* enter the vascular system of the host directly after penetration

through the roots and remain in the conductive tissue until the disease syndrome is well advanced. A unique adaptation of these organisms is that until the advanced stages of vascular colonization, the pathogen is exclusively confined in the xylem, whose fluids contain only low concentrations of sugars, amino acids and various inorganic salts (Green, 1981; Pegg, 1981a).

General symptoms of Verticillium wilts include epinasty, chlorosis, necrosis and wilting of leaves, followed by stunting or death of the plant. Browning, tyloses, deposition of gels and gums may be observed internally in the vessels. Symptomatology varies from host to host and levels of symptoms depends mainly on the concentration of inoculum, pathotype of *Verticillium* present, plant variety and stage of plant development, temperature, soil moisture, and nutrition, notably in relation to potassium content (Temple, DeVay & Forrester, 1973; Pegg, 1981a; Puhalla & Bell, 1981).

No single method is highly effective in controlling *Verticillium* diseases, and very often an integrated management system is necessary to minimize losses (El-Zik, 1985). Control begins with the selection of a cultivar that presents some degree of resistance to the pathogen, apart from good agronomic caracteristics. Then, a combination of cultural practices are adapted to minimize the losses from Verticillium wilt. Chemical control can be effective, particularly with systemic fungicides, but it is generally not used, because of prohibitive costs (Pegg, 1974; Oliveira, 1982; Bell, 1992). However, the adoption

of certain control measures is greatly dependent upon the crop involved. Factors like longevity of the crop (if annual or perennial), area cultivated, profitability and environmental consequences should be taken into account.

1.2.2. Verticillium wilt on cocoa.

Die-back or sudden death of *T. cacao* L. caused by *V. dahliae* has been recognized in Uganda for many years and may well be the reason that cocoa has not become a significant crop in that country (Leakey, 1965). In Brazil, Verticillium wilt has become a increasingly serious problem in the States of Bahia and Espirito Santo, which are responsible for approximately 85% of the Brazilian cocoa production (CEPLAC, 1982; Oliveira, 1982; Lass, 1985; CEPEC/ CEPLAC, 1990). Although the disease has been known in Brazil since the thirties under the name of 'sudden death' (Silva, 1938), it was only in 1980 that *V. dahliae* was registered as the causal agent of this syndrome (Oliveira, 1980). More recently, *V. dahliae* was reported causing wilt on cocoa in Colombia (Granada, 1989).

The following description of symptoms was outlined by Emechebe, Leakey & Banage (1971):

Acute wilting: The commonest external symptoms of Verticillium wilt in field and older seedlings (six-month old or more) in the glasshouse are the sudden wilting and subsequent necrosis of the leaves (Plate 1.1.a). The first symptom is the drooping of leaves without any apparent

loss of turgor. They then start to desiccate, beginning from the tips and margins, and the lamina dries and rolls inwards. Affected leaves eventually become brittle and occasionally shredded but they remain attached to the stem for over five weeks. Gradually, the dead fine branches break off and the leaves fall, so that the affected shoots become completely devoid of leaves and fine branches.

Defoliation: This is a not very common symptom, but it does sometimes occur. This defoliation differs from that associated with the late symptoms of the disease because the fine branches have not died by the time it starts.

Stunting: Stunting was observed on plants which had been inoculated at the seedling stage. Growth of the affected plant is drastically reduced by the almost complete failure of leaves to expand.

Recovery from the disease: Recovery mainly results from growth of axillary buds on the old main stem. In plants inoculated at 4-6 weeks old, growth is usually resumed by buds on the cotyledonary node.

Internal symptoms: As in any other Verticillium hadromycosis, the most distinct internal symptom of infection is the discolouration of xylem vessels of the petiole, pedicel, stem and root system. They become initially light brown but the browning increases in intensity as the disease progresses, so that, before the appearance of any external symptoms, most of the discoloured areas are dark brown (Plate 1.1.b). Transverse and longitudinal sections of affected parts showed that

discolouration is due not only to the browning of walls of xylem vessels, but also to the black or brown gum deposits in the lumina of the vessels. Tyloses and/or fungal mycelium have also been observed partly or completely occluding the lumina of xylem vessels (Plate 1.2.).

Verticillium wilt is the most serious disease of cocoa in Uganda inducing losses of up 30% (Emechebe *et al.*, 1971). In Brazil, the disease is more common in regions subject to rainfall shortages, causing annual plant mortality of up to 10% on unshaded cocoa areas (Almeida, Almeida & Figueiredo, 1989). According to Leakey (1965), severe attacks, following especially dry conditions or waterlogging, can cause the death of a cocoa tree one week after a situation of apparent health and vigour.

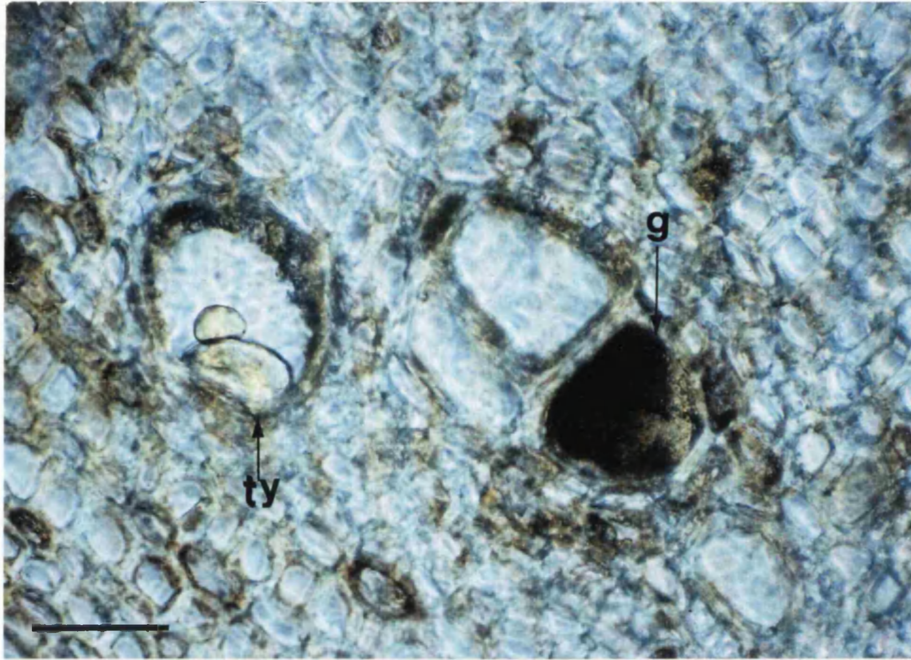
Plate 1.1. Field symptoms of Verticillium wilt on cocoa in Brazil. a) External symptoms: chorosis, necrosis and general wilting of the leaves; b) transverse section of a cocoa branch showing vascular discolouration.

a

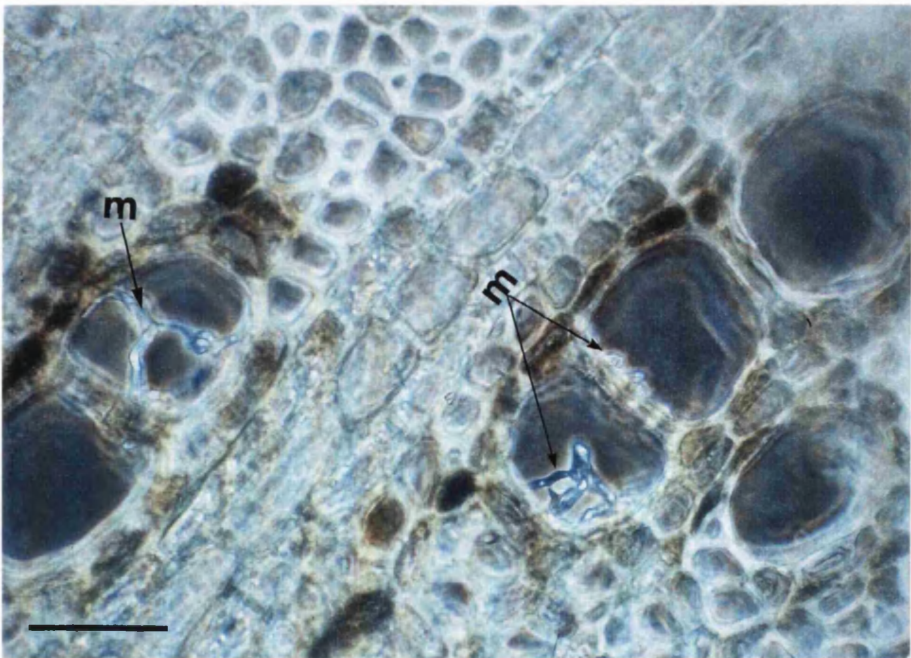
b

Plate 1.2. Transverse section of an infected cocoa stem under light microscopy: a) Dark brown gum deposits (g) and tylosis (ty), produced in response to infection; b) *V. dahliae* mycelia (m) growing in the lumina and through the pit, between xylem vessels. (Bar markers represent 50μm).

a



b



As with other soil-borne wilt diseases of perennial crops, breeding for resistance affords the only realistic means for a durable control of Verticillium wilt on cocoa (Lawrence, Campelo & Figueiredo, 1991). From the geographically vast Amazon basin, the likely centre of origin of the cocoa plant, around 22,000 different accessions of *Theobroma* spp. have been collected and maintained by CEPLAC/ Ministry of Agriculture in Brazil. However, the enormous variation within this population has not been adequately exploited in terms of resistance to diseases, mainly due to the lack of quick and efficient screening procedures. In relation to Verticillium wilt, there is virtually no information on isolate variation and mechanisms of pathogenicity or host resistance, and there is not a quick and reliable method for selection of disease-resistant plants. The current investigation was mainly aimed at these key issues.

2. MATERIAL AND METHODS.

2.1. Plant material and growth conditions.

2.1.1. At the University of Bath, U.K.

Self pollinated cocoa seeds of cultivars ICS-1 and Pound-7 (from the Cocoa Research Centre, CEPEC/ CEPLAC, Bahia, Brazil) and of cultivar West African Amelonado (supplied by the Cocoa Research Group at the University of Reading, U.K.), were germinated in trays containing vermiculite placed in a growth chamber maintained at 28°C and 80-100% relative humidity. Seven days later, the seedlings were individually potted into 13 cm wide and 23.5 cm high polyethylene bags, filled with a mixture of Fisons (Fisons p.l.c., Loughborough, Leicestershire, U.K.) fine (F2) compost, Fisons medium (M2) compost and perlite (1:1:1), and transferred to a glasshouse. To avoid contamination between treatments after inoculation, the seedlings were arranged in plastic troughs (610 x 160 x 140 mm, B-line, Swansea, U.K.), to contain five plants inoculated with the same isolate. Every month, plants within each treatment were randomised between the troughs for that treatment and the troughs were re-randomised in the glasshouse. The glasshouse temperature and relative humidity were monitored daily with a thermohydrograph and adjusted for approximately 25±2°C and 60±10% relative humidity. Supplementary illumination from artificial lights (Camplex 500W metal halide) was used to maintain levels of photosynthetically active radiation (PAR) between 350-450 μmol M^{-2} sec^{-1}, during a daylength of 12-14 h. Seedlings were kept well watered from below and were

fed at monthly intervals with liquid fertilizer (Fisons Liquinure, 1 in 45 dilution, containing N, P and K in the ratio 8:4:4, plus trace elements). The pH of the mixture used as substrate ranged from 5.0 at the beginning of the experiments to 6.4 after 5 months, mainly because the lime contained in the irrigation water was gradually incorporated into the substrate.

The seeds of aubergine (*Solanum melongena* L.) cv. Moneymaker, tomato (*Lycopersicon esculentum* Mill) cvs. GCR-26 and GCR-218, cotton (*Gossypium hirsutum* L.) cv. Deltapine 50 and pepper (*Capsicum annum* L.) cv. California Wonder were germinated in trays containing Fisons F2 compost in the same glasshouse already described. Two weeks later, the seedlings were pricked out into larger trays containing similar compost. After one week, these seedlings were pricked out again into 16 cm diameter pots filled with Fisons M2 compost supplemented with 3 g of Osmocote fertilizer (15% N, 12% P_2O_5, 15% K_2O) per kg of compost. These pots were placed in individual saucer pans and watered daily from below to avoid splashing of water and possible contamination between treatments.

2.1.2. At the Cocoa Research Centre, Brazil.

Weed species of the families Compositae (*Bidens pilosa, Erechtites hieraciifolia, Emilia sonchifolia* and *Synedrella nodiflora*), Solanaceae (*Capsicum chinense* and *Solanum americanum*), Amaranthaceae (*Achryranthes indica*), Labiatae (*Leonurus sibiricus*), Malvaceae (*Sida carpinifolia*), Rubiaceae (*Diodia ocimifolia*), Urticaceae

(*Boehmeria cylindrica*) and Verbenaceae (*Lantana camara*), were grown in a glasshouse at the Cocoa Research Centre, Brazil. Attempts were made to keep the growth and inoculation conditions , as similar as possible to those already described for the crops inoculated at Bath University, U.K.

By contrast, self-pollinated cocoa seeds of cultivars ICS-1, Pound-7, ICS-6, ICS-8, SIC-2, SIC-328, SIC-802, BE-5 and PA-30 were germinated in trays containing vermiculite, potted in polyethylene bags filled with local soil and transferred to a partially shaded nursery (50% shade), with average daily air temperatures ranging from 26-32°C during the experimental period (summer of 1992).

2.2. Fungal isolates, inoculum production and methods of inoculation.

2.2.1. Fungal isolates.

The geographical and host origins of *V. dahliae* isolates used in this study are shown in Table 2.1.. Cultures of all isolates were derived from single spores. Stock cultures were maintained in sterile soil and in potato dextrose agar (Appendix 1) at 5°C.

Table 2.1. Geographical and host origins of *V. dahliae* isolates.

ISOLATE DESIGNATION[+]	ORIGINAL HOST	GEOGRAPHICAL ORIGIN	YEAR OF ISOLATION
BA-1	COCOA	Bahia, BRAZIL	1990
BA-2	COCOA	Bahia, BRAZIL	1990
BA-3	COCOA	Bahia, BRAZIL	1990
BA-4	COCOA	Bahia, BRAZIL	1992
BA-5	COCOA	Bahia, BRAZIL	1992
BA-6	COCOA	Bahia, BRAZIL	1992
ES-1	COCOA	Espirito Santo, BRAZIL	1990
ES-2	COCOA	Espirito Santo, BRAZIL	1992
COL-1	COCOA	Palmira, COLOMBIA	1991
UG-1	COCOA	UGANDA	1962
UG-2	COCOA	UGANDA	1992
OK-1	OKRA	Minas Gerais, BRAZIL	1991
SW*	TOMATO	Wales, U.K.	----
TS-2**	TOMATO	California, U.S.A.	----
RG**	TOMATO	North Carolina, U.S.A.	1981
58*	TOMATO	Queensland, AUSTRALIA	1977
TOM-34*	TOMATO	Athens, GREECE	----
EGP**	AUBERGINE	Athens, GREECE	----
SS-4***	COTTON	California, U.S.A.	----
T-9***	COTTON	California, U.S.A.	----
97	PEPPER	Bari, ITALY	1976
LV-1	------	UGANDA	----

[+]Isolates BA-1, BA-2, BA-3, BA-4, ES-1, ES-2 and OK-1 were collected by the author in the states of Bahia, Espirito Santo, and Minas Gerais, Brazil; BA-5 and BA-6 were supplied by M. L. Oliveira, Cocoa Research Centre, CEPLAC, Ilheus, BA, Brazil; COL-1 by G. Granada, I.C.A., Palmira, Colombia; UG-1 by I.M.I., Kew, England; UG-2 by G. Hakiza, Kawanda Exp. Station, Kampala, Uganda; SW by M. Milton, University of Swansea, U.K.; TS-2 by W. Tolmsoff, USDA, College Station, Texas, U.S.A.; RG by R. Gardner, Horticultural Crops Research Station, N. Carolina, U.S.A.; 58 by R. O'Brien, Department of Primary Industries, Queensland, Australia; TOM-34 and EGP by E. C. Tjamos, Benaki Phytopathological Institute, Athens, Greece; SS-4 and T-9 by C. Boisson, ORSTOM, Montpellier, France; 97, by J. Heale, King`s College, University of London, England, and isolate LV-1 by G.L. Hennebert, Universite Catholique de Louvain, Belgium. Apart from these isolates above listed, We also received 21 Nit1 and NitM tester strains for VCG analysis, from C.A. Strausbaugh, University of California, Berkeley, U.S.A..

* Race 1 isolates on tomato; ** Race 2 isolates on tomato (O'Garro & Clarkson, 1988).

*** SS-4 and T-9 are, respectively, non-defoliating and defoliating isolates on cotton.

2.2.2. Inoculum production.

All isolates were multiplied in a sucrose-salts medium (Cooper & Wood, 1975, Appendix 1). The pH was adjusted to 6.5 using NaOH 0.1M and 150ml of the medium was dispensed into 250ml conical flasks, which were plugged with non-absorbent cotton wool and autoclaved at 121°C for 20 minutes. Five mycelial discs were taken from the active edge of the fungal culture growing on potato-dextrose-agar (PDA) and transferred to each flask. The liquid cultures were grown for seven days in a Gallenkamp rotary incubator running at 150 rpm and maintained at 25°C in a 24h light regime supplied by fluorescent lamps of total light intensity equivalent to 15 W m^{-2}. The resulting conidial suspension of each isolate was filtered through two layers of muslin and its concentration was assessed using a Newbauer improved haemacytometer. The final conidial concentration was adjusted through successive dilutions in sterile distilled water.

2.2.3. Methods of inoculation.

Two alternative methods of inoculation were used depending on the experiment:

Soil drenching: Unless otherwise stated, each plant of a certain species was inoculated with 50 ml of a suspension containing 1 x 10^{7} conidia ml^{-1}, applying the inoculum from a sterile syringe over the soil around the base of the hypocotyl. Uninoculated control seedlings received 50 ml of a 10% sucrose-salts medium. These plants were not stressed as following root-dipping inoculation

(Erwin, Moje & Malca, 1965), nor were their root systems deliberately wounded before inoculation.

Stem puncture: The stem puncture method described by Bugbee & Presley (1967), was used only to inoculate cocoa plants. Seedlings were inoculated using a syringe fitted with a 21-gauge needle. The conidial suspension (generally 1×10^7 ml^{-1}) was delivered from the syringe, to form a bead at the tip of the needle. The needle was then inserted downwards into the hypocotyl at a 45° angle to the stem, until the bevel of the point was just visible. The drop of inoculum that formed in the axis between the stem and the needle disappeared rapidly into the cortex. Approximately 10 μl of the inoculum suspension was absorbed in each puncture. Uninoculated control seedlings were injected with 10% of the sucrose-salts medium solution to simulate depleted culture medium.

2.3. Assessment of plant growth and disease development.

2.3.1. Disease index.

Every leaf of each plant was examined periodically after inoculation and its symptoms were assessed on a 0 to 4 scale, with 0, 1, 2, 3, and 4 representing respectively 0%, 1 to 25%, 26 to 50%, 51 to 75% and more than 75% of reduction in the photosynthetic area due to wilting and necrosis, as adapted from Sidhu & Webster (1977). A disease index was then calculated as the mean of the ratings from individual leaves:

$$\text{Disease Index} = \frac{\text{Sum of leaf ratings per plant}}{\text{Total number of leaves per plant}}$$

2.3.2. Growth measurements.

At the end of the experiments, plant height was taken as the measurement (cm), from soil level to the shoot apex. Final growth of the aerial parts (stem + shoots + leaves) of each plant was also assessed by dry-weight analysis, following 72 hours in a drying oven at 90°C. The number of leaves formed were recorded for all replicates in experiments concerning the effect of plant age on disease incidence.

2.3.3. Browning of xylem vessels.

Cross-sections from the base of the stem or base of the petiole, where appropriate, were obtained using a razor blade. The thinner sections of each sample were observed under the microscope; any brown xylem vessel was noted and the percentage of vessels so affected was thereafter calculated.

2.3.4. Re-isolation and quantification of *V. dahliae*.

Attempts were made to recover *V. dahliae* from the stems of plants in each treatment, using a direct re-isolation technique. Small pieces of stem (about 5 cm long), were cut from different internodes, depending on the plant species involved. The epidermis and, where appropriate, the bark, were aseptically removed and five complete cross-sections of the remaining tissues were plated onto a medium containing absolute ethanol (1% v/v) + agar (1.2% w/v), which was described as selective to *V. dahliae* (adapted from Nadakuvukaren & Hornen, 1959).

A comminution-dilution technique was used for

quantitative recovery of *V. dahliae* from a known fresh weight (ca. 0.5g) of cross-sections aseptically prepared from the middle region of the tap root and/or base of the stem of cocoa seedlings. One ml of acid washed sand and 5 ml of sterile distilled water were added to the plant tissue inside a mortar and the mixture was ground using a pestle. A ten fold dilution series was prepared and plated onto duplicate plates of PCNB-peptone-agar selective medium (Papavizas, 1967, Appendix 1). After incubation for 7 days at 25°C, colonies of *V. dahliae* were counted and the number of colony forming units/ g of fresh weight was calculated for each sample.

2.4. Measurement of water relation parameters and ethylene production by cocoa plants.

In experiments carried out to study water relations on cocoa infected with distinct isolates of *V. dahliae*, the specific parameters, total transpiration, stomatal conductance, leaf water potential and ethylene production were quantified. All measurements were taken under conditions of high light intensity, around midday (from 10 a.m. to 2 p.m.), which is in general, the period of higher physiological activity during the day.

2.4.1. Total transpiration.

Total water loss (evaporation + transpiration) from polyethylene bags containing cocoa seedlings was determined 14, 17 and 21 days after soil inoculation. Under the glasshouse conditions already described, bags

containing soil and growing seedlings were watered to field capacity and then weighed. Following weighing, they were left for 24 hours without watering, and weighed again. To provide estimations of evaporation from soil surface, a similar process was conducted concurrently by weighing bags containing only soil. Transpiration per plant was calculated from the difference between total water loss in each bag and mean evaporation.

2.4.2. Stomatal conductance and leaf water potential.

Stomatal diffusive resistance and leaf water potential were determined 14, 17 and 21 days after inoculation, each time using the oldest and the youngest fully developed leaf present in the canopy of each plant. Especial attention was paid to keep all leaves to be sampled, submitted to direct illumination from the artificial lights. Stomatal resistance (r1) was taken from the abaxial (stomate bearing) surface of each leaf, by water vapour diffusion using a MK-2 automatic porometer (Delta-T Devises Ltd., Cambridge, U.K.), following instructions of the manufacturer. Stomatal conductance was then calculated, as the inverse of r1 (1 / r1). Midday water potential was measured by inserting a freshly excised leaf with only the cut end of the petiole protruding, straight into a standard pressure chamber (Chas. W. Cook & Son, Birmingham, U.K.), which was operated following the principles of Scholander, Hammer, Brabstreet & Hemmingsen (1965).

2.4.3. Ethylene production.

Petioles from the youngest and oldest leaves of each plant were used in ethylene measurements by gas chromatography. Initially, six dilution series were made up in the range of 1-50 ppm of ethylene and a calibration curve was constructed by measurement of peak height. Ethylene was measured in a Pye series 104 gas chromatograph (Pye Unicam Ltd.) fitted with a flame ionization detector and an alumina column. Operating conditions were: Carrier gas (Nitrogen) flow rate= 40 $ml.min^{-1}$; Hydrogen pressure= 15 $lb.in^{-2}$; air pressure= 10 $lb.in^{-2}$; oven temperature= $125^{o}C$ isothermal. The gas chromatography was coupled to a chart recorder set at 10 mV and a chart speed of 10 $mm.min^{-1}$.

Immediately after cutting, the lower third of cocoa petioles were inserted individually in very small glass tubes, with an interior air space of 4 cm^3 and sealed with a rubber serum bottle stopper. Thirty minutes later, gas samples of 1 ml collected with a gas tight-syringe from the upper air space of each tube, were injected into the chromatography chamber. This sampling time aimed to minimize the ethylene produced as a result of wounding, which does not start to accumulate until 1-2 h after cutting (Tzeng & DeVay, 1985).

2.5. Generation of nitrate non-utilizing (*nit*) mutants and assessment of vegetative compatibility groups (VCG's) within *V. dahliae* isolates.

Isolation of nit mutants was performed by the technique of Cove (1976), first modified by Puhalla (1985) and later by Strausbaugh, Schroth, Weinhold & Hancock (1992). A dilution series of each single spore wild-type isolate of *V. dahliae* was prepared on PDA and the plates were incubated for 3 days at 25°C. Mycelial blocks (1 mm^3) from each of twenty colonies (for each isolate) were plated onto a minimal medium (MM) (Puhalla & Spieth, 1983) and incubated for 3-4 days at 25°C. Then, a mycelial block (1 mm^3) from each colony was plated onto chlorate amended medium (MM plus 25 g/l of potassium chlorate and 1.6 g/l of L-asparagine). After incubation for 2-3 weeks at 25°C, fast growing chlorate-resistant sectors were subcultured onto MM, in which the sole nitrogen source was nitrate. On this medium, most chlorate-resistant sectors yielded very thin but normally expansive mycelial growth, indicative of their inability to reduce nitrate. All colonies with this type of growth response in MM, were considered *nit* mutants. All *nit* mutants exhibited wild type growth on PDA and were stored on this medium in a fridge at 5°C.

Phenotype identification of *nit* mutants was determined by their growth in a basal medium that was MM without nitrate, amended with one of the following nitrogen sources: sodium nitrate (2 g/l), sodium nitrite (0.4 g/l) or hypoxanthine (0.5 g/l). Thus, mycelial blocks

were removed from the edge of *nit* mutant colonies, placed on these amended MM, and incubated for 1-2 weeks at 25°C. *Nit* mutants unable to utilize nitrate, but able to use nitrite and hypoxanthine were designated as Nit1, in accordance with Correll, Gordon & McCain (1988). Similarly, *nit* mutants incapable of utilizing nitrate and hypoxanthine, but capable of using nitrite, were designated NitM.

All 22 isolates obtained (Table 2.1.) were tested for vegetative complementation, by pairing Nit1 and NitM mutants on MM. *Nit* mutants were paired by placing a mycelial transfer in the centre of a 9cm-diameter Petri dish facing four transfers of a different mutant type around, and, at least 2 cm apart from it. In order to assign each isolate to an already identified VCG (*sensu* Strausbaugh *et al.*, 1992), 21 Nit1 and NitM testers strains representing 5 different VCGs (kindly supplied by Dr. C.A. Strausbaugh, University of California, Berkeley, U.S.A.), were also paired with, at least, a Nit1 and a NitM mutant from each of our isolates. Paired cultures were observed at weekly intervals for four weeks. Complementation, a result of heterokaryon formation between *nit* mutants, was evident by the prototrophic growth resulting in a dense aerial mycelium or microsclerotial formation at the mycelial interface. Two isolates were considered vegetatively compatible and therefore included in the same VCG, if prototrophic growth appeared at the zone of mycelial contact between Nit1 and NitM mutants from each of them.

2.6. Extraction of antifungal compounds.

2.6.1. Small scale.

These included the extractions of pre-formed (tannins) and post-infectional (phytoalexins) antifungal compounds for slide bioassays using crude plant extracts only.

2.6.1.1. Plant material and sample preparation.

The initial sample size utilized for extraction of pre-formed or post-infectional antifungal compounds consisted of five 100-220-day-old seedlings per treatment (cultivar or date after inoculation). Inoculations, unless otherwise stated, were performed with isolate ES-1, 10^7 spores/ml, applying 20 punctures per hypocotyl. The preparation of samples and the extraction process were carried out under conditions of low light intensity, aiming to avoid possible photo-degradation of such compounds. Small pieces of stem (about 5 cm length), were cut from the hypocotyl of each plant. After washing and rinsing them in distilled water, the bark of each piece was aseptically removed and the remaining tissue was cut longitudinally to remove the inner pith. The woody parts from the cortex were sliced with a post-mortem knife, labelled, plunged into liquid nitrogen and then stored in a deep freezer at -80°C. The frozen samples were subsequently submitted to the specific extraction procedures, either for tannins or phytoalexins.

2.6.1.2. Extraction and quantification of condensed tannins.

Five grams of the frozen tissue (ca. 1 g from each plant) were comminuted with 10g of acid-washed sand, using a pestle and mortar. Then, 25 ml of 2M hydrochloric acid (BDH, Poole, Dorset, U.K.) was added to each mortar containing the ground sample, before covering them with aluminium foil and heating in a water bath at 95°C for one hour. Immediately after, 25 ml of n-butanol (BDH, Poole, Dorset, U.K.), was added to each mortar and the materials were covered and heated again at the same temperature for another hour. The condensed tannins were hydrolysed during this period, as evidenced by the red colour developed in the butanolic phase (upper layer) (Harborne, 1984). An aliquot of 5ml of this phase was then collected from each mortar, centrifuged (3000g., 5min.), and the supernatant taken into a quartz cuvette for determination of the optical density. The condensed tannin (procyanidin) content was estimated by the absorbance of cyanidin extracted into n-butanol, at 545 nanometres (nm) (Harborne, 1984), in a UV-visible recording spectrophotometer (model UV-260, Shimadzu Corporation, Kyoto, Japan). In the absence of precise information on the degree of polymerization of the cocoa procyanidin, it was assumed a extinction coefficient ($E^{1\%}{}_{1cm}$) of 150 for an average procyanidin (Bate-Smith, 1973). Tannin concentration (μg/g) was then calculated as:

Tannin (μg/g)= Absorbance (545nm) X 10 (dilution) /150

After these determinations, the aliquots were used in slide bioassays as original samples for dilution series.

2.6.1.3. Extraction of phytoalexins.

5 g of the frozen tissue were comminuted with 10g of acid-washed sand, using a pestle and mortar. 25 ml of diethyl ether (Aristar, BDH, Poole, Dorset, U.K.) were added to each mortar containing the ground sample. After further grinding with a pestle, the liquid phase was collected in a tube and dried by evaporation of the solvent in an air stream. The crude extracts collected in each tube were resuspended in 1 ml of absolute methanol (HPLC grade, Rathburn Chemicals Limited, Walkerburn, Scotland, U.K.) and used in slide bioassays as original sample for dilution series.

2.6.2. Large scale.

These included bulked extractions of phytoalexins, for attempts at purification, identification and further toxicity tests.

2.6.2.1. Sample preparation and extraction of phytoalexins.

Cocoa seedlings of cultivar Pound-7 were inoculated with four punctures per hypocotyl and were harvested 15 days later. Large samples of sliced stem tissue were prepared and stored as described for small scale extractions. Bulked frozen samples were ground in a mill, using a fine sieve especially devised for grinding cereals (EBC Model, Casella London Ltd., London, U.K.). Then, 570 g of the powdered material was continuously extracted with

diethyl ether in a Soxhlet apparatus for 48 hours. The liquid phase obtained was vacuum dried in a rotary evaporator (Buchi Laboratoriums, 9230, Flawil, Switzerland) at 35°C. Dried samples were kept in a desiccator at 5°C for a short period before separation procedures were undertaken.

2.7. Separation and detection of phytoalexins.

2.7.1. Small scale (thin-layer chromatography).

A combination of preparative (PTLC, 1 or 2 mm thick) and analytical (ATLC, 0.25 mm thick) silica gel thin-layer chromatography plates containing fluorescent particles (F^{254}, E. Merck, Darmstadt, Germany), were used for separation of milligram samples. Fifty microliters of crude ether extracts from cocoa stems were spotted at 2cm from the base of 20 cm x 20cm plates and run for 12-15 cm with different solvent mixtures, previously screened in small pieces of ATLC plates (usually 10 cm high, 5cm wide). All solvents used were HPLC grade and came from BDH, Poole, Dorset, U.K. After evaporation of the solvents, potential antifungal bands were marked under ultra violet (UV) light (254 and 350 nm). Fungitoxicity was determined by spraying the plates with *V. dahliae* (suspension containing 10^7 conidia/ ml diluted in equal volume of sucrose-salts medium, Cooper & Wood, 1975) and incubating them in TLC tanks with moistened filter paper on the side walls for at least 48 hours at 25°C. Specific colour reagents were also tried, to help detection and

characterization of these compounds on those plates not sprayed with *V. dahliae*. Iodine crystal vapour (BDH Chemicals Ltd., Poole, Dorset, U.K.) was confined in a TLC tank and used for detection of all organic substances, especially those that did not absorb or fluoresce under UV light (Ganshirt, 1969). Folin-Ciocalteu (Sigma Chemical Co., St. Louis, MO, U.S.A.) and vanillin-sulphuric acid (BDH Chemicals Ltd., Poole, Dorset, U.K.) were sprayed on plates for the detection of, respectively, phenolics (Harborne, 1984) and phenolics plus terpenoids (Krebs, Heusser & Wimmer, 1969; Banthorpe, 1991). The Rf of each inhibitory substance was determined as the distance moved by the substance/ the distance moved by the solvent front.

2.7.2. Large scale (flash chromatography + thin-layer chromatography).

A combination of successive flash column chromatography and silica gel thin-layer chromatography was adopted to obtain the pure samples for identification and use in bioassays. The sequence of columns, TLC plates and solvent systems developed specifically for the extraction of cocoa phytoalexins is described in Results (7.2.8.).

2.8. Identification of phytoalexins.

Nuclear Magnetic Resonance Spectroscopy (Carbon ^{13}C-NMR and/or Hydrogen ^{1}H-NMR), Gas Chromatography combined with Mass Spectrometry (GC-MS) or Mass Spectrometry (MS) alone, were kindly conducted by Dr. M. H. Beale, at Long Ashton Research Station, University of Bristol, Bristol, BS18 9AF, U.K. and Dr. M. G. Rowan, at the School of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY, U.K. ^{1}H-NMR and ^{13}C-NMR data were obtained from a Jeol JNM-GX400 Spectrometer; mass data from a VG 7070E mass spectrometer with 2000 data system. In the GC-MS procedure, samples were eluted with hydrogen and run in BPX-5 capillary columns.

To confirm the provisional identity of two of the novel phytoalexins, comparative GC-MS analyses were conducted between such samples and authentic pure samples obtained commercially (the case of 4-hydroxyacetophenone from Sigma Chemical Co., St. Louis, MO, U.S.A.) or supplied by other researchers (the case of arjunolic acid, kindly provided by Dr. T. Inoue, Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan).

2.9. Bioassay for assessing toxicity of antifungal compounds.

A slide bioassay technique was devised for the toxicological evaluation of antifungal compounds to spores of *V. dahliae*. Cavity slides were immersed overnight in 2M sulphuric acid, rinsed in sterile distilled water and

dried in an oven at 85°C. Two day old liquid cultures of *V. dahliae* (isolate ES-1) were filtered through muslin and centrifuged at 3000 g for 5 minutes to remove the culture fluids. Conidia were resuspended in sterile distilled water (pH 7.0) and diluted to 1 x 10^6 spores/ ml. Unless otherwise stated, 50μl of the respective alcoholic solution containing the product to be tested (crude plant extract or condensed tannins), or 50μl of sterile distilled water (pH 7.0) or solvent control, were pipetted in each cavity, allowed to evaporate and then, 25μl of the conidial suspension were added. Pure phytoalexins were dissolved in diethyl ether before addition to the cavities. Each slide was transferred to an individual Petri dish containing a moisturized filter paper and incubated for 15 hours at 25°C. Four replicate slides were prepared per treatment in each experiment. Immediately after the incubation period, 10μl of a 0.01% solution of cotton blue in lactophenol (BDH, Poole, Dorset, U.K.) were poured in each cavity to stain conidia and germ tubes and to arrest further growth. Microscopic examination of germination and measurements of germ tube length were followed at X200 magnification. Conidia were considered to have germinated when the germ tube length was longer than the maximum spore diameter. Percentage of germination and mean germ tube length were calculated based on the germination of 100 spores and germ tube length of 20 spores, quantified at random in five microscope fields from each cavity slide.

3.0. Experimental design and statistical analysis.

Fully randomised designs were always utilised combining different treatments into a number of replicates ranging from 4-50, depending on the objectives and methods involved in each experiment. Statistical analyses were carried out using programmes from Minitab (Minitab Inc., State College, PA, U.S.A.) and Statsease (B. Clarke, University of Nottingham, U.K.). Normality of data was tested by the n-scores method. When data were not suitable for analysis of variance, non-parametric Kruskal-Wallis and repeated Mann-Whitney U-test were performed. Examination of data from re-isolation (in the form of ratios), was made by Chi-squared analysis of contingency tables and, as appropriate, by Fisher's Exact test (Sokal & Rohlf, 1981). Data from vegetative compatibility between isolates were submitted to cluster analysis based on Jacquard's similarity coefficient (Sneath, 1962). Linear regression analyses, Pearson's correlation coefficients or graphic representation of the results were also presented, when bringing any other relevant point into the context.

RESULTS AND DISCUSSIONS:

3. HOST SPECIALIZATION OF VERTICILLIUM DAHLIAE, WITH EMPHASIS ON ISOLATES FROM COCOA.

3.1. INTRODUCTION.

Verticillium species are responsible for some of the world's major diseases affecting vegetable, field, tree and ornamental crops. Most of the crop losses are caused by *V. albo-atrum* and *V. dahliae* (Schnathorst, 1981).

As a soil-borne pathogen, *V. dahliae* has been shown to colonize the roots of a wide range of plants, including species which do not become systemically invaded. Evans & Gleeson (1973) showed that a wide range of genera and species were colonized by *V. dahliae*, including members of the families Solanaceae, Malvaceae, Compositae, Gramineae, Convolvulaceae, Papilionaceae, Labiatae and Chenopodiaceae. Brown & Wiles (1970) found notable differences in host reaction to *Verticillium* among species of the same family, or even, among cultivars of the same species.

Unlike *Fusarium* species which exhibit host specificity, *Verticillium* species have not been grouped into formae speciales, because an isolate from one host often attacks several other unrelated plant species (Isaac, 1949). Nevertheless, some isolates of *Verticillium* are rather specialized: *V. dahliae* from peppermint, pepper, strawberry, Brussels sprouts and tobacco and *V. albo-atrum* from lucerne and hops have limited host ranges (Puhalla & Bell, 1981).

Weeds are common hosts for *V. dahliae* (Heale & Isaac, 1963; Harrison & Isaac, 1969; Evans, 1971; Evans & Gleeson, 1973; Busch, Smith & Elango, 1978), although sometimes, symptoms are not apparent on many species (Woolliams, 1966; Brown & Wiles, 1970; Vargas-Machuca, Martin & Galindez, 1987). Symptomless weeds may provide a means of multiplication, dissemination and survival of *V. dahliae* in agricultural lands (Evans, 1971; Schnathorst, 1981) and have frequently contributed to the collapse of crop rotation programmes (Busch, Smith & Elango, 1978).

In relation to alternative hosts, there is evidence from East Africa, that cotton is involved in the multiplication, dissemination and survival of *V. dahliae* in cocoa plantations. Verticillium wilt of cotton is widely distributed in Uganda and this country is perhaps, unique in growing cocoa in the same ecological zone as cotton (Lass, 1985). Moreover, Ugandan isolates of *V. dahliae* from cotton, okra and cocoa were equally pathogenic when inoculated on cocoa. They all induced typical symptoms on okra, cocoa and cotton but with decreasing disease severity, respectively, on these three hosts (Emechebe, 1974). Similarly, in Colombia, two varieties of potato were highly susceptible to a *V. dahliae* isolate from cocoa (Granada, 1989). In Brazil, nothing is known about possible alternative hosts for *V. dahliae* in cocoa growing areas. The main epidemiological consequences concern the spread of the fungus to other crops and the possible introduction of other strains, via contaminated plant material, into cocoa farms of Bahia and

Espirito Santo. The host selectivity of *V. dahliae* was therefore investigated, by assessing the pathogenicity, of isolates mainly from cocoa, to important crops and native weeds from Brazil.

3.2. RESULTS.

3.2.1. Inoculation on cocoa.

Cocoa seedlings were inoculated when 110 days old with isolates BA-2, BA-3, ES-1, (all from cocoa, Brazil); UG-1 (from cocoa, Uganda); SW (race 1 on tomato, from U.K.); TS-2 (race 2 on tomato, from U.S.A.); and SS-4 (non-defoliating pathotype on cotton, from U.S.A.).

On cocoa, the first symptoms of the disease (flagging followed by chlorosis and necrosis of upper leaves) appeared about 20 days after inoculation. Within 16 days from the start of symptoms, the majority of the plants inoculated with isolates BA-2, BA-3, ES-1, UG-1, TS-2 and SS-4 had died or showed a widespread desiccation of the foliage (Plate 3.1.a). At the end of this experiment (36 days after inoculation), significantly higher disease indices were obtained for all isolates compared with isolate SW (race 1 on tomato), which caused general wilting in only a small number of plants (Table 3.1.).

Table 3.1. Effect of different isolates of *V. dahliae* on symptomatology, recovery of the pathogen and growth of cocoa seedlings, cv. ICS-1.

ISOLATE	DISEASE INDEX*	RE-ISOL.** (10th)	DRY WEIGHT* (g)	HEIGHT* (cm)
SS-4	3.15 d	9:1 b	8.18 e	1.75 de
ES-1	3.05 d	10:0 b	9.69 cde	1.75 de
BA-3	2.83 cd	9:1 b	8.85 de	1.50 e
BA-2	2.48 bcd	9:1 b	10.57 bcd	1.70 de
UG-1	2.40 bc	9:1 b	9.05 de	3.70 bc
TS-2	1.86 b	10:0 b	12.42 bc	2.70 cd
SW	0.47 a	3:7 a	12.85 b	5.75 b
Control	0.00 a	0:10 a	16.33 a	9.75 a

* Final disease index as adapted from Sidhu & Webster, (1977); Dry weight of aerial parts (stem + shoots + leaves); Height= Increase in height after inoculation. Values represent the means of 20 replicates. Within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 10th internode of the stem. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

V. dahliae was readily re-isolated from the stems of symptomatic seedlings, with a very low level of contamination on alcohol-agar selective medium (Plate 3.1.b). Since similar rates of recovery of *V. dahliae* occurred from all the positions tested (1st, 4th, 7th and 10th internodes), only data from the topmost internode are shown in Table 3.1. and subsequent tables. These results indicated that a widespread vascular colonization had taken place in susceptible cocoa plants, which was microscopically confirmed by the frequent presence of hyphae growing within the xylem vessels. All cocoa isolates plus the cotton isolate and the race 2 isolate from tomato systemically colonized the stem of the majority of inoculated cocoa seedlings. In contrast, SWANSEA was only re-isolated from those few plants showing external symptoms. A significant positive correlation (Pearson coefficient, r=0.917) was demonstrated between disease index and fungal recovery from cocoa stems.

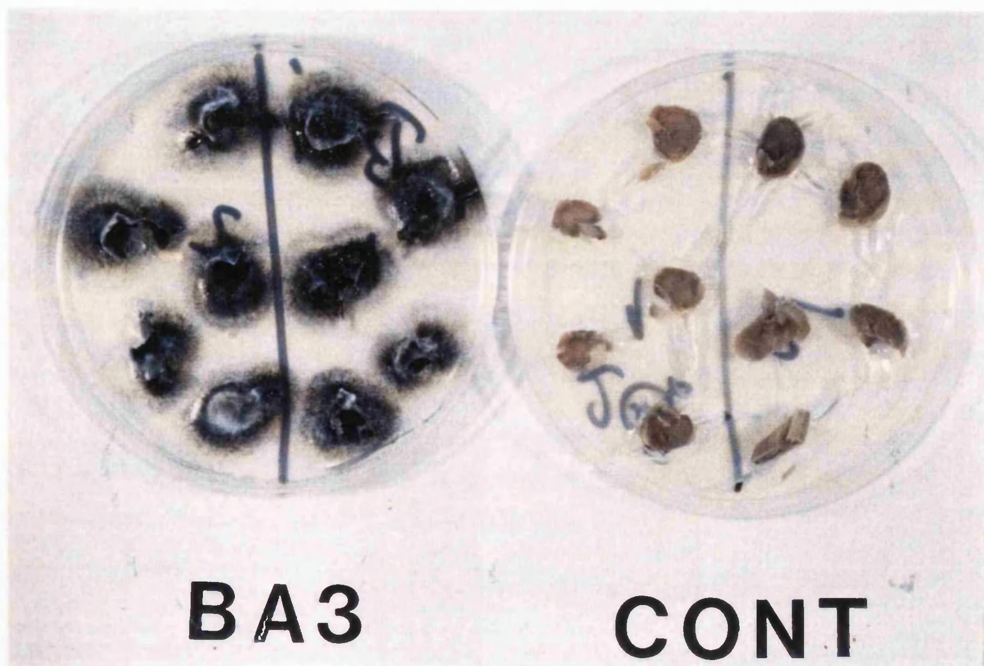
Brazilian isolates from cocoa (BA-2, BA-3 and ES-1), and SS-4 isolate from cotton incited greater reductions in height than the Ugandan isolate from cocoa (UG-1) and the race 1 isolate from tomato (SWANSEA). However, isolates BA-3 and UG-1 from cocoa plus the isolate SS-4 (cotton) caused greater reduction in dry weight than TS-2 and SWANSEA (tomato). Isolates ES-1 and BA-2 showed intermediate behaviour in relation to this parameter (Table 3.1.).

Plate 3.1. a) Typical external symptoms incited by a non-defoliating isolate of *V. dahliae* on cocoa (isolate SS-4, from cotton): Sudden wilting and subsequent desiccation of the leaves. Affected leaves eventually become brittle and occasionally roll inwards, but remain attached to the stem for a long time. (Plants on the left hand side are uninoculated controls).

b) Typical colony morphology of *V. dahliae* re-isolated from cross-sections of cocoa stems on alcohol-agar selective medium. (Petri dishes containing samples from plants inoculated with isolate BA-3 on the left and samples from uninoculated controls on the right hand side).

a

b



3.2.2. Inoculation on aubergine.

Isolates BA-2, BA-3, ES-1 and UG-1 from cocoa, EGP from aubergine, and TS-2 from tomato were inoculated on aubergine cv. Moneymaker. All isolates caused intense symptoms, which started as early as 12 to 15 days after inoculation. By the end of this experiment (22 days), many inoculated plants showed extensive foliar chlorosis, necrosis and wilting followed by partial defoliation and severe stunting (Plate 3.2.a).

Generally, reductions in plant height and dry weight occurred to the same extent, inoculating either isolates from cocoa or from aubergine. Isolate TS-2 (tomato) caused less reduction in growth than other isolates, but this was not significantly different from ES-1 and EGP (dry weight) or ES-1 (height) (Table 3.2.).

Re-isolation of the pathogen was consistently achieved, from the base to the 7th internode of the stems in all inoculated treatments. Some differences among isolates were detected using data from the 10th (topmost) internode, which confirmed a less extensive colonization by TS-2 (Table 3.2.). A significant positive correlation (r=0.929) was demonstrated to occur between external symptoms (final disease indices) and extension of colonization (data from all internodes assessed).

Table 3.2. Effect of different isolates of *V. dahliae* on symptomatology, recovery of the pathogen and growth of aubergine plants, cv. Moneymaker.

ISOLATE	DISEASE INDEX*	RE-ISOL.** (10th)	DRY WEIGHT* (g)	HEIGHT* (cm)
BA-2	2.70 c	6:3 bc	8.53 c	36.94 d
UG-1	2.53 c	8:1 bc	9.18 c	39.28 cd
BA-3	2.39 bc	7:2 bc	9.81 c	39.61 cd
EGP	2.32 bc	9:0 c	10.73 bc	39.00 cd
ES-1	1.91 bc	8:1 bc	10.74 bc	44.11 bc
TS-2	1.60 b	4:5 ab	12.60 b	46.39 b
Control	0.00 a	0:9 a	18.41 a	58.78 a

* Final disease index as adapted from Sidhu & Webster, (1977); Dry weight of aerial parts (stem + shoots + leaves). Values represent the means of 18 replicates. Within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 10th internode of the stem. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

3.2.3. Inoculation on tomato.

Isolates BA-1, BA-2, BA-3, ES-1 and UG-1 from cocoa and SWANSEA and TS-2 from tomato were inoculated on tomato plants cv. GCR-26 and GCR-218 (respectively, without and with Ve gene for resistance against race 1). In all cultivars, epinasty of lower leaves was the first visible symptom, which appeared approximately 15 days after inoculation. Acropetal leaf chlorosis and necrosis, combined with mild stunting were observed for some isolates by harvest time (32 days after inoculation) (Plate 3.2.b).

On GCR-26, isolate SWANSEA (race 1) caused more stunting in height and more reduction in dry weight than any other isolate tested (Table 3.3.). On GCR-218, isolate TS-2 (race 2) caused the highest level of reduction in dry weight (Table 3.4.); this parameter seems to be more discerning than height loss, for differentiation between *V. dahliae* isolates inoculated on tomato.

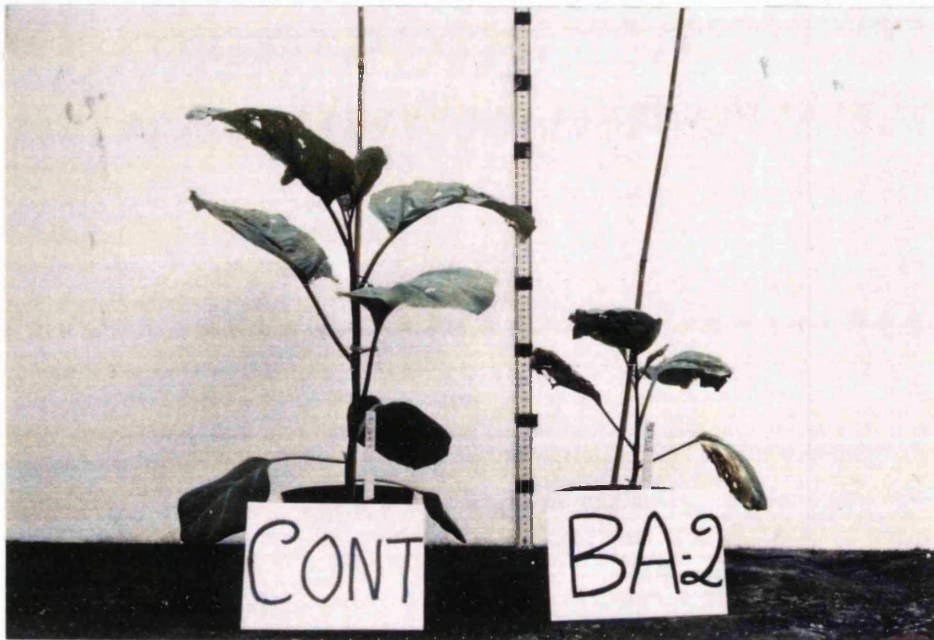
Final disease indices (Tables 3.3. and 3.4.), confirmed that isolates from cocoa were less aggressive on tomato cv. GCR-26 than isolates SWANSEA (race 1) and TS-2 (race 2). But, on tomato cv. GCR-218, TS-2, followed by BA-3, caused more severe symptoms than other isolates tested. Nevertheless, all isolates were readily recovered from the 3rd, 8th, and 13th internodes of tomato plants. Data from the topmost internode assessed (18th), showed that isolates SWANSEA, TS-2 and BA-3 were those most frequently recovered from GCR-26 (Table 3.3.), and isolates TS-2 and BA-3 from GCR-218 (Table 3.4.).

Plate 3.2. Typical external symptoms induced by *V. dahliae* isolates from cocoa on aubergine and tomato (plants on the left hand side are uninoculated controls):

a) Isolate BA-2 inducing foliar chlorosis, necrosis and wilting, with consequent severe stunting on aubergine plants.

b) Isolate BA-3 causing chlorosis and wilting only on the lower leaves, with consequent mild stunting on tomato.

a



b

Table 3.3. Effect of different isolates of *V. dahliae* on symptomatology, recovery of the pathogen and growth of tomato plants, cv. GCR-26.

ISOLATE	DISEASE INDEX*	RE-ISOL.** (18th)	DRY WEIGHT* (g)	HEIGHT* (cm)
SW	2.13 d	7:2 c	27.76 e	116.06 b
TS-2	1.85 d	7:2 c	31.96 d	143.72 a
BA-3	1.22 c	5:4 bc	39.08 c	146.72 a
BA-2	1.22 c	3:6 abc	41.94 bc	149.94 a
ES-1	1.06 bc	3:6 abc	40.57 c	151.50 a
BA-1	0.95 b	3:6 abc	44.70 ab	149.56 a
UG-1	0.91 b	1:8 ab	40.08 c	145.28 a
Control	0.00 a	0:9 a	47.88 a	148.89 a

* Final disease index as adapted from Sidhu & Webster, (1977); Dry weight of aerial parts (stem + shoots + leaves). Values represent the means of 18 replicates. Within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 18th internode of the stem. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

Table 3.4. Effect of different isolates of *V. dahliae* on symptomatology, recovery of the pathogen and growth of tomato plants, cv. GCR-218.

ISOLATE	**DISEASE INDEX***	**RE-ISOL.**** (18th)	**DRY WEIGHT*** (g)	**HEIGHT*** (cm)
TS-2	1.85 d	9:0 c	27.87 e	143.89 b
BA-3	1.31 c	5:4 bc	38.60 c	152.94 ab
BA-2	1.10 b	3:6 ab	43.90 ab	155.44 ab
ES-1	1.09 b	2:7 ab	41.03 bc	158.39 a
BA-1	1.05 b	2:7 ab	41.90 bc	161.11 a
SW	1.00 b	1:8 ab	33.96 d	156.44 ab
UG-1	0.99 b	3:6 ab	41.29 bc	159.28 a
Control	0.00 a	0:9 a	45.66 a	158.56 a

* Final disease index as adapted from Sidhu & Webster, (1977); Dry weight of aerial parts (stem + shoots + leaves). Values represent the means of 18 replicates. Within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 18th internode of the stem. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

3.2.4. Inoculation on cotton.

Isolates BA-1, BA-2, BA-3, ES-1 and UG-1 from cocoa, plus SS-4 and T-9 (respectively, non-defoliating and defoliating from cotton) were inoculated on cotton, cv. Deltapine 50. As previously reported, T-9 caused defoliation on cotton, while SS-4 did not. These isolates and the Ugandan isolate from cocoa (UG-1) were more aggressive than the Brazilian isolates from cocoa (Table 3.5., disease index), inducing severe symptoms and eventually death of many inoculated plants (Plate 3.3.a).

Isolate BA-1, a hyaline variant of BA-3, produced no symptoms on leaves, nor was re-isolated from the stem of cotton plants; all other isolates were recovered from all internodes examined, indicating successful systemic colonization (Table 3.5., re-isolation).

Brazilian isolates from cocoa, with the exception of BA-1, caused severe reductions in height, which were not significantly different from those induced by the most aggressive isolates. Dry weights of aerial parts were greatly reduced, particularly by isolates T-9, SS-4, and UG-1 (Table 3.5.).

Table 3.5. Effect of different isolates of *V. dahliae* on symptomatology, recovery of the pathogen and growth of cotton plants, cv. Deltapine 50.

ISOLATE	DISEASE INDEX*	RE-ISOL.** (10th)	DRY WEIGHT* (g)	HEIGHT* (cm)
SS-4	3.25 c	9:0 c	7.95 d	56.33 b
T-9	3.23 c	8:1 bc	5.01 e	55.11 b
UG-1	2.88 c	9:0 c	7.25 d	56.78 b
ES-1	1.92 b	5:4 bc	12.12 c	58.89 b
BA-3	1.73 b	6:3 bc	10.76 c	56.61 b
BA-2	1.52 b	4:5 ab	11.19 c	58.33 b
BA-1	0.00 a	0:9 a	14.13 b	74.22 a
Control	0.00 a	0:9 a	16.56 a	77.39 a

* Final disease index as adapted from Sidhu & Webster, (1977); Dry weight of aerial parts (stem + shoots + leaves). Values represent the means of 18 replicates. Within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 10th internode of the stem. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

3.2.5. Inoculation on pepper.

Isolates BA-1, BA-2, BA-3, ES-1 and UG-1 from cocoa, and 97 from pepper were inoculated on pepper cv. California Wonder. The Ugandan isolate from cocoa (UG-1) showed an intermediate response between the pepper isolate (most aggressive) and Brazilian isolates from cocoa (least aggressive) (Table 3.6., disease index). All Brazilian isolates caused very few or no symptoms on pepper, but were re-isolated from the 1st, 4th and 7th internodes to the same extent as 97 and UG-1. Only at the topmost internode assessed (10th), was it possible to differentiate BA-1 from all other isolates, which colonized more extensively pepper stems (Table 3.6., re-isolation).

Although causing severe wilting and subsequent death of almost all inoculated plants, the pepper isolate did not differ from UG-1 in relation to dry weight of aerial parts, or from UG-1, BA-3 and BA-2 in height (Table 3.6.) (Plate 3.3.b). Therefore, reduction in height seems to be a less useful parameter than reduction in dry weight to measure the effect of *V. dahliae* on pepper.

Table 3.6. Effect of different isolates of *V. dahliae* on symptomatology, recovery of the pathogen and growth of pepper plants, cv. California Wonder.

ISOLATE	DISEASE INDEX*	RE-ISOL.** (10th)	DRY WEIGHT* (g)	HEIGHT* (cm)
97	3.59 d	9:0 c	6.34 c	31.50 d
UG-1	1.85 c	9:0 c	5.94 c	34.50 bcd
BA-2	0.47 b	7:2 c	8.27 b	34.00 cd
ES-1	0.12 ab	6:3 bc	9.11 a	36.72 bc
BA-3	0.00 a	7:2 c	7.89 b	32.50 cd
BA-1	0.00 a	2:7 ab	8.79 ab	37.22 b
Control	0.00 a	0:9 a	9.38 a	39.83 a

* Final disease index as adapted from Sidhu & Webster, (1977); Dry weight of aerial parts (stem + shoots + leaves). Values represent the means of 18 replicates. Within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

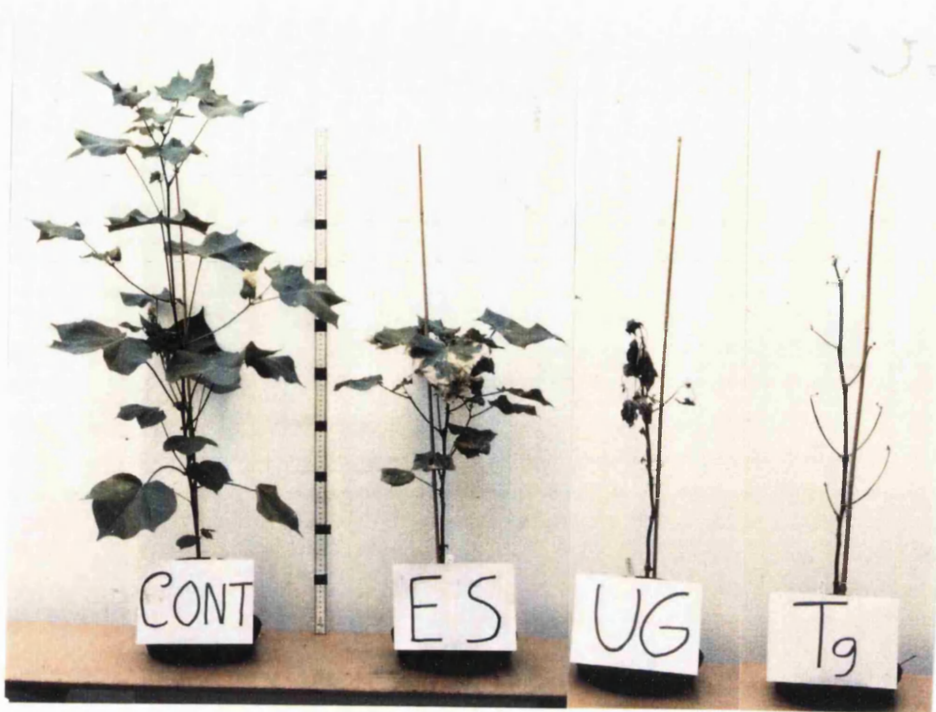
** Re-isolation from the 10th internode of the stem. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

Plate 3.3. Typical external symptoms induced by *V. dahliae* isolates from cocoa (ES and UG) on cotton and pepper, compared to isolates from these crops (T-9 and PEP, respectively). (Plants on the left hand side are uninoculated controls).

a) Symptoms on cotton: Isolate ES-1 from Brazil inducing foliar necrosis and stunting; isolate UG-1 from Uganda and isolate T-9 from cotton, inducing death of plants (note the complete defoliation caused by T-9.)

b) Symptoms on pepper: Isolate UG-1 from Uganda causing severe wilting and isolate 97 (PEP) from pepper causing severe wilting and consequent death of plants.

a



b

3.2.6. Inoculation on native weeds species from Brazil.

Fourteen weed species generally widespread in the cocoa region of Bahia, Brazil, were inoculated with isolate BA-3, isolated from a cocoa tree in the same region. 18 plants were inoculated and the same number remained as uninoculated controls within each species.

Symptomatology and growth parameters were assessed 45 days after inoculation. Final disease indices obtained (Table 3.7.) revealed that only four species showed external symptoms. *Erechtites hieraciifolia* was the only weed killed by *V. dahliae*, after developing severe wilting and stunting (Plate 3.4.a). *Solanum americanum*, followed by *Sida carpinifolia* and *Emilia sonchifolia* also exhibited significant wilt symptoms and stunting. *Sida carpinifolia* presented in addition, a typical intense chlorosis on the lower leaves, which was often observed in the field and sometimes confounded with nutritional deficiency (Plate 3.4.b). *V. dahliae* was successfully re-isolated not only from the weeds previously cited, but also from the stems of the following symptomless hosts: *Diodia ocimifolia*, *Achryranthes indica*, *Capsicum chinense* and *Boehmeria cylindrica*. Other weed species tested were immune to systemic infection. Significant reductions in dry weight and height of *Erechtites hieraciifolia*, *Solanum americanum*, and *Sida carpinifolia* and reduction only in height of *Emilia sonchifolia* were also detected. Growth of other species was not affected by inoculation (Table 3.7.).

Table 3.7. Effect of a Brazilian isolate of *V. dahliae* from cocoa (BA-3), on symptomatology, recovery of the pathogen and growth of weed species.

SCIENTIFIC NAME	DISEASE INDEX*	RE-ISOL.** (10th)	DRY WEIGHT* (%)	HEIGHT* (%)
Erechtites hieraciifolia	3.45 d	9:0 b	31.93 c	46.13 d
Solanum americanum	2.49 c	9:0 b	19.26 b	19.15 c
Sida carpinifolia	1.10 b	9:0 b	17.30 b	9.14 bc
Emilia sonchifolia	1.08 b	8:1 b	1.26 a	7.81 bc
Diodia ocimifolia	0.00 a	7:2 b	0.18 a	2.94 abc
Achryranthes indica	0.00 a	6:3 b	2.67 a	2.70ab
Capsicum chinense	0.00 a	5:4 b	1.89 a	2.93 abc
Boehmeria cylindrica	0.00 a	5:4 b	-1.33 a	-7.70 a
Bidens pilosa	0.00 a	0:9 a	-0.26 a	-3.13 ab
Synedrella nodiflora	0.00 a	0:9 a	3.39 a	2.94 abc
Leonurus sibiricus	0.00 a	0:9 a	3.73 a	2.17 ab
Lantana camara	0.00 a	0:9 a	-3.89 a	-3.54 ab

* Final disease index as adapted from Sidhu & Webster (1977); % of reduction in dry-weight of aerial parts and % of reduction in height, both of inoculated plants in relation to the respective controls for each species. Values represent the means of 18 replicates and within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 10th internode of the stem. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

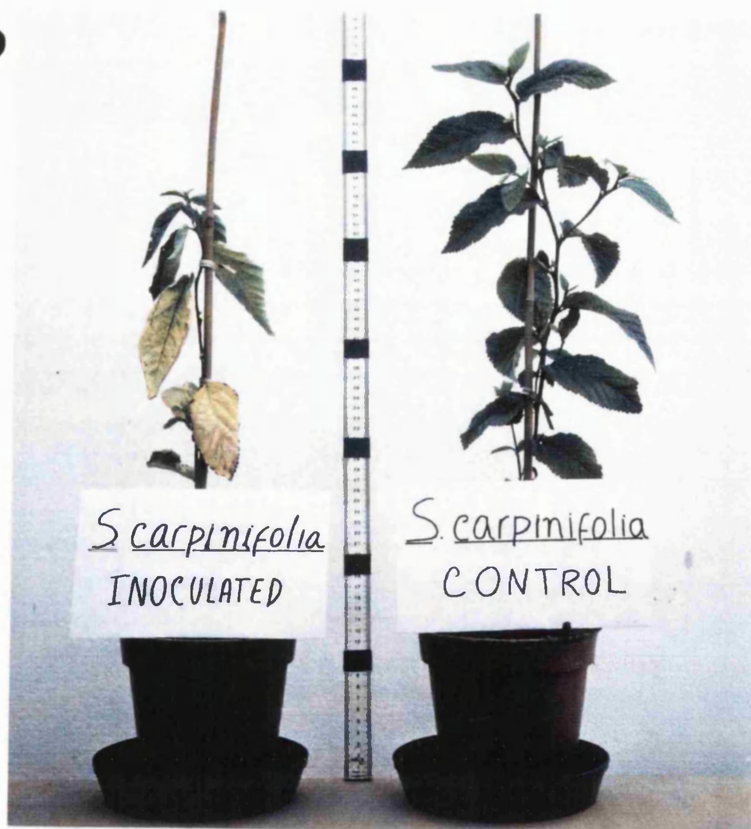
Plate 3.4. Typical external symptoms induced by a *V. dahliae* isolate from cocoa (BA-3) on native weeds from Brazil:

a) *Erechtites hieraciifolia*, control and inoculated plots in the glasshouse. Note the uniformity of symptoms, including severe wilting, stunting and consequent death of some plants.

b) *Sida carpinifolia*, inoculated and control plants. Apart from developing wilt on leaves and general stunting, inoculated plants also showed an intense chlorosis of the lower leaves.

a

b



3.3. DISCUSSION.

The current study clearly demonstrates that isolates of *V. dahliae* vary in pathogenicity on different hosts. Vigouroux (1971) pointed out that isolates from a region of permanent monoculture are similar and all display high virulence against this particular crop (preferential host), but generally, a weak virulence against other species which they can nevertheless infect (occasional hosts). *T. cacao* L., growing in northeastern Brazil, may be considered a preferential host for *Verticillium*, according to the Vigouroux concept, since all Brazilian isolates from cocoa were very destructive to cocoa, but not so to most other plant species. Emechebe (1974) found that Ugandan isolates from cocoa, okra and cotton were equally pathogenic to cocoa. In the current study, American isolates from cotton (non-defoliating) and tomato (race 2) were also highly pathogenic on cocoa, which suggests that a large number of *V. dahliae* isolates can cause symptoms on cocoa seedlings.

Isolates from cocoa induced different responses on the three solanaceous crops inoculated. All Brazilian isolates caused severe symptoms on aubergine, but few or no symptoms on pepper. These results agree with those of Vigouroux (1971), who postulated that aubergine is a preferential host, whilst pepper is an occasional host for *V. dahliae*. In addition, Ciccarese, Frisullo & Cirulli (1987) reported that Italian isolates of *V. dahliae* from chicory, broccoli and tomato were highly pathogenic to aubergine, but caused no symptoms on pepper, suggesting

that isolates pathogenic to pepper occur less frequently in nature. Tomato seems to be an occasional host for cocoa isolates of *V. dahliae*, because these isolates caused only mild symptoms on both varieties (with and without Ve gene). However, Fordyce & Green (1963) reported that peppermint isolates initially avirulent to tomato, became virulent after passages through a susceptible tomato cultivar, and Tjamos (1981) suggested that the broadening of host range could occur in the field after introduction of other potential hosts into the same area. Tomato varieties have not been extensively cultivated in the cocoa region of the States of Bahia and Espirito Santo in Brazil and this may explain the low level of aggressiveness of cocoa isolates on tomato.

The higher level of aggressiveness of the Ugandan isolate from cocoa to cotton, compared with Brazilian isolates from cocoa, suggests that selection or adaptation of the pathogen to the host may have occurred, since cotton has been cultivated in the same region as cocoa in Uganda, but not in Brazil. However, this consideration should be interpreted with caution, as just one isolate from Uganda was tested. Vigouroux (1971) concluded that the apparent *de novo* appearance of a host-specific strain is most probably due to selection from the population of soil strains, rather than adaptation. The hypothesis of selection was later supported by Tjamos (1981) studying pathogenicity of *V. dahliae* isolates from areas of single and diversified cropping systems in Greece. Nevertheless, according to Pegg (1974) it is difficult to be categoric

on this subject, dealing with a fungus that exhibits heterokaryosis and parasexuality.

The introduction of a preferential host in a crop rotation would be expected to increase greatly the amount of inoculum of a wide range of strains. Such inoculum would be available for successive crops, some of which might be occasional hosts (Vigouroux, 1971). The inoculations of different cocoa isolates in various plant species demonstrated that they are not specific in terms of host range. All crops and eight out twelve weed species tested were systemically invaded and therefore can be considered as possible reservoirs of those isolates. Such crops, especially aubergine, should be avoided in cases of intercropping or replacing cocoa in areas devastated by Verticillium wilt.

There have been numerous reports on weeds infected by different isolates of *V. dahliae* (e.g. Engelhard, 1957; Heale & Isaac, 1963; Woolliams, 1966; Harrison & Isaac, 1969; Brown & Wiles, 1970; Evans, 1971; Evans & Gleeson, 1973; Busch, Smith & Elango, 1978). However, none of the eight weed species infected in the current study have been reported to date as *Verticillium* hosts. In this study, *Bidens pilosa*, a South American widespread species, was not systemically colonized, although *V. dahliae* had been recovered from its stems in Peru (Vargas-Machuca *et al.*, 1987). These authors found a complete agreement in the presence or absence of external symptoms between artificially inoculated and naturally infected weed species. Our investigation was performed under glasshouse

conditions, but complementary work should include a survey of infected plants in the field.

Previous researchers (Visser & Hattingh, 1980; Bender & Shoemaker, 1984), demonstrated that stunting is an unreliable indicator of *Verticillium* pathogenicity on tomato and O'Garro & Clarkson (1988), later confirmed that race-1 and race-2 could not be distinguished in terms of pathogenicity on cultivars Roma and Roma VF, when analyzing only reductions in height. Our study has also provided evidence that plant height is not a very useful parameter to compare the effects of different isolates of *V. dahliae*, not only on tomato, but also on pepper and cotton. By contrast, disease index appeared to be a reliable parameter to assess the response to Verticillium wilt, considering all of the inoculated hosts.

The method of re-isolation currently utilized in this work was not quantitative, but provided an indication of the internal distribution of the pathogen. Significant positive correlations were demonstrated between external symptoms (disease index) and extent of stem colonization (re-isolation data) recorded from cocoa, aubergine, tomato and cotton. These results might be expected since they represent the most frequent response of plant species infected with *Verticillium* (Brandt, Lacy, & Horner, 1984; Townsend, Schreiber, Hall & Bentz, 1990; Papadopoulos, Christie, Boland & Bush, 1991). However, non-significant correlation between symptoms and colonization (r=0.391) was observed on pepper, mainly because Brazilian isolates from cocoa did not cause symptoms, but were re-isolated

even from the top of the stem. Also, four weed species (*Diodia ocimifolia*, *Achryranthes indica*, *Capsicum chinense* and *Boehmeria cylindrica*), showed a similar pattern when inoculated with isolate BA-3 from Brazil. Such findings are in agreement with other reports on symptomless hosts of *Verticillium* (Brown & Wiles, 1970; Evans, 1971; Evans & Gleeson, 1973; Krikun & Bernier, 1987; Mathre, 1989) and implies a possible tolerance to certain isolates of *V. dahliae*. Mussel (1981) considered that tolerance is an incomplete form of resistance, i.e. a plant is able to produce an acceptable yield, whilst providing, at least, a limited habitat for growth and reproduction of the pathogen. Tolerant hosts may also provide raised inoculum levels in the field, resulting in substantial losses for a perennial or subsequent crop. Therefore, an adequate control of all weed species is also required as part of an integrated disease management programme in cocoa growing areas.

4. WATER RELATIONS AND ETHYLENE PRODUCTION BY COCOA SEEDLINGS INFECTED WITH DEFOLIATING AND NON-DEFOLIATING ISOLATES OF *V. DAHLIAE*.

4.1. INTRODUCTION.

Turgid cells contain sufficient water to exert pressure against the cell wall and maintain the rigidity of the cell. If the water content of the cell is reduced, a point is reached at which, the vacuole no longer exerts pressure against the wall. The cell wall loses rigidity and tissue consisting of such cells wilts (Hall & Machardy, 1981).

Plant diseases that result from invasion of the vascular system by fungi, generally exhibit, as a major part of their syndrome, the development of water stress in leaves. Wilt often occurs first on the lower leaves and on one side of the plant. It is commonly observed first around midday, i.e., the time of maximum transpiration. The wilted tissue recovers turgor later in the day or, more often during the ensuing dark period. This pattern of wilting and recovery, might be repeated several times. Such wilt is said to be recoverable or reversible. Eventually the wilted tissue can no longer recover turgor at night. The tissue is then, irreversibly wilted, at least when attached to the plant (Hall & Machardy, 1981).

Sudden and acute water stress is expressed by the wilting and collapse of leaves and shoots, death of leaves while still green, and failure of the abscission mechanism. Death of the whole plant may occur, but die-

back of shoots and branches may constitute intermediate stages in woody perennials. Less acute water stress causes marginal necrosis and defoliation. Prolonged subacute stress results in mottled chlorosis of the leaves. These do not become flaccid and are eventually shed by normal abscission. Non-lethal water stress also leads to reduction in leaf size, shoot extension and cambial growth (Talboys, 1968).

The evidence indicates that vascular wilt diseases are associated with varying degrees of host water stress, however few authors have explained where and how such water stress could occur (Mepsted, 1993). According to Hall & Machardy (1981), resistance to water flow occurs within both stems and petioles of disease plants, but disturbances in petioles appear to contribute more to water stress and symptom expression. Dimond & Edgington (1960) and Dimond (1966) explain this in terms of hydraulics. Hence, resistance is greater in small rather than in large bundles of xylem vessels, and the greatest resistance within bundles occurs in the small, independent petiole bundles. In stems, the interconnections and by-passes within and between xylem bundles ensures that a given amount of interference with water flow within a portion of a bundle will have relatively little effect on the transpiration stream; a similar percentage of interference within a petiole bundle has a large effect.

Previous workers (Keen, Long & Erwin, 1972; Duniway, 1973; Misaghi, DeVay & Duniway, 1978), demonstrated that an increase of resistance to water flow in the xylem,

rather than the effects of toxins on cell permeability is the main cause of water stress symptoms in cotton infected with *V. dahliae*. Pathogen-produced protein-lipopolysaccharide complex and gelation in xylem vessels are factors that might contribute to increased resistance to water flow (Keen *et al.* 1972). Misaghi *et al.* (1978) found a positive correlation between the number of occluded xylem elements and the degree of chlorosis in cotton plants infected with *V. dahliae*, non-defoliating pathotype SS-4. On the other hand, working in a naturally infested cotton field, Hampton, Wullschleger & Oosterhuis (1990) argued that *V. dahliae* reduced photosynthesis initially through non-stomatal processes, which were not mediated by water-deficit stress. According to these authors, disease progression is the product of two mechanisms of pathogenicity, toxins and water stress. In addition, Pegg (1981a) stated that the long-standing dialogue between the proponents of toxin-induced versus vascular occlusion-induced wilting is to some extent irrelevant, when it is faced with the evidence that both causes can be seen operating simultaneously.

Cotton plants infected with *Verticillium* manifest a number of different symptoms. Schnathorst & Mathre (1966) described *V. dahliae* pathotypes on cotton as defoliating or non-defoliating, but other authors (Bell, 1973; Ashworth Jr., 1983) supported the existence of a continuum of aggressiveness among strains of *V. dahliae*, rather than the occurrence of distinct pathotypes. Two of the more likely suppositions made from symptomatology are that wilt

reactions are mediated by water stress and defoliation is mediated by imbalances in growth regulators or both. Talboys (1968) first suggested that defoliation is related to the level of water stress; Wiese & Devay (1970) and Tzeng & DeVay (1985) demonstrated enhanced production of ethylene from cotton plants inoculated with a defoliating isolate compared to a non-defoliating one.

Apparently, a number of physiological disturbances also occur during Verticillium wilt development on cocoa plants, but the mechanisms underlying pathogenesis are not understood. As the physiological bases of the symptoms caused by different isolates is unclear, the main purpose of this chapter of the thesis was to compare the effect of inoculations with defoliating and non-defoliating isolates of *V. dahliae* on water stress and ethylene production by cocoa seedlings in relation to the development of wilt symptoms.

4.2. RESULTS.

4.2.1. Water relations and ethylene production by cocoa seedlings infected with defoliating and non-defoliating isolates of *V. dahliae*.

The impact of a defoliating (T-9) and a non-defoliating (BA-3) isolate on water stress and other physiological changes in cocoa was assessed during Verticillium wilt development. Each seedling of the cv. ICS-1 was inoculated when 110 days old with 50 ml of a conidial suspension (1 x 10^7 conidia/ ml) from one of these isolates. Nine replicates were used for each treatment (isolate) and for uninoculated controls. Total transpiration, stomatal diffusive resistance, leaf water potential, ethylene production and disease index were assessed 14, 17 and 21 days after inoculation. In order to compare the physiological and pathological changes between distinct positions of the canopy, measurements of stomatal conductance, water potential, ethylene production, number of CFU's and percentage of vascular browning were made from the oldest and youngest fully developed leaf within the plant canopy at each time (referred to in the text as, respectively, lower or base and upper or top of the canopy). Statistical analysis for comparisons between treatments were based on repeated Mann-Whitney U-test, carried out independently for samples from the upper and lower canopy, where applicable.

The effect of infection on cocoa transpiration was first detected 14 days after inoculation, even before the

detection of the initial external symptoms, which did not occur until 17 days (Table 4.1., treatment BA-3). Total transpiration per plant was markedly reduced from that time until the end of the experiment (21 days), whether considering BA-3 or T-9 isolates, compared to the control. The experiment was concluded 21 days post-inoculation, because the onset of defoliation in treatment T-9 would affect the sampling of leaves from the top of the plant, where defoliation usually begins.

Stomatal closure in leaves of inoculated plants was indicated by significantly lower levels of stomatal conductance from 17 days post-inoculation, particularly at the upper canopy of plants inoculated with BA-3 (Table 4.2.). Twenty one days after inoculation, such reduction in stomatal conductance was still more pronounced on leaves from plants inoculated with BA-3 as compared with T-9. Independent of the isolate inoculated, reductions in stomatal conductance appeared first and reached a lower level on leaves from the top compared to leaves from the base of the canopy. A similar pattern was observed in relation to water uptake as determined by the midday leaf water potential. Significant reductions in leaf water potential were detected at the top of the canopy as early as 14 days after inoculation with isolate BA-3. At the base of the canopy such difference was detected later, 21 days after inoculation. Leaf water potential was reduced progressively from 14 to 21 days, and again, this reduction was more intense with isolate BA-3 compared with T-9. Extremely high pressures (generally above 100

lb.in^{-2}) were needed to remove water from petioles from the upper canopy of diseased plants.

Table 4.1. Effect of defoliating (T-9) and non-defoliating (BA-3) isolates of *V. dahliae* on total transpiration and symptomatology of cocoa seedlings, cv. ICS-1.

PARAMETER	**TREATMENT (Isolate)**	**TIME (Days after inoculation) 14**	**17**	**21**
TOTAL TRANSPIRATION PER PLANT*	T-9	40.78 ab	31.24 a	22.80 b
	BA-3	31.89 b	25.28 b	16.33 b
	Control	43.32 a	37.07 a	39.48 a
DISEASE INDEX*	T-9	0.00 a	1.106 b	2.184 b
	BA-3	0.00 a	0.552 ab	1.637 b
	Control	0.00 a	0.00 a	0.00 a

* Total transpiration rates are shown in g of water/ plant/ 24 h; disease index as adapted from Sidhu & Webster, (1977). Means followed by the same letter in each column, are not significantly different. (Mann-Whitney U-test; $p \leq 0.05$). Values represent means of 9 replicates.

Table 4.2. Effect of defoliating and non-defoliating isolates of *V. dahliae* on stomatal conductance and midday leaf water potential of leaves from the upper and lower canopy of cocoa seedlings, cv. ICS-1.

PARAMETER	TREATMENT (Isolate/ Position)	TIME (Days after inoculation) 14	17	21
STOMATAL CONDUCTANCE*	T-9 / Top	0.186 a	0.137 ab	0.125 b
	BA-3 / Top	0.174 a	0.085 b	0.048 c
	Control / Top	0.208 a	0.153 a	0.204 a
	T-9 / Base	0.169 a	0.130a	0.147 ab
	BA-3 / Base	0.177 a	0.094 a	0.097 b
	Control / Base	0.190 a	0.131 a	0.155 a
LEAF WATER POTENTIAL*	T-9 / Top	-0.41 ab	-0.83 b	-1.04 b
	BA-3 / Top	-0.48 b	-1.14 b	-1.96 c
	Control / Top	-0.37 a	-0.40a	-0.38 a
	T-9 / Base	-0.70 a	-0.60 a	-0.55a
	BA-3 / Base	-0.63 a	-0.88 a	-1.16 b
	Control / Base	-0.56 a	-0.56 a	-0.48 a

* Stomatal conductance is given in $cm.s^{-1}$, and leaf water potential in MPa (Megapascal). Means followed by the same letter in each column and for each position (top or base of the canopy), are not significantly different. (Mann-Whitney U-test; $p \leq 0.05$). Values represent means of 9 replicates.

However, although less water stressed, plants inoculated with T-9 produced more ethylene than plants inoculated with BA-3, as detected on petioles from the upper canopy 17 days after inoculation onward (Table 4.3.). Twenty one days post-inoculation, ethylene production determined on petioles from the upper canopy of plants infected with T-9 and BA-3 was increased, respectively, 4.4- and 2.3-fold over uninoculated controls. Within the lower canopy, no significant increase in ethylene production was detected on inoculated treatments compared to the control, indicating once again that all physiological changes caused by disease were concentrated in the upper canopy at the time of assessment.

Immediately after harvest, the number of CFU's and the percentage of vascular browning were assessed on petioles from the upper and the lower canopy. (Table 4.4.). BA-3 and T-9 did not differ statistically in terms of CFU's and browning, whether considering top or base of the canopy. Nevertheless, a greater number of CFU's and a higher percentage of browning were detected with both isolates at the top of the canopy compared with the base. These results revealed that *V. dahliae* colonization was more effective on the upper part of the plant, which coincided with more intense physiological disturbance in this region.

Table 4.3. Effect of defoliating and non-defoliating isolates of *V. dahliae* on ethylene production by petioles of the upper and lower canopy of cocoa seedlings, cv. ICS-1.

PARAMETER	TREATMENT (Isolate/ Position)	TIME (Days after inoculation) 14	17	21
ETHYLENE PRODUCTION*	T-9 / Top	5.77 a	18.56 b	21.79 c
	BA-3 / Top	5.38 a	6.22 a	11.52 b
	Control / Top	4.93 a	5.13 a	4.94 a
	T-9 / Base	3.25 a	3.70 a	3.64 a
	BA-3 / Base	3.84 a	3.71 a	3.32 a
	Control / Base	3.84 a	3.32 a	3.12 a

* Ethylene production was measured at the base of the petiole and is given in nl/ g of fresh weight/ hour. Means followed by the same letter in each column and for each position (top or base of the canopy), are not significantly different. (Mann-Whitney U-test; $p \leq 0.05$). Values represent means of 9 replicates.

A complementary experiment was then conducted in order to understand the relation between the patterns of internal distribution of *V. dahliae* and xylem vessel architecture; this was determined by the uptake of a dye solution via the transpiration stream. One of the lateral roots from 110-day-old healthy cocoa seedlings was severed under water and immediately submerged in a 0.23% eosin solution (water soluble yellow shade, BDH, Poole, Dorset, U.K.), contained in a small plastic vial. After 24 hours, five fully mature leaves were removed from the upper and other five from lower canopy of those plants. The presence of eosin internally and the number of vessels that had taken up this dye was assessed in cross-sections from the base of each petiole under the microscope.

Data from ten plants (Table 4.5.), showed that eosin was present in 2/3 of the petioles from the upper canopy and in approximately 1/4 of those from the lower canopy. Thus, significantly more vessels were stained in petioles from the upper canopy than in those from the lower canopy. This pattern of dye distribution suggested the presence of an increasing number of interconnections within and between xylem bundles from the base to the top of the stem, which may be responsible for the similar pattern of *V. dahliae* distribution observed in the experiment previously described.

Table 4.4. Effect of defoliating and non-defoliating isolates of *V. dahliae* on vascular browning and recovery of the pathogen from petioles of the upper and lower canopy of cocoa seedlings, cv. ICS-1.

TREATMENT (Isolate / Position)	COLONY FORMING UNITS (CFU's) *	PERCENTAGE OF VASCULAR BROWNING
T-9 / Top	2887 b	68.08 b
BA-3 / Top	1413 b	60.75 b
Control / Top	0 a	0.00 a
T-9 / Base	757 b	20.20 b
BA-3 / Base	580 b	26.13 b
Control / Base	0 a	0.00 a

* All parameters were measured at the base of the petiole; CFU's= colony forming units/ g of fresh weight. Means followed by the same letter in each column and for each position (top or base of the canopy), are not significantly different. (Mann-Whitney U-test; $p \leq 0.05$). Values represent means of 9 replicates.

Table 4.5. Pattern of eosin distribution in petioles from the upper and lower canopy of uninoculated cocoa seedlings, cv. ICS-1.

PARAMETER	LOWER CANOPY	UPPER CANOPY
% OF PETIOLES STAINED	28.00 a*	66.00 b
% OF VESSELS STAINED	11.62 a	48.33 b

* Data are means of 50 replicates. Within each row, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

4.2.2. Effect of silver thiosulphate on the patho-physiology of cocoa seedlings infected by a defoliating isolate of ***V. dahliae*****.**

Silver thiosulphate (STS) is a well known inhibitor of ethylene in plants and has been used to prevent foliar abscission (Sexton & Roberts, 1982; Cameron & Reid, 1983; Veen, 1983). Sometimes, disease severity has also been reduced by STS applications (Boller, 1991).

With the aim of identifying the role of ethylene on defoliation caused by the isolate T-9, cocoa plants were sprayed with STS every 7 days, during three consecutive weeks following soil inoculation. The STS solution was prepared and stored following the method described by Reid, Paul, Farhoomand, Kofranek & Staby (1980) (Appendix 3). Thirty cocoa seedlings were inoculated, and from these, 15 were sprayed with 0.1M STS and 15 remained as inoculated controls, sprayed with distilled water only. The same number of uninoculated plants were sprayed with STS to verify the occurrence of any phytotoxic effect compared with control healthy plants. Vascular browning, ethylene production and number of CFU's were determined 20 days after inoculation using the lower third of petioles from leaves located within the upper canopy (1 petiole sampled/ plant). Defoliation and disease index were also recorded for each plant, 30 days after inoculation.

Defoliation was almost completely avoided as a result of STS spraying on inoculated plants. Nevertheless, the level of disease was not affected, since the leaves,

although remaining attached to the plant, suffered from chlorosis, wilting and necrosis, as expressed by the disease severity index (Table 4.6. and Plate 4.1.). The percentage of vascular browning was not significantly affected by applications of STS, and, although 6.1 times more CFU's were recovered from petioles of STS-sprayed plants, this difference was not significant, indicating great variability of these data. In addition, no significant difference was detected in ethylene production, irrespective of spraying inoculated plants with STS. Vascular browning and CFU's were directly correlated ($r=0.878$; $p\leq 0.01$), but no correlation was found between CFU's or browning and ethylene production.

Table 4.6. Effect of silver thiosulphate on general symptoms, fungal colonization in and ethylene production by petioles of cocoa seedlings inoculated with *V. dahliae*, isolate T-9.

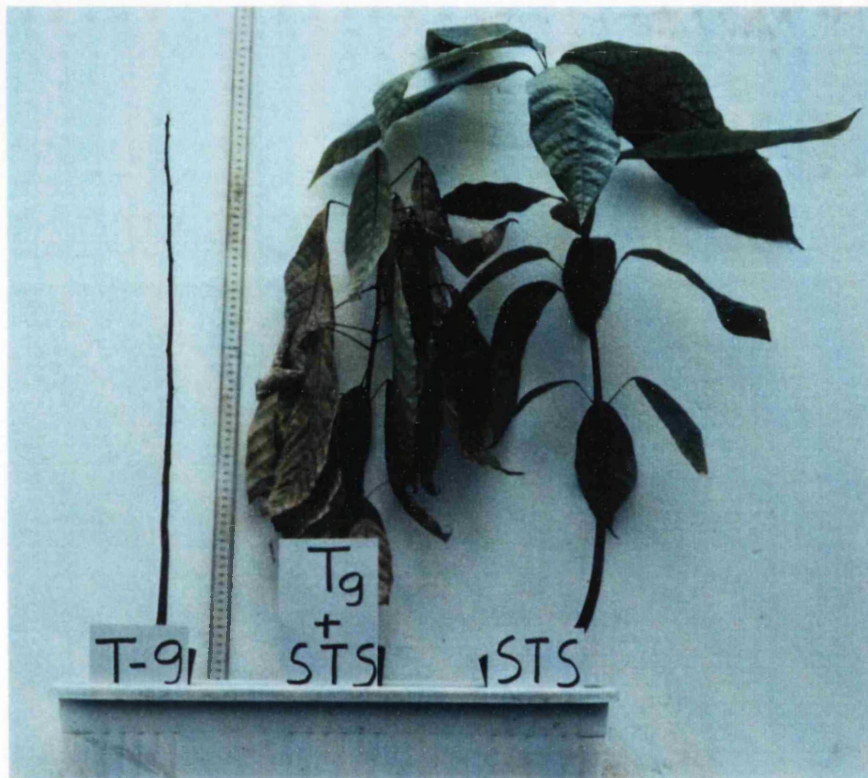
PARAMETER	--TREATMENT---	
	T-9*	T-9 + STS*
DEFOLIATION (%)	68.50 b***	2.93 a
DISEASE INDEX**	2.74 a	2.39 a
VASCULAR BROWNING (%)	64.25 a	73.58 a
CFU's**/ g fresh weight	261.4 a	1597.8 a
ETHYLENE nl/ g fresh weight/ h	20.84 a	53.52 a

* Treatment T-9 + STS means that the seedlings were inoculated with isolate T-9 and sprayed three times (5, 12 and 19 days after inoculation) with silver thiosulphate; treatment T-9 was inoculated with isolate T-9 and sprayed at the same times with distilled water only.

** CFU's stands for colony forming units; disease index as adapted from Sidhu & Webster, (1977).

*** Data are means of 15 replicates. Within each row, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Plate 4.1. Typical effect of weekly sprayings with a 0.1M solution of silver thiosulphate (STS) on cocoa plants inoculated with a defoliating isolate of *V. dahliae* (T-9 + STS, in the centre of the plate): There was a complete avoidance of defoliation compared to plants inoculated but not sprayed (left hand side), however other symptoms of the disease (foliar chlorosis, necrosis and wilting) were not prevented. Plant on the right hand side is an uninoculated control sprayed with STS, showing no apparent symptom of toxicity due to this tratment.


T-9
Tg
+
STS
STS

4.3. DISCUSSION.

The rapid decrease in total transpiration, stomatal conductance and midday leaf water potential of cocoa seedlings, closely associated with the onset of foliar symptoms, indicates that water stress is a major cause of the development of sudden wilt caused by *V. dahliae* on cocoa. Furthermore, when inoculated with the non-defoliating isolate (BA-3), significant decreases in total transpiration and midday leaf water potential were detected 14 days post-inoculation even before the evidence of the first symptoms on the leaves (17 days), which suggests a cause and effect relationship. Hall & Machardy (1981) stated that internal water potential may be low enough to limit growth and development, before they are low enough to cause wilt. Due to the speed of disease development on cocoa, such a effect on growth could not be assessed.

At the end of the experiment (21 days), plants inoculated with isolate BA-3 were much more water stressed than plants inoculated with the defoliating isolate (T-9). These results confirmed Talboys claims (1968), who first postulated that sudden and acute water stress is expressed by the wilting and collapse of leaves and shoots, death of leaves while still green and the failure of the abscission mechanism; while less acute water stress can cause marginal necrosis and defoliation.

Results obtained regarding transpiration are in agreement with those of Harrison (1971), who found that

potato plants infected with *V. dahliae* and *V. albo-atrum*, transpired more slowly than healthy ones and this difference increased as disease progressed. These reductions in transpiration have been reported in many other wilt diseases, including Fusarium wilt of banana (Page, 1959) and Verticillium wilt of tomato (Threlfall, 1959) and chrysanthemum (MacHardy, Busch & Hall, 1976). Water stress produced by vascular fungi has also been expressed in other ways such as reduced growth, reduced leaf water potential, reduced water flow through the plant, low relative water content, reduced stomatal conductance and reduced anabolism in general (Dimond, 1970; Harrison, 1971; Duniway, 1973; Hall & Machardy, 1981; Tzeng, Wakeman & DeVay, 1985; Hampton *et al*., 1990; Mepsted, 1993). However, the physiological responses depend on the host and pathogen involved in the interaction. Tzeng & DeVay (1985) working under glasshouse conditions found that leaves of cotton plants inoculated with T-9 (defoliating pathotype) had higher leaf water potentials than the healthy controls until the occurrence of defoliation, about 10-12 days after inoculation. Conversely, in the current study a decrease in water potential was detected following soil inoculation of cocoa plants with isolate T-9. Furthermore, independent of isolate inoculated, leaves of cocoa from the upper canopy became water-stressed before the leaves from the lower canopy in contrast to cotton where older leaves showed water-stress symptoms (abscission or wilt, depending on isolate) before younger leaves (Tzeng & DeVay, 1985).

When infected with the defoliating isolate from cotton, tomato plants usually present only mild symptoms, like epinasty of the leaves. The apparent hormonal disturbance that is associated with bud breaking and epinasty on tomato is likely to be related to the rapid defoliation of cotton plants (Schnathorst & Evans, 1971). Wiese & DeVay (1970) suggested that epinasty, chlorosis and finally defoliation are related to ethylene production, which in turn, has been demonstrated to increase in water stressed plants (McMichael, Jordan & Powell, 1972). However, in the current study, although more water stressed, petioles from plants inoculated with isolate BA-3 produced lower amounts of ethylene in relation to similar petioles from plants inoculated with T-9. Despite fungal development and ethylene production being more intense in petioles from the upper canopy as compared to the lower canopy, no significant correlation was found to occur between these two parameters, irrespective of isolate tested. Pegg (1981b) suggested that there is little evidence to indicate that the pathogen-produced ethylene contributes much to the total ethylene pool in the plant. Furthermore, a non-defoliating isolate from cotton was demonstrated to produce significantly more ethylene *in vitro* than did the defoliating isolate (Tzeng & DeVay, 1984). Therefore, the isolate T-9 seems to be triggering the production of ethylene by plant tissue, which is certainly involved in defoliation, as demonstrated by spraying STS on cocoa seedlings.

The ethylene inhibitor STS almost completely prevented defoliation when sprayed on cocoa seedlings, but did not affect fungal colonization or general symptoms of vascular wilt, apart from defoliation. Also, no significant difference in ethylene production was detected, whether the inoculated plants were sprayed or not. At first, a reduction in ethylene production was expected, following STS sprayings. However, it is considered that the the ion Ag^{+} (from STS) interferes with the binding sites for ethylene (Sisler, 1982), and this model fits into a positive feedback mechanism, in which the binding of ethylene to its receptor would tend to trigger its own biosynthesis (Veen, 1983). Thus, STS inhibits ethylene action (expressed as defoliation in this case), not its synthesis.

When the eosin solution was absorbed via the roots and the number of stained vessels assessed, it was demonstrated that this dye was more concentrated in petioles from the upper canopy compared with petioles from the lower canopy. As dye and pathogen presented a similar pattern of internal distribution, it is reasonable to suggest that the more intense colonization developed by *V. dahliae* in upper vascular tissues of cocoa plants is closely related to vessel anatomy (increase in number of interconnections within and between xylem bundles higher up in the stem). Another hypothesis to explain such a phenomenon is the ability of older tissues to produce more phytoalexins or other antifungal compounds that can retard the development of the pathogen. Bell (1969) and Hunter,

Halloin, Veech & Carter (1978) found higher levels of phytoalexin accumulation in older than in younger tissue from cotton stems, following inoculation with *V. dahliae* and *Rhizoctonia solani*, respectively. The presence of antifungal compounds in different parts of cocoa stems should be investigated in the future.

5. EFFECT OF METHOD OF INOCULATION, INOCULUM DENSITY AND SEEDLING AGE AT INOCULATION ON THE EXPRESSION OF RESISTANCE OF COCOA TO *V. DAHLIAE*.

5.1. INTRODUCTION.

Chemical control of Verticillium wilt of cocoa is not effective or economical mainly because the pathogen is soil-borne and this host is a perennial crop; thus, breeding for resistance is the only realistic means of long-term disease control. From the vast Amazon basin, the likely centre of origin of the cocoa tree, around 22,000 assumed accessions of *Theobroma* spp. have been collected and maintained by CEPLAC/ Ministry of Agriculture in Brazil. However, the enormous variation within this population has not been adequately exploited for disease resistance, mainly due to the lack of efficient screening procedures. Little is known about the interaction of pathogen, host and environment on the incidence and severity of Verticillium wilt on cocoa. This study was concerned primarily with the improvement of screening procedures by considering the effect of inoculation method, inoculum density and host age on the expression of resistance.

Several methods for inoculation of plants with vascular pathogens have been used, with varying success. These include inoculation into stem punctures, soil drenching, root dipping or spraying the roots with spore suspensions and growing plants in an already infested soil (Barrow, 1973). Stem puncture inoculation has been successful with other woody shrubs, such as cotton (Erwin

et al., 1965; Bugbee & Presley, 1967; Bell, 1992) and pigeon pea (Marley & Hillocks, 1993). Erwin *et al.* (1965) found that stem puncture gave a more predictable infection with *V. dahliae* whereas root dipping caused a physiological stunting of cotton plants. Since the inoculum is placed directly into the stem, one disadvantage of stem puncture would be its failure to detect a mechanism of resistance operating only in the root system. However, there is evidence from cotton, that resistance to *Verticillium* occurs after root infection (Garber & Houston, 1966). Although causing severe damage and sometimes death of uninoculated control plants, root spraying (Emechebe, 1974) and root dipping (Braga & Silva, 1989; CEPLAC, 1991) have been the most widely used methods to inoculate cocoa seedlings, with assessment of symptoms being carried out three to four months later. Stem puncture, although highly effective on cotton, has not previously been tested on cocoa.

Resistance to wilt diseases depends in part on the inoculum density of the pathogen. Even susceptible cultivars may show few, if any symptoms, with very low levels of inoculum. But, with increased inoculum density, a decrease in incubation periods and an increase in incidence and severity of symptoms, including mortality, is expected. Even resistant cultivars may become progressively more susceptible as inoculum concentrations exceed certain critical levels (Bell & Mace, 1981), although there is likely to be a smaller proportional increase in symptom development in a resistant than in a

susceptible cultivar (Mathre & Johnston, 1975).

Inoculum density can also play a significative role in the incidence of Verticillium wilt on cocoa (Emechebe, 1974; CEPLAC, 1991). The numerical threshold of infection, below which disease did not occur in 16-week-old root-inoculated susceptible seedlings, was ca. 5 x 10^4 conidia/ ml (Emechebe, 1974). Although CEPLAC (1991) has suggested 1 x 10^7 conidia/ ml to select cocoa cultivars, no investigation has been carried out on the effect of different inoculum densities on resistant cultivars. In the current study, resistant and susceptible cocoa cultivars were inoculated using soil drench and stem puncture methods with increasing concentrations of conidia of *V. dahliae*.

Resistance to fungal vascular wilts may change during plant growth and development, and these changes, if not adequately considered, might lead to mistaken conclusions (Bell & Mace, 1981). Reports on the effect of host age on the incidence of Verticillium wilts are conflicting. Presley & Taylor (1969), Emechebe (1975) and O'Garro & Clarkson (1988), working respectively on cotton, cocoa and tomato, found that infection increases with host age, while Parker (1959) and Evans, Snyder & Wilhelm (1966) found the opposite on deciduous fruit trees and cotton. After damaging the roots of cocoa seedlings prior to inoculation, Emechebe (1975) found that the incidence of Verticillium wilt increased from 0 to 80% with increasing plant age from two to 25 weeks. In order to investigate the effect of seedling age on the incidence of

Verticillium wilt on cocoa, soil drench and stem puncture inoculation methods were tested in glasshouse experiments at Bath University, U.K. The most suitable method and age for inoculation obtained in these conditions, were subsequently assessed in nursery screenings for resistance at CEPLAC in Brazil.

5.2. RESULTS.

5.2.1. Effect of method of inoculation and inoculum density of *V. dahliae* on disease expression by two cultivars of cocoa.

135-day-old cocoa seedlings of the cultivars ICS-1 and Pound-7, were soil drench or stem puncture inoculated with ten fold dilutions of *V. dahliae* ranging from 10^8 to 10^4 conidia/ ml. 50 ml of the conidial suspension/bag or 2 punctures/ stem were used on 20 or 15 replicates/ inoculum concentration, for respectively, soil drench or stem puncture inoculation. Disease symptoms were assessed periodically from 10 to 90 days, depending on the speed of symptom development. No visual symptoms of stress were apparent throughout the experiment on the respective uninoculated control plants, whether they had been punctured or not.

Independent of the inoculum density, disease progressed more rapidly and reached higher levels when seedlings were stem punctured (Tables 5.1. and 5.2.).

Plate 5.1.a. shows the typical browning of vascular tissue in the woody stem of Pound-7 and ICS-1, 10 days after puncture inoculation with 1 x 10^7 conidia/ ml. The first external symptoms (flaccidity followed by chlorosis and necrosis of upper leaves) generally appeared during the second week following stem puncture inoculation, or by the fourth week following soil drench inoculation. By 25 days after inoculation, symptoms were severe (mean disease indices were >2.50) in the majority of stem puncture inoculated treatments and low (<1.00) in all soil drench treatments, i.e., when external symptoms were reaching the maximum level on stem inoculated plants, they were just beginning to be expressed on soil inoculated plants. Also, even after 90 days, symptoms in plants that had been soil inoculated were not as severe as those in stem inoculated plants. In addition, assessment of individual records for each plant revealed that almost all stem puncture inoculated plants (94.6%) showed symptoms, whereas only a few (13.5%) soil inoculated plants became diseased.

The susceptible (ICS-1) and the resistant (Pound-7) cultivars could be differentiated using either inoculation method. Significant differences between cultivars appeared earlier with higher inoculum concentrations; with 10^8 or 10^7 conidia/ ml, such differences were evident as early as 15 days after inoculation by stem puncture, or by 48 days following soil drenching. However, with 10^6, 10^5 or 10^4 conidia/ ml, significant differences between these cultivars were detected 25 days after stem puncture but were never detected following soil drenching, because

disease levels remained low throughout the period of observation (Tables 5.1. and 5.2.).

Table 5.1. Effect of inoculum concentration of *V. dahliae* on the susceptibility of two cocoa cultivars following stem puncture inoculation.

	DAYS AFTER INOCULATION					
	10 DAYS		15 DAYS		25 DAYS	
CULTIVAR	**ICS-1**	**POUND-7**	**ICS-1**	**POUND-7**	**ICS-1**	**POUND-7**
CONIDIA/ ml						
10^8	0.41* a	0.06 a	1.82 b	0.86 a	3.55 b	3.06 a
10^7	0.38 a	0.02 a	1.79 b	0.86 a	3.52 b	2.97 a
10^6	0.13 a	0.02 a	1.30 a	0.72 a	3.40 b	2.76 a
10^5	0.00 a	0.00 a	0.03 a	0.09 a	2.42 b	1.00 a
10^4	0.00 a	0.00 a	0.00 a	0.07 a	1.56 b	0.86 a

* Final disease indices as adapted from Sidhu & Webster, (1977). Values represent the means of 15 replicates, and means followed by the same letter in each row and for each date after inoculation are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Table 5.2. Effect of inoculum concentration of *V. dahliae* on the susceptibility of two cocoa cultivars following soil drench inoculation.

	DAYS AFTER INOCULATION					
	25 DAYS		48 DAYS		90 DAYS	
CULTIVAR	**ICS-1**	**POUND-7**	**ICS-1**	**POUND-7**	**ICS-1**	**POUND-7**
CONIDIA/ ml						
10^8	0.63* a	0.17 a	1.68 b	0.67 a	2.19 b	0.67 a
10^7	0.26 a	0.00 a	1.25 b	0.15 a	1.78 b	0.25 a
10^6	0.00 a	0.00 a	0.00 a	0.25 a	0.48 a	0.25 a
10^5	0.00 a	0.00 a	0.09 a	0.00 a	0.00 a	0.00 a
10^4	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a

* Final disease indices as adapted from Sidhu & Webster, (1977). Values represent the means of 20 replicates, and means followed by the same letter in each row and for each date after inoculation are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

By 25 days after stem puncture inoculation, differences between Pound-7 and ICS-1 were not as evident at higher inoculum density as at lower inoculum densities. Nevertheless, by 60 days after inoculation, independent of the inoculum concentration used, almost all plants of the resistant cultivar Pound-7 had recovered from infection, as evidenced by the development of new green symptomless shoots and leaves from which *V. dahliae* could not be reisolated. By contrast, the ratio of recovery of the susceptible cultivar ICS-1 was significantly lower at all inoculum densities studied (Table 5.3. and Plate 5.1.b).

Table 5.3. Ratio of recovered: dead plants, 60 days after stem puncture inoculation of two cocoa cultivars.

CULTIVAR	**ICS-1**	**POUND-7**
INOCULUM DENSITY (conidia/ ml)		
10^8	4:11* b	13:2 a
10^7	5:10 b	14:1 a
10^6	5:10 b	13:2 a
10^5	9:6 b	15:0 a
10^4	7:8 b	14:1 a

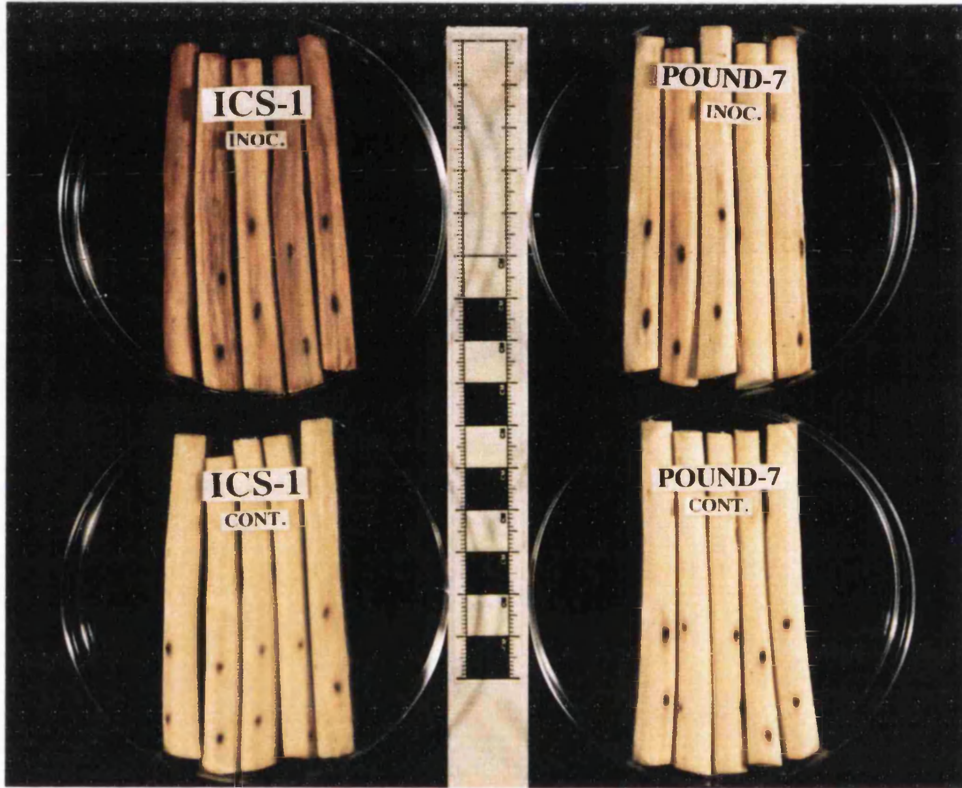
* Ratios obtained refer to a YES:NO response according to the presence of new shoots and leaves. Ratios followed by the same letter in each row, are not significantly different (Fisher's exact test, $p \leq 0.05$).

Plate 5.1. Typical internal and external symptoms induced on susceptible (ICS-1) and resistant (Pound-7) cocoa cultivars, by stem puncture inoculation (2 punctures/ plant, isolate ES-1, 10^7 conidia/ ml):

a) Segments of inoculated stems of cultivars ICS-1 and Pound-7, showing browning of vascular tissue in the woody stem near the punctured area, 10 days after inoculation (top of plate); note the more intensive symptoms in the susceptible cultivar ICS-1. Segments on the botton of the plate are from the respectives uninoculated punctured controls.

b) Foliar recovery of cultivar Pound-7 (right hand side), compared to dead ICS-1 plants, 60 days after inoculation; note the normal growth of the respective punctured controls.

a



b

5.2.2. Effect of seedling age at inoculation on disease incidence.

Cocoa seedlings of different ages were submitted to two different methods of inoculation as follows:

5.2.2.1. Inoculation by soil drenching.

Seedlings of cultivar ICS-1, when 15, 60 and 102 days old, were inoculated by drenching the soil with 50 ml of an aqueous suspension containing 1 x 10^7 conidia/ ml. Twenty seedlings were inoculated and twenty were kept as uninoculated controls for each age. Increase in height, number of leaves and disease index were assessed for each plant. Re-isolation of the pathogen was attempted from the base of the stems at the end of the experiment (90 days after inoculation).

With 15-day-old plants, no significant differences between inoculated and control treatments were found for any parameter (Table 5.4.). However, significant differences in height and disease index occurred with 60-day-old seedlings. On 102-day-old seedlings, significant differences were observed not only for increase in height and disease index, but also in the number of leaves produced. Re-isolation from the stem was achieved for only one seedling inoculated when 15 days old, from six inoculated when 60 days and from 14 inoculated when 102 days old. These data show that *V. dahliae* frequently does not reach the vascular system of stems of immature seedlings, following this inoculation procedure.

Table 5.4. Effect of seedling age at inoculation on cocoa growth and disease symptoms following inoculation with *V. dahliae*, by soil drenching.

AGE AT INOCULATION	TREATMENT	INCREASE IN HEIGHT (cm)	NUMBER OF LEAVES	DISEASE INDEX
15 days	Control	26.3* a	12.9 a	0.00 a
	Inoculated	23.0 a	12.1 a	0.20 a
60 days	Control	29.3 a	13.5 a	0.00 a
	Inoculated	18.6 b	11.7 a	1.23 b
102 days	Control	27.6 a	11.5 a	0.00 a
	Inoculated	17.2 b	8.05 b	2.33 b

* Data were collected 90 days after inoculation. Values represent the means of 20 replicates, and means followed by the same letter in each column and for each age are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

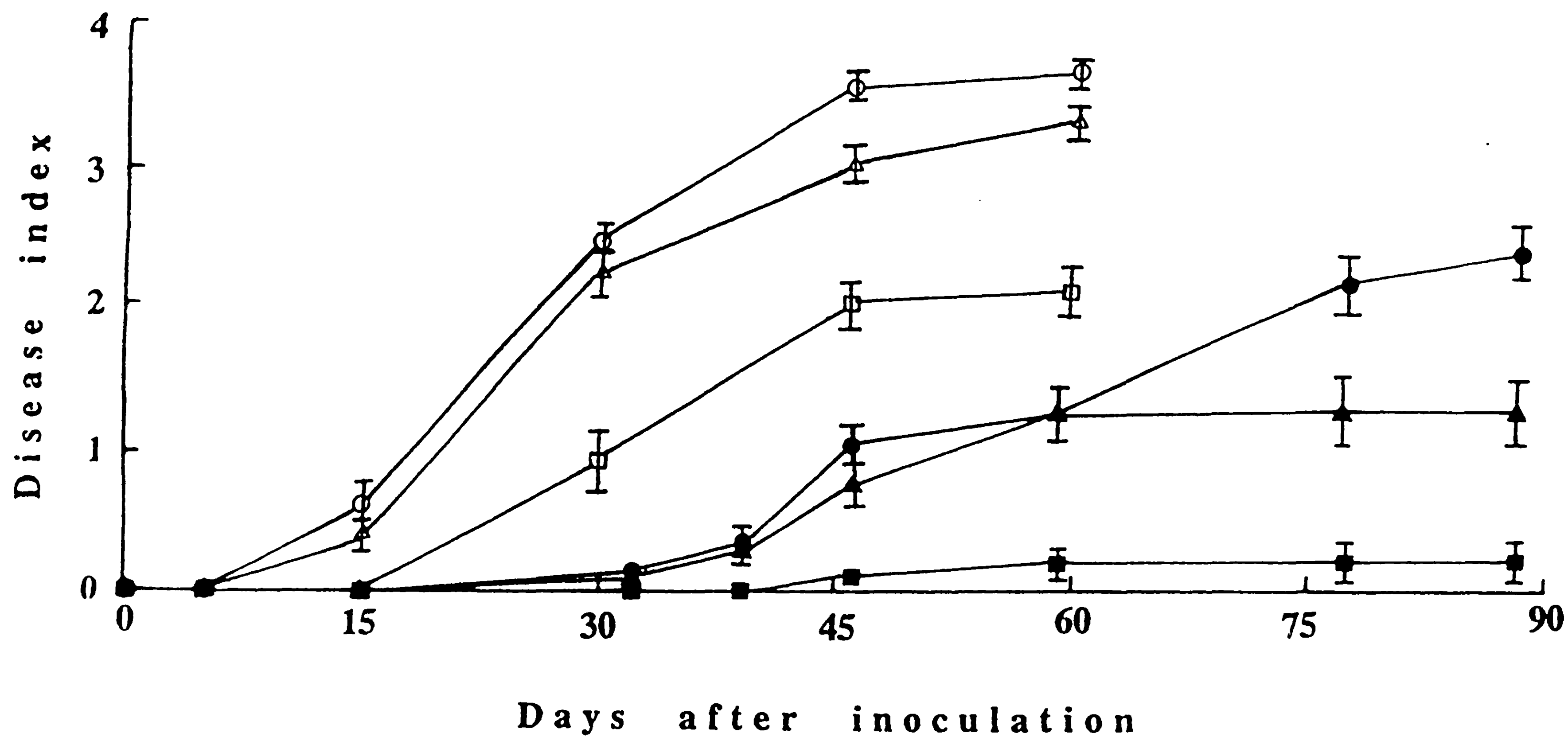
5.2.2.2. Stem puncture inoculation.

Seedlings of the cultivar West African Amelonado were simultaneously inoculated via stem punctures at the base of the hypocotyl, when 15, 45 and 100 days old, with 1×10^7 conidia/ ml. The stem diameter at the base of the hypocotyl of ten plants from each age group was measured prior to inoculation, and plants in each group received a different number of punctures, proportional to the mean area of their stem sections. Thus, 15-day-old plants (mean stem section area of 7.06 mm^2) received 2 punctures; 45-day-old plants (mean 19.95mm^2) received 6 punctures and 100-day-old plants (mean 43.68 mm^2) received 12 punctures.

Considering the disease progress curves for soil and stem inoculation (Figure 5.1.), it was again clear that, symptoms appeared earlier, disease progressed more rapidly, and consequently, much more extensive symptoms were recorded from 30 days after inoculation by stem puncture than by soil drenching. Similar patterns of disease progress occurred for different ages, independent of the method of inoculation; i.e., disease progressed more rapidly and reached higher levels in older plants.

Significant differences between inoculated and uninoculated stem punctured seedlings, in height, number of leaves and disease index were detected for all ages tested (Table 5.5.). Re-isolation of the pathogen after 60 days, from a region 5 cm above the inoculation area of all inoculated seedlings, independent of their age, provided further evidence of successful colonization.

Figure 5.1. Effect of plant age on the severity of vascular wilt on cocoa following inoculation by stem puncture or soil drenching. (Bars represent standard error of means).



□ 15-day-old seedlings, stem puncture inoculated;
■ 15-day-old seedlings, soil drench inoculated;
△ 45-day-old seedlings, stem puncture inoculated;
▲ 60-day old seedlings, soil drench inoculated;
○ 100-day-old seedlings, stem puncture inoculated;
● 102-day-old seedlings, soil drench inoculated.

Therefore, younger seedlings are susceptible to inoculation by stem puncture but not by soil drenching.

Table 5.5. Effect of seedling age at inoculation on cocoa growth and disease symptoms following inoculation with *V. dahliae*, via stem punctures.

AGE AT INOCULATION	TREATMENT	INCREASE IN HEIGHT (cm)	NUMBER OF LEAVES	DISEASE INDEX
15 days	Control	20.7* a	12.5 a	0.00 a
	Inoculated	12.4 b	5.3 b	2.10 b
45 days	Control	34.3 a	17.3 a	0.00 a
	Inoculated	15.5 b	7.2 b	3.28 b
100 days	Control	51.9 a	28.9 a	0.00 a
	Inoculated	30.1 b	17.2 b	3.63 b

* Data were collected 45 days after inoculation. Values represent the means of 10 replicates and means followed by the same letter in each column and for each age are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

5.2.3. Effect of stem puncture inoculation on 15-day-old seedlings from cultivars with different levels of resistance to *V. dahliae*.

Results from the previous experiments suggested that stem puncture inoculation of 15-day-old cocoa seedlings would save time and space in a screening programme for resistance to *V. dahliae*. However, no information was available on the efficiency of stem puncture inoculation on very young seedlings in distinguishing between resistant and susceptible lines. This was investigated in an experiment conducted at CEPLAC in Brazil, with 15-day-old seedlings inoculated with 1 x 10^7 conidia/ ml delivered into 2 punctures/ stem. Seven cultivars with already known levels of resistance to Verticillium wilt based on root dipping inoculations on 15-day-old seedlings (Braga & Silva, 1989; CEPLAC, 1991) were included, along with two cultivars not tested before (SIC-328 and ICS-8).

Ranking cultivars for resistance was similar, whether based on results obtained 30 or 60 days after stem inoculations or on previously reported ratings from root inoculations (Table 5.6.). SIC-328 was ranked as wilt resistant along with Pound-7 and SIC-2; ICS-8 was considered wilt susceptible along with ICS-1, BE-5 and ICS-6. Cultivar SIC-802 demonstrated an intermediate response, following either method of inoculation.

Table 5.6. Effect of stem puncture on symptoms of Verticillium wilt expressed by nine cultivars of cocoa inoculated when 15 days old.

CULTIVAR	RESISTANCE RATING*	-D I S E A S E 30 days**	I N D E X- 60 days**
SIC-2	R	0.00 a	0.13 a
SIC 328	ND	0.22 a	0.22 ab
POUND-7	R	0.00 a	0.33 ab
PA-30	R	0.40 ab	0.66 ab
SIC-802	R/S	0.44 ab	0.71 bc
ICS-6	S	0.80 bc	1.33 cd
BE-5	S	1.15 cd	1.51 d
ICS-1	S	0.98 cd	1.68 d
ICS-8	ND	1.33 d	1.92 d

* CEPLAC's classification of cocoa cultivars for resistance to *V. dahliae*, based on screenings carried out between 1988-1990 using root dipping inoculation on 15-day-old seedlings (Braga & Silva, 1989; CEPLAC, 1991): R= Resistant; R/S Moderately resistant; S= Susceptible; ND= not determined. A cultivar was considered as resistant if its disease index was not significantly different from that of Pound-7, and susceptible if not significantly different from that of ICS-1.

** Data were collected 30 and 60 days after inoculation and values represent the means of at least fifteen replicates (minimum of 15 and maximum of 25 replicates). Within each column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

5.3. DISCUSSION.

The success of a breeding programme for disease resistance depends upon the methods employed for inoculation, evaluation and selection within the target host population. These methods must minimize the number of susceptible plants that escape infection and be indicative of the population performance in natural epidemics (Grau, 1991); i.e., the successful invasion of the host by the pathogen is imperative before any genetic analysis can be made (Barrow, 1973) and a direct correlation is required between glasshouse and field experiments (Bell, 1992). In the current study, resistant and susceptible cultivars could be differentiated by either soil drench or stem puncture inoculation under glasshouse and nursery conditions. Although just nine cultivars have been checked so far, it seems that the resistance mechanism(s) preventing root colonization also operates in the stems of cocoa seedlings, as previously reported for Verticillium wilt of cotton (Garber & Houston, 1966).

Fewer plants escaped systemic infection and more normally distributed disease index data were obtained when plants were stem rather than root inoculated. As a consequence, very low inoculum densities were capable of differentiating between resistant and susceptible cultivars when plants were inoculated by stem puncture. Erwin *et al.* (1965), comparing root and stem inoculations, found that the more uniform response of cotton cultivars to stem puncture in glasshouse conditions also more

closely correlated to field results. Subsequently, Devey & Rosielle (1986) obtained excellent phenotypic and genotypic correlations between resistance ratings following natural infection in the field and artificial stem inoculations with defoliating isolates of *V. dahliae*. Cocoa lines selected as resistant to Verticillium wilt at the Cocoa Research Centre are now being distributed to producers in affected areas in Brazil and comparisons between nursery and field ratings are planned for the near future.

The stability of plant resistance to Verticillium wilts can also vary considerably according to inoculum densities of the pathogen. For example, high inoculum concentrations largely overcame the resistance of cotton (Schnathorst & Mathre, 1966) and completely overcame the best resistance available in potato to *V. dahliae* (Frank, Webb & Wilson, 1975). In the current study, there was a trend for the resistance of Pound-7 to be overcome by inoculum densities above 10^6 conidia/ ml, 25 days after inoculation. However, plants of this resistant cultivar later recovered, producing new disease-free shoots and leaves, regardless of the inoculum pressure they had been submitted to. Recovery of Pound-7 appeared to be long-lived, because even more than one year after inoculation, most plants remained symptomless and the new xylem formed did not show the vascular discolouration characteristic of the disease. Similar results were obtained by Melo & Costa (1985) with Verticillium wilt of aubergine, and the persistent recovery occurring in some infected lines was

described as a resistant reaction. Consequently, selection of cocoa cultivars for wilt resistance should be based on more than one date of disease assessment, in order to avoid the rejection of potentially resistant or even tolerant material.

Inoculum densities as low as 10^4 conidia/ ml were sufficient to differentiate resistant and susceptible cultivars inoculated via stem puncture, whereas with the soil drench method, 10^7 conidia/ ml were necessary. Baayen & Schrama (1990) compared five methods of inoculation of *Fusarium oxysporum* f. sp. *dianthi* in carnation and suggested that stem injection was more effective mainly because a lower inoculum dosage was required to obtain the same symptom levels as in other methods. In fact, inoculum production for large screenings has been a problem when selecting cocoa cultivars for resistance to Verticilium wilt in Brazil, and the possibility of using a lower inoculum density than that previously recommended (10^7 conidia/ml), should be taken in account when opting for an inoculation method.

It was clearly demonstrated that, apart from the method of inoculation and inoculum density, the age of the host also had a considerable effect on the incidence and severity of the disease. Independent of the method of inoculation, Verticillium wilt symptoms intensified with increasing age, which agreed with the previous results of Presley & Taylor (1969), Emechebe (1975) and O'Garro & Clarkson (1988).

The lower frequency of re-isolations obtained from

younger cocoa seedlings inoculated by soil drenching, might reflect difficulties of invasion and colonization in an immature root system. Following penetration, the length of vascular elements can strongly influence the rate at which spores of pathogens are distributed through the vascular system and, thereby, the ability of the plant to seal off infection (Beckman, Mace, Halmos & McGahan, 1961; Presley & Taylor, 1969; Mace, Veech & Hammerschlag (1971); Beckman, 1987). Beckman *et al*. (1961) found that longer, older vessels commonly allowed the passage of spores of *Fusarium oxysporum* f.sp. *cubense* into the rhizome of banana plants, whereas the resistance of younger seedlings was associated with the presence of shorter vessels in their root system. Also, during the early stages of infection in cotton, end walls in the hypocotyls may prevent the spread of *V. dahliae* upward in the xylem vessels of young cotton seedlings; these end walls progressively disappear with age as the plants become more susceptible (Presley & Taylor, 1969). Mace *et al*. (1971) shown that, apart from enlarged openings in the xylem end walls of older tomato seedlings, the increase in vessel diameter with age could also account for the more rapid spore uptake in mature seedlings. The effect of plant age on anatomy aspects of xylem vessels should be investigated in cocoa seedlings.

Less severe symptoms occurred in very young seedlings inoculated under nursery conditions in Brazil compared to similar seedlings inoculated in a more controlled environment in the U.K.. It is well known that

high temperature (above 28°C) is a major factor reducing the rate of infection and the severity of symptoms caused by *V. dahliae* (Garber & Presley, 1971; Schnathorst, 1981). The occurrence of high temperatures in nursery trials during the summer greatly hampered progress on the study of Verticillium wilt on cocoa in Brazil (CEPLAC, 1991). However, stem puncture inoculation resulted in increased symptom expression such that the level of disease obtained on 15-day-old seedlings was sufficient to differentiate resistant and susceptible cultivars, even in conditions relatively unsuitable for infection. These results therefore suggest that, stem puncture inoculation of 15-day-old seedlings is the best method of screening cocoa cultivars for resistance to Verticillium wilt and would allow economy of space, time and other resources, particularly in a large scale breeding programme, where hundreds of genotypes require critical assessment for diverse parameters.

6. VARIATION IN PATHOGENITICY TO COCOA AND VEGETATIVE COMPATIBILITY AMONG *V. DAHLIAE* ISOLATES.

6.1. INTRODUCTION.

Selection for resistance to Verticillium wilt diseases should take into consideration the possible genetic variation within the pathogen population. Among isolates from a host plant, variation in aggressiveness may be found and disease symptoms are sometimes expressed in different ways. In the U.K., isolates of *V. albo-atrum* that vary in their pathogenicity to hops have been implicated in the occurrence of fluctuating and progressive symptoms on this crop (Isaac & Keyworth, 1948), while in the U.S.A., some isolates of *V. dahliae* cause a severe defoliation on cotton, whereas others do not (Schnathorst & Mathre, 1966 ; Puhalla & Bell, 1981). Puhalla & Hummel (1983), Joaquim & Rowe (1990) and Strausbaugh *et al.* (1992), based on vegetative compatibility analyses, showed that defoliating and non-defoliating strains from cotton, belong to genetically isolated, subspecific populations. Isolates belonging to these two groups were later designed as P-1 and P-2, corresponding to defoliating and non-defoliating pathotypes, respectively (Bell, 1992). ln Verticillium wilt of tomato, two different races occur. Resistance to both *V. dahliae* (race 1) and *V. albo-atrum* is conferred by a single gene, first introduced into commercial varieties by Schaible, Cannon & Waddoups (1951). Subsequently, Grogan, Ioannou, Schneider, Small & Kimble (1979)

described *V. dahliae* isolates pathogenic to these resistant lines, which were designated race 2. So far, physiological races of *V. dahliae* have only been defined on tomato.

In contrast, although Verticillium wilt is a major disease in certain cocoa producing countries such as Brazil, Colombia and Uganda, little attention has been given so far to the variation in pathogenicity of *V. dahliae* towards this host. In a series of screening trials carried out in the state of Bahia, Brazil, the cultivar Pound 7 has frequently been ranked as the most resistant material to Verticillium wilt (Braga & Silva, 1989; CEPLAC, 1991) and the inheritance of this character is apparently conferred by a recessive pair of alleles (Braga & Silva, 1989). However, selection procedures have been based on inoculations with a single non-catalogued isolate of unidentified geographical origin, which may have implications as to the conclusions made. In order to investigate what effect pathogen variation might have on resistance expression, putative resistant and susceptible cocoa cultivars were inoculated with isolates from different geographical origins and hosts.

Vegetative compatibility analysis has been established as an useful tool to examine genetic diversity among populations of wilt fungi. Molecular marker approaches also demonstrated that isolates within a vegetative compatibility group (VCG) tend to be more similar than isolates in different VCG's (Bosland & Williams, 1987; Jacobson & Gordon, 1990). Puhalla &

Hummel (1983) identified 16 different vegetative compatibility groups of *V. dahliae*, based on the production of UV-light colour mutants among a worldwide collection from several hosts. Joaquim & Rowe (1990) proposed a revised VCG system for *V. dahliae* based on the production of nitrate non-utilizing (*nit*) mutants and assigned isolates into 4 VCG's (1, 2, 3, 4A and 4B). By including more tester strains and doing a more comprehensive pairing of testers, Strausbaugh *et al.* (1992) found that some of the strains classified as self-incompatible by Joaquim & Rowe were able to produce heterokaryons. Thus, a new group (VCG-5) was identified from one of those strains. Recently, Chen (1994), studied the VCG diversity of 42 isolates of *V. dahliae* from ornamental wood plants, using the tester strains developed by Joaquim & Rowe (1990). Isolates were assigned into three groups (VCG's 1, 2 and 4), with the great majority of isolates falling into VCG-1. The narrow range of VCG diversity found was in sharp contrast to the wide range of hosts plants from which the isolates came from.

Heterokaryosis and parasexuality allow many *Verticillium* species to exchange genetic information and strains that do not form heterokaryons with each other, would be genetically isolated (Hastie, 1962; Fordyce & Green, 1964; Puhalla, 1979; Heale, 1988; Strausbaugh *et al.*, 1992). The fact that strains within a VCG are interactive and isolated from members in other VCG's, may result in the association of specific genetic traits such as pathogenicity with certain VCG's but not with others

(Flood, Whitehead & Cooper, 1992; Leslie, 1993). At present, however, there is no general rule about correlation between pathogenicity and compatibility group, with some systems showing useful correlation, while others show little or no correlation (Leslie, 1993). Therefore, it is necessary to characterize our *V. dahliae* isolates more precisely, to allow a better understanding of pathogen variability, which is likely to play a crucial role in screening for resistance. Apart from evaluating the range of aggressiveness of some *V. dahliae* isolates to cocoa cultivars, the current investigation was also concerned with the assessment of VCG diversity amongst isolates and the determination of any possible relationship between pathogenicity on cocoa and VCG's.

6.2. RESULTS.

6.2.1. Pathogenicity of isolates from different hosts on a cocoa cultivar, selected as resistant to *V. dahliae*.

To investigate the effect of different isolates on symptom expression, 110-day-old cocoa seedlings, cv. Pound-7, were inoculated with isolates from cocoa (BA-3 and ES-1, from Brazil, COL-1 from Colombia and UG-1 from Uganda), cotton (SS-4 and T-9, from the U.S.A.), tomato (SW from the U.K. and TS-2 from the U.S.A.), pepper (97 from Italy), aubergine (EGP from Greece) and okra (OK-1 from Brazil).

Cultivar Pound-7 exhibited distinct levels of resistance to different isolates. Based on final disease indices and re-isolation data (Table 6.1.), this cultivar appeared to be resistant to isolates SW, OK-1 and ES-1, moderately resistant to isolates UG-1 and BA-3 and susceptible to isolates SS-4, 97, TS-2, COL-1, T-9 and EGP. Thus, many isolates from other crops were capable of overcoming the resistance of Pound-7, but considering the group of isolates from cocoa, only COL-1 did so.

The effect of these different isolates on defoliation of cocoa was for the first time experimentally assessed (Table 6.1.). Isolate T-9, was very aggressive and also produced extensive defoliation on cv. Pound-7. Isolate SS-4 induced little defoliation on this cultivar, despiting causing severe wilting and even death of seedlings. Such results concur with the effects of these isolates on cotton (Resende, Flood & Cooper, 1994a); therefore, isolates not statistically different from T-9 or SS-4 in terms of defoliation were considered, respectively, as defoliating or non-defoliating on cocoa. Consequently, the Colombian isolate (COL-1), was the only one from cocoa, assigned as defoliating on Pound-7; all other isolates did not differ statistically from SS-4 and were considered as non-defoliating.

Table 6.1. Effect of different isolates of *V. dahliae* on symptomatology, recovery of the pathogen and defoliation of cocoa seedlings, cv. Pound-7.

ISOLATE	DISEASE INDEX*	RE-ISOLATION** (10th)	DEFOLIATION*** (%)
SS-4	2.57 d	8:2 d	36.53 ab
97	2.16 cd	7:3 cd	49.85 bc
TS-2	1.97 cd	6:4 bcd	26.50 a
COL-1	1.92 cd	5:5 bcd	58.92 cd
T-9	1.90 cd	6:4 bcd	83.00 d
EGP	1.54 bc	5:5 bcd	43.78 abc
UG-1	0.64 ab	2:8 abc	48.90 bc
BA-3	0.63 ab	2:8 abc	26.40 a
OK-1	0.17 a	1:9 ab	-----
SW	0.00 a	0:10 a	-----
ES-1	0.00 a	0:10 a	-----
Control	0.00 a	0:10 a	-----

* Final disease index data were collected 90 days after inoculation, as adapted from Sidhu & Webster (1977). Values represent the means of 20 replicates. Within this column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 10th internode of stems. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae*; ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

*** Percentage of defoliation, based on the number of leaves and foliar scars, remaining on each diseased plant at the end of the experiment. Within this column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$); ----- statistical analysis was not applicable to these treatments, due to small size samples (occurrence of few diseased plants).

6.2.2. Pathogenicity of cocoa isolates from different geographical origins on resistant and susceptible cocoa cultivars.

6.2.2.1. Isolates from different states in Brazil, inoculated by soil drenching.

Verticillium wilt is a very destructive disease in the cocoa region of the states of Bahia and Espirito Santo and, despite the selection of cultivars Pound-7 and ICS-1 as, respectively, standards of resistance and susceptibility for screening trials in Brazil, no attention has been given to the choice of a standard isolate to be used in such trials. In order to investigate possible differential host responses to isolates from those states, 280-day-old seedlings of cultivars ICS-1 and Pound-7 were soil drench inoculated with isolates BA-3 (from Bahia) and ES-1 (from Espirito Santo), which came from distinct growing areas at least 500 km apart.

These standard cultivars could be differentiated in susceptibility to *V. dahliae*, only when inoculated with isolate ES-1. This isolate induced high levels of infection and symptoms on ICS-1 but not on Pound-7 (Table 6.2.). In contrast, isolate BA-3 induced low levels of infection and symptoms on both cultivars. Low ratios of re-isolation were obtained from the 5th internode of those plants in general; thus, quantitative recovery measurements expressed by colony forming units (CFU's) were performed from lower parts (base of the stem just above the hypocotyl and middle region of the tap root) of all plants. However, patterns of fungal colonization at

both sites were similar to those obtained with qualitative re-isolation; i.e. isolate ES-1, inoculated on cultivar ICS-1, produced significantly more CFU's than the other combinations of isolate and cultivar. Therefore, plants not showing symptoms were not systemically colonized, which accounted for the similar responses obtained with all parameters tested.

Table 6.2. Effect of *V. dahliae* isolates from two different states in Brazil, on wilt incidence and recovery of the pathogen from two cultivars of cocoa inoculated by soil drenching.

ISOLATE	CULTIVAR	DISEASE INDEX*	--C O L O N I Z A T I O N-- RE-ISOL.** (5th)	CFU's*** (Root)	CFU's*** (Stem)
ES-1	ICS-1	2.19 b	11:9 b	74.8 b	73.8 b
ES-1	POUND-7	0.20 a	1:19 a	0.00 a	0.00 a
BA-3	ICS-1	0.39 a	2:18 a	0.09 a	1.35 a
BA-3	POUND-7	0.53 a	3:17 a	0.00 a	0.39 a

* Final disease index data were collected 90 days after inoculation, as adapted from Sidhu & Webster, (1977). Values represent the means of 20 replicates and within this column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** Re-isolation from the 5th internode of stems. Ratios obtained refer to a YES:NO response according to recovery of *V. dahliae* and ratios followed by the same letter in this column, are not significantly different (Fisher's exact test, $p \leq 0.05$).

***CFU's represents the number of colony forming units/ g of fresh weight at the middle of the tap root (Root) or base of the stem just above the hypocotyl (Stem). All values represent the means of 20 replicates and within these columns, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

A significant interaction ($p \leq 0.01$) was detected between isolates and cultivars, following an analysis of variance of transformed disease index data (Table 6.3.). Thus, variability in pathogenicity is evident within the *Verticillium* population at cocoa plantations in the States of Bahia and Espirito Santo. More isolates should be included in future trials, aiming a better understanding of such variability.

Table 6.3. Analysis of variance of the disease index data obtained for Table 6.2., transformed in $\log_{10} + 0.001$.

SOURCE OF VARIATION	d.f.+	s.s.	m.s.	v.r.	F.pr.
CULTIVAR	1	40.78	40.78	4.53	0.036*
ISOLATE	1	66.99	66.99	7.44	0.008**
CULTIVAR X ISOLATE	1	98.16	98.16	10.91	0.001**
RESIDUAL	76	684.07	9.00		
TOTAL	79	890.02			

+ d.f= degrees of freedom, s.s.=sum of squares, m.s.=mean square, v.r.=variance ratio, F. pr.=probability of F.

* Significant at 0.05.

** Significant at 0.01.

6.2.2.2. Isolates from different countries in South America, inoculated by stem puncture.

Stem puncture proved to be a more effective inoculation technique for Verticillium wilt on cocoa when compared to soil drench inoculation (previous chapter), which led to the investigation of possible differentiation of isolates on resistant and susceptible cultivars, by that new method. In this experiment, along with the isolates from Brazil previously compared (BA-3 and ES-1), isolate COL-1 from Colombia was also used to inoculate cultivars ICS-1 and Pound-7. 150-day-old seedlings (15 plants from each cultivar) were stem puncture inoculated (two punctures/ plant) and disease index was assessed periodically for 20 days, when all plants were harvested and the number of CFU's/ g of fresh weight was determined at the base of the stem, 5cm above the inoculation point.

Disease incidence and severity were much more highly expressed for all isolates here than in the previous soil inoculation experiment, as demonstrated by the disease index and CFU data obtained (Table 6.4.). Using either parameter, only isolate ES-1 could differentiate the tested cultivars in terms of resistance to *V. dahliae*. This isolate induced significantly higher levels of external symptoms and colonization on ICS-1 than on Pound-7. Isolate COL-1 produced severe defoliation and widespread colonization on both cultivars, and was therefore unable to differentiate them. In spite of the general occurrence of higher levels of disease following stem puncture, isolate BA-3 induced milder symptoms and

less extensively colonized both cultivars, when compared to the other isolates. Thus, similarly to the previous experiment, it was unable to distinguish the resistance levels of the two cultivars.

The results obtained in this chapter showed that each isolate of *V. dahliae* presented similar patterns of aggressiveness towards cocoa cultivars, following either, soil drench or stem puncture inoculation. The importance of choice of isolate for screening for disease resistance was also emphasized, since the resistance of cultivar Pound-7 only became apparent with isolate ES-1.

Table 6.4. Effect of *V. dahliae* isolates from two South American countries on wilt incidence and recovery of the pathogen from two cultivars of cocoa inoculated by stem puncture.

ISOLATE	ES-1	BA-3	COL-1	ES-1	BA-3	COL-1
CULTIVAR	DISEASE INDEX*			NUMBER OF CFU's**		
ICS-1	3.71 bB	2.84 aA	3.35 aAB	1986.0 bB	185.3 aA	1353.0 aB
POUND-7	2.82 aA	2.63 aA	3.66 aB	81.9 aA	212.8 aA	1496.0 aB

* Final disease index data were collected 20 days after inoculation, as adapted from Sidhu & Webster, (1977). Values represent the means of 15 replicates and means followed by the same low-case letter in each column or by the same capital letter in each row and for each parameter, are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** CFU's represents the number of colony forming units/ g of fresh weight determined 20 days after inoculation at the base of the stem, 5cm above the inoculation area. Values represent the means of 15 replicates and means followed by the same low-case letter in each column or by the same capital letter in each row and for each parameter, are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

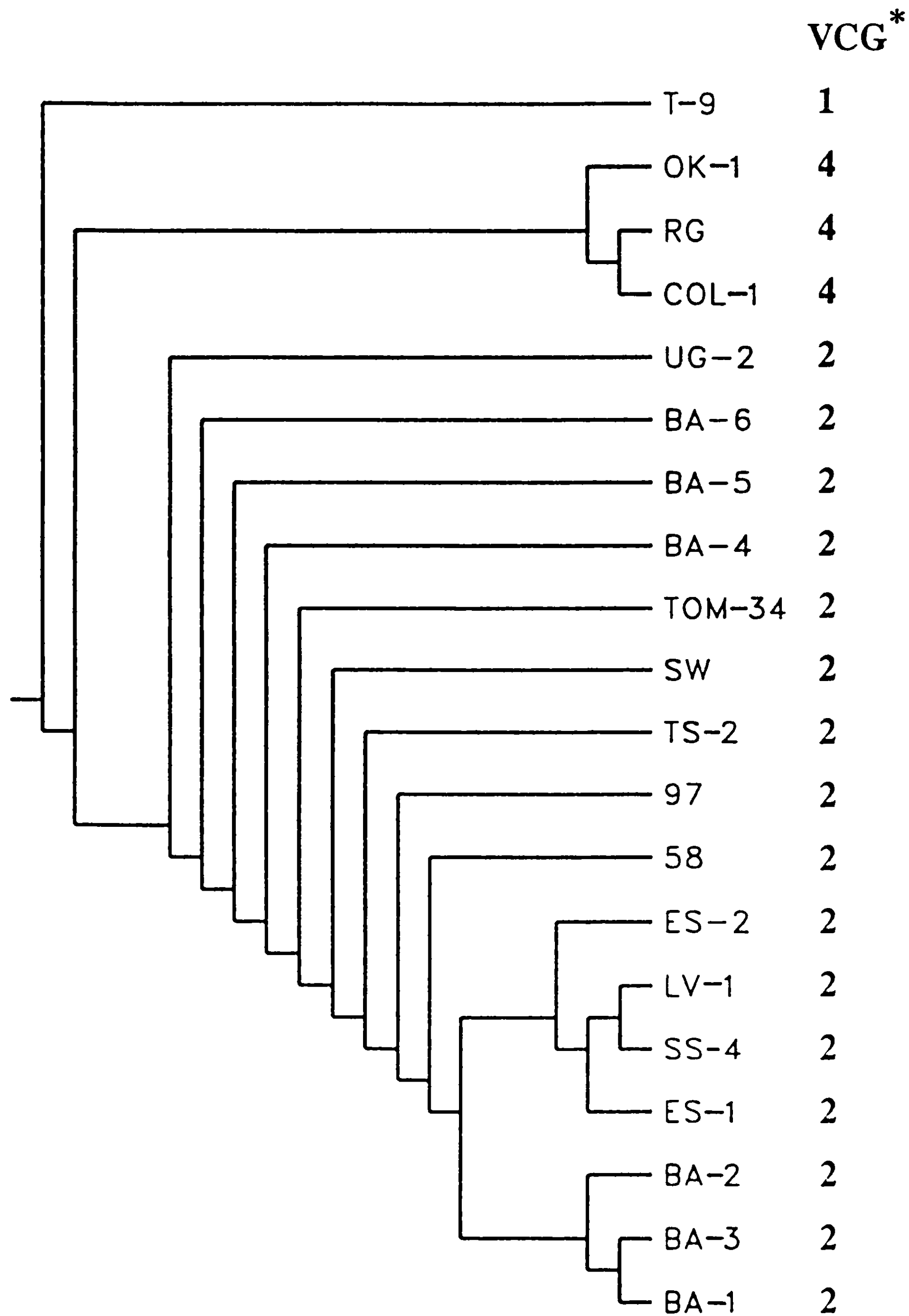
6.2.3. Assessment of vegetative compatibility within *V. dahliae* isolates.

Twenty two isolates of *V. dahliae* from eight different countries and from six different hosts, were tested for vegetative compatibility using *nit* mutants. These comprised the 11 isolates previously inoculated on cocoa (Table 2.1.) and additional isolates from cocoa and tomato. A complicating factor in assessing vegetative compatibility was that the extent of prototrophic growth in inter-isolates pairings often varied among isolates and individual *nit* mutants. This problem was partially reduced by increasing the time of assessment after pairing from 21 days (Joaquim & Rowe, 1990) to 30 days. Nevertheless, only strong compatibility reactions (prototrophic growth, with profuse microsclerotial formation or abundant aerial mycelium at the mycelial interface), were considered as compatible reactions (Plate 6.1.). Weak reactions characterized by only growth within the agar or scattered growth on the agar surface at the mycelial interface were considered incompatible, following the criteria established by Strausbaugh *et al.* (1992).

Only three VCG's were identified among the isolates tested and VCG-2 was the largest group, containing about 80% of all isolates tested (Figure 6.1.). All Brazilian isolates from cocoa were assigned to VCG-2, independent of their state of origin in Brazil or level of pathogenicity towards cocoa (as in the case of isolates BA-3 and ES-1) (Plate 6.1.). However, the dendrogram based on Jacquard's

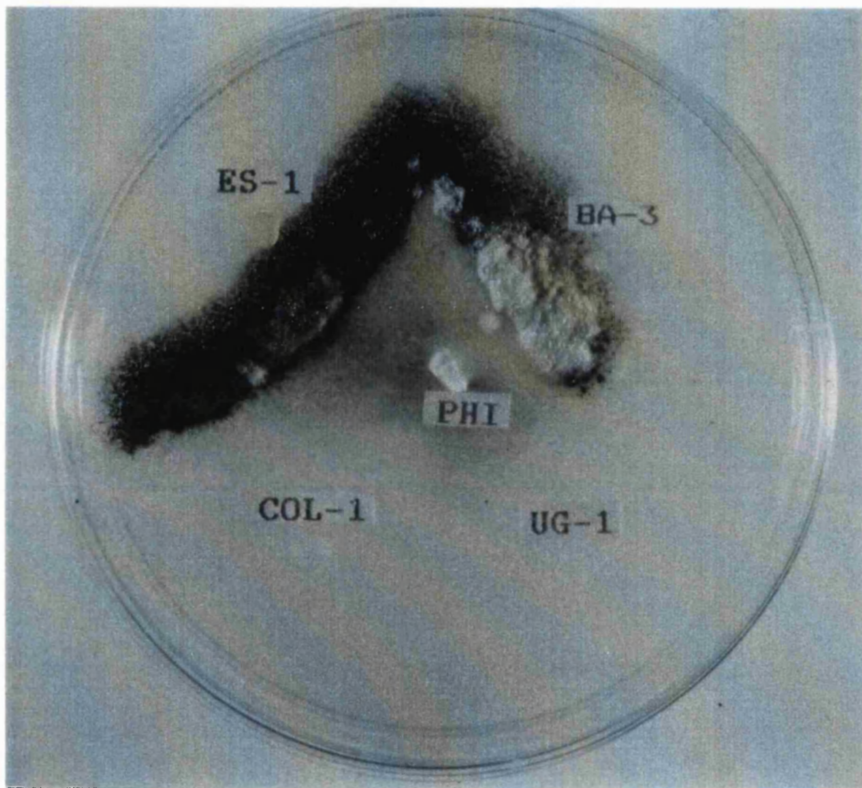
similarity coefficient (Sneath, 1962) demonstrated that three subgroups were apparently present within VCG-2.

Figure 6.1. Dendrogram constructed on the basis of relatedness of *V. dahliae* isolates, as revealed by VCG analysis.



* Vegetative compatibility groups, *sensu* Strausbaugh *et al.* (1992).

Plate 6.1. Vegetative compatibility of *V. dahliae* cocoa isolates from different countries. When paired with the tester strain PHI (VCG-2, *sensu* Strausbaugh *et al.*, 1992), only the isolates BA-3 and ES-1 formed the typical compatibility reaction (prototrophic growth, with profuse microsclerotial formation or abundant aerial mycelium at the mycelial interface). Isolates COL-1 from Colombia or UG-1 from Uganda were incompatible with PHI (and other strains in VCG-2), developing no visible reaction at the mycelial interface.


ES-1
BA-3
PHI
COL-1
UG-1

Cocoa isolates, BA-1, BA-2 and BA-3, from Bahia, Brazil, showed 100% similarity when paired against the tester strains for this group. They were closely related to ES-1 and ES-2, from Espirito Santo, Brazil, although these strains appeared to be in another subgroup in VCG-2. A third possible subgroup was that containing the cocoa isolates BA-4, BA-5, BA-6 from Brazil and UG-2 from Uganda.

Most isolates from tomato (SW, TS-2, TOM 34 and 58), although characterized as different races on this host, were included within the same subgroup of VCG-2. An Italian isolate from pepper (97), an American isolate from cotton (SS-4), two Ugandan isolates (UG-2 from cocoa and LV-1 with unidentified original host) were also allocated to VCG-2. Only the defoliating isolate T-9, was compatible with tester strains from VCG-1, and therefore allocated to this group. The Colombian isolate, although inducing defoliation on cocoa, was assigned to another group (VCG-4), which also contained a Brazilian isolate from okra (OK-1) and an American isolate from tomato (RG). Finally, the Greek isolate from aubergine (EGP) and an Ugandan isolate from cocoa (UG-1) could not be assigned to any VCG, because of the failure of their *nit* mutants to form heterokaryons with complementary *nit* mutants from the tester strains. Furthermore, UG-1 was considered self-incompatible, because Nit1 and NitM mutants from this isolate were also unable to complement one another.

6.3. DISCUSSION.

Cultivar Pound-7 has been selected for resistance to *V. dahliae* and has been incorporated as a resistant standard in all screening trials conducted at the Cocoa Research Centre in Brazil (Braga & Silva, 1989; CEPLAC, 1991). However, throughout these trials, a single not yet characterized isolate of unidentified geographical origin has been used. From the current study, it would appear that the resistance of Pound-7 is greatly dependent upon the choice of isolate for inoculation. Many isolates from other crops and a Colombian isolate from cocoa induced significantly more severe symptoms on Pound-7 than the Brazilian isolates from cocoa. Further investigation of these South American isolates revealed that isolate BA-3 could not differentiate Pound-7 from the standard susceptible ICS-1, as it induced low levels of disease in both cultivars, especially when compared to isolates ES-1 and COL-1. By contrast, isolate COL-1 could not differentiate Pound-7 from ICS-1, since it induced high disease incidence and severity in both cultivars. The high susceptibility of Pound-7 to COL-1, suggests that this cultivar should not be recommended to cocoa areas affected by *Verticillium* in Colombia. ES-1 was the most discriminating isolate among the three isolates from cocoa so far compared; it demonstrated the relative resistance of Pound-7, whichever method of inoculation utilized (soil drenching or stem puncture). However, it is not yet known if this isolate is representative of the *V. dahliae* population in cocoa growing areas of Brazil. More cocoa

isolates were recently collected and will be included in further screens for resistance.

A differential response was produced by *V. dahliae* isolates with regard to defoliation on cocoa, cv. Pound-7. Among the cocoa isolates tested, only COL-1 from Colombia induced a level of defoliation statistically similar to T-9 (standard defoliating isolate). Schnathorst & Mathre (1966) have described *V. dahliae* pathotypes on cotton as defoliating or non-defoliating, but other authors (Bell, 1973; Ashworth Jr., 1983) support the existence of a continuum of aggressiveness among strains of *V. dahliae* on cotton, rather than the occurrence of distinct pathotypes. A situation similar to the later one may occur with Verticillium wilt of cocoa in Brazil, where intermediate levels of defoliation seems to be present. In cocoa growing areas of Northeastern Brazil, it is common to observe diseased trees with or without leaves, but to date, no attention has been given to the pattern of defoliation caused by isolates from such distinct trees. The use of stem puncture for inoculation of cocoa seedlings (Resende, Flood & Cooper, 1994b), will certainly facilitate rapid characterization of a larger number of *Verticillium* isolates in relation to the severity of symptoms they can induce on cocoa plants.

Puhalla (1979) has hypothesized that the study of genetically isolated populations within *V. dahliae* should help to determine the origin of new pathotypes or the spread of known ones. When the cotton defoliating type was first identified in California, it was believed that it

had arisen as a variant of the then prevalent non-defoliating SS-4 type (Schnathorst & Mathre, 1966). However, defoliating and non-defoliating types were allocated into different vegetative compatibility groups, based on either, colour mutants (Puhalla & Hummel, 1983) or *nit* mutants (Joaquim & Rowe, 1990; Strausbaugh *et al.*, 1992). Thus, the theory of a common genetic origin for T-9 and SS-4 is no longer acceptable. Results presented here are in agreement with those of previous authors; T-9 was assigned to VCG-1 and SS-4 to VCG-2. In addition, although isolates from different crops were studied, the majority of them were allocated to VCG-2; similar findings were reported by Joaquim & Rowe (1990) and Strausbaugh *et al.* (1992).

One out of the 22 isolates examined exhibited heterokaryon self-incompatibility, which occurs when two phenotypically distinct *nit* mutants (Nit1 and NitM), derived from the same parent are unable to complement one another, due to the inability of their hyphae to anastomose (Correll, Gordon & McCain, 1988). This phenomenon had been found previously with *Verticillium* (Correll *et al.*, 1988; Joaquim & Rowe, 1990) and *Fusarium* species (Correll, Klittich & Leslie, 1987; Jacobson & Gordon, 1990). Since the self incompatible isolate (UG-1) has been stored over a long period of time (I.M.I. accession in 1962), self-incompatibility might be a result of a prolonged storage in culture, as suggested by Jacobson & Gordon (1990). In contrast, isolate EGP was not self incompatible, but was unable to form heterokaryons

with complementary mutants from the tester strains. This isolate should be tested again and may well be assigned to a new VCG.

Differences concerning the geographical and pathogenic distribution of VCG's of *V. dahliae* have been found (Joaquim & Rowe,1991; Strausbaugh *et al.*, 1992). Joaquim & Rowe (1991) revealed the presence of three VCG's within *V. dahliae* isolates from potato fields in Ohio; most isolates in VCG-4A were significantly more virulent than those in VCG's 2 and 4B, which led to the recognition of two different pathotypes causing severe or mild symptoms on potato cv. 'Superior'. Strausbaugh *et al.* (1992) found that, among strains from potatoes in California, those from the Tulelake region were in VCG-4, whereas strains from the Bakersfield region were in VCG-1. In the current study, all cocoa isolates from Brazil were assigned to VCG-2 and the Colombian isolate to VCG-4. The fact that COL-1 induced severe symptoms, including defoliation on both, Pound-7 and ICS-1, may indicate that distinct pathotypes exist in Colombia and Brazil. However, some variation in pathogenicity was also detected between isolates from the states of Bahia (BA-3) and Espirito Santo (ES-1). Although in the same VCG (VCG-2), isolates from these states fell into different sub-groups within this VCG, based on Jaccard's similarity coefficients. Such sub-groups within VCG-2 were previously detected by Strausbaugh *et al.* (1992) when comparing percentages of similarity between isolates. New isolates from cocoa will be included in future complementary tests, and it is

likely that more VCG's within *Verticillium* populations will be identified, as more isolates are examined (Correll *et al.*, 1988).

In the light of the degree of VCG complexity that has been observed in other vascular wilt fungi (Ploetz & Correll, 1988; Jacobson & Gordon, 1990; Correll, 1991; Elias & Schneider, 1991), it was not surprising in this study, to allocate some race-1 and race-2 isolates from tomato to the same group (VCG-2). The occurrence of two races from diverse locations (Australia, Greece, U.K. and U.S.A.) in the same VCG, only indicates that genetic determinants responsible for host specificity can exist within genetically isolated populations. Also, many authors (e.g. Bosland & Williams, 1987; Ploetz & Correll, 1988; Jacobson & Gordon, 1990) have found no consistent correlation between VCG's and races of various formae speciales of *Fusarium oxysporum*. Correll (1991) presented models to explain the race-VCG diversity identified so far, and based on these models he argued that virulence and VCG's phenotypes are evolving independently one from another. Clearly, more isolates and additional genetic markers such as restriction fragment length polymorphisms (RFLP) and/or polymerase chain reaction (PCR) need to be examined in order to determine genetic and pathogenic relationships within and between VCG's.

7. BIOCHEMICAL MECHANISMS INVOLVED IN THE RESISTANCE OF COCOA TO VERTICILLIUM WILT.

7.1. INTRODUCTION.

Genetic resistance, conferring the ability of the host to prevent a pathogen from becoming established, is considered the ideal method of controlling many diseases in cultivated plant species, especially fungal wilt diseases of perennial crops. Consequently, many researchers have attempted to determine the mechanisms that are controlled by the genetic system, aiming to facilitate the development and use of resistant cultivars (Bell & Mace, 1981; Tenkouano, Miller, Hart, Frederiksen & Nicholson, 1993).

In order to understand the possible mechanisms of resistance to vascular wilt diseases, it is essential to consider the environment in which vascular pathogens operate. Two sets of events have to take place before a vascular wilt disease can develop: The pathogen must gain access to the xylem of the host (usually following penetration of the roots) and it must continue to colonize the xylem more or less extensively. Even if these pre-requisites are fulfilled, the disease may not develop, because other circumstances may not be conducive to symptom expression (Beckman & Talboys, 1981). The mechanisms of resistance to vascular diseases can often be related to the occurrence of structural or anatomical barriers, or to the presence of inhibitory compound(s) within the tissues of the host. In addition, the broad categories of structural and chemical defence are often

subdivided according to whether a particular mechanism is operative before infection (constitutive, passive or pre-infectional resistance) or develops as a direct consequence of physiological interaction between the plant and the potential pathogen (induced, active or post-infectional resistance). A third and somewhat intermediate division, can be regarded as semi-constitutive resistance. (Ingham, 1973).

Several hypotheses have been presented to explain resistance to Verticillium wilts, whether based on anatomical or biochemical mechanisms. For example, Talboys (1958) reported that in hops, the formation of mechanical barriers (including tyloses), occurred more frequently in roots of tolerant than of susceptible cultivars. On the biochemical side, high concentrations of constitutive phenolic compounds have been often correlated with host resistance to Verticillium wilt; e.g. chlorogenic acid in potatoes (Patil, Powelson, & Young, 1964) and tannins in cotton (Bell, 1991). Induced low-molecular weight antifungal compounds or phytoalexins have been widely studied in member of the families Leguminosae, Solanaceae and Malvaceae (Bailey & Mansfield, 1982) and have also been implicated in the resistance of certain crops to Verticillium wilt, such as alfalfa, tomato and cotton (Bell, 1969; Khan & Milton, 1978; Bailey & Mansfield, 1982; Mace, 1989; Mace, Stipanovic & Bell, 1990). Over 200 species of plants from at least fifteen families have been shown to produce phytoalexins and this substantial number *a priori* indicates that they are widely distributed,

physiologically important components of plant disease defence (Mansfield, 1983; Keen, 1990). Despite detailed studies in certain species, a very large proportion of the plant kingdom still awaits investigation (Bailey, 1987).

Host defence to vascular wilt pathogens is very often composed of multiple mechanisms and these appear to occur concomitantly or interactively in time and space (Bell & Mace, 1981; Mace, 1989; Bell, 1992). In cotton, morphological differences alone were not adequate to explain the distinct levels of resistance presented by varieties or species of *Gossypium* to Verticillium wilt (Garber & Houston, 1966, 1967). Bell (1969) reported that the resistance of cotton cultivars to *V. dahliae* appeared to be determined by the speed of formation of phytoalexins in the infected tissue. The more recent version to explain this resistance, is that it is dependent on the sequential, co-ordinated formation of physical occlusion (gels, gums and tyloses) and phytoalexins in xylem vessels, plus lignins and tannins in xylem walls and surrounding xylem ray cells (Bell, 1992).

Very little is known of the biochemical mechanisms of resistance of cocoa to diseases. Capriles de Reys, Schulz & Munoz (1964) and Capriles de Reys & Reys (1968) screened a wide range of phenolic compounds, possibly related to the resistance of cocoa cultivars to *Ceratocystis fimbriata*. Capriles de Reys *et al.* (1964) found in the resistant cultivar IMC-67, a higher polyphenolic content than in the susceptible OC-61. The chlorogenic acid content in this resistant cultivar was

two times greater than in the susceptible one; however, its level in resistant cocoa plants was still well below levels required for toxicity *in vitro*. Capriles de Reys & Reys (1968) demonstrated the presence of other phenolic compounds occurring in higher levels in this resistant cultivar, whether plants had been inoculated or not. Amongst these compounds, gentisolyl glycoside, a gentisic acid ester, was detected at high fungitoxic levels in the stems of IMC-67 plants. In a search for a *in vitro* method for assessing resistance of cocoa plants to *Crinipellis perniciosa*, Evans & Bastos (1980) examined the effects of alcoholic extracts of cocoa flush tissues on basidiospores in culture. Extracts from more resistant cocoa clones completely inhibited basiodiospore germination; extracts from susceptible clones induced shorter and swollen germ tubes compared to those produced in standard agar media. The antifungal activity present in these tissues was later associated with a high molecular weight procyanidin fraction, also known as condensed tannin (Brownlee, McEuen, Hedger & Scott, 1990). This widespread and abundant class of polyphenols, have a range of molecular structures consisting of two or more flavan-3-ol units. Brownlee, Hedger & Scott (1992) compared the fungitoxic activity of a range of procyanidins (dimer, trimer and polymer) and monomeric flavan-3-ols (catechin and epicatechin) to *C. perniciosa*; they found an increase in antifungal activity with increasing molecular weight of the compounds tested. The biochemical mechanisms of resistance of cocoa to *Phytophthora* remain unresolved; a

post-infectional defence compound or phytoalexin has been isolated, but not characterized, from inoculated stems of *Theobroma grandiflora*, a species which shows resistance to *P. palmivora* (Daguenet & Parvais, 1981). In relation to Verticillium wilt of cocoa, there is virtually no information on the mechanisms controlling the resistance of the few cultivars already selected (Braga & Silva, 1989; CEPLAC, 1991). The main aim of this chapter was the study of potential biochemical mechanisms of resistance, either constitutive or induced, of *T. cacao* to *V. dahliae*.

7.2. RESULTS.

7.2.1. Estimation of the condensed tannin content of resistant and susceptible cultivars.

Based on the literature available on mechanisms of resistance of cocoa to diseases, it was initially hypothesized, that the antifungal compounds involved in the resistance of cocoa to *V. dahliae* would be tannins or other phenolic compounds. In the first experiment based upon these possibilities, forty cocoa seedlings from each of the cultivars ICS-1, Pound-7 and West African Amelonado were stem puncture inoculated with *V. dahliae* (10^7 conidia/ ml, 20 punctures/ plant). Twenty plants of each cultivar remained as unpunctured controls and the same number of plants received similar punctures but with no inoculum. Twenty days after puncture, the stems of half of the inoculated and all punctured control plants from each

cultivar were harvested, processed and stored at -80°C. The unpunctured controls and the remaining twenty inoculated plants from each cultivar were harvested after 60 days. Tannin extraction was carried out at the same time for all treatments, based on groups of five plants per replicate. The levels of condensed tannins was estimated by the absorbance at 545 nm of cyanidin extracted into n-butanol following the acid treatment of the samples (Bate-Smith, 1973; Harborne, 1984).

A build-up in tannin content was detected from 3 weeks after stem puncture, whether plants had been inoculated or not. However, no significant difference among cultivars was detected at any of the dates assessed (Table 7.1.). Fifty microlitres of the hydrolysed tannin solution from each sample was spotted on TLC plates and run in a mixture of diethyl ether : methanol (6:1). Inhibitory spots where *V. dahliae* did not grow on the plates were detected for all treatments but only at the point of application of the solution, indicating no movement of the toxic compound(s) (Plate 7.1.). No significant difference in the size of the inhibitory zones produced by each treatment was observed; however, spots from samples collected 60 days after inoculation presented more intense red colour than those from samples harvested earlier (20 days), confirming the accumulation of tannins after puncture.

Table 7.1. Condensed tannin content of cocoa stem samples, based on the measurement of absorbance at 545nm (adapted from Bate-Smith, 1973; Harborne, 1984).

TREATMENT/ CULTIVAR	WITHOUT STEM PUNCTURE	TIME AFTER STEM PUNCTURE 20 days CONTROL	20 days INOCULATED	60 days INOCULATED
ICS-1	366 aA*	520 aA	580 aAB	773 aB
Pound-7	506 aA	693 aAB	706 aAB	880 aB
W.A.A.**	480 aA	710 aAB	746 aAB	886 aB

* Values (in $\mu g/g$) are means of four determinations, based on bulked extracts of five plants each. Means followed by the same low-case letter in each column or by the same capital letter in each row are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

** West African Amelonado.

Plate 7.1. TLC plate showing the effect of hydrolysed tannin extracts from different cocoa cultivars on *V. dahliae* growth. Fifty microliters of the following butanolic red solutions were spotted at the bottom of this plate, before developing in a mixture of diethyl ether : methanol (6:1):

60D-I: 60 days after inoculation, cultivar ICS-1

60D-P: 60 days after inoculation, cultivar Pound-7

60D-A: 60 days after inoculation, cultivar West African Amelonado.

20D-I: 20 days after inoculation, cultivar ICS-1

20D-P: 20 days after inoculation, cultivar Pound-7

20D-A: 20 days after inoculation, cultivar West African Amelonado.

Note a visual increase in colour intensity of the spots produced by the samples collected 60 days after inoculation, compared to those harvested 20 days after inoculation.

60D-I 60D-P 60D-A 20D-I 20D-P 20D-A

Samples of hydrolysed tannin extracts from cultivars Pound-7 and ICS-1 (inoculated and unpunctured control treatments) harvested 60 days after inoculation, were submitted to a dilution series in n-butanol (2, 4, 7 and 10 fold) before toxicity was checked by the slide bioassay with *V. dahliae*. No differences in terms of spore germination or germ tube length were detected between treatments within each dilution, whether plants had been inoculated or not. For all treatments, the toxic effects of these extracts remained above 1/7 dilution in relation to germ tube length, or above 1/4 dilution, considering the percentage of germination (Tables 7.2. and 7.3.). Highly concentrated extracts completely inhibited conidial germination while more dilute extracts resulted in shorter and swollen germ tubes, compared to those produced in the water control treatment (Plate 7.2.).

Thus, the increased condensed tannin levels found in cultivars ICS-1 and Pound-7, 60 days after inoculation compared to the respective unpunctured controls (Table 7.1.), did not correspond to a higher toxicity to *V. dahliae* (Tables 7.2. and 7.3.). Also, no significant differences in relation to both tannin content (Table 7.1.) and toxicity to *V. dahliae* (Tables 7.2. and 7.3.), were evident between susceptible and resistant cultivars.

Table 7.2. Effect of hydrolysed tannin extracts from two cultivars of cocoa on the germ tube length of *V. dahliae* conidia.

CULTIVAR/ TREATMENT	GERM TUBE LENGTH (μm) / DILUTIONS 1/2X	1/4X	1/7X	1/10X
POUND-7 INOCULATED	0.00**b	0.75 b	7.25 b	18.02 a
ICS-1 INOCULATED	0.00 b	1.38 b	10.85 b	19.05 a
POUND-7 CONTROL*	0.00 b	1.25 b	11.43 b	21.35 a
ICS-1 CONTROL*	0.00 b	2.43 b	13.82 b	21.10 a
WATER CONTROL	21.18 a	21.18 a	21.18 a	21.18 a
SOLVENT CONTROL	22.52 a	22.52 a	22.52 a	22.52 a

* Unpunctured control treatment.
** Values represent the means of 4 replicates. Within each column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

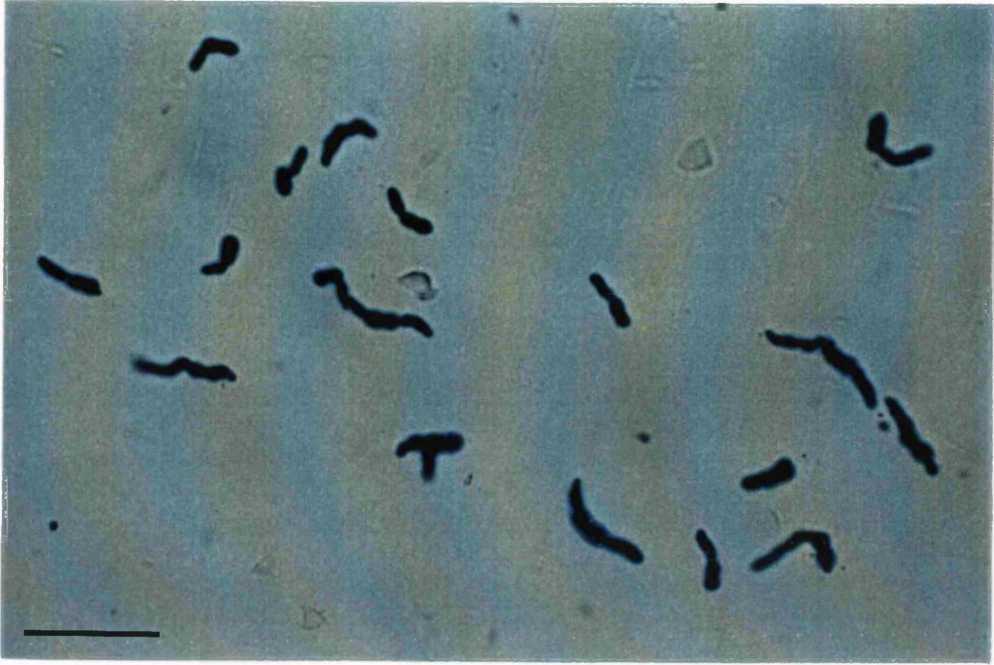
Table 7.3. Effect of hydrolysed tannin extracts from two cultivars of cocoa on the percentage of germination of *V. dahliae* conidia.

CULTIVAR/ TREATMENT	GERM TUBE LENGTH (μm) / DILUTIONS 1/2X	1/4X	1/7X	1/10X
POUND-7 INOCULATED	0.00**b	8.75 b	88.00 a	90.70 a
ICS-1 INOCULATED	0.00 b	11.00 b	97.50 a	97.00 a
POUND-7 CONTROL*	0.00 b	14.50 b	95.50 a	99.00 a
ICS-1 CONTROL*	0.00 b	15.75 b	98.00 a	97.50 a
WATER CONTROL	96.50 a	96.50 a	96.50 a	96.50 a
SOLVENT CONTROL	98.00 a	98.00 a	98.00 a	98.00 a

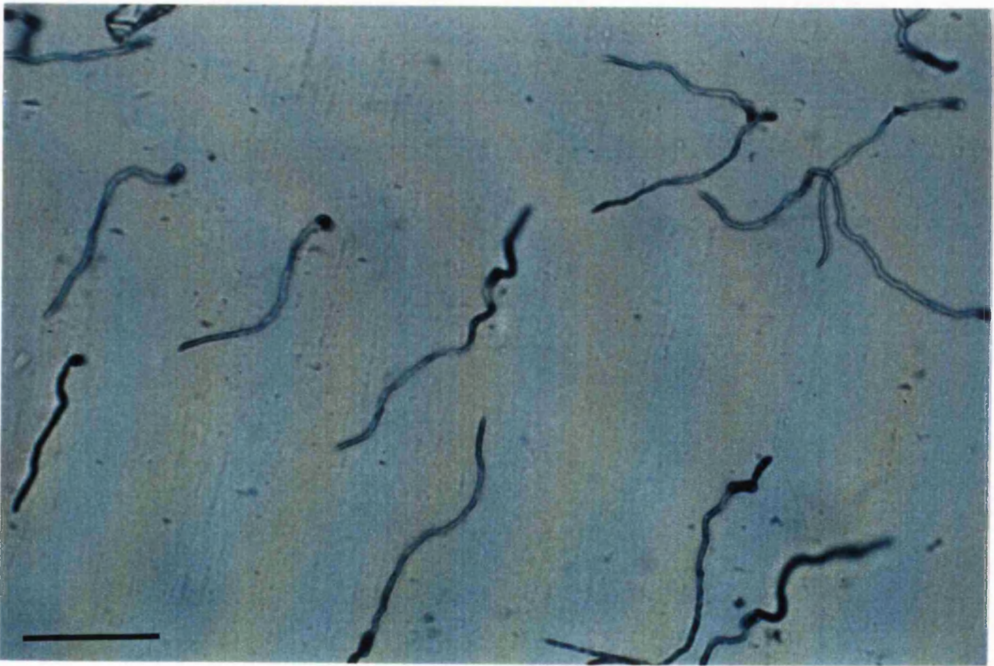
* Unpunctured control treatment.
** Values represent the means of 4 replicates. Within each column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Plate 7.2. Germinating conidia of *V. dahliae*, after incubation for 15 hours at 25°C: a) swollen and shorter germ tubes produced in the presence of hydrolysed tannin extract from cocoa (1/7X dilution); b) normal germ tubes produced in sterile distilled water (pH 7.0). Photomicrographs were taken after staining with a 0.1% solution of cotton blue in lactophenol. (Bar markers represent 20μm).

a



b



7.2.2. Toxicity of a range of procyanidins to V. dahliae conidia.

The diverse class of procyanidins or condensed tannins in plant tissues are a mixture of structures consisting of various numbers of linked flavan-3-ol units. The major lower flavonoids of *T. cacao* are catechin, epicatechin, the dimers B-1 and B-2 and the trimer C-1 (Figure 7.1., adapted from Porter, Ma & Chan, 1991). The following pure procyanidin compounds, (kindly supplied by Prof. E. Haslam, Department of Chemistry, University of Sheffield, U.K.), were assayed for toxicity to *V. dahliae* conidia on cavity slides: Epicatechin, molecular weight (mol. wt.)= 290; dimer B-2, mol. wt.= 578, from *Crataegus monogyna*; trimer, mol. wt.= 862-864, from *Calluna vulgaris*; polymer, mol. wt. approx. 1700-2000 from *Calluna vulgaris*. A two-fold dilution series, ranging from 1000 μg/ml to 62.5 μg/ml, was prepared dissolving the compounds in a mixture of methanol and water (1:1, v/v).

All procyanidins tested inhibited the germination and growth of *V. dahliae*, depending on concentration. Considering the effect on conidial germination (Table 7.4.), significant toxicity in relation to the water control was evident at concentrations ≥1000 μg/ml for the monomer and dimer and ≥500 μg/ml for the trimer and polymer. However, against germ tubes (Table 7.5.), significant reduction in growth in relation to the water control was found at concentrations ≥500 μg/ml for the monomer and dimer, ≥250 μg/ml for the trimer and ≥125 μg/ml for the polymer. Thus, inhibition of germ tube growth was a more sensitive parameter than inhibition of

germination and revealed a tendency for the larger molecules to be more toxic to *V. dahliae* than the smaller ones. The effective concentrations, which reduced germination or germ tube growth by 50% (ED_{50}) and inhibited germination completely (ED_{100}), were determined by linear regression, transforming the concentrations data in Log_{10} (Table 7.6.). ED_{50}s for germ tube length were always lower than ED_{50}s for germination and ED_{100}s decreased with increasing molecule sizes, which confirmed the trends observed in Tables 7.4. and 7.5.

Figure 7.1. Procyanidins and monomeric flavan-3-ols commonly found in *Theobroma cacao* L.

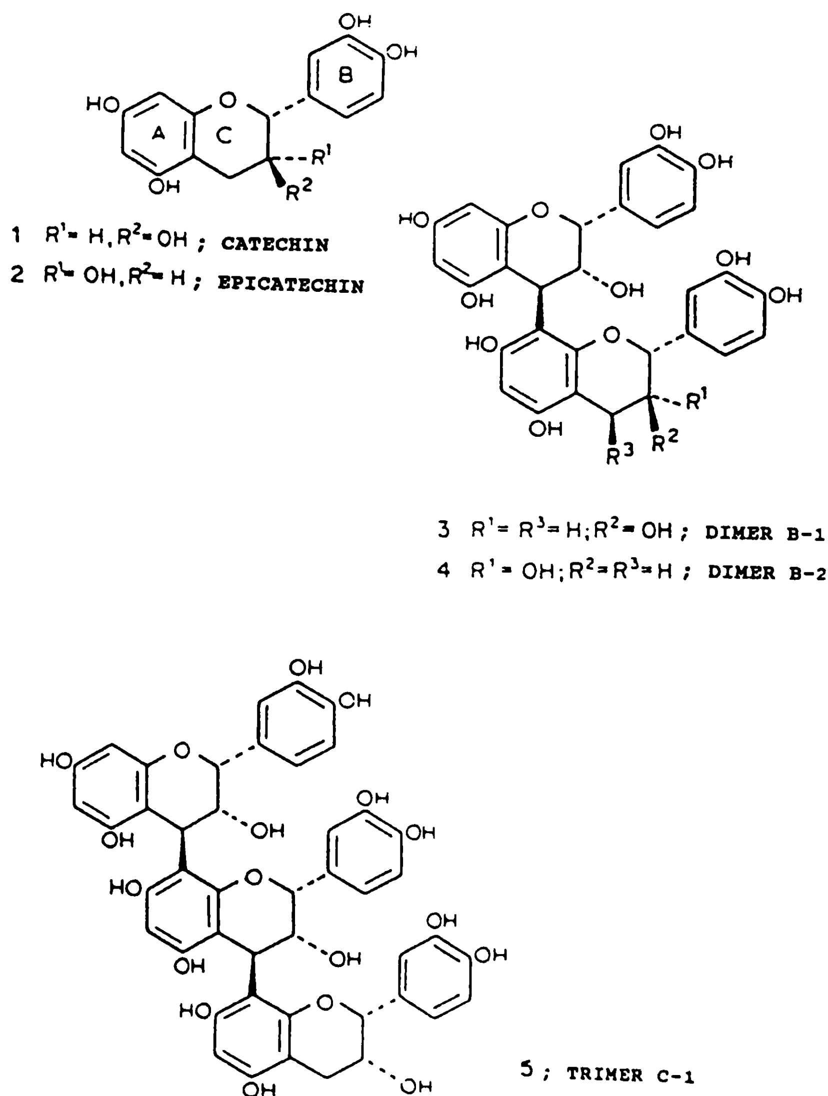


Table 7.4. Effect of different sizes of procyanidin molecules on the percentage of germination of *V. dahliae* conidia.

PROCYANIDIN*	CONCENTRATIONS ($\mu g/ml$) 1000	500	250	125	WATER CONTROL
Monomer	43.50**b	83.30ab	93.75a	98.00a	97.50a
Dimer B2	36.20b	89.75a	93.50a	97.50a	97.50a
Trimer	0.50c	24.50b	89.50a	97.75a	97.50a
Polymer	1.00c	23.00b	91.75a	96.25a	97.50a

* Procyanidin sources: Monomer= Epicatechin from Sigma Co., dimer B2 from *Crataegus monogyna*, trimer and polymer from heather (*Calluna vulgaris*).

** Values represent the means of 4 replicates. Means followed by the same letter in each row are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Table 7.5. Effect of different sizes of procyanidin molecules on the germ tube length of *V. dahliae* conidia.

PROCYANIDIN*	CONCENTRATIONS ($\mu g/ml$) 1000	500	250	125	WATER CONTROL
Monomer	4.57**b	8.90b	14.29ab	18.12a	28.40a
Dimer B2	2.80c	13.12b	18.82ab	22.12a	28.40a
Trimer	0.05c	2.17c	17.75b	28.92a	28.40a
Polymer	0.05c	1.96c	15.57b	17.12b	28.40a

* Procyanidin sources: Monomer= Epicatechin from Sigma Co., dimer B2 from *Crataegus monogyna*, trimer and polymer from heather (*Calluna vulgaris*).

** Germ tube length values in μm, representing the means of 4 replicates. Means followed by the same letter in each row are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Table 7.6. Toxicity of the procyanidins compounds to *V. dahliae* conidial germination and germ tube growth, in terms of ED_{50} and ED_{100} estimated by linear regression.

PROCYANIDIN+ (µg/ml)	**ED_{50}* GERMINATION (µg/ml)**	**ED_{50}* GERM TUBE LENGTH (µg/ml)**	**ED_{100}** GERMINATION (µg/ml)**
Monomer	954.99	237.13	3677.35
Dimer B2	776.25	354.81	2754.23
Trimer	379.75	304.43	909.91
Polymer	382.82	208.93	903.65

+ Procyanidin sources: Monomer= Epicatechin from Sigma Co., dimer B2 from *Crataegus monogyna*, trimer and polymer from heather (*Calluna vulgaris*).

* ED_{50}= Effective concentration of the compound, which reduced germination or germ tube growth of *V. dahliae* conidia by 50%.

** ED_{100}= Effective concentration of the compound, which completely inhibited germination of *V. dahliae* conidia.

7.2.3. Selection of a solvent for optimal extraction of phytoalexins from cocoa stems.

Since no significant differences in tannin content were detected between susceptible and resistant cultivars, the extraction of other possible antifungal compounds was considered. Four organic solvents with increasing polarities (petroleum ether, diethyl ether, ethyl acetate and methanol) were tested to extract antifungal substances from cv. Pound-7 (0, 30 and 75 hours after inoculation).

Any residues from these solvents left on the cavity slides after evaporation had no toxic effect on germination or germ tube growth of *V. dahliae* (Table 7.7.). There was also no significant inhibition of spore germination or growth in extracts from cocoa plants prior to inoculation. However, beyond 30 hours after inoculation, significant reductions of germination and germ tube length were demonstrated in plant tissues extracted with diethyl ether. By contrast, methanol apparently extracted some substances stimulatory for mycelial growth, as demonstrated by the longer germ tubes produced in all extracts. Diethyl ether was therefore selected for use in further extractions.

Table 7.7. Effect of different solvents on the extraction of antifungal compounds effective against *V.dahliae*, from cocoa stems, cv. Pound-7.

		BEFORE INOCULATION		TIME AFTER INOCULATION 30 HOURS		75 HOURS	
	SOLVENT+	GERMN.* (%)	GERM TUBE* (μm)	GERMN. (%)	GERM TUBE (μm)	GERMN. (%)	GERM TUBE (μm)
PLANT EXTRACT	P. E.	98.00**a	21.95b	97.75a	46.50a	96.25ab	49.37ab
	D. E.	78.50a	14.82b	27.75c	3.21c	37.25c	3.99d
	E. A.	88.00a	17.87b	97.75a	42.52a	96.50ab	36.05b
	MET.	99.00a	52.50a	98.50a	58.10a	99.25a	65.90a
CONTROLS	WATER	93.25a	20.50b	94.25b	23.37b	87.25b	18.28c
	P. E.	92.25a	21.50b				
	D. E.	87.00a	20.87b				
	E. A.	92.25a	23.31b				
	MET.	88.00a	21.87b				

+ P.E. stands for petroleum ether, D.E. for diethyl ether, E.A. for ethyl acetate and MET. for methanol.
* GERMN.(%)= percentage of germination and GERM TUBE (μm)= germ tube length.
** Values represent the means of 4 replicates. Within each column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

7.2.4. Temporal accumulation of phytoalexins in the stems of cultivar Pound-7, following inoculation.

The standard of resistance (cultivar Pound-7) was stem puncture inoculated with *V. dahliae* (10^7 conidia/ ml, 20 punctures/ plant). Samples from the base of the stem were harvested 0, 5, 8 and 11 days after inoculation and immediately stored at -80°C. A control without punctures (day zero) and a control with punctures (day 11) were also included in the experiment. Extractions procedures and germination tests were carried out at the same time for all samples, after harvesting and freezing the last samples (11 days).

A gradual decrease in germination and germ tube length was detected from 0 to 11 days post-inoculation, demonstrating an increasing antifungal activity of Pound-7 extracts with time (Table 7.8.). When compared with the water control treatment, it was again demonstrated that antifungal activity was not present at significant levels in plants prior to inoculation or wounding; however accumulation was stimulated by either, inoculation or wounding. Fifty microliters of each crude extract sample were also spotted on analytical silica gel thin-layer chromatography (ATLC) plates, run in diethyl ether : methanol (6:1) and when dried, the plates were sprayed with *V. dahliae* conidia. Inhibitory zones were observed 48 hours after spraying, only for those samples collected 5 or more days after puncture (Plate 7.3.). These substance(s), which appear only after inoculation or

physical stress, fit into the broad definition of phytoalexins (Paxton, 1981).

Table 7.8. Accumulation of compounds toxic to *V.dahliae* in cocoa stems, cultivar Pound-7, expressed by percentage of germination of conidia, germ tube length and germ tube length reduction in relation to the water control treatment.

DAYS AFTER PUNCTURE	TREATMENT	GERMINATION (%)	GERM TUBE LENGTH (μm)
0	(CONTROL*)	94.25*** a	38.35 a
5	INOCULATED	61.00 b	16.12 b
8	INOCULATED	28.50 c	4.09 c
11	INOCULATED	4.25 d	0.46 d
11	(CONTROL**)	6.25 d	0.81 d
--	WATER CONTROL	97.50 a	48.35 a

* Control without punctures; ** Control with punctures (20 punctures/ plant).

*** Values represent the means of 4 replicates. Within each column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Plate 7.3. TLC plate showing the effect of crude extracts from cultivar Pound-7 on *V. dahliae* growth after a 2-day incubation period in a humid chamber at 25°C. Fifty microliters of the following extracts dissolved in methanol were spotted at the bottom of this plate, before running in diethyl ether : methanol (6:1):

0: Control without punctures.
5: Five days after inoculation.
8: Eight days after inoculation.
11 I: Eleven days after inoculation.
11 C: Control, eleven days after puncture.

Note that only extracts from inoculated or punctured plants produced inhibitory spots at the top of the plate (DG= dark grey spot under UV light).

DG DG DG DG

0 5 8 11 11

I C

7.2.5. Temporal accumulation of phytoalexins in susceptible and resistant cultivars.

Having detected significant accumulation of antifungal compounds over time in the resistant Pound-7, the next approach was to compare the level of accumulation in this cultivar to that of the susceptible cultivar ICS-1. Plants which were stem puncture inoculated with *V. dahliae* (10^7 conidia/ ml, 20 punctures/ plant) had the base of their stems harvested at successive days (3, 6, 10 and 15 days) following inoculation and were preserved in a deep freezer (-80°C). Unpunctured controls were harvested at day zero and stored in the same way. All samples were submitted to extraction and toxicity tests, at the same time.

Samples from crude extracts were diluted two and five times for the slide bioassays with *V. dahliae*. Two times diluted samples from stems of Pound-7 significantly reduced the germination and elongation of *V. dahliae* conidia, compared to ICS-1 extracts or to distilled water only (Tables 7.9. and 7.10.). Differences between extracts from Pound-7 and ICS-1 expressed by reduction of germ tube length were detected from 6 days after inoculation, and differences in percentage of germination of conidia from 10 days onwards. Maximum accumulation of antifungal compounds as indicated by these parameters, apparently occurred 15 days after inoculation on cultivar Pound-7. Cultivar ICS-1 probably accumulated much less phytoalexins than Pound-7 during this period of time, since only slight, not statistically significant reductions in germination and germ tube length were detected for ICS-1

extracts post-inoculation, compared to the water control treatment. However, all differences between treatments disappeared when 5-fold diluted samples were compared.

Table 7.9. Accumulation of antifungal compounds in crude extracts from cocoa stems, cultivars Pound-7 and ICS-1, expressed by the germ tube length of *V. dahliae* conidia.

CULTIVAR/ TREATMENT	---BEFORE--- --INOCULATION--	-----TIME 3 DAYS	AFTER 6 DAYS	INOCULATION----- 10 DAYS	15 DAYS
POUND-7	17.92 a*	11.29 a	9.72 b	8.83 b	5.03 b
ICS-1	20.53 a	16.75 a	17.84 a	15.41 a	13.82 a
WATER CONTROL	19.85 a	19.85 a	19.85 a	19.85 a	19.85 a

* Germ tube length values in μm, representing the means of 4 replicates. Within each column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Table 7.10. Accumulation of antifungal compounds in crude extracts from cocoa stems, cultivars Pound-7 and ICS-1, expressed by the percentage of germination of *V. dahliae* conidia.

CULTIVAR/ TREATMENT	---BEFORE--- --INOCULATION--	-----TIME 3 DAYS	AFTER 6 DAYS	INOCULATION----- 10 DAYS	15 DAYS
POUND-7	98.50 a*	92.00 a	72.50 a	45.25 b	42.00 b
ICS-1	97.25 a	96.50 a	89.00 a	92.50 a	87.55 a
WATER CONTROL	96.75 a	96.75 a	96.75 a	96.75 a	96.75 a

* Values represent the means of 4 replicates. Within each column, means followed by the same letter are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

7.2.6. Detection and characterization of phytoalexins from cocoa stem crude extracts.

Different mixtures of solvents (solvent systems) were tested, aiming for a better separation for further characterization of the phytoalexins detected in the bioassays with crude extracts. Initially, a solvent mixture consisting of diethyl ether : methanol (6:1) was tested to run the samples on TLC plates. A single inhibition zone (mean Rf 0.90-1.00) was then detected 48 hours after spraying the plate with *V. dahliae* conidia. However, aiming to lower the Rf of the antifungal compound, three parts of petroleum ether were added to the mixture. This was achieved and it also greatly increased the separation of compounds that were co-running with the initial inhibitory zone. As a result, three new inhibition zones appeared on the plates sprayed with *V. dahliae* (Plate 7.4.). From now on in the text, these substances will be referred to as compounds 1, 2, 3 and 4, respectively from the least polar to the most polar one. A better separation of these compounds was achieved after modifications suggested by Prof. J.W. Mansfield (Wye College, University of London, Ashford, Kent, U.K.), personal communication), which resulted in a two-dimensional solvent system including diethyl ether : petroleum ether : methanol (6:3:1) in the first direction and chloroform : hexane : acetone (3:3:1) in the second one. The Rf values of compounds 1, 2, 3 and 4 separated in this 2D system are given in Table 7.11.

Plate 7.4. Analytical TLC plate showing the four inhibitory compounds as clear zones, after spraying with *V. dahliae* conidia and incubating for 5 days in a humid chamber at 25°C. Five hundred microliters of the crude extract dissolved in methanol were spotted at the bottom of this plate, before running in a mixture of diethyl ether : petroleum ether : methanol (6:3:1). 1= compound-1, 2= compound-2, 3= compound-3, 4= compound-4. Complete inhibition zones were caused by compounds 1, 2 and 4, in contrast with compound-3, which only resulted in weak inhibition of *V. dahliae* mycelial growth.

1
2
3
4

Specific colour reagents were tested on TLC plates, to improve the detection and characterization of the phytoalexins after observation under UV light (Table 7.12.). Compound-2, hardly visible as a pale grey spot under UV, could be visualized on the TLC plate, with iodine vapour. The other phytoalexins (compounds 1 and 4 visible as dark grey spots and compound-3 as a grey spot under UV), also developed the orange colouration with iodine. Colour reactions typical of phenolic compounds were not observed for the cocoa phytoalexins when the Folin-Ciocalteu reagent was sprayed on the TLC plates; no positive reaction was observed, even by fuming the plates with ammonia vapour inside a TLC tank, after the Folin-Ciocalteu application. However, when sprayed with Vanillin-H_2SO_4, a clear pink colour, indicative of phenolic compounds was observed for compound-3 and a dark blue colour indicative of terpenoids for compound-4 (Harborne, 1984) (Plate 7.5.). The putative terpenoid phytoalexin (number 4) could also be visualized by spraying the plate with water only, which revealed its high concentration and milky-white colour.

Table 7.11. Rf values of the phytoalexins from cocoa stems, in silica gel TLC plates.

SOLVENT SYSTEM	INHIBITORY POST-INFECTIONAL SUBSTANCE			
	COMPOUND-1	COMPOUND-2	COMPOUND-3	COMPOUND-4
1st (D.E.: P.E.:M*) (6 : 3 : 1)	0.73-0.80	0.42-0.50	0.35-0.40	0.21-0.30
2nd (C : H : A*) (3 : 3 : 1)	0.68-0.80	0.41-0.48	0.04-0.13	0.00-0.10

* Diethyl ether : petroleum ether : methanol (6:3:1) in the first direction and chloroform : Hexane : Acetone (3:3:1) in the second one.

Table 7.12. Colour reactions of the cocoa phytoalexins when submitted to different chemical reagents and UV light.

REAGENT	INHIBITORY POST-INFECTIONAL SUBSTANCE			
	COMPOUND-1+	COMPOUND-2	COMPOUND-3	COMPOUND-4
IODINE	ORANGE	PALE ORANGE	ORANGE	DARK ORANGE
VANILLIN-H_2SO_4*	-----	-----	PINK	DARK BLUE
FOLIN-CIOCALTEU**	-----	-----	-----	-----
UV LIGHT (254nm)	DARK GREY	PALE GREY	GREY	DARK GREY

+ Colour under UV light (254 nm): Compound-1= dark grey, compound-2= pale grey, compound-3= grey, compound-4= dark grey.

*Specific for phenolic and terpenoid compounds.

**Specific for phenolic compounds.

----- No visible colour reaction.

Plate 7.5. Colour reactions displayed by compounds 3 and 4 after spraying with the Vanillin-H_2SO_4 reagent. Fifty microliters of partially purified compounds 3 and 4 were applied individually and side by side at the bottom of this plate, before running in a mixture of diethyl ether : petroleum ether : methanol (6:3:1). Two strips, each one 2cm wide, were cut from the lateral sides of the plate, sprayed with this reagent and heated in an oven at $80^{o}C$ for 5 minutes. Shortly after, these strips were matched to the original plate already colonized by *V. dahliae* and the photograph was taken. Note the pink colour developed for compound-3 and the dark blue colour for compound-4.

3
4

7.2.7. Large scale separation and purification of cocoa phytoalexins.

Initially, a column of 6.0 cm diameter and 15 cm depth of silica gel (60-120 mesh, from Fisons p.l.c., Loughborough, Leicestershire, U.K.), was used to elute a sample of 12.5 g of dried, ether-soluble material previously extracted from 2,280 g of fresh plant material in a Soxhlet apparatus. Mixtures of diethyl ether : petroleum ether (2:1) followed by diethyl ether : petroleum ether : methanol (6:3:1) were utilized as eluents. Pressure was applied from a manual pump to give a driven flow rate of approximately 5cm/ min and fractions of 50 ml were collected each time in small flasks. Monitoring of these fractions was performed on aluminium backed ATLC plates, using diethyl ether : petroleum ether : methanol (6:3:1) as solvent system to run the 50 μl samples applied. After evaporating the solvent mixture, these plates were sprayed with *V. dahliae* conidia and incubated for 48 hours at 25°C. Based on the inhibition zones detected on them (e.g., Plate 7.6.), those flasks containing each one of the four inhibitory substances were chosen, and had their contents bulked and concentrated under vacuum by rotary evaporation at 35°C. Further purification was attempted for each of the phytoalexins in narrower columns (2cm and 1cm diameter, successively), collecting fractions of 10ml and 5ml respectively, and monitoring each time as described above. In the 2 cm diameter column, hexane was used as eluent for compound-1, petroleum ether : ethyl acetate (3:2) for compound-2,

petroleum ether : ethyl acetate (1:1) for compound-3 and a succession of chloroform : hexane : acetone (3:3:1) and diethyl ether : petroleum ether : methanol (12:3:2) for compound-4. In the 1cm diameter column, hexane was used for eluting compound-1, chloroform : hexane : acetone (3:3:1) for compounds-2 and 3, and hexane : acetone (3:2) for compound-4. After eluting from the 1cm column for each compound and monitoring for fungitoxicity, samples of the inhibitory substances were spotted on ATLC plates and run successively in chloroform : hexane : acetone (3:3:1), hexane : acetone (3:2) and diethyl ether : petroleum ether : methanol (6:3:1). Flasks containing the substances that produced single bands after running the plates in all these three solvents and monitoring for purity under UV light and in iodine vapour, were considered pure phytoalexins and therefore rotary evaporated to dryness and kept in a freezer at -80°C for further assays. Compound-4 was considered pure at this stage, but compounds 1, 2 and 3 still needed further purification. Compound-1, which was a mixture of fatty acids and other substances at this stage, was dissolved in diethyl ether and shaked in a separation funnel with a 2M aqueous solution of sodium hydroxide, aiming to saponify the fatty acids. These immiscible solutions were then collected in different flasks for monitoring. Compound-1 was retained in the ether fraction. Compounds 1, 2 and 3 were then submitted to preparative thin-layer chromatography on fluorescent 1mm thick silica gel plates with a 4 cm wide magnesium sulphate concentration zone (E. Merck,

Darmstadt, Germany). After reassessing the efficacy of all solvent systems already tested during the previous steps of the separation process, hexane was chosen for developing compound-1, chloroform : hexane : acetone (3:3:1) for compound-2 and hexane : acetone (3:2) for developing compound-3 on these TLC plates. A one inch wide strip was cut from each plate and sprayed with *V. dahliae* for monitoring the toxicity of these compounds. By matching these strips to the original plates, the phytoalexins could be detected as single bands on silica gel, and then scraped off from the plates. These substances were recovered from the silica gel samples by eluting with diethyl ether; the liquid phase was collected in small flasks, monitored for purity, then dried and stored as already described.

Figure 7.2. shows a diagram of the whole purification procedure, as well as the amounts obtained at the initial and final stages of the process. Based on these data and on the assumptions that purity was achieved and no losses occurred during the process, the amount of each compound in mg/ kg of fresh weight of stem tissue (or ppm), 15 days after inoculation, was estimated as: Compound-1: 39.3 ppm; compound-2= 1.9 ppm, compound-3= 4.9 ppm and compound-4= 167.9 ppm.

Plate 7.6. Monitoring system for the phytoalexins from cocoa in silica gel TLC plates. After running the crude extracts in a flash chromatography column, 50 μl samples from each flask were spotted side by side on the bottom of the plate. Diethyl ether : petroleum ether : methanol (6:3:1) was used as solvent system to develop the plate, which was thereafter sprayed with *V. dahliae* conidia and incubated for 48 hours at 25°C. This picture represents the monitoring of a 6 cm diameter column product, where mixtures of diethyl ether : petroleum ether (2:1) followed by diethyl ether : petroleum ether : methanol (6:3:1) were utilized as eluents. Compounds 2, 3 and 4 were recovered in flasks numbered from 12 to 25 (note the distribution of the inhibitory spots where *V. dahliae* did not grow).

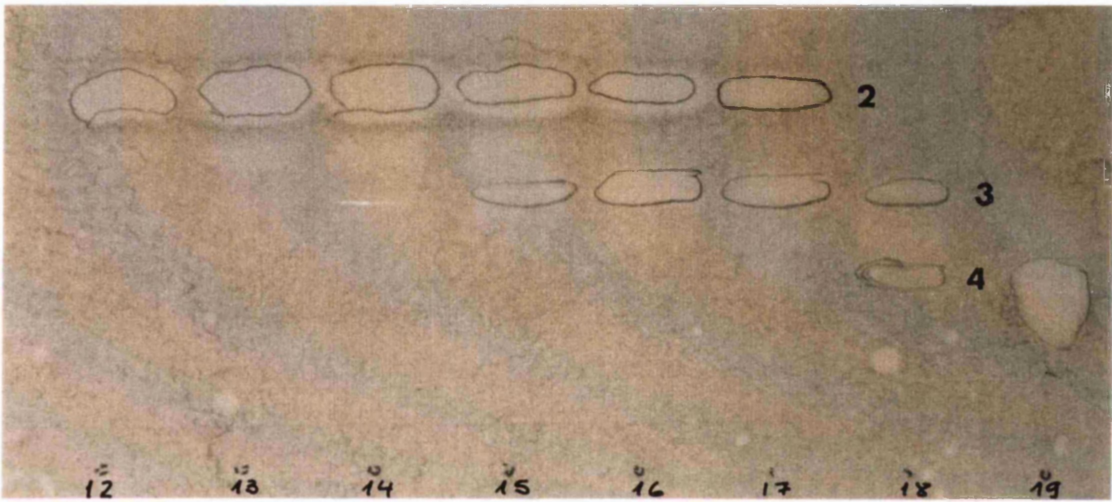

2
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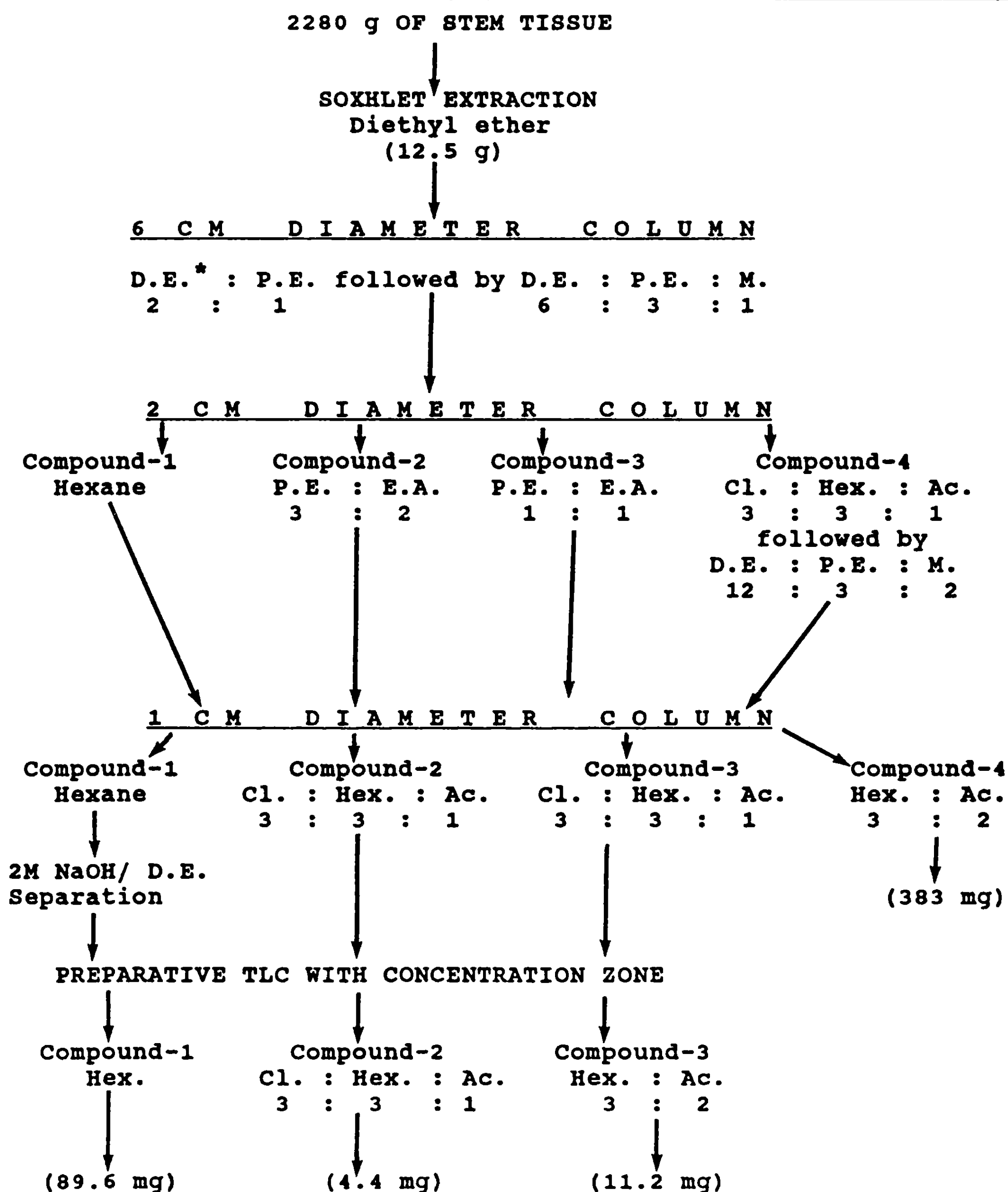
Figure 7.2. Diagramatic representation of the large scale procedure for separation and purification of phytoalexins from cocoa, including the amounts obtained (in milligrams) for each compound.



* D.E.= Diethyl ether, Ac.= Acetone, Cl.= Chloroform, E.A.= Ethyl acetate, Hex.= Hexane, M.= Methanol, P.E.= Petroleum ether.

7.2.8. Identification of the phytoalexins from cocoa.

Samples obtained from the large scale purification process were used for this purpose. Compounds 2 and 3 were identified by Dr. M. G. Rowan at the University of Bath, using ^{1}H-NMR and Electron Impact Mass Spectrometry (IEMS).

Compound-2, molecular weight=136, was identified as 4-hydroxyacetophenone (molecular ion (M^{+}) in Figure 7.3.). The NMR spectrum of the sample containing this molecule, revealed the presence of at least two additional related substances (represented by two methoxy singlets in Figure 7.4.). Methoxy derivatives of 4-hydroxyacetophenone were confirmed by low e.V. EIMS, which revealed enhanced molecular ions not only at m/z=136 but also at m/z=166 (Figure 7.5.). Further purification of these compounds were not attempted due to the small sample size obtained (4.4 mg). However, it was later estimated from the NMR integrations that the original sample, here designated compound-2, contained about 70% of 4-hydroxyacetophenone. Compound-2 showed almost identical inhibitory activity towards *V. dahliae* conidia when compared to an authentic sample of 4-hydroxyacetophenone (ED_{100} of 37.2μg/ml for compound-2 and 29.5 μg/ml for pure 4-hydroxyacetophenone).

Compound-3 was obtained in its pure form after the purification procedure described. Its E.I. mass spectrum showed a clear molecular ion at m/z=152 (Figure 7.6.) and the compound was identified as 3,4 dihydroxyacetophenone (NMR spectrum in Figure 7.7.).

Compound-4, also obtained in its pure form, was identified by Dr. M. H. Beale at the University of

Bristol, as arjunolic acid (2α,3ß,23-trihydroxyolean-12-en-28-oic acid). Identification was achieved after ^{1}H-NMR (Figure 7.8.), ^{13}C-NMR (Figure 7.9.) and comparative GC-MS analyses between the cocoa phytoalexin and an authentic sample of arjunolic acid, isolated from *Myrica rubra* (Myricaceae) in Japan. Both samples, derivatised as methyl esters (CH_2N_2) of trimethylsilyl ethers (MeTMSi), had identical Kovat's Retention Index (3886) and mass spectra as MeTMSi derivatives (Figure 7.10.), which confirmed the identity of compound-4.

After the whole purification procedure, compound-1 was still a mixture of sulphur and long chain methylenic compounds as demonstrated by its ^{1}H-NMR spectrum (Figure 7.11.) and GC-MS analysis (Figure 7.12). GC spectrum of this sample revealed that elementary sulphur was the major component. TLC bioassays demonstrated similar Rf's and toxicities levels for a pure sample of sulphur and 'compound-1'. Also, it was later demonstrated that the inhibitory spot corresponding to this compound was only detected in TLC plates, when the samples applied came from stems harvested eight or more days after puncture inoculation (not from intact or puncture-control plants). Attempts to extract sulphur from *V. dahliae* cultures or culture filtrates also failed, indicating that it is not from fungal origin. However, it is not yet known if this element is synthesized *in planta* or it is derived from a sulphur-containing organic compound that decomposes during the extraction and separation processes.

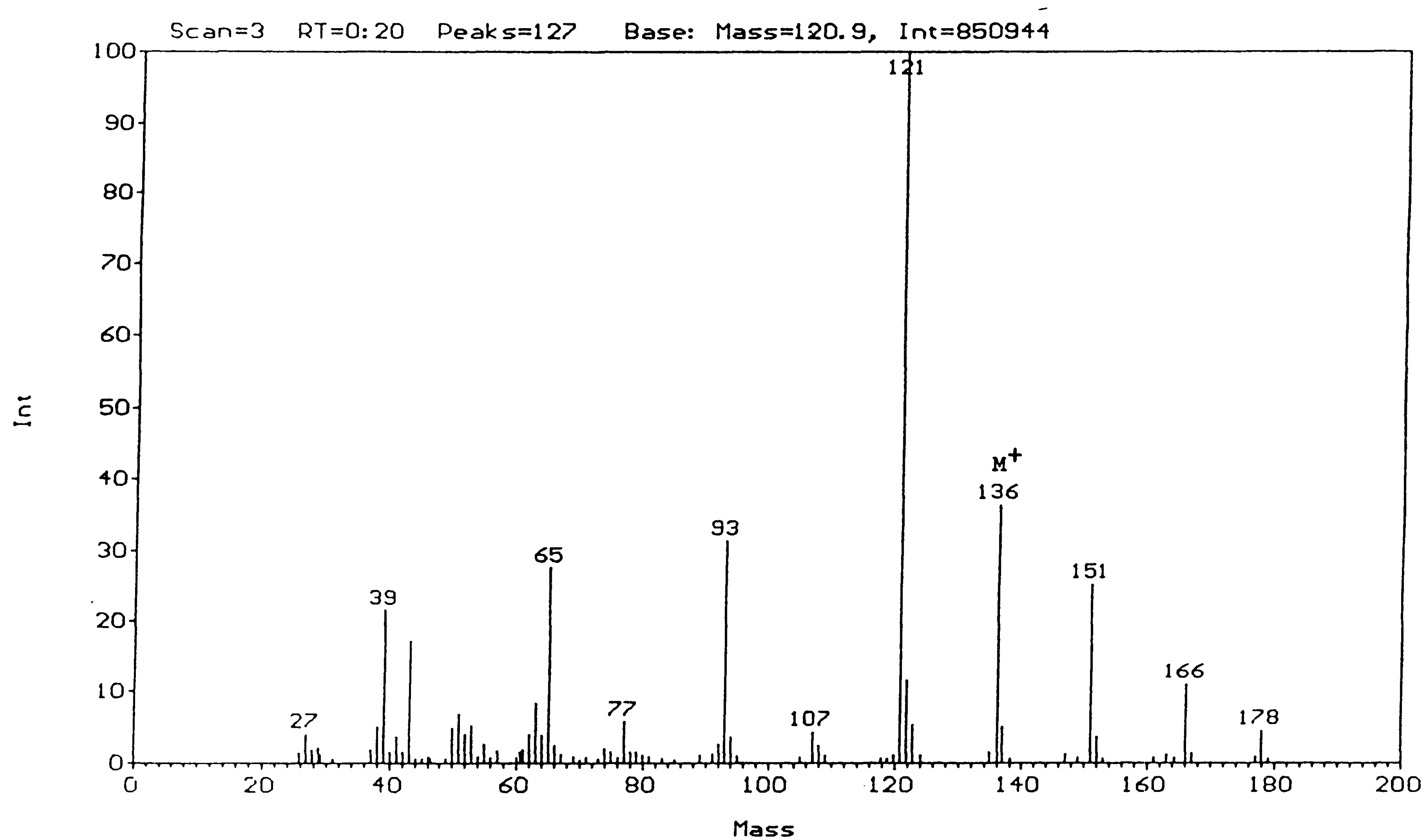
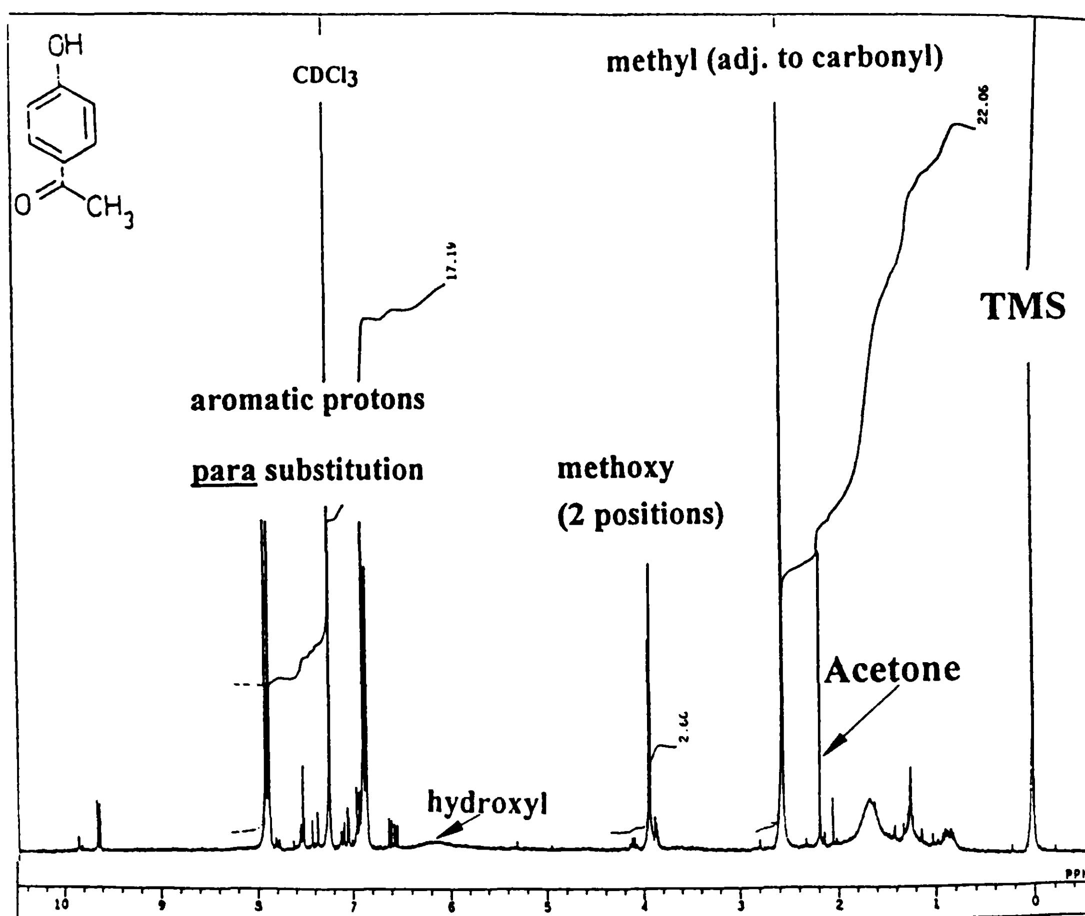


Figure 7.3. Mass spectrum of compound 2 (electron impact, 70 e.V.).

Figure 7.4. ^{1}H-NMR spectrum of compound 2 (270 MH_Z using deuterochloroform ($CDCl_3$) as solvent and tetramethyl silane (TMS) as internal standard).



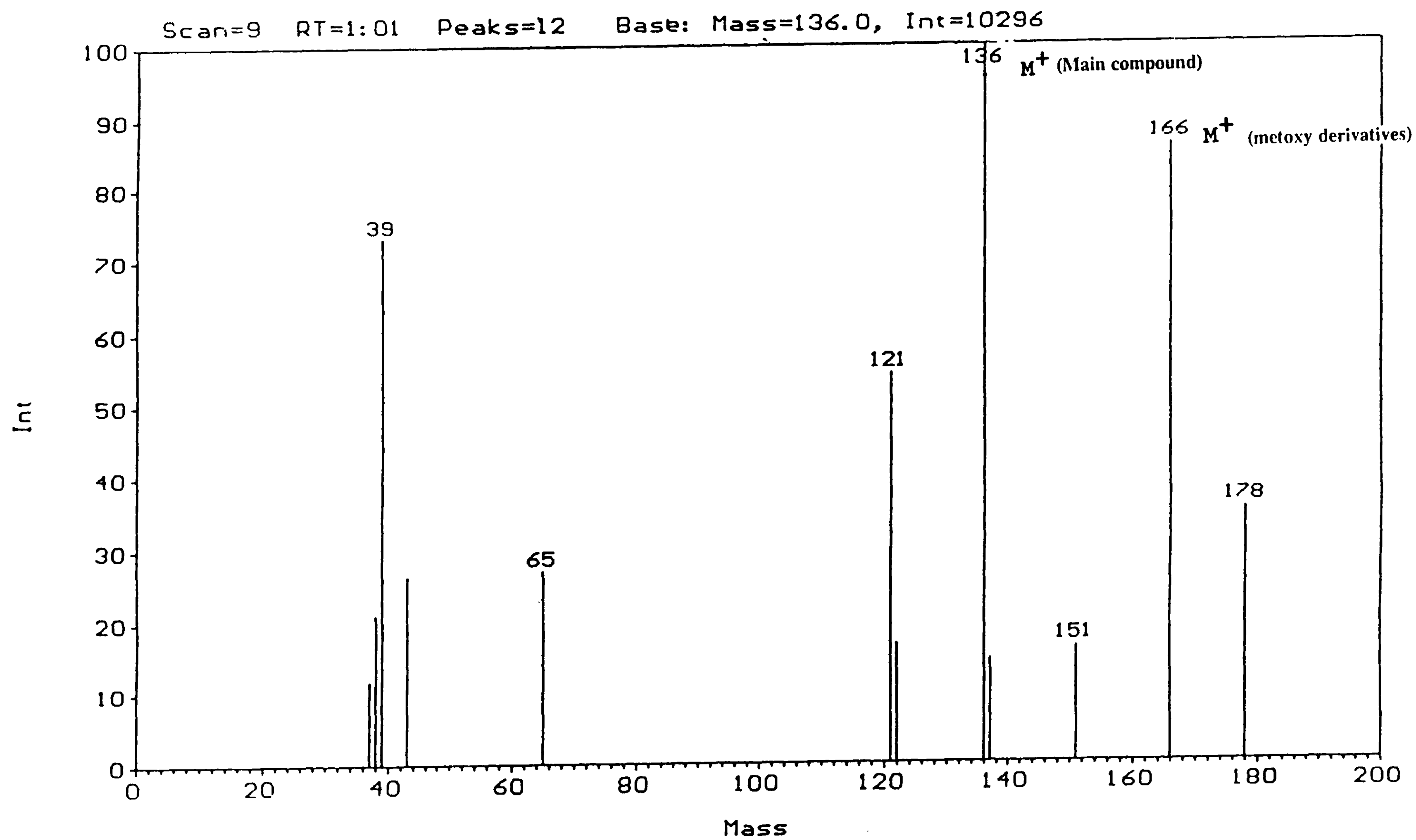


Figure 7.5. Mass spectrum of compound 2 (electron impact, low e.V.).

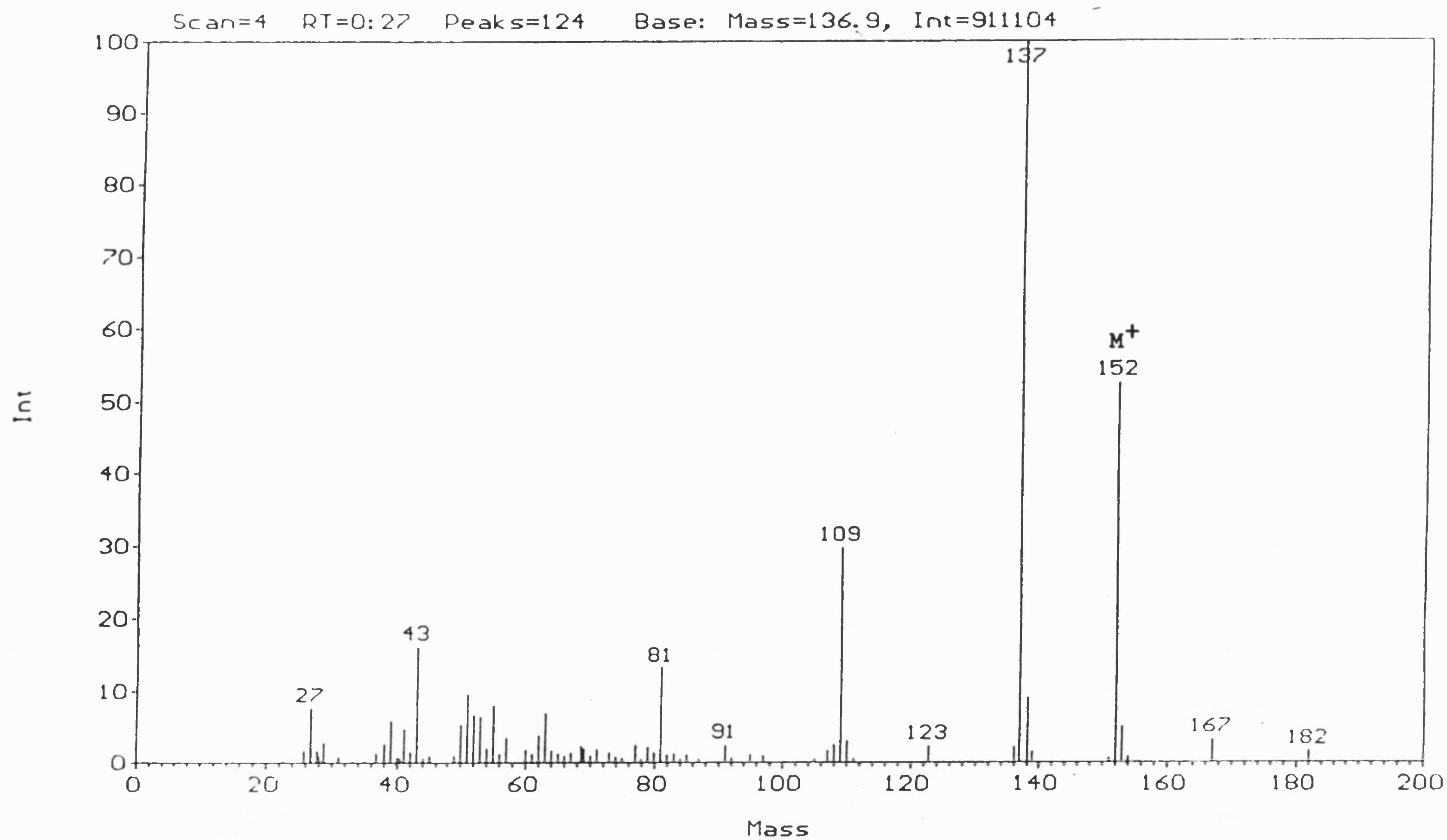
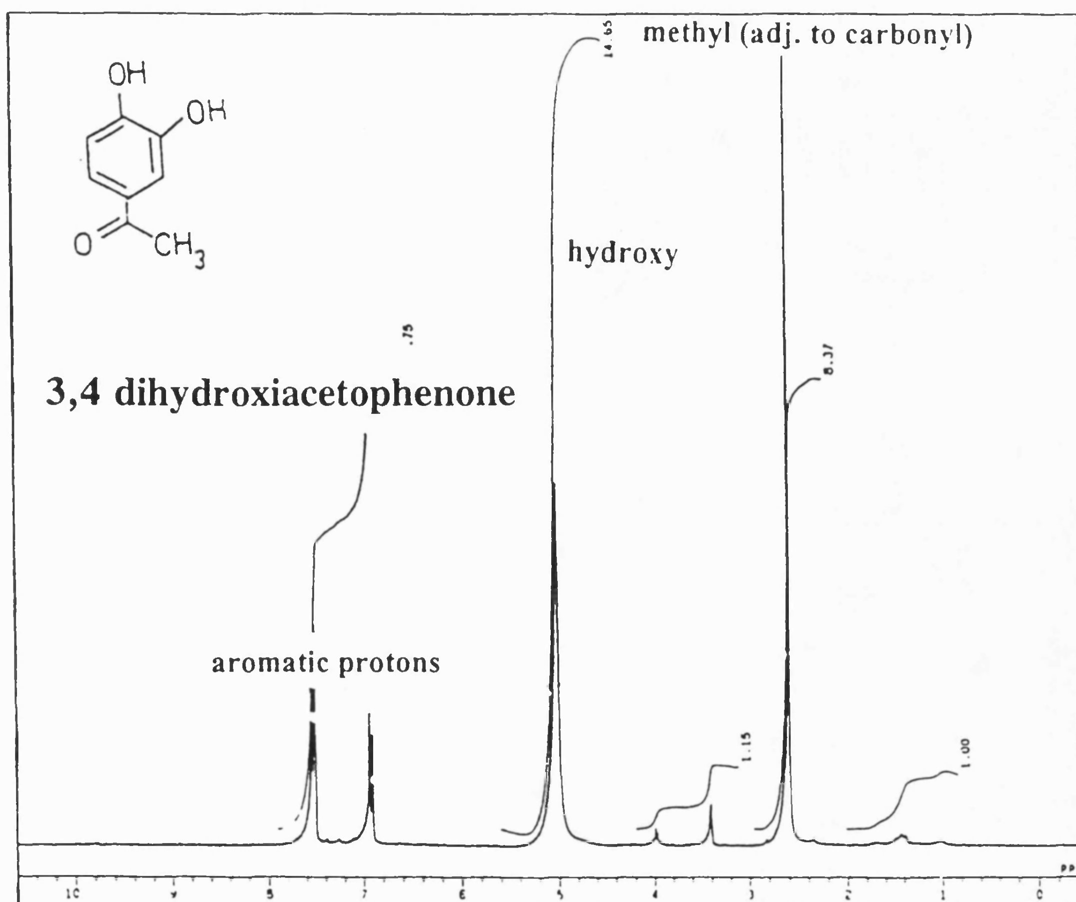


Figure 7.6. Mass spectrum of compound 3 (electron impact, 70 e.V.).

Figure 7.7. ^{1}H-NMR spectrum of compound 3 (270 MH_Z in deuteromethanol).



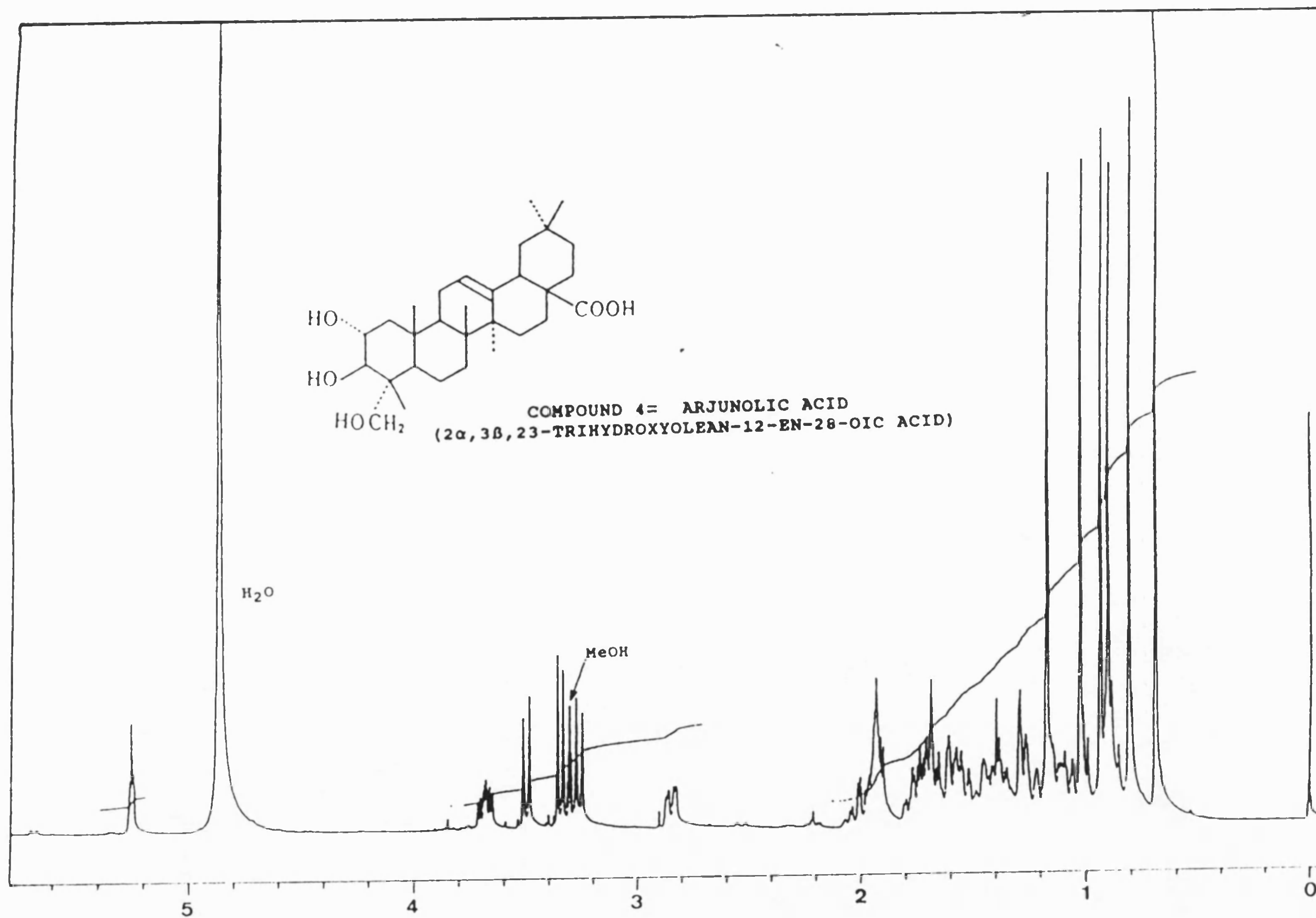


Figure 7.8. ^{1}H-NMR spectrum of compound 4 (400 MH_Z in deuteromethanol).

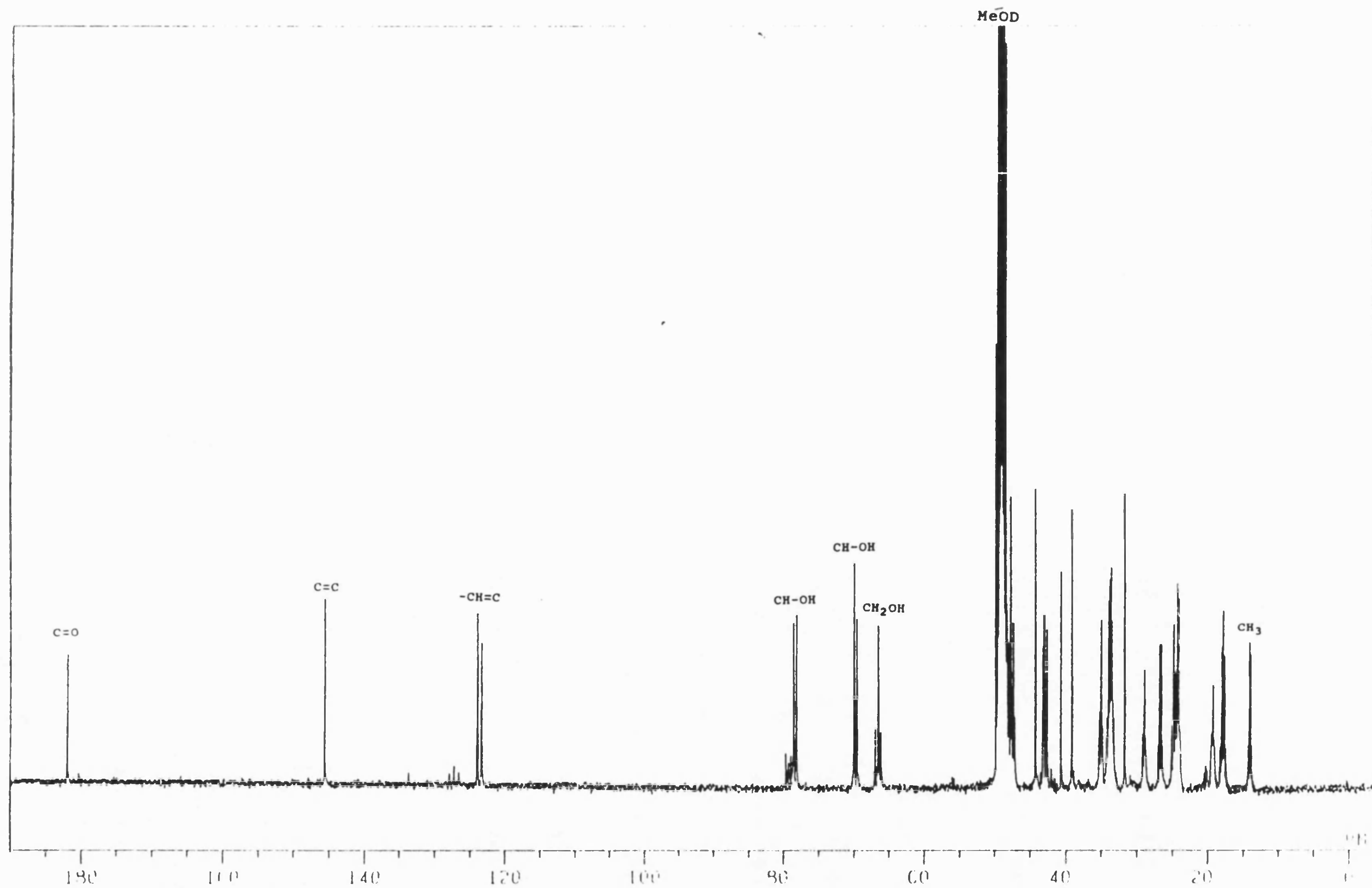
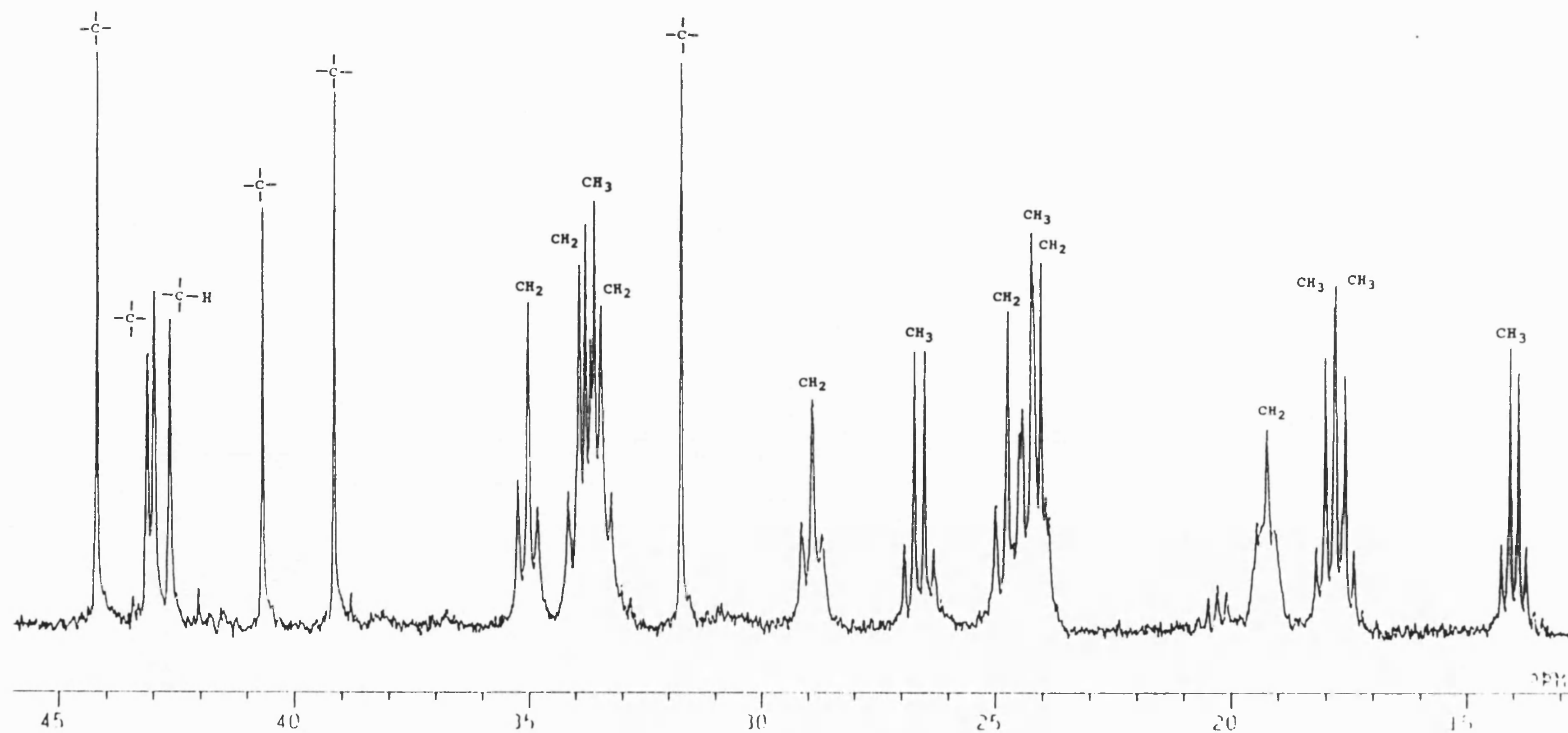
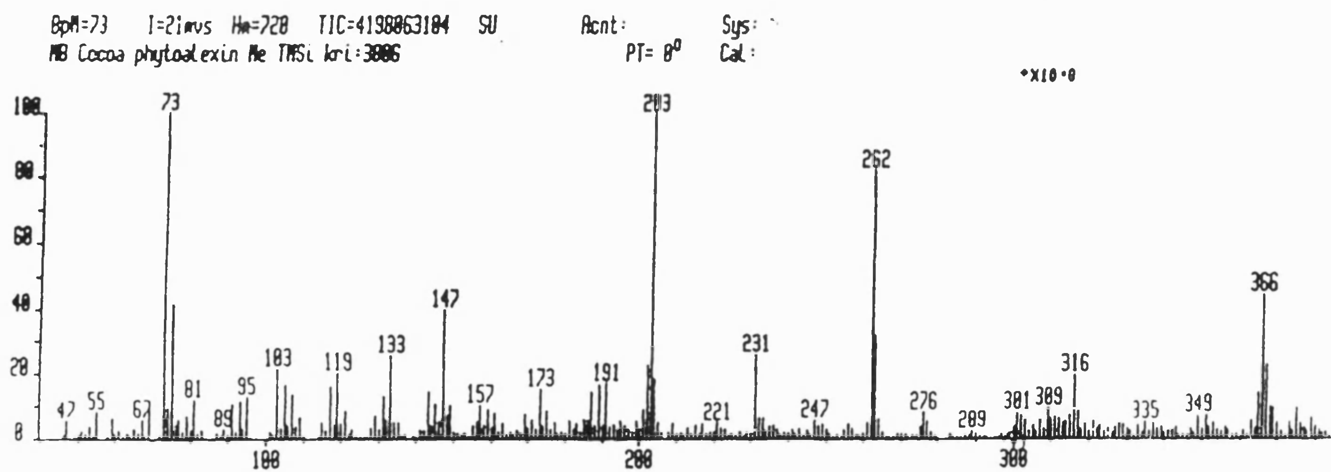


Figure 7.9. ^{13}C-NMR OFR spectrum of compound 4 (100 MH_Z in deuteromethanol).



Continuation of Figure 7.9. (^{13}C-NMR OFR spectrum of compound 4).



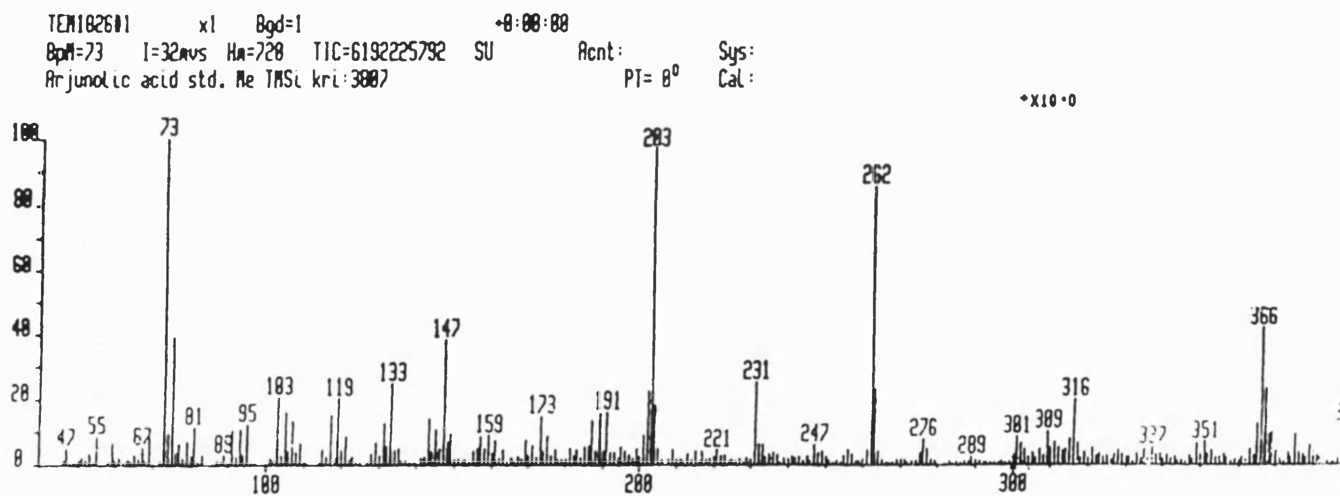
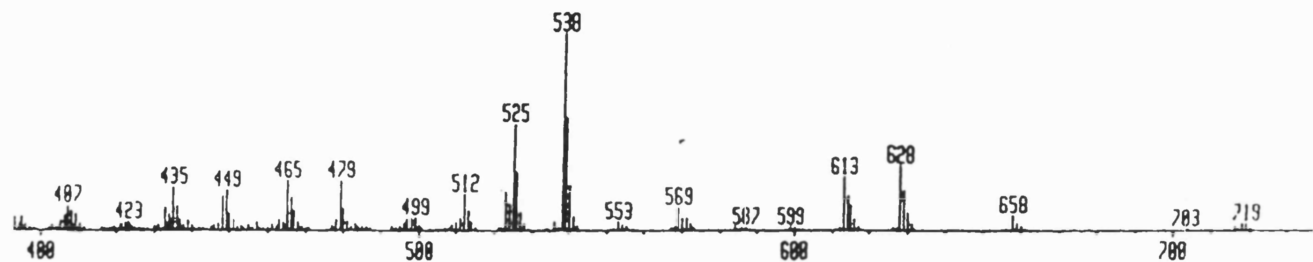
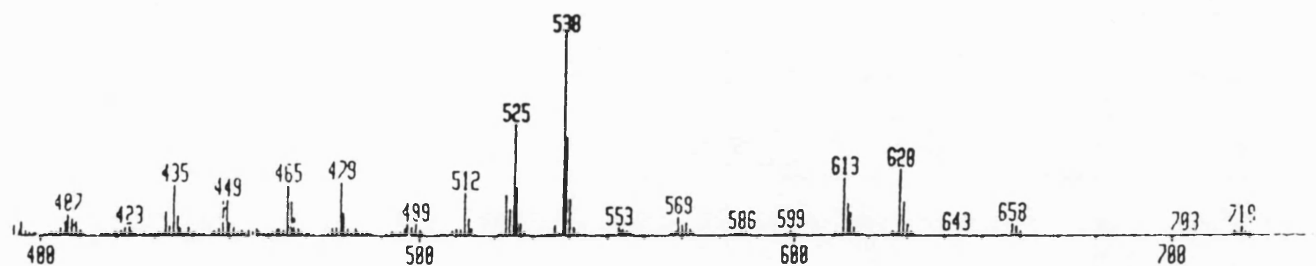


Figure 7.10. GC-MS spectrum of compound 4 (top) compared to that of an authentic sample of arjunolic acid (bottom), (electron impact, 70 e.V.).





Continuation of Figure 7.10. (GC-MS of compound 4 compared to that of an authentic sample of arjunolic acid).

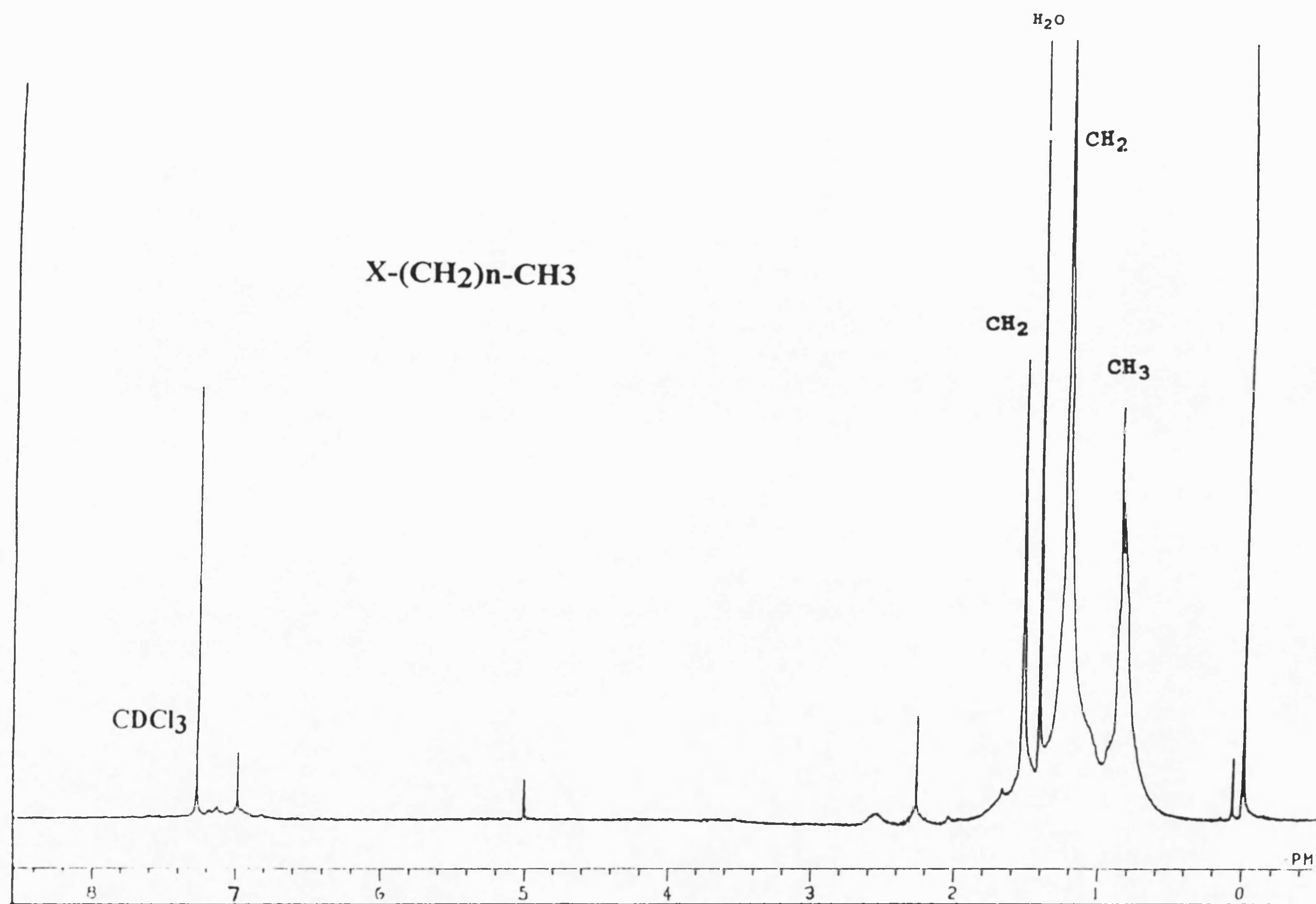
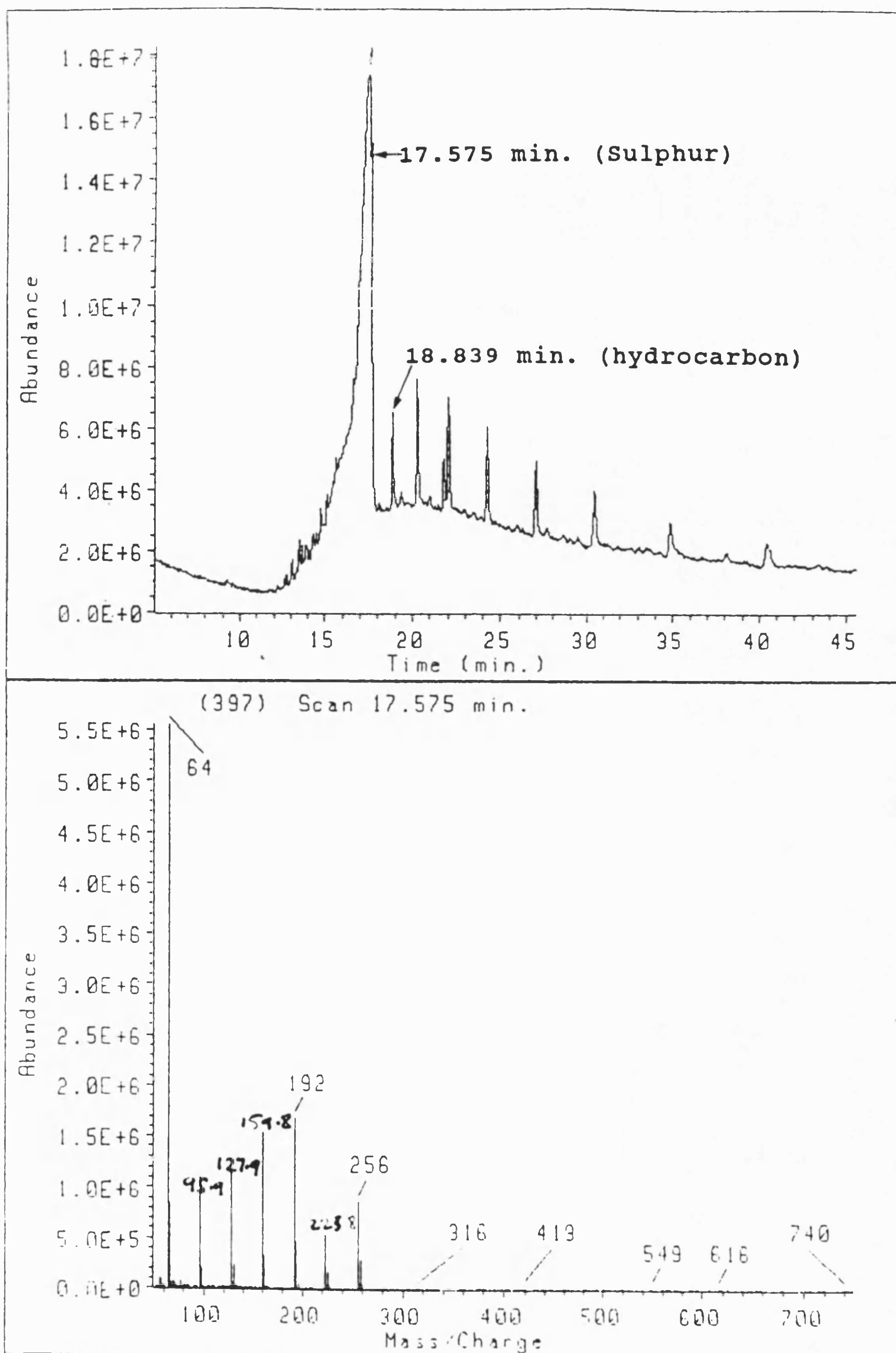


Figure 7.11. ^{1}H-NMR spectrum of compound 1 (400 MH_{Z} in deuterochloroform).

Figure 7.12. GC-MS spectrum of compound 1 (electron impact, 70 e.V.).



7.2.9. Toxicity of the cocoa phytoalexins to *V. dahliae* conidia.

The toxicities of the samples obtained from the large scale purification process were tested by bioassay with *V. dahliae* conidia in cavity slides. A two-fold dilution series was prepared in diethyl ether.

All phytoalexins, with the exception of compound-3, were highly toxic to *V. dahliae*. Significant reductions in germination in relation to the water control were detected at low concentrations for compounds 1, 2 and 4 (≥3.1 μg/ml for compound-1, ≥6.2 μg/ml for compound-2, ≥12.5μg/ml for compound-4), but at high concentrations for compound-3 (≥100 μg/ml) (Table 7.13.). Toxicity was even more pronounced when germ tube length was considered; significant reductions in germ tube growth in relation to the water control were detected at concentrations as low as ≥1.5 μg/ml for compound-1 and ≥6.2 μg/ml for compounds 2 and 4. However, the effect of compound-3 on germ tube remained detectable only at concentrations ≥100 μg/ml (Table 7.14.).

Table 7.13. Effect of the cocoa phytoalexins on the percentage of germination of *V. dahliae* conidia.

PHYTOALEXIN*	CONCENTRATIONS (µg/ ml) 50.0	25.0	12.5	6.2	3.1	1.5	WATER CONTROL
Compound-1	0.0**b	6.0 b	10.5 b	39.0 b	44.7 b	88.2 a	98.7 a
Compound-2	0.0 d	4.2 cd	41.7 bcd	45.0 bc	86.0 ab	95.2 a	98.7 a
Compound-4	0.0 b	0.5 b	41.4 b	96.0 a	97.2 a	96.7 a	98.7 a

PHYTOALEXIN*	CONCENTRATIONS (µg/ml) 800	400	200	100	50	WATER CONTROL
Compound-3	0.0 b	0.0 b	13.7 b	24.7 b	97.2 a	98.5 a

* Compound-1= sulphur + hydrocarbons; compound-2= 4-hydroxyacetophenone; compound-3= 3,4 dihydroxyacetophenone; compound-4= arjunolic acid.

** Values represent the means of 4 replicates. Means followed by the same letter in each row are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

Table 7.14. Effect of the cocoa phytoalexins on the germ tube length of *V. dahliae* conidia.

PHYTOALEXIN*	CONCENTRATIONS (µg/ml) 50.0	25.0	12.5	6.2	3.1	1.5	WATER CONTROL
Compound-1	0.00**c	0.47 c	0.50 c	3.80 bc	5.84 bc	13.20 b	22.10 a
Compound-2	0.00 b	0.25 b	2.95 b	3.45 b	16.12 a	16.36 a	22.10 a
Compound-4	0.00 c	0.00 c	2.95 c	13.55 b	16.14 ab	18.00 ab	22.10 a

PHYTOALEXIN*	CONCENTRATIONS (µg/ml) 800	400	200	100	50	WATER CONTROL
Compound-3	0.00 b	0.00 b	0.90 b	4.12 b	18.86 a	22.73 a

* Compound-1= sulphur + hydrocarbons; compound-2= 4-hydroxyacetophenone; compound-3= 3,4 dihydroxyacetophenone; compound-4= arjunolic acid.

** Germ tube length values in µm, representing the means of 4 replicates. Means followed by the same letter in each row are not significantly different (Mann-Whitney U-test, $p \leq 0.05$).

The ED_{50} and ED_{100} determinations (Table 7.15.) also revealed the increasing toxicity from compound-3 to compounds 4, 2 and 1, respectively. The lower ED_{50}'s found for germ tube length than for germination, confirms that the first parameter is more sensitive for the detection of toxicity of compounds to *V. dahliae*.

Table 7.15. Toxicity of the purified cocoa phytoalexins to *V. dahliae* conidial germination and germ tube growth, in terms of ED_{50} and ED_{100} estimated by linear regression.

PHYTOALEXIN+ (µg/ml)	ED_{50}* GERMINATION (µg/ml)	ED_{50}* GERM TUBE LENGTH (µg/ml)	ED_{100}** GERMINATION (µg/ml)
Compound-1	4.55	1.74	27.73
Compound-2	7.76	4.43	37.15
Compound-3	118.71	95.50	370.68
Compound-4	10.26	6.01	35.56

+ Compound-1= sulphur + hydrocarbons;
compound-2= 4-hydroxyacetophenone;
compound-3= 3,4 dihydroxyacetophenone;
compound-4= arjunolic acid.

* ED_{50}= Effective concentration of the compound, which reduced germination or germ tube growth of *V. dahliae* conidia by 50%.

** ED_{100}= Effective concentration of the compound, which completely inhibited germination of *V. dahliae* conidia.

7.3. DISCUSSION.

The research described in this chapter was focused upon the elucidation of substances linked with the resistance of cocoa to *V. dahliae*. Condensed tannins or procyanidins were recently described as the possible main antifungal factors present in crude extracts from cocoa flush stems and responsible for the *in vitro* toxicity to *Crinipellis perniciosa* (Brownlee, 1990). The antimicrobial properties of tannins were also recently related to the resistance of cotton to *V. dahliae* (Bell, 1991; 1992). Nevertheless, when condensed tannin content was estimated in cocoa cultivars susceptible and resistant to *V. dahliae*, no significant difference was observed amongst them. Also, the toxicity levels of these crude extracts against *V. dahliae* conidia in cavity slides were similar for the cultivars tested.

An accumulation of procyanidins over a period of 60 days after stem puncture was detected for all cultivars, as judged by the spectrophotometric assay and colour intensity of the spots on TLC plates. However, the slide biossay technique did not reveal increased toxicity to *V. dahliae* in response to the accumulation of these compounds. Bell (1973) reported a rapid increase in polyphenol content after infection of cotton with *V. dahliae*; the major phenolics were believed to be tannins, however lignins and quinones also increased during infection. Nicholson, Kollipara, Vincent, Lyons & Cadena-Gomes (1987) discovered the accumulation of two toxic

anthocyanidinic pigments in sorghum mesocotyls in response to inoculation with a pathogenic and a non-pathogenic fungus; later, Tenkouano *et al.* (1993), found that these compounds accumulated to greater concentrations in resistant than in susceptible sorghum inbreds. Although the ratio of accumulation of procyanidins over time appeared to be very similar for the three cocoa cultivars examined, a far greater range of cultivars needs to be assayed and the extensive collection of cocoa cultivars at CEPLAC in Brazil, could be utilized.

The reports on the toxic effect of different molecular sizes of procyanidins on cocoa pathogens are conflicting. Brownlee (1990) compared the activity of a range of procyanidins against *C. perniciosa* and found that the toxicity of these compounds was directly proportional to their molecular weight. The polymeric procyanidin from sorghum, reproduced the toxic effects at concentrations similar to the purified cocoa procyanidin and ≥80 μg/ml produced complete inhibition of spore germination. By contrast, Bell (1991; 1992) found that, independent of the isolate tested, tannin concentrations greater than 2000 μg/ml were necessary to kill *V. dahliae* and suggested that small tannin oligomers are apparently more toxic to this fungus. The results presented here seem to agree with Brownlee's in the sense that larger procyanidin molecules are more toxic than small ones; complete inhibition of germination of *V. dahliae* conidia (ED_{100}) ranged from ca. 3677μg/ml for the monomer epicatechin to 904μg/ml for the polymer from heather (*C. vulgaris*). In a review on the

antimicrobial properties of tannins, Scalbert (1991) argues that the binding properties of these polymers to proteins and polysaccharides would explain their generally higher toxicity *in vitro* when compared to related oligomers, which lack this characteristic.

The ED_{100} values mentioned seem to be too high to account for the involvement of tannins in defence; however, large amounts of procyanidins and precursors can be found in certain organs of several plant species: Leaves of primitive cotton varieties contain about 20% (w/w) of condensed tannins (Lane & Schuster, 1981), cocoa flush stem tissue comprises up to 10% (w/w) of these substances (Brownlee *et al.*, 1990) and fresh, unfermented cocoa seeds contain 2% (w/w) of epicatechin (Porter *et al.* 1991). In the current work, extractable polymeric procyanidin from the hypocotyls of cocoa seedlings was estimated by the amount of cyanidins produced after submitting the samples to acid hydrolysis (Bate-Smith, 1973; Harborne, 1984). The levels found *in planta* were not as high as those reported in the literature, which may due to the method of extraction utilized; Brownlee *et al.* (1990) extracted cocoa stems with de-gassed solvents to minimize the oxidative browning processes before acid hydrolysis. Nevertheless, the amounts of tannins found here in cocoa stems were in general above the ED_{50} levels to *V. dahliae* conidia determined for a pure sample from heather. This comparison is based on the assumption that tannins from different sources are equally toxic, as suggested by Feeny (1976) and confirmed by Brownlee

(1990).

Despite the occurrence of potentially toxic concentrations in cocoa stems, the localization and complexation of these polymers may limit their availability and therefore toxicity to the pathogen *in vivo*. Most tannins are generally confined within cellular vacuoles (Haslam, 1979; Stafford, 1988) and may be already complexed to other polymers (Scalbert, 1991).

It was initially hypothesized, that the antifungal compounds involved in the resistance of cocoa to *V. dahliae* would be tannins or other phenolic compounds. The fact that tannin content could not differentiate cocoa cultivars in relation to resistance to *V. dahliae* led to the investigation of other possible antifungal compounds. Unlike tannins, phytoalexins have never been identified before from cocoa plants; thus, new techniques had to be developed for extraction and detection of these compounds. Initially, diethyl ether was selected as the most appropriate solvent to extract post-infectional compounds toxic to *V. dahliae* from cocoa stems. In addition to the slide biossay, toxicity of these substances was confirmed in TLC bioassays by spraying *V. dahliae* (the pathogen itself) over the plate, which must be advantageous in relation to the conventional use of *Cladosporium* spp. to develop the plates, since sensitivity may depend on the microorganism involved (Smith, 1982).

Using the complementary techniques, slide and TLC bioassays, a build-up of antifungal compounds was demonstrated in the punctured stems of 120-day-old

seedlings of the resistant cultivar Pound-7, whether they had been inoculated or not. In many cases, phytoalexins accumulate in plants exposed to various types of stress in the absence of infection, which suggests that their synthesis may be part of a co-ordinated response to injury, leading to wound healing (Kuhn & Hargreaves, 1987). When plants of the same cultivar were punctured approximately a year later (500-days old) with a 10% sucrose-salts medium solution, they failed to produce phytoalexins. Perhaps, the injury caused by the punctures alone on the stems of these much older plants did not cause enough cell damage necessary to stimulate the production of phytoalexins at detectable levels. The effect of age and maturity of cocoa tissues on phytoalexin synthesis remains to be investigated.

Cultivar Pound-7 appeared to accumulate significantly larger amounts of these post-infectional antifungal compounds within the period from 10 to 15 days following inoculation than the susceptible cultivar ICS-1, as judged by the slide bioassays. Both the rate and speed of phytoalexin synthesis together with the occlusion of vessels by tyloses, have been often correlated with the resistance of cultivars to Verticillium wilt diseases. Tjamos & Smith (1974), demonstrated that approximately four times more rishitin accumulated in stem segments of a tomato cultivar with monogenic resistance to Verticillium wilt than in a susceptible cultivar. Hutson & Smith (1980) showed that in addition to phytoalexins, tyloses were more frequent in resistant than in susceptible tomato plants.

The combined effect of rapid tylose formation and synthesis of terpenoid aldehydes is apparently needed for the expression of resistance of the cotton cultivar 'SBSI' to *V. dahliae* (Mace, 1989; Bell, 1992). Preliminary observations on the extent of tylosis in xylem vessels of ICS-1 and Pound-7 indicated that this defence response was not very common in either cultivar. Other alternative mechanisms able to localize the pathogen and concentrate the phytoalexins in the infection zone of cocoa plants, such as gummosis in vessels and lignification and suberization of cell walls in roots, should also be investigated in resistant and susceptible cultivars.

The use of successive flash column chromatography and silica gel thin-layer chromatography, combined with the monitoring system utilized in this work, proved to be reasonably efficient for detection and purification of milligramal samples of phytoalexins required for structural determinations. ^{1}H-NMR, ^{13}C-NMR and comparative GC-MS analyses led to the identification, at least partial, of four phytoalexins accumulated in response to *V. dahliae* inoculation in resistant cocoa seedlings. These inducible antimicrobial substances belong to three quite different chemical groups, ranging from a sulphur-containing compound, to acetophenones and to a particularly complex triterpenoid. All are novel potential phytoalexins in *T. cacao*, but some of them were already reported as antimicrobial substances from other plant species. Kuhn & Hargreaves (1987) suggested that a particular compound may occur as a constitutive inhibitor

in a certain plant species but as a phytoalexin in other, which is here demonstrated.

Compound-2: 4-hydroxyacetophenone, occurs in the temperate tree species of the Pinaceae family, genera *Abies*, *Picea*, *Larix* and *Pinus* (Gottstein & Gross, 1992). It exhibited fungitoxic activity towards *Cladosporium cucumerinum* and the forest pathogen *Rhizosphaera kalkhoffii*, although its role as a phytoalexin was not clear (Osswald, Zieboll, Schutz, Firl & Elstner, 1987). 4-hydroxyacetophenone may also function as a chemical indicator of general stress (Jensen & Lokke, 1990).

Compound-3: 3,4-dihydroxyacetophenone is reported as a natural product from *Picea maximowiczil* (Pinaceae) and *Ilex pubescens* (Aquifoliaceae). It was recently isolated from coffee residue and proved to have antimicrobial activity against a range of bacteria, yeasts and filamentous fungi. There is a possibility for using this antimicrobial compound as a preservative agent in foods, cosmetics and drugs (Nishina, Kajishima, Matsunaga, Tezuka, Inatomi & Osawa, 1994).

Compound-4, the triterpene arjunolic acid, has been isolated previously from many plant species, such as *Myrica rubra* (Yaguchi, Sakurai, Nagai & Inoue, 1988), *Terminalia brassii, T. complanata* (Collins, Pilotti, & Wallis, 1992), *T. arjuna*, *Mitragyna ciliata*, *Tristania conferta*, *Psidium guaijava* and *Cochlospermum tinctorium* (Diallo, Vanhaelen, Vanhaelen-Fastre, Konoshima, Kosuka & Tokuda, 1989; Collins, Pilotti & Wallis, 1992). Arjunolic acid and derivatives are reported to have high anti-viral

and bactericidal activity. Its inhibitory effect on skin tumor promoters was found to be greater than those of previously studied natural products (Diallo *et al.*, 1989) and in Africa, the tropical tree *Musanga cecropioides*, which contains various pentacyclic triterpenes, including methyl arjunolate, is known in folk medicine for its potent activity against many bacterial and other pathological infections (Lontsi, Sondengam, Ayafor, Tsoupras & Tabacchi, 1990). Since these previous reports refer to antimicrobial effects in animals, this is the first one on the toxicity of arjunolic acid to a plant pathogen.

Compound-1: Although the toxic effect of sulphur upon phytopathogenic fungi has been known since the last century, this substance remained very effective and it is still sold in larger quantities than any other fungicide (Feichtmeir, 1949; Tweedy, 1969; Hassall, 1982). Certain genera of bacteria (e.g., *Thiobacillus*, *Beggiatoa*, *Thiothrix*, *Chlorobium* and *Chromatium*) play a key role in the interconversion of sulphur compounds in nature since they are able to metabolize and produce either, intracellular or extracellular elementary sulphur. By contrast, plants are suppose to convert inorganic sulphate only to organic sulphur compounds (Young & Maw, 1958). Some sulphur compounds from mustard, radish and other vegetables are recognized for their antimicrobial properties (Jocelyn, 1972). Recently, several sulphur-containing phytoalexins were reported from cruciferae species (Devys, Barbier, Loiselet, Rouxel, Sarniguet,

Kollmann & Bousquet, 1988). The lack of evidence for the production of elementary sulphur by plants, along with the experimental results where sulphur was isolated from puncture-inoculated stems only, suggest that 'compound-1' is a sulphur-containing phytoalexin(s), which reacts during the extraction and separation processes, producing sulphur and other possible derivatives.

The toxicity of the cocoa phytoalexins to *V. dahliae* was easily demonstrated by inhibition of conidial germination or reduction of germ tube elongation. Minimum inhibitory concentrations that completely inhibited germination of *V. dahliae* conidia (ED_{100}), ranged from 27.7μg/ml for compound-1 to 370.7μg/ml for compound-3. Compounds 2 and 4 were nearly as toxic as compound-1 (ED_{100} of 37.2 and 35.6μg/ml, respectively). The toxicity of these compounds, with the exception of compound-3 (3,4-dihydroxyacetophenone), fell within the order of magnitude noted by Smith (1982), as generally common for phytoalexins (10^{-5} to 10^{-4} M). Bioassays comparing 3,4-dihydroxyacetophenone to some catechol analogues demonstrated that the antimicrobial activity of catechols were always lowered when a highly polar group (e.g., hydroxyl) was incorporated into them (Nishina *et al.*, 1994). These findings may help to explain the much greater activity found for compound-2 (4-hydroxyacetophenone) when compared to compound-3 (3,4-dihydroxyacetophenone).

In earlier investigations, some plant species were characterized by the formation of a single phytoalexin (Hargreaves, 1976). Nowadays, it is known that most

species produce several closely related phytoalexins, but in certain species, the accumulation of one compound may predominate over others. Some plants like broad beans (Hargreaves, 1976), tomato (De wit & Kodde, 1981) and now cocoa, are exceptions to this rule, since they accumulate at least two different groups of phytoalexins. However, it should be considered that a substance found to be inhibitory after isolation from the host tissue, does not necessarily assist in its defence *in vivo*. To begin with, the substance must at least, be present in the host tissue in sufficiently toxic concentration and must be contacted by the pathogen. Estimation of the quantities of the phytoalexins per gram of woody stem, 15 days after stem puncture inoculation of cultivar Pound-7 with *V. dahliae*, indicated that only compounds 1 and 4 were present in amounts well above their toxic levels obtained *in vitro*, which suggests that they are potentially more important in cocoa defence than compounds 2 and 3. However, this evidence should be interpreted with caution, since no data are available on which of these compounds is produced in the right place, at the right time and in an appropriate form available to the pathogen. In cotton plants infected with *V. dahliae*, terpenoid phytoalexins are formed in paratracheal parenchyma cells, exuded into xylem vessels and absorbed by hyphae, conidia, cell walls and tyloses (Mace, Bell & Beckman, 1976). Although no information is available specifically on the presence of acetophenones in the vascular system of plants, it is well documented that exudation of phenolics compounds by paratracheal cells is

very common amongst various plant species infected by vascular pathogens (Bell & Mace, 1981; Beckman & Talboys, 1981).

Potential phytoalexins were isolated, partially purified and identified, and bioassayed for toxicity against *V. dahliae*; nevertheless, clarification of the role of each of those compounds in cocoa defence mechanisms will require further investigation. Attempts to reveal the definitive identity of 'compound-1' are still under way. After that, the determination of the relative amounts and rates of accumulation of each compound in susceptible and resistant cultivars would be a crucial stage, which is dependent upon the development of quantitative methodologies, possibly based on high pressure liquid chromathography (HPLC). In addition, detailed *in vivo* studies of the time-space relationship involving fungal development and phytoalexin accumulation, will be relevant particularly during the critical early stages of infection. Chromatographic techniques may be not sufficiently sensitive to measure, for example, the amounts of phytoalexins induced in a specific trap site (i.e., a vessel end wall) within the vascular system. Moesta, Hahn & Grisebach (1983) developed a radioimmunoassay for the soybean phytoalexin glyceollin I, which was capable of detecting a few picomoles (or nanograms) of this substance in infected tissue. The study of the antigenicity of cocoa phytoalexins constitutes the first step towards the development of immunocytochemical techniques for their localization and quantification *in*

planta.

The discovery of phytoalexins in cocoa plants and the preliminary assessments based on crude extracts from susceptible and resistant cultivars, raises the possibility of using these parameters in the selection of cultivars resistant to *V. dahliae*. Also, the study of the possible induction and degradation of phytoalexins by isolates of *V. dahliae* with distinct levels of aggressiveness towards cocoa or by other pathogens from cocoa would be facilitated in the future, by the establishment of the basic techniques for identification and quantification of these compounds. However, one should bear in mind that disease defence in plants is generally a complex of mechanisms and chemical and anatomical characteristics may interact to constitute the overall resistance.

8. GENERAL DISCUSSION.

In comparison with many temperate crops and their associated diseases, cocoa is still very much under-researched. Because of the nature of their occurrence in developing countries with limited resources, the study of cocoa diseases has not, on the whole, received the scientific attention and inputs otherwise justified by their importance to national or local economies (Holderness, 1993). In addition, sudden death caused by *V. dahliae* may be considered a relatively new problem in the main cocoa producing areas of the new world; therefore there was virtually no information on host range, isolate variation and physiology of infected plants along with methods for selection and mechanisms of cocoa resistance to the disease. The elucidation of these aspects constituted the intrinsic aims of this thesis.

Initially, studies on *V. dahliae* variability in terms of pathogenicity were carried out considering different geographical origins and hosts. Isolates from *T. cacao* were pathogenic, albeit to different degrees, to other crops such as aubergine, tomato, pepper and cotton and to native weeds from cocoa plantations. Also, some plant species were colonized, but did not show symptoms, when soil drench inoculated. Symptomless carriers of the disease are likely to be important in the multiplication and survival of the fungus in the field. Future studies should include a monitoring of natural infection in the cocoa growing area of the state of Bahia, Brazil,

targeting mainly those potential alternative hosts revealed in glasshouse inoculations.

Isolates from diverse origins were also tested against the cultivar Pound-7, selected in Brazil as a standard of resistance to *V. dahliae*. The resistance of this cultivar proved to be vulnerable to isolates from different solanaceous and malvaceous crops along with a Colombian isolate from cocoa. Cocoa isolates from the states of Bahia (BA) and Espirito Santo (ES) in Brazil were shown to differ in their aggressiveness to cocoa; e.g. isolate BA-3, being less aggressive on cocoa than isolate ES-1, was unable to differentiate susceptible and resistant cultivars. This result led to the choice of isolate ES-1 for further screenings for resistance and studies on the mechanisms of host resistance. However, it is still not know if isolate ES-1 is representative of the population of *V. dahliae* in cocoa growing areas of Brazil. Clearly, more isolates need to be tested, and this aspect should receive top priority when reassessing the CEPLAC's breeding programme to *V. dahliae* in the near future.

The inability to identify subspecific groups within Verticillium wilt species has made detection and quantification of pathogenic strains problematic. However, since the last decade several new techniques have been developed and are now being widely used to understanding genetic diversity within *Verticillium* species (Rowe, 1994). In the current study, vegetative compatibility based on the heterokaryon formation between nitrate non-utilizing mutants was assessed in a sample of 22 isolates.

No consistent correlation was found between pathogenic variation, geographical origin and vegetative compatibility groups. All eight Brazilian isolates from cocoa tested were assigned into VCG-2. In VCG-2 were also assigned a cocoa isolate from Uganda and several other from various hosts and countries. In general, VCG diversity within *V. dahliae* appears to be fairly low, when assessed by the *nit* mutant system (Chen, 1994; Rowe, 1994), but amongst the molecular biology techniques, RFLP and PCR based assays have been proving very useful in discriminating between *Verticillium* strains (Rowe, 1994). To conclude, a combination of different techniques should be used to evaluate the genetic diversity of *Verticillium* from cocoa.

Following the discovery of *V. dahliae* causing sudden death in cocoa in Brazil (Oliveira, 1980), several precious years of research were spent on the chemical control of the disease; systemic fungicides were selected but they proved to be uneconomical (Lawrence, Campelo & Figueiredo, 1991). Priorities on the study of Verticillium wilt of cocoa have been changing since 1987, and it is now common sense amongst CEPLAC researchers that breeding for resistance is the only realistic means for a long-lived control of this disease. A facile inoculation technique that gives reproducible results with critical differentiation of resistant and susceptible cultivars has been a goal in the breeding programme of CEPLAC since then. In the current study, resistant and susceptible cocoa genotypes could be differentiated by either,

traditionally used soil drenching or novel stem puncture inoculation. Although only nine cultivars were tested, it seems that a similar or related mechanism(s) that prevent root colonization is also effective in the stem. Disease susceptibility was previously assessed 90 days following soil inoculation; however with stem puncture, symptom development was more uniform and disease could be assessed as early as 15 days after inoculation. Also, very young seedlings were susceptible to stem-puncture but not to soil inoculation, which will represent economy in time and space in future screenings.

Stem puncture inoculation has been adopted routinely for studies of Verticillium wilt at CEPLAC in Brazil following its successful use to screen cocoa cultivars in nursery conditions there (Lopes, 1994, personal communication). This technique will certainly allow new studies on this disease along with the screening for resistance; the aggressiveness of a wide range of isolates can be tested within a shorter period of time and experiments involving rootstock-scion combinations can now be implemented in nursery or in field conditions. However, further calibration of this method may be necessary, dependent on the environmental conditions and the objectives of each experiment. As previously demonstrated, the inoculum concentration (Resende *et al.*, 1994b) and the number of punctures delivered per plant (Bugbee & Sappenfield, 1968), can markedly influence the frequency and extent of infection.

Some isolates of *V. dahliae* can induce severe

defoliation on cocoa plants and others lead to a complete wilt of the leaves, without defoliation. The underlying mechanisms that precede the occurrence of these two distinct responses were studied in seedlings inoculated by soil drenching under glasshouse conditions. Water stress proved to be a major cause of the wilt symptoms and was even more pronounced when seedlings were inoculated with a 'non-defoliating' isolate. By contrast, a 'defoliating' isolate induced the accumulation of higher levels of ethylene in newly developed leaves, where the first symptoms generally appeared. This plant hormone was apparently responsible for the accelerated senescence and defoliation, as demonstrated by the application of an ethylene synthesis inhibitor. Defoliation as a response to Verticillium wilt may also represent a type of tolerance in woody perennial plants such cocoa. By abscissing the infected leaves, the plant reduces transpiration and inoculum at the same time; thus, it may be able to contain infections in the stem tissue and then give rise to new growth from axillary or top buds, with the formation of new vascular tissue from the cambium.

Symptoms obtained with non-defoliating isolates following stem puncture were later found to be much more similar to field symptoms than those obtained by soil inoculation. With soil inoculation a yes/no response in terms of death/no disease was observed for these isolates. By contrast, the general picture after stem puncture was that of infected plants struggling to survive; the resistant cultivars were eventually successful in recovery

from infection. This is reminiscent of the field situation and might suggests that in cocoa plantations the mechanical transmission of the fungus *via* infested tools after pruning and harvest operations, may be more efficient for disease dispersal than the dissemination *via* soil and debris. It is suggested that, for a better understanding of the recovery phenomenon, water relations and other physiological parameters should be measured also in those stunted leaves, which form following either, stem puncture inoculation or natural infection in the field.

It is well documented that taxonomically related plant species are probably more similar in terms of chemical response than species far apart in the genealogical tree; e.g., Ingham & Harborne (1976) proposed that phytoalexin production may be used as a novel approach for the study of systematic relationships among higher plants. No previous study was available on the biochemistry of the resistance of cocoa (or other species from the same family) to Verticillium wilt; cotton was the closest studied relative to cocoa (both are placed in the order Malvales). Some evidence from this thesis suggested that similar biochemical and physiological responses may be shared by *G. hirsutum* and *T. cacao* when challenged by *V. dahliae*, as follows. Cocoa is one of the few species so far reported in which defoliating isolates from cotton induce the same pattern of symptoms; stem inoculation is now considered the best method for differentiating levels of resistance on cocoa, after being successful for many years in selecting cotton cultivars. However, regarding

secondary products with biological activity, the situation is rather complex and the main coincidence is that both species produce terpenoid compounds as their major phytoalexins, at least in quantitative terms. Cotton is a much more investigated crop than cocoa and more than fifty antimicrobial substances have been isolated from it, including terpenes, terpenoids, phenols, ketones, tannins, lignin, cyclopropanoid and cyclopropenoid fatty acids (Bell & Stipanovic, 1983). From cocoa, only a few compounds like phenolics (Capriles de Reys *et al.* 1964; Capriles de Reys & Reys, 1968), tannins (Brownlee, 1990), and now a triterpenoid and two acetophenones, have been identified as antifungals so far.

Constitutive tannins, lignin and terpenoid phytoalexin production have been correlated with resistance to *V. dahliae* on cotton (Bell, 1992) and tannins have also been suggested as indicators of resistance of cocoa to *C. perniciosa* (Brownlee, 1990). The toxicity of condensed tannins to *V. dahliae* was demonstrated, but no differences in their levels were detected when comparing resistant and susceptible cocoa cultivars. By contrast, the phytoalexins isolated from inoculated stems apparently were present in larger amounts in the standard resistant cultivar Pound-7 than in the standard susceptible ICS-1. The quantification of individual phytoalexins in these and other cultivars with known levels of resistance to the disease is crucial at this stage. However, it is dependent upon the development of appropriate methodologies; HPLC analyses will be the first to be attempted in CEPLAC's

laboratories.

The study on the mechanisms of resistance of cocoa to *V. dahliae* is far from over. Much time and effort had to be allocated in order to separate, purify and characterize the four novel phytoalexins. As a strategy from now on, it is proposed that further studies should be concentrated on those phytoalexins that seems to play major roles after infection. Preliminary evidence suggests that, despite phenolic phytoalexins being the most relevant group in many plants species, they seem not to be that important in cocoa, since estimates of the quantities of these compounds present in cv. Pound-7 were well below the toxic level to *V. dahliae* obtained *in vitro*. *Prima facie* it appears that in cocoa, the sulphur-containing and the triterpenoid phytoalexins are more relevant to disease resistance, considering both toxicities and levels found *in planta*.

The discovery of phytoalexins in cocoa plants and the preliminary assessments based on crude extracts from susceptible and resistant cultivars, raise the possibility of using these compounds as tools to select cultivars resistant to *V. dahliae*. A better knowledge of the genetics of the resistance of cocoa to *V. dahliae*, as well as more information on pathogen variability would certainly help to fulfil this objective. Braga & Silva (1989) suggested based on diallelic crosses involving Pound-7 and ICS-1 that resistance of cocoa to Verticillium wilt was controlled primarily by a pair of recessive alleles. However, this hypothesis was tested under

conditions of low infection following root inoculation and further attempts to confirm the monogenic nature of the resistance have failed. Possibly, there are also other minor genes controlling the expression of resistance in Pound-7, which makes the interpretation of the results more difficult (Lopes, 1994, personal communication).

Successful pathogens have evolved methods for dealing with phytoalexins, including suppression of their production, detoxification, tolerance and in some cases avoiding elaboration of substances called elicitors, that would otherwise initiate the defence reaction (Mansfield 1983; 1986; Keen, 1990). Thus, there is the possibility of isolating elicitors from *V. dahliae* that would activate phytoalexin synthesis. The use of elicitors as agents to select plants for phytoalexin production would be advantageous in comparison to intact pathogens, since there is no risk of disease transmission by injecting elicitors, into for example, single branches of cocoa trees. This non-destructive method would be ideal for selection of potentially resistant plants from CEPLAC's germplasm bank. On the other hand, it is now apparent that differentiation between aggressive and non-aggressive strains involves an initial recognition event, which in resistant tissues may lead to subsequent accumulation of phytoalexins. There are grounds for optimism that a deeper understanding of the recognition process will allow the development of rational approaches to durable disease control (Mansfield & Bailey, 1982).

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10. APPENDICES.

Appendix 1.

Preparation of media.

All media were sterilized at 121°C for 15 minutes.

1. Sucrose-salts Medium.
(Cooper & Wood, 1975)

	$g\ l^{-1}$
$NaNO_3$	2
KH_2PO_4	1
$MgSO_4.7H_2O$	0.5
Sucrose	15
Trace elements stock solution	10ml l^{-1}

Trace elements stock solution:	mg l^{-1} distilled water
$FeSO_4.7H_2O$	20
$ZnSO_4.7H_2O$	100
$Na_2MoO_4.7H_2O$	2
$CuSO_4.5H_2O$	2
$MnCl_2.4H_2O$	2

The medium was made up to 1 litre in distilled water and adjusted to pH 6.5 before sterilization.

2. Papavizas (PCNB-Peptone-Agar) Medium.
(Papavizas, 1967)

	$g\ l^{-1}$
KH_2PO_4	1
Peptone	5
$MgSO_4.7H_2O$	0.5
Botrilex (20% a.i. Pentachloranitrobenzene)	5
Agar	12

The medium was made up to 1 litre in distilled water. The following antimicrobial agents were added to the medium after sterilization, as it cooled: 0.05 g chloramphenicol, 0.3 g penicillin and 0.134 g streptomycin sulphate.

3. Potato Dextrose Agar.

39g of PDA powder (London Analytical & Bacteriological Media Ltd., London) was added to 1 litre of distilled water.

4. Minimal Medium.
(Puhalla & Spieth, 1983)

	$g\ l^{-1}$
$NaNO_3$	2
KH_2PO_4	1
$MgSO_4.7H_2O$	0.5
KCl	0.5
Sucrose	30
Agar Difco	20
Trace elements stock solution	0.2 ml l^{-1}

Trace elements stock solution: g/ 95 ml distilled water

Citric acid	5
$ZnSO_4.7H_2O$	5
$FeSO_4.7H_2O$	4.75
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1
$CuSO_4.5H_2O$	0.25
$MnSO_4.H2O$	0.05
H_3BO_3	0.05
$Na_2MoO_4.2H_2O$	0.05

The medium was made up to 1 litre in distilled water.

Appendix 2.

Preparation of silver thiosulphate (STS) solutions.
(Reid *et al.*,1980).

Stock solutions of silver nitrate ($AgNO_3$, 0.1 M) and of sodium thiosulphate ($Na_2S_2O_3.5H_2O$, 0.1M), were prepared with deionized water were stored in the dark. Silver thiosulphate complex ($Ag(S_2O_3)_2^{3-}$) was prepared as needed on the day of spraying by combining these solutions in the proportion of 1:4 respectively. Since nearly all the silver reacted to form the ($Ag(S_2O_3)_2^{3-}$) complex, STS concentrations are reported in this study as the total $AgNO_3$ concentration.

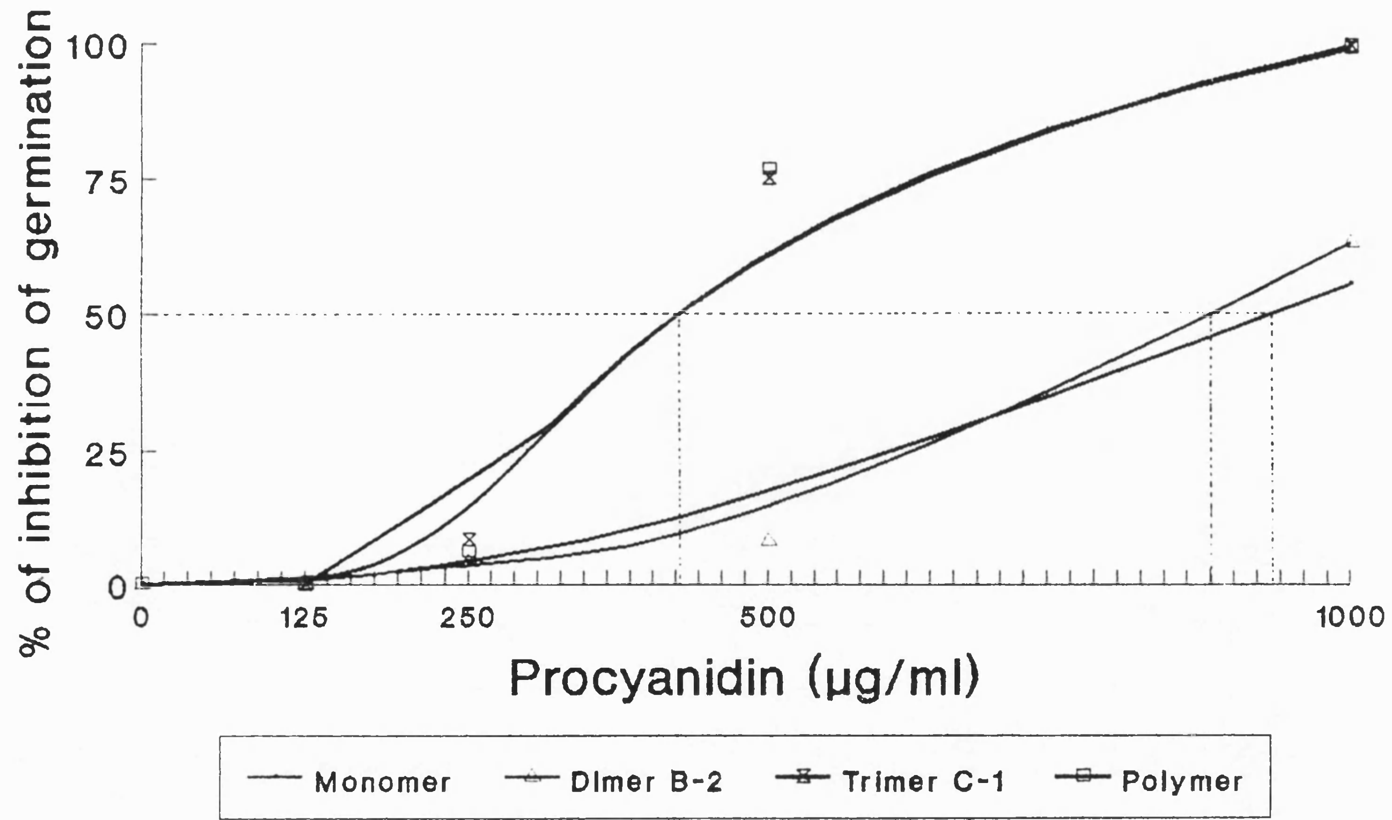
Appendix 3.

Spray reagent for TLC plates.

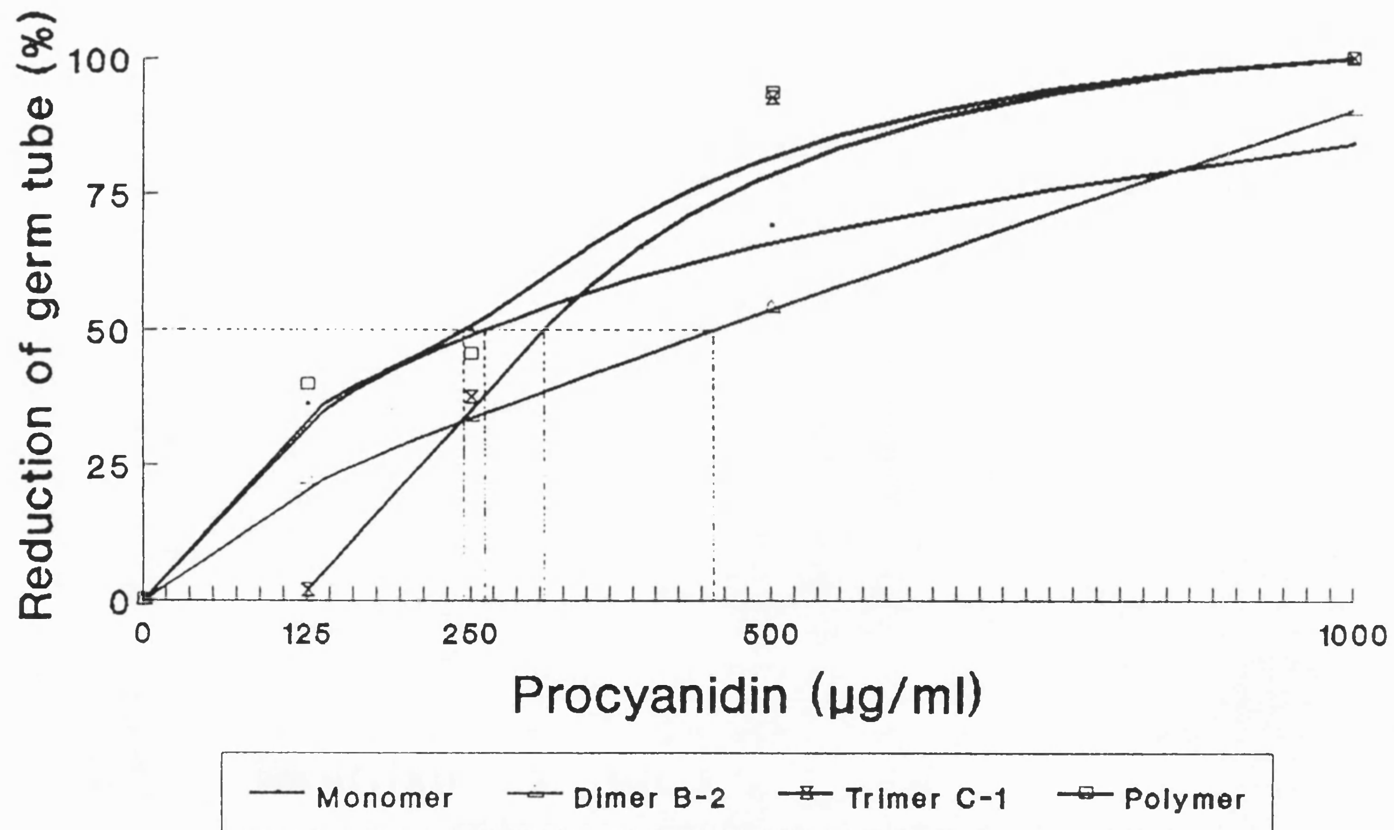
Vanillin-sulphuric acid reagent.

0.5 g of vanillin is dissolved in 100 ml of a mixture of sulphuric acid-ethanol (4:1). After spraying, the TLC plate is heated at 80°C for 5 minutes.

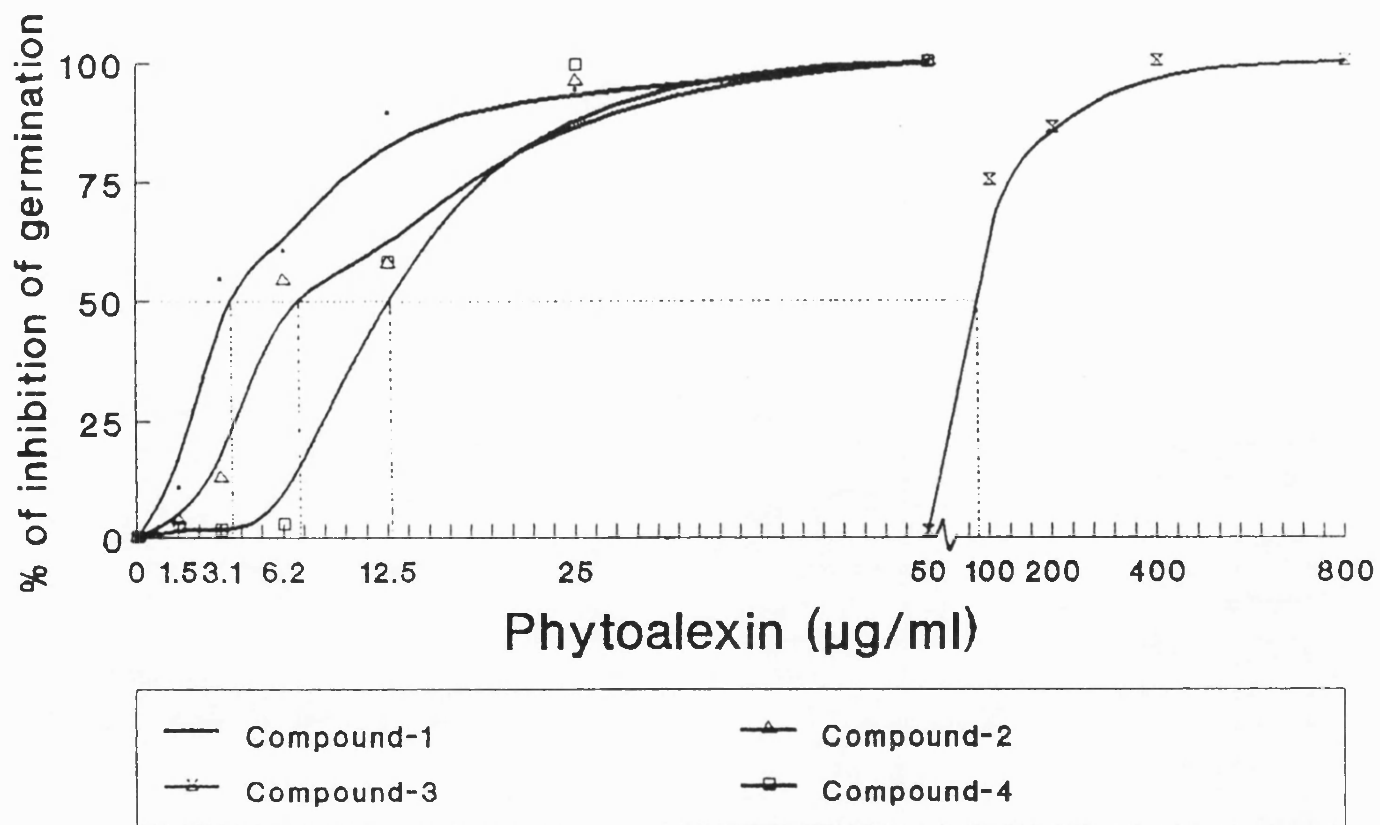
APPENDIX 4. EFFECT OF INCREASING CONCENTRATIONS OF DIFFERENT PROCYANIDINS MOLECULES ON *V. DAHLIAE*, EXPRESSED BY THE PERCENTAGE OF INHIBITION OF GERMINATION.



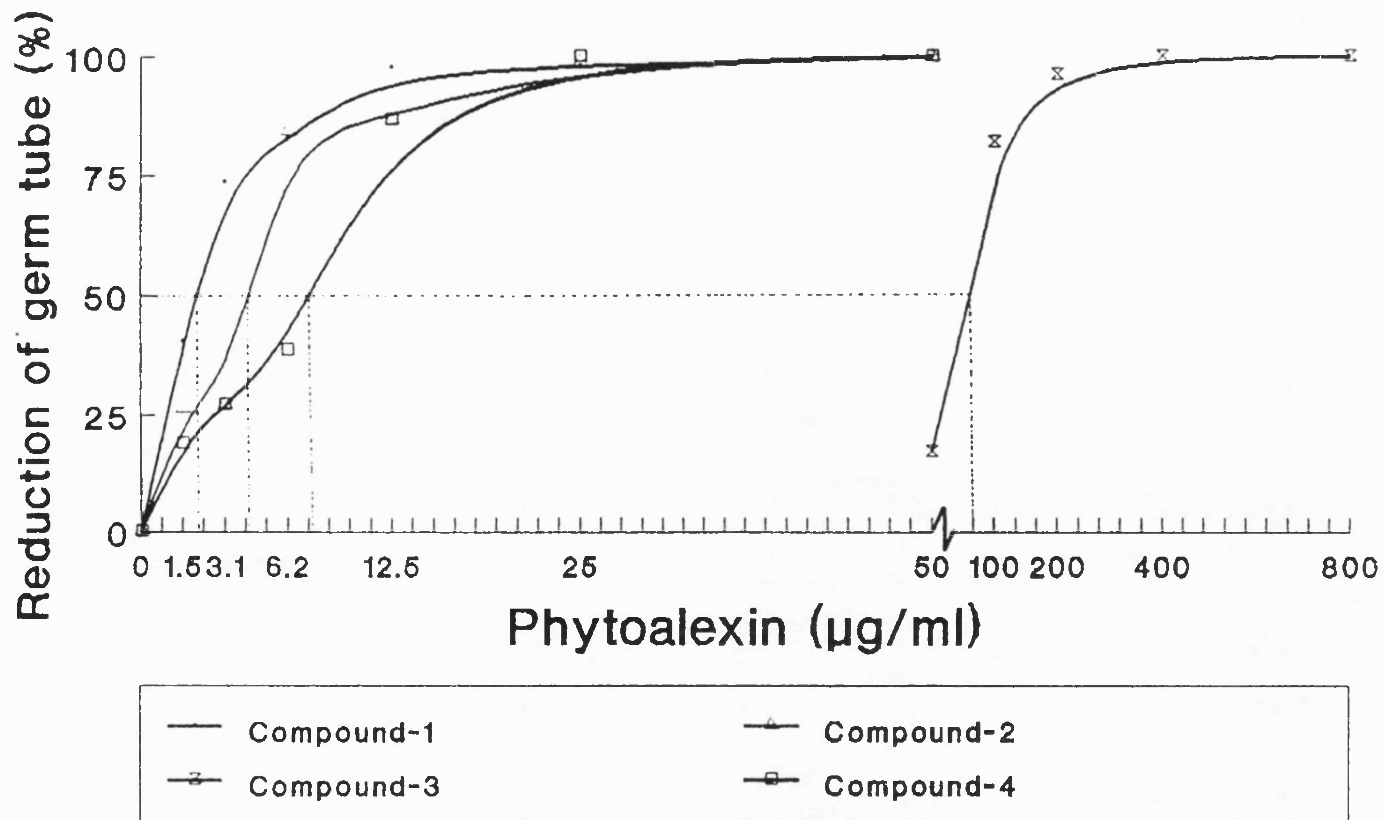
APPENDIX 5. EFFECT OF INCREASING CONCENTRATIONS OF DIFFERENT PROCYANIDINS MOLECULES ON *V. DAHLIAE*, EXPRESSED BY THE PERCENTAGE OF REDUCTION OF GERM TUBE LENGTH.



APPENDIX 6. EFFECT OF INCREASING CONCENTRATIONS OF THE COCOA PHYTOALEXINS ON *V. DAHLIAE*, EXPRESSED BY THE PERCENTAGE OF INHIBITION OF GERMINATION.



APPENDIX 7. EFFECT OF INCREASING CONCENTRATIONS OF THE COCOA PHYTOALEXINS ON *V. DAHLIAE*, EXPRESSED BY THE PERCENTAGE OF REDUCTION OF GERM TUBE LENGTH.



Appendix 8. Equations obtained from linear regressions for the estimation of LD_{50}'s and LD_{100}'s to *V. dahliae* in response to tannins and phytoalexins. Concentrations (Conc.) of the compounds were transformed in $\log_{10}$.

TANNINS: % of germination of spores:

E Q U A T I O N:	**R-sq(%)**
Conc.= 2.29 + 0.0138 Monomer	80.9
Conc.= 2.34 + 0.0110 Dimer	70.4
Conc.= 2.20 + 0.00759 Trimer	92.1
Conc.= 2.21 + 0.00746 Polymer	90.1

Tannins: % of reduction of germ tube length:

E Q U A T I O N:	**R-sq(%)**
Conc.= 1.45 + 0.0185 Monomer	99.6
Conc.= 1.92 + 0.0126 Dimer	94.2
Conc.= 2.08 + 0.00807 Trimer	93.5
Conc.= 1.74 + 0.0116 Polymer	88.1

Phytoalexins= % of germination of spores:

E Q U A T I O N:	**R-sq(%)**
Conc.= -0.127 + 0.0157 Compound-1	88.6
Conc.= 0.210 + 0.0136 Compound-2	95.0
Conc.= 1.58 + 0.00989 Compound-3	73.1
Conc.= 0.471 + 0.0108 Compound-4	86.1

Phytoalexins: % of reduction in germ tube length:

E Q U A T I O N:	**R-sq(%)**
Conc.= -0.864 + 0.0221 Compound-1	81.0
Conc.= -0.094 + 0.0148 Compound-2	82.3
Conc.= 1.43 + 0.0110 Compound-3	67.5
Conc.= 0.069 + 0.0142 Compound-4	90.8

Appendix 9.

Isolates of *Verticillium dahliae* were imported and retained under the licence No. PHF 343A/ 41/22 issued by the Ministry of Agriculture Fisheries and Food.

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Host specialization of *Verticillium dahliae*, with emphasis on isolates from cocoa (*Theobroma cacao*)

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Isolates of *Verticillium dahliae* from cocoa (four Brazilian and one Ugandan) were screened against cocoa, aubergine, tomato, cotton, and pepper. Isolates originating from hosts other than cocoa were also inoculated. In general, all inoculated crops were systemically invaded by isolates from cocoa. Isolates from each host tended to be more aggressive on their original host. All isolates from cocoa were pathogenic to cocoa, but exhibited various degrees of aggressiveness to other crops. They induced severe symptoms on aubergine, but few symptoms on tomato, although colonization occurred up to the stem apex in both cases. Likewise, symptomless invasion of pepper was found with some Brazilian isolates. The Ugandan isolate was significantly more aggressive on cotton and pepper than the Brazilian isolates.

A Brazilian isolate from cocoa from the State of Bahia was also used to inoculate 12 common weed species from the same geographical area. Four species showed wilt symptoms, while *V. dahliae* was readily recovered from the stems of a further four species. The role of alternative hosts on disease spread in cocoa growing areas is discussed.

INTRODUCTION

Verticillium species are responsible for some of the world's major diseases affecting vegetable, field, tree and ornamental crops. Most of the crop losses are caused by *V. albo-atrum* and *V. dahliae* (Schnathorst, 1981).

As a soil-borne pathogen, *V. dahliae* has been shown to colonize the roots of a wide range of plants, including species that do not become systemically invaded. Evans & Gleeson (1973) showed that a wide range of genera and species were colonized by *V. dahliae*, including members of the families Solanaceae, Malvaceae, Compositae, Gramineae, Convolvulaceae, Papilionaceae, Labiatae and Chenopodiaceae. Brown & Wiles (1970) found notable differences in host reaction to *Verticillium* among species of the same family, or even among cultivars of the same species.

Unlike *Fusarium* species, which exhibit host specificity, *Verticillium* species have not been grouped into special forms, because an isolate from one host often attacks several other unrelated plant species (Isaac, 1949). Nevertheless, some isolates of *Verticillium* are rather specialized: *V. dahliae* from peppermint, pepper, strawberry, Brussels sprouts and tobacco and *V. albo-atrum* from lucerne and hops have limited host ranges (Puhalla & Bell, 1981).

Weeds are common hosts for *V. dahliae* (Heale & Isaac, 1963; Harrison & Isaac, 1969; Evans, 1971; Evans & Gleeson, 1973; Busch *et al.*, 1978), although sometimes symptoms are not apparent on many species (Woolliams, 1966; Brown & Wiles, 1970; Vargas-Machuca *et al.*, 1987). Symptomless weeds may provide a means of multiplication, dissemination and survival of *V. dahliae* in agricultural lands (Evans, 1971; Schnathorst, 1981) and have frequently contributed to the collapse of crop rotation programmes (Busch *et al.*, 1978).

Die-back or sudden death of cocoa (*Theobroma cacao*), caused by *V. dahliae*, has been recognized in Uganda for many years and may well be the reason that cocoa has not become a significant crop there (Leakey, 1965). In Brazil, the second largest producer of cocoa in the world, *Verticillium* wilt has become an increasingly serious problem in the states of Bahia and Espirito Santo, which are responsible for 85% of Brazilian cocoa production (CEPLAC, 1982; Oliveira, 1982; Wood & Lass, 1985). More recently, *V. dahliae* was reported causing wilt on cocoa in Colombia (Granada, 1989).

In relation to alternative hosts, there is evi-

dence from East Africa, that cotton is involved in the multiplication, dissemination and survival of *V. dahliae* in cocoa plantations. *Verticillium* wilt of cotton is widely distributed in Uganda and this country is perhaps unique in growing cocoa in the same ecological zone as cotton (Wood & Lass, 1985). Moreover, Ugandan isolates of *V. dahliae* from cotton, okra and cocoa were equally pathogenic when inoculated on cocoa. They all induced typical symptoms on okra, cocoa and cotton but with decreasing disease severity, respectively, on these three hosts (Emechebe, 1974). Similarly, in Colombia, two varieties of potato were highly susceptible to a *V. dahliae* isolate from cocoa (Granada, 1989). In Brazil, nothing is known about possible alternative hosts for *V. dahliae* in cocoa-growing areas. The main epidemiological consequences concern the spread of the fungus to other crops and the possible introduction of other strains, through contaminated plant material, into cocoa farms of Bahia and Espirito Santo. The host selectivity of *V. dahliae* was therefore investigated by assessing the pathogenicity of isolates, mainly from cocoa, to important crops and native weeds from Brazil.

MATERIALS AND METHODS

Plant material and growth conditions

The seeds of cocoa (*Theobroma cacao*), cultivar ICS-1, were germinated in trays containing vermiculite placed in a growth chamber maintained at 28°C and 80–100% relative humidity. Seven days later, the seedlings were individually potted into polyethylene layflat bags, filled with a mixture of Fisons F2 compost, Fisons M2 compost and perlite (1:1:1), and transferred to a glasshouse maintained at 25 ± 2°C and 60 ± 10% relative humidity. Supplementary illumination from artificial lights was used to maintain levels of photosynthetically active radiation (PAR), between 350–450 μmol/m^2.s, during a daylength of 12–14 h.

The seeds of aubergine (*Solanum melongena*) cv. Moneymaker, tomato (*Lycopersicon esculentum*) cvs GCR-26 and GCR-218, cotton (*Gossypium hirsutum*) cv. Deltapine 50 and pepper (*Capsicum annum*) cv. California Wonder were germinated in trays containing Fisons F2 compost. Two weeks later, the seedlings were pricked out into larger trays containing similar compost. After 1 week, these seedlings were transferred to 16-cm diameter pots filled with Fisons M2 compost and placed in the glasshouse. All seedlings were kept well watered from below and were fed at monthly intervals with liquid fertilizer (Fisons Liquinure, 1 in 45 dilution, containing N, P and K in the ratio 8:4:4+trace elements).

Weed species of the families Compositae (*Bidens pilosa*, *Erechtites hieraciifolia*, *Emilia sonchifolia* and *Synedrella nodiflora*), Solanaceae (*Capsicum chinense* and *Solanum americanum*), Amaranthaceae (*Achyranthes indica*), Labiatae (*Leonurus sibiricus*), Malvaceae (*Sida carpinifolia*), Rubiaceae (*Diodia ocimifolia*), Urticaceae (*Boehmeria cylindrica*) and Verbenaceae (*Lantana camara*), were grown and inoculated at the Cocoa Research Centre (CEPLAC), Itabuna-BA, Brazil, under similar environmental conditions to those already decribed for the crops inoculated at Bath University, UK.

Fungal isolates, inoculum production and standard inoculation

The geographical and host origins of the *V. dahliae* isolates used in this study are shown in Table 1. Cultures of all isolates were derived from single spores. Stock cultures were maintained on potato dextrose agar (PDA) at 7°C. Five disks of mycelium taken from the active edge of each fungal colony growing on PDA were transferred to conical flasks containing 150 ml of a sucrose–salts medium (Cooper & Wood, 1975). The cultures were incubated for 7 days on a rotary incubator (150 r.p.m., 25°C, 24 h light regime supplied by fluorescent lamps of total light intensity equivalent to 15 W/m^2). The resulting fungal growth in each flask was filtered through two layers of muslin and the conidial suspension obtained was adjusted to 1×10^7 spores/ml. Inoculation was carried out by applying 50 ml of this suspension onto the soil surface around the base of each seedling. The plants were not stressed (for example, from root disturbance when using the root-dipping method) nor were their root systems deliberately wounded before inoculation. Uninoculated control seedlings received 50 ml of a 10% sucrose–salts medium. Cocoa seedlings were inoculated when 110 days old (optimum age for disease development; unpublished data), and the other plant species (herbaceous), when 30 days old.

Disease assessment

Every leaf of each plant was examined periodically after inoculation and symptoms were assessed visually based on a 0 to 4 scale adapted

Table 1. Geographical and host origins of *Verticillium dahliae* isolates

Isolate[a] designation	Original host	Geographical origin
BA-1	Cocoa	Bahia, Brazil
BA-2	Cocoa	Bahia, Brazil
BA-3	Cocoa	Bahia, Brazil
ES-1	Cocoa	Espirito Santo, Brazil
UG-1	Cocoa	Uganda
Swansea[b]	Tomato	Wales, UK
TS-2[b]	Tomato	California, USA
EGP	Aubergine	Athens, Greece
SS-4[c]	Cotton	California, USA
T-9[c]	Cotton	California, USA
97	Pepper	Bari, Italy

[a]Isolates BA-1, BA-2, BA-3 and ES-1 came from CEPLAC, Cocoa Research Centre, Ilheus, BA, Brazil; isolate UG-1 from International Mycological Institute, Kew, UK; isolate Swansea from M. Milton, University of Swansea, UK; isolate TS-2 from W. Tolmsoff, ARS, College Station, Texas, USA; isolate EGP from E. Tjamos, Benaki Phytopathological Institute, Athens, Greece; isolates SS-4 and T-9 from C. Boisson, ORSTOM, Montpellier, France; and isolate 97, from J. Heale, King's College, University of London, UK.
[b]Swansea is a race 1 and TS-2 is a race 2 isolate on tomato.
[c]SS-4 is a non-defoliating and T-9 is a defoliating isolate on cotton.

from Sidhu & Webster, (1977), where 0, 1, 2, 3, and 4 represent, respectively, 0%, 1 to 25%, 26 to 50%, 51 to 75% and more than 75% reduction in the photosynthetic area owing to wilting and/or necrosis. A disease index was then calculated as the mean of the ratings from individual leaves:

$$\text{Disease index} = \frac{\text{sum of leaf ratings per plant}}{\text{total number of leaves per plant}}$$

At the end of each experiment, re-isolation of the fungus was attempted from the stems of half the number of plants in each treatment, using a direct isolation technique. After surface decontamination with ethanol (70% v/v), small pieces of the stem were cut from the internodes 3, 8, 13 and 18 of tomato plants and from the internodes 1, 4, 7 and 10 of other species. The epidermis and, where appropriate, the bark of each piece, were aseptically removed and five complete cross-sections of the remaining tissues were plated onto a medium containing absolute ethanol (1% v/v)+agar (1·2% w/v), which was selective to *V. dahliae* (adapted from Nadakuvukaren & Hornen, 1959).

Experimental design and statistical analysis

A fully randomized design was utilized combining different isolates (treatments) and 20 replicates of one plant each for cocoa or 18 replicates for other species tested. Cocoa seedlings were inoculated with all cocoa isolates plus isolates from tomato and cotton. Each of the other crops was inoculated with all isolates from cocoa and one or two isolates from the same crop, as standard controls. Weeds were inoculated only with the isolate BA-3, from cocoa. Statistical analyses were carried out using programs from Minitab (Minitab Inc., State College, PA, USA) and Statsease (B. Clarke, University of Nottingham, UK). Normality of data was tested by the *n*-scores method. Since data were not suitable for analysis of variance, non-parametric Kruskal–Wallis and repeated Mann–Whitney U-test were performed. Examination of data from re-isolation (in form of ratios), were made by χ-squared analysis of contingency tables and, as appropriate, by Fisher's Exact test (Sokal & Rohlf, 1981).

RESULTS

Inoculation on cocoa

Cocoa seedlings were inoculated when 110 days old with isolates BA-2, BA-3, ES-1, (all from cocoa, Brazil); UG-1 (from cocoa, Uganda); Swansea (race 1 on tomato, from UK); TS-2 (race 2 on tomato, from USA); and SS-4 (non-defoliating pathotype on cotton, from USA).

The first symptoms of disease (flaccidity followed by chlorosis and necrosis of upper leaves) appeared about 20 days after inoculation. Within 16 days of the start of symptoms, the majority of the plants inoculated with isolates BA-2, BA-3, ES-1, UG-1, TS-2 and SS-4 had died or showed widespread desiccation of the foliage. At the end of this experiment (36 days after inoculation), significantly higher disease indices were obtained for all isolates compared with isolate Swansea (race 1 on tomato), which caused general wilting in only a small number of plants (Table 2).

V. dahliae was readily re-isolated from the stems of seedlings with symptoms, with a very low level of contamination on the selective medium. Since similar rates of recovery of *V. dahliae*

Table 2. Effect of different isolates of *Verticillium dahliae* on symptom development and recovery of the pathogen from cocoa and cotton plants

	Inoculation on cocoa		Inoculation on cotton	
Isolate	disease index[a]	re-isolation[b] (10)	disease index[a]	re-isolation[b] (10)
SS-4	3·15 d	9:1 b	3·25 c	9:0 c
T-9	–	–	3·23 c	8:1 bc
ES-1	3·05 d	10:0 b	1·92 b	5:4 bc
BA-3	2·83 cd	9:1 b	1·73 b	6:3 bc
BA-2	2·48 bcd	9:1 b	1·52 b	4:5 ab
UG-1	2·40 bc	9:1 b	2·88 c	9:0 c
TS-2	1·86 b	10:0 b	–	–
Swansea	0·47 a	3:7 a	–	–
BA-1	–	–	0·00 a	0:9 a
Control	0·00 a	0:10 a	0·00 a	0:9 a

[a] Final disease index as adapted from Sidhu & Webster (1977); values represent the means of 20 replicates for cocoa and 18 replicates for cotton. Within these columns, means followed by the same letter are not significantly different (Mann–Whitney U-test, $P \leqslant 0{\cdot}05$).
[b] Re-isolation from internode 10 of the stem. Ratios obtained refer to a yes:no response according to recovery of *V. dahliae*. Within these columns, ratios followed by the same letter are not significantly different (Fisher's Exact test, $P \leqslant 0{\cdot}05$).

occurred from all the positions tested (internodes 1, 4, 7 and 10), only data from the uppermost internode are shown in Table 2. These results indicate that a widespread vascular colonization had taken place in susceptible cocoa plants. This was confirmed, using a microscope, by the presence of hyphae growing within the xylem vessels. All cocoa isolates plus the cotton isolate and the race 2 isolate from tomato systemically colonized the stem of the majority of inoculated cocoa seedlings. In contrast, isolate Swansea was only re-isolated from those few plants showing external symptoms. A significant positive correlation (Pearson coefficient, $r = 0{\cdot}917$) was demonstrated between disease index and fungal recovery from cocoa stems.

Inoculation on cotton

Isolates BA-1, BA-2, BA-3, ES-1 and UG-1 from cocoa, plus SS-4 and T-9 from cotton were used to inoculate cotton, cv. Deltapine 50. T-9 caused defoliation on cotton, while SS-4 did not, as previously reported by Tzeng & DeVay (1985). These isolates and the Ugandan isolate from cocoa (UG-1) were more aggressive than the Brazilian isolates from cocoa (Table 2, disease index), inducing severe symptoms and eventually death of many inoculated plants.

Isolate BA-1, a hyaline variant of BA-3, produced no symptoms on leaves, nor was it re-isolated from the stem of cotton plants; all other isolates were recovered from all internodes examined, indicating successful systemic colonization (Table 2).

Inoculation on tomato

Isolates BA-1, BA-2, BA-3, ES-1 and UG-1 from cocoa and Swansea and TS-2 from tomato were used to inoculate tomato plants cv. GCR-26 and GCR-218 (without and with *Ve* gene for resistance against race 1, respectively). In all cultivars, epinasty of lower leaves was the first visible symptom, which appeared approximately 15 days after inoculation. Acropetal leaf chlorosis and necrosis, combined with general stunting were observed for some isolates by harvest time (32 days after inoculation).

Final disease indices (Table 3) confirmed that isolates from cocoa were less aggressive on tomato cv. GCR-26 than isolates Swansea (race 1) or TS-2 (race 2). On tomato cv. GCR-218, TS-2, followed by BA-3, caused more severe symptoms than other isolates tested. Nevertheless, all isolates were readily recovered from the 3rd, 8th, and 13th internodes of tomato plants. Data from the uppermost internode assessed (18) showed that isolates Swansea, TS-2 and BA-3 were those

Table 3. Effect of different isolates of *Verticillium dahliae* on symptom development and recovery of the pathogen from tomato plants, cv. GCR-26 and GCR-218

	Tomato cv. GCR-26		Tomato cv. GCR-218	
Isolate	disease index[a]	re-isolation[b] (18)	disease index[a]	re-isolation[b] (18)
Swansea	2·13 d	7:2 c	1·00 b	1:8 ab
TS-2	1·85 d	7:2 c	1·85 d	9:0 c
BA-3	1·22 c	5:4 bc	1·31 c	5:4 bc
BA-2	1·22 c	3:6 abc	1·10 b	3:6 ab
ES-1	1·06 bc	3:6 abc	1·09 b	2:7 ab
BA-1	0·95 b	3:6 abc	1·05 b	2:7 ab
UG-1	0·91 b	1:8 ab	0·99 b	3:6 ab
Control	0·00 a	0:9 a	0·00 a	0:9 a

[a] Final disease index as adapted from Sidhu & Webster (1977); values represent the means of 18 replicates. Within these columns, means followed by the same letter are not significantly different (Mann–Whitney U-test, $P \leqslant 0{\cdot}05$).
[b] Re-isolation from internode 18 of the stem. Ratios obtained refer to a yes:no response according to recovery of *V. dahliae*. Within these columns, ratios followed by the same letter are not significantly different (Fisher's Exact test, $P \leqslant 0{\cdot}05$).

most frequently recovered from GCR-26, and isolates TS-2 and BA-3 were those most frequently recovered from GCR-218 (Table 3). Thus, the *Ve* gene was only effective against the race 1 isolate of *V. dahliae*; all other isolates were equally pathogenic to GCR-26 and GCR-218.

Inoculation on aubergine

Isolates BA-2, BA-3, ES-1 and UG-1 from cocoa, EGP from aubergine and TS-2 from tomato were used to inoculate aubergine cv. Moneymaker. All isolates caused intense symptoms, which started

Table 4. Effect of different isolates of *Verticillium dahliae* on symptom development and recovery of the pathogen from aubergine and pepper plants

	Inoculation on aubergine		Inoculation on pepper	
Isolate	disease index[a]	re-isolation[b] (10)	disease index[a]	re-isolation[b] (10)
BA-2	2·70 c	6:3 bc	0·47 b	7:2 c
97	–	–	3·59 d	9:0 c
UG-1	2·53 c	8:1 bc	1·85 c	9:0 c
BA-3	2·39 bc	7:2 bc	0·00 a	7:2 c
EGP	2·32 bc	9:0 c	–	–
ES-1	1·91 bc	8:1 bc	0·12 ab	6:3 bc
TS-2	1·60 b	4:5 ab	–	–
BA-1	–	–	0·00 a	2:7 ab
Control	0·00 a	0:9 a	0·00 a	0:9 a

[a] Final disease index as adapted from Sidhu & Webster (1977); values represent the means of 18 replicates. Within these columns, means followed by the same letter are not significantly different (Mann–Whitney U-test, $P \leqslant 0{\cdot}05$).
[b] Re-isolation from internode 10 of the stem. Ratios obtained refer to a yes:no response according to recovery of *V. dahliae*. Within these columns, ratios followed by the same letter are not significantly different (Fisher's Exact test, $P \leqslant 0{\cdot}05$).

Table 5. Effect of a Brazilian isolate of *Verticillium dahliae* from cocoa (BA-3) on symptom development and recovery of the pathogen from weed species

Scientific name	Disease index[a]	Re-isolation[b] (10)
Erechtites hieraciifolia	3·45 d	9:0 b
Solanum americanum	2·49 c	9:0 b
Sida carpinifolia	1·10 b	9:0 b
Emilia sonchifolia	1·08 b	8:1 b
Diodia ocimifolia	0·00 a	7:2 b
Achyranthes indica	0·00 a	6:3 b
Capsicum chinense	0·00 a	5:4 b
Boehmeria cylindrica	0·00 a	5:4 b
Bidens pilosa	0·00 a	0:9 a
Synedrella nodiflora	0·00 a	0:9 a
Leonurus sibiricus	0·00 a	0:9 a
Lantana camara	0·00 a	0:9 a

[a] Final disease index as adapted from Sidhu & Webster (1977); values represent the means of 18 replicates and means followed by the same letter in this column are not significantly different (Mann–Whitney U-test, $P \leqslant 0{\cdot}05$).
[b] Re-isolation from internode 10 of the stem. Ratios obtained refer to a yes:no response according to recovery of *V. dahliae* and ratios followed by the same letter in this column are not significantly different (Fisher's exact test, $P \leqslant 0{\cdot}05$).

as early as 12 to 15 days after inoculation. By the end of this experiment (22 days), many inoculated plants showed extensive foliar chlorosis, necrosis and wilting followed by partial defoliation and severe stunting.

Re-isolations of the pathogen were consistently made from the stem base to internode 7 in all inoculated treatments. Some differences among isolates were detected using data from the (uppermost) internode 10, which confirmed a less extensive colonization by TS-2 (Table 4). A significant positive correlation ($r = 0{\cdot}929$) was demonstrated between external symptoms (final disease indices) and extent of colonization (data from all internodes assessed).

Inoculation on pepper

Isolates BA-1, BA-2, BA-3, ES-1 and UG-1 from cocoa, and 97 from pepper were used to inoculate pepper cv. California Wonder. The Ugandan isolate from cocoa (UG-1) showed a response intermediate between the pepper isolate (most aggressive) and Brazilian isolates from cocoa (least aggressive) (Table 4). All Brazilian isolates caused few or no symptoms on pepper, but were re-isolated from the internodes 1, 4 and 7 to the same extent as 97 and UG-1. Even at the uppermost internode assessed (10), it was possible to differentiate only the isolate BA-1 from all other isolates, which more extensively colonized pepper stems (Table 4).

Inoculation on weeds

Fourteen weed species present in the cocoa region of Bahia, Brazil, were inoculated with isolate BA-3, isolated from a cocoa tree in the same region. Symptom development and re-isolation of the pathogen were assessed 45 days after inoculation. Final disease indices obtained revealed that only four species showed external symptoms (Table 5). *Erechtites hieraciifolia* was the only weed killed by *V. dahliae*. *Solanum americanum*, followed by *Sida carpinifolia* and *Emilia sonchifolia* also exhibited significant wilt symptoms. The pathogen was successfully re-isolated from these weeds and from the stems of the following symptomless hosts: *D. ocimifolia*, *A. indica*, *C. chinense* and *B. cylindrica*. Other weed species were immune to systemic infection; no external symptoms were apparent and the pathogen was not re-isolated from stem tissue.

DISCUSSION

The current study demonstrates that isolates of *V. dahliae* vary in pathogenicity on different hosts. Vigouroux (1971) pointed out that isolates from a region of permanent monoculture are similar and all display high virulence against this particular crop (preferential host) but, generally, a weak virulence against other species which they can, nevertheless, infect (occasional hosts). *Theobroma cacao*, growing in north-eastern Brazil, may be considered a preferential host for *Verticillium*, according to the Vigouroux concept, since all Brazilian isolates from cocoa were very destructive to cocoa, but not so to most other plant species. Emechebe (1974) found that Ugandan isolates from cocoa, okra and cotton were equally pathogenic to cocoa. In the current study, American isolates from cotton (non-defoliating) and tomato (race 2) were also highly pathogenic on cocoa, which suggests that a large number of *V. dahliae* isolates can cause symptoms on cocoa seedlings.

Isolates from cocoa induced different responses on the three solanaceous crops inoculated. All Brazilian isolates caused severe symptoms on

aubergine, but few or no symptoms on pepper. These results agree with those of Vigouroux (1971), who postulated that aubergine is a preferential host, while pepper is an occasional host for *V. dahliae*. In addition, Ciccarese *et al.* (1987) reported that Italian isolates of *V. dahliae* from chicory, broccoli and tomato were highly pathogenic to aubergine, but caused no symptoms on pepper, suggesting that isolates pathogenic to pepper occur less frequently in nature. Tomato seems to be an occasional host for cocoa isolates of *V. dahliae*, because these isolates caused only mild symptoms on both varieties (with and without the *Ve* gene). However, Fordyce (1963) reported that peppermint isolates, initially avirulent to tomato, became virulent after passages through a susceptible tomato cultivar, and Tjamos (1981) suggested that the broadening of host range could occur in the field after introduction of other potential hosts into the same area. Tomato varieties have not been extensively cultivated in the cocoa region of the States of Bahia and Espirito Santo in Brazil and this may explain the low level of aggressiveness of cocoa isolates on tomato.

The higher level of aggressiveness of the Ugandan isolate from cocoa to cotton, compared with Brazilian isolates from cocoa, suggests that selection or adaptation of the pathogen to the host may have occurred, as cotton has been cultivated in the same region as cocoa in Uganda, but not in Brazil. However, this consideration should be interpreted with caution, as just one isolate from Uganda was tested. Vigouroux (1971) concluded that the apparent *de novo* appearance of a host-specific strain is most probably due to selection from the population of soil strains, rather than adaptation. The hypothesis of selection was later supported by Tjamos (1981), studying pathogenicity of *V. dahliae* isolates from areas of single and diversified cropping systems in Greece. Nevertheless, according to Pegg (1974) the problems of dealing with a fungus that exhibits heterokaryosis and parasexuality make it difficult to be categoric on this subject.

The introduction of a preferential host in a crop rotation would be expected to increase greatly the amount of inoculum of a wide range of strains. Such inoculum would be available for successive crops, some of which might be occasional hosts (Vigouroux, 1971). The results obtained with inoculations of different cocoa isolates are evidence that they are not specific in terms of host range. All crops and eight out of 12 weed species tested were systemically invaded and therefore can be considered as possible reservoirs of those isolates. Such crops, especially aubergine, should be avoided in cases of intercropping or replacing cocoa in areas devastated by *Verticillium* wilt.

The method of re-isolation currently utilized in this work was not quantitative, but provided an indication of the internal distribution of the pathogen. Significant positive correlations were demonstrated between external symptoms (disease index) and extent of stem colonization (re-isolation data) recorded from cocoa, aubergine, tomato and cotton. These results might be expected since they represent the most frequent response of plant species infected with *Verticillium* (Brandt *et al.*, 1984; Townsend *et al.*, 1990; Papadopoulos & Christie, 1991). However, non-significant correlation between symptoms and colonization ($r = 0{\cdot}391$) was observed on pepper, mainly because Brazilian isolates from cocoa did not cause symptoms, but were re-isolated even from the top of the stem. Also, four weed species (*D. ocimifolia*, *A. indica*, *C. chinense* and *B. cylindrica*), showed a similar pattern when inoculated with isolate BA-3 from Brazil. Such findings are in agreement with other reports on symptomless hosts of *Verticillium* (Brown & Wiles, 1970; Evans, 1971; Evans & Gleeson, 1973; Krikun & Bernier, 1987; Mathre, 1989), implying a possible tolerance to certain isolates of *V. dahliae*. Mussel (1981) considered that tolerance is an incomplete form of resistance, i.e. a plant is able to produce an acceptable yield while providing, at least, a limited habitat for growth and reproduction of the pathogen. Tolerant hosts may also provide raised inoculum levels in the field, resulting in substantial losses for a perennial or subsequent crop. Therefore, an adequate control of all weed species is also required as part of a disease management programme in cocoa-growing areas.

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